Rapport de la réunion de la Commission des normes biologiques de l'OMSA

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9 au 13 septembre 2024

Paris

Introduction et contribution des Membres

Ce rapport présente les travaux de la réunion de la Commission des normes biologiques de l'OMSA (ci-après désignée en abrégé : « la Commission ») qui s'est tenue à Paris (France) du 9 au 13 septembre 2024.

Au cours de cette réunion, 27 chapitres du *Manuel des tests de diagnostic et des vaccins pour les animaux terrestres* (ci-après, *Manuel terrestre*) de l'OMSA ont été approuvés en vue d'être distribués aux Membres pour un premier cycle de consultations ; un autre chapitre a été approuvé en vue d'être distribué pour un troisième cycle de consultations ; en outre, six candidatures au statut de Centre de référence ont été examinées, ainsi que les désignations proposées pour le remplacement de huit experts.

La Commission souhaite remercier les Membres suivants pour les commentaires reçus par écrit concernant les projets de textes destinés au *Manuel terrestre* : Canada, Chine (Rép. pop. de), États-Unis d'Amérique, Japon, Nouvelle-Zélande, Royaume-Uni, Suisse, États membres de l'Union européenne. La Commission exprime également sa gratitude aux nombreux experts du réseau scientifique de l'OMSA pour leurs précieux conseils et leurs contributions.

Tous les commentaires soumis dans les délais prévus et étayés par des explications argumentées ont été examinés par la Commission. La Commission remercie les Membres qui ont soumis leurs commentaires en suivant le Guide d'information pour les Membres de l'OMSA et les Organisations internationales à des fins de soumission des commentaires pendant la procédure d'élaboration des normes internationales de l'OMSA. La Commission a tenu à souligner qu'aucun commentaire sur les textes distribués avec le présent rapport qui ne suivrait pas les spécifications du Guide d'information susnommé ne sera pris en compte ni publié. La Commission a recouru à la présentation habituelle pour faire ressortir les amendements introduits aux projets de texte, à savoir un double soulignement pour les ajouts et une ligne de rature pour les suppressions. Dans les annexes contenant des textes modifiés, les amendements proposés dans le cadre de cette réunion sont surlignés en jaune afin de les différencier de ceux introduits précédemment.

Comme annoncé dans le rapport de septembre 2023 de la Commission, la Directrice générale a décidé de mettre en place une approche par étapes pour améliorer la transparence du processus d'élaboration des normes de l'OMSA et en assurer une meilleure mise à l'écrit et traçabilité.

La première étape de cette approche a été la publication (en avril 2024) sur le site web des Délégués des commentaires soumis par les Membres et les partenaires de l'OMSA et examinés par la Commission lors de sa réunion de février 2024. Les commentaires ont été téléchargés sur le site web des Délégués au moment même de la publication du rapport de la Commission. Les commentaires sont publiés dans la langue dans laquelle ils ont été rédigés et soumis.

L'étape suivante a été la publication des commentaires pris en compte par la Commission en même temps que les réponses de la Commission, dans une annexe spécifique présentée exclusivement pour information (voir l'annexe 4). L'annexe 4 présente les réponses de la Commission aux commentaires soumis par les Membres lors du deuxième cycle de consultations et reçus avant la Session générale de mai. À l'avenir, tous les commentaires des Membres relatifs aux projets de chapitre seront présentés sous forme de tableau comme c'est déjà le cas dans l'annexe 4. Dans cette annexe, les commentaires pris en compte sont publiés dans la langue dans laquelle ils ont été soumis. Les réponses de la Commission sont présentées en anglais, français ou espagnol suivant la version linguistique. Les commentaires et les réponses de la Commission sont insérés dans la version anglaise du texte tel que distribué en février 2024 pour commentaires. En raison des contraintes de ressources, les textes contextuels ne sont présentés qu'en anglais dans les trois versions linguistiques (anglais, espagnol et français). Veuillez noter que les textes présentés pour recueillir des commentaires figurent aux annexes 5 à 33.

Votre participation au processus d'élaboration des normes de l'OMSA est précieuse. Nous vous remercions pour votre mobilisation dans ce processus !

Annexes

L'annexe 4 est présentée pour information uniquement et contient les commentaires pris en compte ainsi que les réponses de la Commission.

Les textes constituant les annexes 5 à 32 de ce rapport sont présentés pour un premier cycle de consultations et ceux constituant l'annexe 33 sont présentés pour un troisième cycle de consultations.

Modalités de soumission des commentaires

La Commission des normes biologiques encourage vivement les Membres de l'OMSA ainsi que les organisations internationales ayant conclu un accord de coopération avec l'OMSA à participer à l'élaboration des normes internationales de l'OMSA en lui soumettant des commentaires sur les projets de textes annexés au présent rapport.

Il est essentiel que les Membres et les organisations internationales participent au processus d'élaboration des normes en soumettant des commentaires, car cela consolide le fondement scientifique des normes, garantit la prise en compte des diverses situations rencontrées par les Membres et parties prenantes et contribue à l'applicabilité effective des normes. Pour être pris en compte, les commentaires devront être soumis avant la date limite proposée, et rédigés en utilisant le modèle de formulaire figurant dans le Guide d'information pour les Membres de l'OMSA et les Organisations internationales à des fins de soumission des commentaires pendant la procédure d'élaboration des normes internationales de l'OMSA (ci-après, Guide d'information) et dans la Procédure opérationnelle standard pour les Membres de l'OMSA et les organisations internationales à des fins de soumission des commentaires pendant la procédure d'élaboration des normes internationales de l'Organisation (ci-après, POS), consultables sur le site web des Délégués et le site web public de l'OMSA.

Les commentaires dont la présentation ne serait pas conforme au modèle décrit dans le Guide d'information et les SOP ne seront pas pris en compte par la Commission. Toute question relative aux exigences de mise en forme et à la soumission des commentaires doit être adressée au BSC.Secretariat@woah.org.

Comme l'a souligné le président de la Commission lors de la Session générale, le premier cycle de consultations est l'occasion pour les Membres de soumettre les conclusions de leurs experts suite à un examen approfondi des textes proposés, dans le but d'améliorer l'exactitude et la pertinence scientifiques des chapitres. Après la réunion de février, les chapitres tels que préparés en vue de leur adoption sont distribués pour un second cycle de consultations. Les commentaires soumis pendant ce second cycle devraient se limiter à des propositions d'amendements jugés essentiels pour la mise à jour du chapitre.

La Commission des normes biologiques souhaite rappeler que lorsqu'une discussion de la Commission repose sur les contributions d'un Groupe ad hoc, les Membres sont encouragés à examiner le rapport du Groupe ad hoc en question en même temps que le rapport de la Commission. Les rapports des Groupes ad hoc peuvent être consultés sur les pages dédiées du site web de l'OMSA, à l'adresse : https://www.woah.org/fr/ce-que-nous-faisons/normes/processus-detablissement-des-normes/groupes-ad-hoc/

Délai de soumission des commentaires

Les commentaires sur les textes présentés dans ce rapport devront être transmis au Siège de l'OMSA avant le 27 décembre 2024 afin d'être examinés par la Commission des normes biologiques.

Où adresser les commentaires

Les commentaires sont à envoyer au Service scientifique à l'adresse suivante : BSC.Secretariat@woah.org

Dates de la prochaine réunion de la Commission

La Commission a proposé de tenir sa prochaine réunion aux dates suivantes : du 3 au 7 février 2025.

Sommaire

Liste des annexes

1. Mots de bienvenue des directrices

1.1. Directrice générale

Le 9 septembre, la Docteure Emmanuelle Soubeyran, Directrice générale de l'OMSA, et la Docteure Montserrat Arroyo, Directrice générale adjointe de l'OMSA pour les Normes internationales et la science, ont rejoint les membres de la Commission des normes biologiques, de la Commission scientifique pour les maladies animales (en abrégé ci-après, Commission scientifique) et de la Commission des normes sanitaires pour les animaux terrestres (en abrégé ci-après, Commission du Code), afin d'accueillir officiellement les Commissions spécialisées dans leur nouvelle composition suite aux élections qui se sont déroulées pendant la 91e Session générale en mai 2024.

La Docteure Soubeyran a félicité les membres élus et exprimé sa gratitude aux institutions d'origine et gouvernements nationaux des membres des Commissions pour leur soutien. La Docteure Soubeyran a donné un aperçu de sa vision de l'innovation, du développement stratégique et de la visibilité accrue de l'OMSA, en mettant en exergue la collaboration, la numérisation et le renforcement des programmes d'envergure mondiale. La Docteure Soubeyran a informé les membres de la Commission que l'OMSA entendait poursuivre les travaux engagés pour réviser les *Textes fondamentaux* de l'Organisation, en s'attachant particulièrement à l'examen de sa gouvernance, celle-ci étant garante de la crédibilité de l'OMSA auprès de ses Membres et parties prenantes.

La Docteure Soubeyran a mis en avant le rôle crucial joué par les Commissions spécialisées en tant que chefs de file de la gouvernance technique de l'Organisation et souligné l'importance que revêt l'expertise des Commissions pour la réputation et la reconnaissance de l'OMSA. Elle a également insisté sur l'importance de la collaboration entre les différentes Commissions spécialisées. La Docteure Soubeyran a réaffirmé son engagement à promouvoir l'inclusivité et la transparence et noté qu'il était d'une importance capitale non seulement de promouvoir la participation active de tous les Membres au processus d'élaboration des normes, mais aussi de s'assurer que les normes de l'OMSA répondent aux besoins de tous des Membres et qu'elles sont mises en œuvre dans le monde entier.

La Docteure Soubeyran a évoqué les efforts entrepris par l'OMSA pour améliorer la transparence en procédant à la publication des commentaires formulés par les Membres. Elle a ensuite rappelé à la Commission que la numérisation des normes de l'OMSA est en cours, qui se concrétisera dans un outil de navigation en ligne dédié aux normes et offrant aux utilisateurs un accès et une navigation rationalisés. L'inclusion et la participation des membres des Commissions ont également été évoquées en tant qu'aspects essentiels de la gouvernance de l'OMSA. La Docteure Soubeyran a détaillé les projets visant à accroître la participation des Membres dans les procédures d'élaboration des normes ; elle a également annoncé que les prochaines Conférences des Commissions régionales comporteront des séances dédiées à cette question pour que les Membres puissent faire connaître leurs priorités parmi les points inscrits au programme d'élaboration des normes. Dans sa conclusion, la Docteure Soubeyran a réaffirmé l'engagement de l'OMSA à garantir la transparence, la crédibilité et l'inclusivité de toutes ses opérations.

La Docteure Arroyo a relevé l'importance du renouvellement des Commissions et s'est réjouie de l'arrivée de nouveaux membres, de la composition géographiquement équilibrée des Commissions et de la gestion plus efficiente de la charge de travail. Elle a également souligné l'importance de l'inclusivité, de la transparence et de la continuité des travaux au sein de chaque Commission. Pour conclure, la Docteure Arroyo a fait ressortir les principaux points du Cadre de gestion des performances des Commissions spécialisées et souligné son importance pour garantir le perfectionnement continu des travaux des Commissions.

Les membres des Commissions ont pris bonne note de ces informations et exprimé à la Docteure Soubeyran leurs meilleurs souhaits de réussite dans ses fonctions de Directrice générale de l'OMSA.

1.2. Directrice générale adjointe, Normes internationales et science

La Docteure Montserrat Arroyo, Directrice générale adjointe de l'OMSA pour les normes internationales et la science a rejoint la Commission des normes biologiques le 10 septembre 2024 et remercié les membres réélus ainsi que ceux nouvellement élus pour leur contribution à cet aspect important des travaux de l'OMSA.

La Docteure Arroyo a évoqué le rôle des Laboratoires de référence et Centres collaborateurs de l'OMSA en soulignant leur importance et leur intérêt pour l'Organisation. Elle a notamment insisté sur la nécessité pour ces institutions d'offrir un soutien solide aussi bien aux Membres qu'à l'Organisation elle-même, en garantissant un niveau homogène d'assistance couvrant la totalité du réseau.

Les membres de la Commission ont exprimé à la Docteure Arroyo leur gratitude pour l'excellent soutien apporté par le secrétariat de l'OMSA.

Après l'allocution de la Docteure Arroyo, la Docteure Gillian Mylrea, cheffe du Service des Normes a animé une séance d'information spécifique dans le contexte du récent renouvellement des Commissions spécialisées engagées dans un nouveau mandat. Cette séance était la dernière du programme d'initiation destiné aux Commissions spécialisées, qui comportait également des séances de prise de contact destinées aux nouveaux membres des Commissions, à leurs Présidents, aux autres membres des Commissions et à leurs secrétariats afin qu'ils se rencontrent et examinent ensemble les informations importantes en ce début de mandat.

2. Adoption de l'ordre du jour

L'ordre du jour proposé a été examiné et adopté. Le Docteur Emmanuel Couacy-Hymann a présidé la réunion et le secrétariat de l'OMSA a exercé la fonction de rapporteur. L'ordre du jour et la liste des participants figurent respectivement aux annexes 1 et 2.

3. Relations avec les autres Commissions spécialisées

3.1. Questions transversales intéressant les Commissions spécialisées

3.1.1. Travaux relatifs aux animaux hôtes pris en compte dans les normes de l'OMSA pour une maladie listée des animaux terrestres

Lors de sa réunion de septembre 2023, la Commission du Code avait examiné avec la Commission scientifique un projet visant à définir une approche claire et cohérente pour la mention dans le *Code terrestre* et *le Manuel terrestre* des animaux hôtes d'une maladie, infection ou infestation listée. En février 2024, ces deux Commissions ont répondu favorablement à la proposition formulée par leurs secrétariats d'avancer sur cette question en réunissant un groupe de réflexion constitué de membres des trois Commissions.

Lors de la présente réunion, la Commission des normes biologiques a pris connaissance des recommandations du groupe de réflexion, qui s'était réuni en quatre occasions pour déterminer de manière cohérente quels étaient les animaux hôtes d'une maladie listée qu'il convenait de mentionner dans les normes de l'OMSA relatives aux maladies des animaux terrestres. Ces recommandations avaient également été transmises pour avis au président du Groupe de travail sur la faune sauvage.

La Commission des normes biologiques a souscrit aux recommandations du groupe de réflexion quant à la nécessité d'une bonne coordination des travaux d'élaboration des normes sur des maladies spécifiques destinées au *Manuel terrestre* comme au *Code terrestre*. La Commission est également convenue que les chapitres du *Manuel terrestre* dédiés à des maladies particulières devraient comporter une description générale de l'épidémiologie de la maladie en question, mentionnant les animaux sensibles et plus particulièrement ceux chez qui la maladie se déclare naturellement. Les chapitres du *Manuel terrestre* devraient également indiquer quels sont les animaux sensibles effectivement identifiés, les échantillons à prélever à partir de ces animaux et les tests de diagnostic applicables.

La Commission évaluera les recommandations formulées par le groupe de réflexion avec les experts chargés de réviser les chapitres du *Manuel terrestre* dédiés à des maladies particulières et transmettra ses observations au groupe de réflexion.

3.2. Commission scientifique pour les maladies animales

3.2.1. Définitions d'un cas : paratuberculose, arthrite/encéphalite caprine et maedi-visna

La Commission des normes biologiques a examiné les définitions d'un cas pour la paratuberculose, l'arthrite/encéphalite caprine et le maedi-visna et a transmis ses recommandations à la Commission scientifique.

Concernant la définition d'un cas de paratuberculose, la Commission a pris connaissance du projet de définition d'un cas d'infection par *Mycobacterium avium* subsp. *paratuberculosis* (paratuberculose). La Commission a estimé que la paratuberculose satisfait le critère 3 : il existe des méthodes de détection et de diagnostic fiables ainsi qu'une définition de cas suffisamment précise pour identifier clairement les cas et les distinguer d'autres maladies, infections ou infestations.

Des informations plus détaillées sur la définition d'un cas figurent aux points 9.3.1 et 9.3.2 du rapport de la réunion de la Commission scientifique, 9-13 septembre 2024.

3.2.2. Évaluation de la maladie des moutons de Nairobi au regard des critères d'inclusion dans la liste

Voir le point 9.2.1 du rapport de la réunion la Commission scientifique pour les maladies animales, 9–13 septembre 2024.

3.2.3. Recommandations du Groupe ad hoc sur la tremblante

Voir le point 5.1.3 du rapport de la réunion la Commission scientifique pour les maladies animales, 9–13 septembre 2024.

3.3. Commission des normes sanitaires pour les animaux terrestres

3.3.1. Réunion des Bureaux

Voir le point 3.2 du rapport de la réunion de la Commission des normes sanitaires pour les animaux terrestres, 10–19 septembre 2024.

3.4. Commission des normes sanitaires pour les animaux aquatiques

Pas de question examinée.

4. Programme de travail

Le programme de travail actualisé a été adopté et figure à l'annexe 3 du présent rapport.

5. *Manuel des tests de diagnostic et des vaccins pour les animaux terrestres*

Pour l'examen de ce point de l'ordre du jour, le Docteur John Pasick, consultant rédacteur du *Manuel terrestre* de l'OMSA, s'est joint à la Commission.

5.1. Format du rapport et système de soumission des commentaires

Lors de sa réunion de février 2024, la Commission a décidé d'adopter le même format que celui utilisé par la Commission des normes pour les animaux aquatiques et de présenter les commentaires des Membres dans des tableaux, ce qui permet de mieux rendre compte de la justification scientifique de chaque amendement apporté au *Manuel terrestre*. Les Membres peuvent ainsi visualiser et comprendre les décisions de la Commission concernant les changements apportés et les réponses aux commentaires.

5.2. Examen et approbation des projets de chapitre destinés à être distribués aux Membres pour un premier cycle de consultations

La Commission a examiné 30 projets de chapitre et en a approuvé 29, dont certains sous réserve d'une clarification de certains points par les experts, en vue de leur distribution aux Membres pour un premier cycle de consultations avant de les soumettre à l'Assemblée pour adoption en mai 2025. La Commission a décidé de supprimer le chapitre 2.3.2, *Rôle des autorités officielles dans la réglementation internationale des produits biologiques à usage vétérinaire*, estimant qu'il n'a pas sa place dans le *Manuel terrestre*. La question de savoir s'il revient au *Manuel terrestre* de fournir une norme sur la réglementation des produits biologiques devra faire l'objet de discussions plus approfondies.

1.1.3. « Transport de matériel biologique » : la Commission a révisé le chapitre 1.1.3, « Transport de matériel biologique », dont la mise à jour avait été réalisée par un expert d'un Centre collaborateur de l'OMSA. La mise à jour visait à intégrer les changements introduits dans les réglementations de l'IATA depuis la dernière adoption du chapitre en 2018.

Le chapitre 1.1.3 révisé, « Transport de matériel biologique », est présenté à l'annexe 5 à l'intention des Membres pour un premier cycle de consultations.

Les principaux amendements portent sur les points suivants :

Section/paragraphe	Changement
A.1. Expéditeur (chargeur, consignateur)	Ajout d'un alinéa sur l'importance de prévoir à l'avance les mesures permettant de maintenir la chaîne du froid, le cas échéant.
Tableau 1. Synthèse de la classification, la catégorisation, l'identification et l'emballage des matières infectieuses	Mise à jour des dispositions relatives au triple emballage applicables aux marchandises dangereuses de classe 6, division 6.2.
B.4. Matériel biologique non soumis au Règlement sur les marchandises dangereuses	Ajout d'une note précisant qu'il sera peut-être nécessaire de procéder à une évaluation du risque lorsque le matériel considéré n'est pas soumis au Règlement sur les marchandises dangereuses.
Tableau 3. Exemples de matières infectieuses classées dans la catégorie A	Actualisation des listes de micro-organismes classés UN 2814, Matière infectieuse pour l'homme et UN 2900, Matière infectieuse pour les animaux uniquement.
C. Emballage	Actualisation des spécifications de l'ONU relatives à l'emballage, chaque fois que nécessaire.
E. Références et lectures complémentaires	Actualisation de la liste de références.

1.1.7. « Normes pour le séquençage à haut débit, la bioinformatique et la génomique computationnelle » : la Commission a révisé le chapitre 1.1.7, dont la mise à jour avait été réalisée par des expert d'un Centre collaborateur de l'OMSA.

Le chapitre 1.1.7 révisé, « Normes pour le séquençage à haut débit, la bioinformatique et la génomique computationnelle », est présenté à l'annexe 6 à l'intention des Membres pour un premier cycle de consultations.

Les principaux amendements portent sur les points suivants :

Section/paragraphe	Changement
Introduction	Clarification des explications relatives à la « nouvelle procédure », en la comparant à d'autres essais diagnostiques normalisés.
A. Considérations générales	Modification rédactionnelle afin de prendre en compte la temporalité, la technologie du séquençage ne cessant pas d'évoluer.
B. Conduite d'investigations vétérinaires incorporant le SHD-BCG	Ajout d'un texte sur l'importance d'une étroite collaboration avec les experts de l'analyse de données et d'un traitement approprié des données.
C.1. Normes pour l'utilisation de SHD-BCG	Ajout d'informations sur les dispositions spécifiques applicables au choix des plateformes technologiques, y compris la prise en compte du taux d'erreur inhérent à la technologie, les coûts et l'infrastructure associés aux meilleures pratiques pour le déroulement des opérations de séquençage.
C.2. Échantillonnage et production de rapports	Suppression de la répétition par rapport à l'énoncé de la section B concernant l'intégration de vétérinaires ; ajout de la mention de l'importance que le laboratoire dispose de lignes directrices internes.
	Ajout d'informations supplémentaires sur les stratégies d'enrichissement, y compris sur le moment où les appliquer afin d'améliorer la sensibilité de la

Section/paragraphe	Changement
C.3. Échantillons et préparations des échantillons	technique ; mention également des spécificités et des précautions à prendre lors du SHD.
	Suppression de la répétition des informations concernant la séparation des zones de travail afin de prévenir les risques de contamination croisée.
	Ajout d'informations sur les bonnes pratiques et mention de l'utilisation d'un index.
C4. Création de données de séquençage	Les plateformes de SHD présentant des différences dans leur chimie et leurs protocoles, il est nécessaire de suivre des principes de base de contrôle qualité ; précision ajoutée concernant la couverture des différents témoins positifs et négatifs, qui devrait correspondre à différents emplacements de la plaque multi-puits.
	Suppression de la disposition visant à déterminer le biais GC dans une zone cible car la manière dont l'utilisateur peut se servir de cette information pour suivre la performance du test n'est pas claire ; ajout d'une mesure supplémentaire de la qualité pour évaluer la performance analytique des tests basés sur le SHD dans des zones dont la complexité est faible ; ajout d'une description sur la manière d'écourter les lectures.
C.5. Bioinformatique	Ajout d'informations détaillées sur la transparence de lignes directrices claires sur la gestion des métadonnées
C.6. Gestion des données	Texte révisé indiquant que les systèmes de gestion des données devraient spécifier quelles sont les données à soumettre aux archives ou aux bases de données nationales ou internationales, tout en soulignant l'importance d'apporter le plus grand soin à la sélection des plateformes d'intégration des données.
C.7. Validation des systèmes de test aux fins prévues	Ajout d'une précision sur l'importance de diluer les échantillons dans la même matrice.
E. Références et lectures complémentaires	Actualisation de la liste de références.

1.1.9. « Contrôle de la stérilité et de l'absence de contamination des matériels biologiques à usage vétérinaire : la Commission a révisé le chapitre 1.1.9, dont la mise à jour avait été effectuée par les expert d'un Centre collaborateur de l'OMSA.

Le chapitre 1.1.9 révisé, « Contrôle de la stérilité et de l'absence de contamination des matériels biologiques à usage vétérinaire » est présenté à l'annexe 7 pour un premier cycle de commentaires par les Membres.

Les principaux amendements portent sur les points suivants :

Section/paragraphe	Changement
E. Vaccins à parasites vivants	Ajout d'une nouvelle section sur les vaccins contre les maladies parasitaires.
H.5. Example de procédure pour tester un vaccin antiparasitaire vivant	Ajout d'une nouvelle section sur les procédures pour les vaccins antiparasitaires.

2.1.1. « Méthodes de laboratoire utilisées pour les tests de sensibilité des bactéries aux antimicrobiens » : la Commission a révisé le chapitre 2.1.1, mis à jour par des expert de trois Centres collaborateurs de l'OMSA suite à la demande de la Commission du Code que ce chapitre soit révisé pour déterminer si les informations qu'il contient concernant l'établissement des seuils cliniques étaient suffisantes et à jour.

Le chapitre 2.1.1 révisé, « Méthodes de laboratoire utilisées pour les tests de sensibilité des bactéries aux antimicrobiens », est présenté à l'annexe 8 pour un premier cycle de commentaires par les Membres.

Les principaux amendements portent sur les points suivants :

Section/paragraphe	**Changement**
2. Choix des antimicrobiens pour les tests et les rapports	Mise à jour de cette section pour y intégrer davantage de bonnes pratiques en matière de sélection d'antimicrobiens appropriés pour les tests de sensibilité aux agents antimicrobiens.
5.1.1. Considérations concernant l'utilisation des méthodes de diffusion en disque	Ajout d'une description des inconvénients de la méthode de diffusion en disque.
5.2.1. Dilution en bouillon	Ajout d'une description des avantages et des inconvénients des méthodes de dilution en bouillon.
5.4. Orientations futures de la détection de la sensibilité/résistance aux antimicrobiens	Modification du titre de la section en « Méthodes génétiques de détection de la résistance », actualisation de la sous-section sur les tests moléculaires et ajout d'une nouvelle sous-section sur le séquençage du génome entier.
6. Seuils de sensibilité aux antimicrobiens et critères pour la zone d'inhibition	Modification du titre de la section en « Critères d'interprétation des tests de sensibilité aux antimicrobiens » et actualisation du texte, y compris sur les principaux facteurs permettant d'interpréter les données relatives à un isolat bactérien pour le qualifier de sensible, d'intermédiaire, de résistant, de type sauvage ou de type non sauvage vis-à-vis d'un agent antimicrobien donné.
Tableau 1. Méthodes phénotypiques existantes de tests de sensibilité et leurs caractéristiques	Modification du titre du Tableau 1 : « Méthodes phénotypiques existantes de tests de sensibilité aux antimicrobiens », mise à jour du tableau et suppression des colonnes Utilisation dans les tests de sensibilité à des fins thérapeutiques, Seuils pouvant être utilisés, Comparabilité des résultats et Caractéristiques.
Références	Actualisation exhaustive de la liste de références.

2.2.1. « Mise au point et optimisation des méthodes de détection des anticorps » : la Commission a révisé le chapitre 2.2.1, dont la mise à jour avait été réalisée par des expert d'un Centre collaborateur de l'OMSA.

Le chapitre 2.2.1 révisé, « Mise au point et optimisation des méthodes de détection des anticorps », est présenté à l'annexe 9 pour un premier cycle de commentaires par les Membres.

Les principaux amendements portent sur les points suivants :

Section/paragraphe	**Changement**
Introduction	Ajout d'informations sur les normes et les documents d'orientation publiés pour chacune des étapes de la validation des tests de diagnostic et des essais d'aptitude interlaboratoires, et rappel soulignant que les principes sous-jacents de la mise au point d'un essai constituent le fondement de tests

Section/paragraphe	Changement
	de diagnostic de grande qualité et adaptés à l'objectif ou aux objectifs prévus.
A.1. Objectif(s) prévu(s) de l'essai de détection des anticorps	Texte simplifié avec de nouvelles références et références croisées en remplacement d'anciens passages et du tableau ; rappel de la consigne aux utilisateurs du *Manuel terrestre* de bien vouloir lire préalablement la section B.4, « Étape 4 – Mise en œuvre des programmes » car elle décrit les relations entre la sensibilité et la spécificité diagnostiques, les résultats faux positifs et faux négatifs erronés, ainsi que les valeurs prédictives positives et négatives.
A.2.1.3. Panels de référence positifs et négatifs	Ajout de recommandations sur le stockage à long terme d'échantillons de sérum et des stocks de travail.
B.1.2. Spécificité analytique	Ajout d'informations sur les échantillons à évaluer pour déterminer la spécificité analytique.
B.3. Étape 3 – Reproductibilité et estimation élargie de la répétabilité	Insertion de nouvelles références sur la reproductibilité, assorties d'exemples concrets d'essais d'aptitude et d'essais comparatifs interlaboratoires, ajout d'une étude de cas concernant la sélection et l'utilisation des panels de référence, et mention de l'importance à des fins de transparence des biobanques virtuelles pour ce qui concerne les réactifs et les échantillons utilisés pendant l'élaboration et la validation des tests.
B.4.1. Interprétation des résultats et détermination des valeurs prédictives	Changement du titre de la section « Interprétation des résultats » et ajout de références relatives à l'interprétation des résultats d'un test au moyen de la valeur prédictive et du rapport de vraisemblance.
B.5. Suivi de la performance de l'essai	Ajout d'une référence à des aspects de la validation des kits pour les tests effectués à des fins de diagnostic et de surveillance des maladies du bétail : responsabilités respectives du producteur et de l'utilisateur final. Ajout d'un alinéa à la liste des points de contrôle lors du suivi de la performance, posant la question de savoir si des procédures sont en place pour déterminer les actions à mener lorsque les témoins se situent en dehors des limites acceptables.
Références	Actualisation de la liste de références.

2.2.2. « Mise au point et optimisation des méthodes de détection des antigènes » : la Commission a révisé le chapitre 2.2.2, dont la mise à jour avait été réalisée par des experts d'un Centre collaborateur de l'OMSA.

Le chapitre 2.2.2 révisé, « Mise au point et optimisation des méthodes de détection des antigènes », est présenté à l'annexe 10 pour un premier cycle de commentaires par les Membres.

Les principaux amendements portent sur les points suivants :

Section/paragraphe	Changement
Introduction	Ajout d'informations sur les normes et les documents d'orientation publiés relatifs à la validation des tests de diagnostic et aux essais d'aptitude interlaboratoires, y compris concernant leur conception, leur analyse, et la préparation de rapports clairs, complets et transparents sur les études de validation.
A.1. Objectif(s) prévu(s) de l'essai de détection des antigènes	Texte simplifié avec de nouvelles références et références croisées en remplacement d'anciens passages et du tableau.

Section/paragraphe	Changement
A.1.1. Objectif 1a : Contribuer à la démonstration de l'absence d'infection dans une population donnée, a) statut « indemne » avec ou sans vaccination	Ajout d'un nouveau paragraphe expliquant comment minimiser la probabilité de résultats faux-négatifs lors de la conception de l'essai ou de l'algorithme du test.
A.1.1. Objectif 1b : Contribuer à la démonstration de l'absence d'infection dans une population donnée, b) recouvrement du statut indemne après des foyers.	Ajout d'informations sur les tests adaptés ou mis au point après un foyer en tant qu'exemples de tests « démontrant l'absence de maladie ».
A.1.2. Objectif 2 : Certifier l'absence d'infection ou la présence de l'agent pathogène chez un animal ou une marchandise à des fins de commerce/déplacements	Ajout d'un paragraphe contenant des exemples et précisant que lorsque l'objectif concerne la certification à des fins de déplacements d'animaux, il sera préférable d'utiliser un test ou une combinaison de tests présentant une sensibilité diagnostique (SeD) élevée, notamment en recourant à des tests multiples effectués en parallèle, où un échantillon sera considéré positif dès lors que l'un des tests est positif.
A.2.1.1. Échantillons de tests	Mise en garde concernant le fait que le transport d'échantillons de tissus dans du glycérol et du tampon phosphate peut affecter la sensibilité des essais de détection des antigènes ; il est recommandé de rincer les échantillons de tissus avec une solution tampon phosphate salin avant de les préparer pour le test, car cela contribue à empêcher l'inhibition de la fluorescence.
A.2.1.3. Panels de référence positifs et négatifs	Ajout d'un texte explicatif et d'une référence sur la mise au point et la validation d'un test de détection de l'antigène à l'immunoperoxydase pour améliorer le diagnostic de la rage en Indonésie.
A.2.1.4. Antigènes purifiés et antigènes bruts pour la production d'anticorps	Actualisation exhaustive des informations fournies dans cette section afin de mieux couvrir le sujet.
A.2.1.5. Anticorps monoclonaux et polyclonaux pour la détection des antigènes	Ajout d'une nouvelle section contenant des informations sur ce thème important.
B.1.1. Répétabilité	Ajout d'une nouvelle section expliquant ce qu'est la répétabilité et pourquoi elle est importante.
B.1.2. Spécificité analytique	Cette section sur la spécificité analytique (SpA) a été étoffée pour décrire la sélectivité, l'exclusivité et l'inclusivité, trois composantes de la SpA.
B.1.4. Comparaison des méthodes de test de référence avec la méthode de test candidate	Changement du titre de la section en « Comparaison entre les méthodes de test de référence et le test soumis à évaluation », et ajout d'une explication sur la manière de conduire les comparaisons entre méthode de test.
Références	Actualisation de la liste de références.

2.2.3. « Mise au point et optimisation des méthodes de détection des acides nucléiques » : la Commission a révisé le chapitre 2.2.3, dont la mise à jour avait été réalisée par des experts d'un Centre collaborateur de l'OMSA.

Le chapitre 2.2.3 révisé, « Mise au point et optimisation des méthodes de détection des acides nucléiques », est présenté à l'annexe 11 pour un premier cycle de commentaires par les Membres.

Les principaux amendements portent sur les points suivants :

Section/paragraphe	Changement
Introduction	Ajout d'informations sur les normes et les documents d'orientation publiés relatifs à un certain nombre d'aspects de la validation des tests de diagnostic et des essais d'aptitude interlaboratoires, et sur la mise au point et l'optimisation des essais de détection des acides nucléiques
A.2.1 Assurance qualité	Ajout d'un texte soulignant que les laboratoires devraient conserver des registres exacts et les archiver dans des bases de données communes et sécurisées. En vue de l'homologation de l'essai, des rapports complets devront être préparés décrivant les échantillons utilisés, les copies et les analyses statistiques correspondant à chaque étape de la validation.
A.2.2. Matériel de référence	Ajout d'un texte soulignant l'importance de bien comprendre la biologie de l'agent pathogène considéré et des tissus qui seront infectés, afin de sélectionner le type d'échantillon approprié ; le choix du matériel de référence doit tenir compte des configurations probables du diagnostic ; ajout d'exemples d'échantillons non invasifs.
A.2.3.1. Choix du test	Changement du titre en « Objectif du test et séquence cible » et ajout d'informations complémentaires y compris sur les caractéristiques essentielles de la conception du test afin de modéliser les séquences cibles appropriées et d'optimiser la conception de l'amorce et de la sonde.
A.2.3.2. Tests auxiliaires ou secondaires	Ajout de cette nouvelle section.
A.2.3.3. Tests utilisables sur le lieu d'intervention (POC)	Ajout de cette nouvelle section.
A.2.4. Étude de faisabilité	Ajout d'un texte soulignant l'importance de tester différentes souches/lignées génétiques de l'agent pathogène cible, ainsi que d'autres pathogènes génétiquement apparentés avec celui-ci, afin de déterminer respectivement l'inclusivité et l'exclusivité préliminaires, avec des exemples.
A.2.5.1. Témoin de l'espèce hôte	Suppression de cette section.
A.2.5.3. Témoin d'inhibition	Cette section a fait l'objet d'une mise à jour exhaustive et deux nouveaux alinéas ont été ajoutés : i) témoins endogènes internes, et ii) témoins endogènes externes.
B.1. Étape 1 – Critères de performance analytique	Les informations contenues dans cette section ont fait l'objet d'une mise à jour exhaustive, en particulier les sous-sections sur la répétabilité (PCR qualitative conventionnelle), la spécificité analytique (SpA) et la sensibilité analytique (SeA).
B.4. Étape 4 – Mise en œuvre des programmes	Ajout d'un paragraphe sur l'assemblage des échantillons, car celui-ci peut accroître l'efficacité de l'analyse d'un grand nombre d'échantillons.
Références	Actualisation de la liste de références.

2.2.5. « Méthodes statistiques de validation » : la Commission a révisé le chapitre 2.2.5, dont la mise à jour avait été réalisée par des experts d'un Centre collaborateur de l'OMSA.

Le chapitre 2.2.5 révisé, « Méthodes statistiques de validation », est présenté à l'annexe 12 pour un premier cycle de commentaires par les Membres.

Les principaux amendements portent sur les points suivants :

Chapitre intégralement mis à jour
Ce chapitre a été intégralement mis à jour, l'essentiel du texte existant ayant été actualisé ou remplacé, de même que les figures et tableaux. L'ordre des sections a également été modifié. Elles sont désormais présentées dans l'ordre suivant : Section A. Sensibilité analytique ; B. Spécificité analytique ; C. Répétabilité ; D. Sensibilité diagnostique et spécificité diagnostique ; E. Reproductibilité – au lieu de : A. Répétabilité d'un essai dans un laboratoire unique ; B. Reproductibilité de l'essai entre laboratoires ; C. Sensibilité analytique (SeA, synonyme = limite de détection : LD) ; D. Spécificité analytique (SpA) ; E. Performance diagnostique de l'essai. La section bibliographique a également été intégralement mise à jour.

2.2.8. « Comparabilité des épreuves suite à des changements introduits dans une méthode de test validée » : la Commission a révisé le chapitre 2.2.8, dont la mise à jour avait été réalisée par des experts d'un Centre collaborateur de l'OMSA.

Le chapitre 2.2.8 révisé, « Comparabilité des épreuves suite à des changements introduits dans une méthode de test validée », est présenté à l'annexe 13 pour un premier cycle de commentaires par les Membres.

Les principaux amendements portent sur les points suivants :

Chapitre intégralement mis à jour
Ce chapitre a été intégralement mis à jour, l'essentiel du texte existant ayant été actualisé ou remplacé, de même que les figures et tableaux. L'ordre des sections a également été modifié. Elles sont désormais présentées dans l'ordre suivant : Section A. Détermination des critères d'évaluation et de conclusion ; B. Limite de détection ; C. Répétabilité ; D. Caractéristiques diagnostiques ; E. Comparaison des courbes ROC ; F. Discussion ; G. Analyse des données – au lieu de : A. Mise sur pied d'expériences comparatives ; B. Inspection visuelle ; C. Répétabilité ; D. Graphique de Bland–Altman ; E. Expérience pour la limite de détection ; F. Comparaison des courbes ROC ; G. Discussion et conclusions ; H. Analyse des données. La section bibliographique a également été intégralement mise à jour.

3.1.2. « Maladie d'Aujeszky (infection par le virus de la maladie d'Aujeszky) » : la Commission a révisé le chapitre 3.1.2, dont la mise à jour avait été réalisée par l'expert du Centre collaborateur de l'OMSA.

Le chapitre 3.1.2 révisé, « Maladie d'Aujeszky (infection par le virus de la maladie d'Aujeszky) », est présenté à l'annexe 14 pour un premier cycle de commentaires par les Membres

Les principaux amendements portent sur les points suivants :

Section/paragraphe	**Changement**
Résumé	Actualisation de la taxonomie et suppression d'une phrase sur la possibilité de distinguer les anticorps induits par une infection naturelle de ceux résultant de la vaccination avec des vaccins obtenus par délétion de gènes.
A. Introduction	Actualisation de la taxonomie et ajout d'une précision et de références concernant l'encéphalite humaine associée aux nouvelles souches variantes.
Tableau 1. Méthodes d'essai disponibles pour le diagnostic de la maladie d'Aujeszky et emplois	Modification de la notation attribuée à l'épreuve d'agglutination au latex et à l'ELISA de « +++ » en « ++ » pour l'emploi « déterminer le statut immunitaire des animaux au niveau de l'individu ou d'une population (suite à une vaccination) » car ces tests ne permettent pas de quantifier les anticorps chez les animaux pris individuellement ; qualification de l'ELISA en tant que gB et ajout d'une ligne pour l'ELISA gE.

Section/paragraphe	Changement
B.1.2. Identification du virus par amplification en chaîne par polymérase	Actualisation exhaustive de cette section et ajout de tableaux sur les séquences d'amorce/de sonde et les paramètres des cycles pour la PCR en temps réel et conventionnelle.
B.2.2. Dosage immuno-enzymatique	Actualisation des références correspondant à cette section.
C.2.3. Exigences relatives à l'autorisation	Modification de l'intitulé de la section en « Exigences relatives à l'approbation réglementaire » et ajout d'une précision indiquant que les épreuves de réversion vers la virulence devraient être conformes à la ligne directrice GL41 du VICH (Examen des vaccins vétérinaires vivants chez les animaux cibles pour contrôler l'absence de réversion vers la virulence, 2008[1]).
Références	Actualisation de la liste de références.
Annexes 1 à 6	Ajout de six annexes donnant la justification des épreuves choisies et des notations attribuées dans le Tableau 1.

3.1.8. « Fièvre aphteuse (infection par le virus de la fièvre aphteuse) » : la Commission a révisé le chapitre 3.1.8, tel que mis à jour par les experts des Centres collaborateurs de l'OMSA. Cette mise à jour a principalement porté sur la section dédiée aux épreuves de diagnostic ; les experts procéderont par la suite à la mise à jour des sections dédiées aux vaccins et à l'adéquation des vaccins, avec l'ajout des tableaux justificatifs.

Le chapitre 3.1.8 révisé, « Fièvre aphteuse (infection par le virus de la fièvre aphteuse) », est présenté à l'annexe 15 pour un premier cycle de commentaires par les Membres

Les principaux amendements portent sur les points suivants :

Section/paragraphe	Changement
A. Introduction	Clarification précisant que la dernière détection du sérotype C remonte à 2004 et qu'il est désormais considéré comme n'étant plus en circulation.
Tableau 1. Méthodes d'essai disponibles pour le diagnostic de la fièvre aphteuse et emplois	Suppression de l'épreuve de fixation du complément, qui manque de sensibilité.
B. Tests de diagnostic	Modification de la composition recommandée pour le milieu de transport en soulignant qu'en cas de non-disponibilité de celui-ci, l'expédition des échantillons vers le laboratoire dans des conditions réfrigérées ou sur de la glace, sans milieu de transport, peut être envisagée ; ajout de recommandations pour la collecte d'écouvillons pharyngés de porcs, et ajout d'autres matrices d'échantillons possibles ; étoffement des instructions concernant l'utilisation du godet probang pour prélever le fluide œsophagien-pharyngé. Ajout d'une mention au Senecavirus A tout au long de cette section en tant que virus vis-à-vis duquel il est recommandé de réaliser un diagnostic différentiel.
B.1.1. Isolement viral	Ajout d'informations sur le choix de la lignée cellulaire et les essais systématiques de sensibilité des cellules.
B.1.2.3. Dispositifs d'essai à flux latéral	Ajout d'informations sur le kit d'essai à flux latéral certifié par l'OMSA, en soulignant que les résultats obtenus avec des dispositifs d'essai à flux latéral

[1] https://www.ema.europa.eu/en/documents/scientific-guideline/vich-gl41-target-animal-safety-examination-live-veterinary-vaccines-target-animals-absence-reversion_en.pdf.

Section/paragraphe	Changement
	non certifiés devraient être confirmés au moyen de tests de laboratoire validés.
B.1.2.4. Épreuve de fixation du complément	Suppression de l'épreuve de fixation du complément, en raison de sa sensibilité médiocre.
B.1.3.4. Épreuve RT-PCR en temps réel	Actualisation du protocole et ajouts de références à des méthodes qui contribuent à réduire les résultats faux négatifs.
B.1.3.6. Épidémiologie moléculaire	Ajout d'un lien vers une liste de souches prototypes du virus de la fièvre aphteuse qui peuvent constituer les points de référence de base d'un arbre phylogénétique ; ajout d'une phrase expliquant que dans les zones où les lignées du virus de la fièvre aphteuse en circulation sont bien connues et où l'accès à des capacités de séquençage est difficile, les épreuves RT-PCR en temps réel spécifiques des lignées sont une solution permettant une caractérisation rapide à un coût relativement modeste ; ajout de mentions aux méthodes permettant d'obtenir des séquences entières ou partielles du virus de la fièvre aphteuse par séquençage de Sanger ou à haut débit.
B.2. Épreuves sérologiques	Confirmation de la possibilité d'une réactivité croisée entre différents sérotypes avec les épreuves ELISA de détection des anticorps dirigés contre la protéine non structurale et mise en garde sur la prudence avec laquelle celles-ci doivent être utilisées dans les zones où de nombreux sérotypes du virus de la fièvre aphteuse peuvent être présents, lorsque l'objectif est d'exercer une surveillance.
B.2.1. Neutralisation virale	Mise à jour de la procédure d'essai.
B.2.2.1. Dosage immuno-enzymatique de compétition en phase solide	Clarification sur le mode de calcul de la valeur seuil.
C.5.3.1. Test de validité de la DP_{50} et test de protection contre la généralisation podale (PGP)	Texte corrigé afin de confirmer que les animaux vaccinés ne sont pas considérés comme étant protégés dès lors qu'ils présentent des lésions podales de fièvre aphteuse sur au moins un pied dans les sept jours suivant l'inoculation.
D.4.1.4. Test d'adéquation des souches vaccinales par fixation du complément	Suppression de cette section.
Références	Actualisation de la liste de références.

3.1.9. « Cowdriose » : la Commission a révisé le chapitre 3.1.9, dont la mise à jour avait été réalisée par l'expert du Centre collaborateur de l'OMSA.

Le chapitre 3.1.9 révisé, « Cowdriose », est présenté à l'annexe 16 pour un premier cycle de commentaires par les Membres.

Les principaux amendements portent sur les points suivants :

Section/paragraphe	Changement
B.3.4. ELISA indirect MAP1-B pour toutes espèces	Ajout d'un nouvel ELISA indirect pour toutes espèces.

Section/paragraphe	Changement
C.2. Préparations de vaccins atténués	Ajout d'informations sur un vaccin atténué contre la cowdriose administré par voie intramusculaire chez les bovins, les ovins et les chèvres Angora, ainsi que d'une référence.
Annexes 1, 2 et 3	Ajout de trois annexes donnant la justification des épreuves choisies et des notations attribuées dans le Tableau 1, Méthodes d'essai disponibles pour le diagnostic de la cowdriose et emplois.

3.1.21. « Peste bovine (infection par le virus de la peste bovine) » : la Commission a révisé le chapitre 3.1.21, dont la mise à jour avait été réalisée par les experts du Centre collaborateur de l'OMSA.

Le chapitre 3.1.21 révisé, « Peste bovine (infection par le virus de la peste bovine) », est présenté à l'annexe 17 pour un premier cycle de commentaires par les Membres.

Les principaux amendements portent sur les points suivants :

Section/paragraphe	Changement
Résumé	Ajout d'un texte soulignant que les analyses diagnostiques utilisant du matériel contenant le virus de la peste bovine devraient être réalisées par un Laboratoire de référence de l'OMSA et que la production de vaccin et les activités de recherche utilisant le virus de la peste bovine ou du matériel en contenant requièrent l'autorisation expresse préalable de la FAO[2] et l'OMSA et ne pourront être effectuées que dans un établissement habilité à détenir le virus de la peste bovine.
Tableau 1. Méthodes d'essai disponibles pour le diagnostic de la peste bovine et emplois	Suppression de l'épreuve d'immunodiffusion en gélose (AGID) en raison de son absence de spécificité et de sensibilité, et de l'épreuve de dosage immuno-enzymatique de compétition (C-ELISA) qui n'est plus disponible sous forme de kit ; ajout de l'amplification en chaîne par polymérase conventionnelle couplée à une transcription inverse (RT-PCR) ; modification de la notation de l'isolement viral pour l'emploi « confirmation des cas cliniques » de « +++ » à « + » car sa faible sensibilité interdit de la qualifier de test recommandé, et modification de la notation de la neutralisation virale de « +++ » à « ++ » pour les emplois « populations indemnes d'infection », « contribution aux politiques d'éradication », « prévalence de l'infection – surveillance » et «déterminer le statut immunitaire d'animaux pris individuellement ou de populations (après vaccination) » en raison des limitations induites par la difficulté de réaliser cette épreuve et de la quasi-impossibilité de la réaliser à l'échelle requise pour une surveillance effective.
Remarque spécifique à la situation de post-éradication	Texte clarifiant que les échantillons à analyser pour un diagnostic définitif devront être expédiés à l'un des Laboratoires de référence de l'OMSA pour la peste bovine et non à un établissement habilité par la FAO-OMSA à détenir le virus de la peste bovine.
B.1. Détection et identification de l'agent pathogène	Texte clarifiant que l'isolement viral devra être réalisé par un Laboratoire de référence de l'OMSA uniquement et non par un établissement habilité par la FAO-OMSA à détenir le virus de la peste bovine.
B.1.2 Détection de l'antigène par épreuve d'immunodiffusion en gélose	Suppression de l'AGID.

[2] FAO : Organisation des Nations unies pour l'alimentation et l'agriculture

Section/paragraphe	Changement
B.1.2. Méthodes de détection et de caractérisation de l'acide nucléique	Ajout d'un texte soulignant que les méthodes de diagnostic moléculaire nécessitent des témoins positifs et négatifs appropriés pendant leur réalisation, et qu'en l'absence de réactions concluantes avec les témoins les résultats seront à interpréter avec prudence.
B.2.1. Dosage immuno-enzymatique de compétition	Suppression du C-ELISA et annonce qu'une nouvelle épreuve est en cours de mise au point.
C.1.1. Principes et utilisation prévue du produit	Information concernant l'existence d'un stock de semences vaccinales, disponible sous réserve d'autorisation expresse de la FAO et l'OMSA.
C.2.2.2. Spécifications applicables aux substrats et aux milieux	Texte ajouté pour clarifier, d'une part, que le sérum bovin utilisé doit provenir de pays indemnes de fièvre aphteuse et dont le statut au regard du risque d'encéphalopathie spongiforme bovine est négligeable et qu'il doit aussi avoir été trouvé indemne du virus de la diarrhée virale bovine et d'autres virus adventices et, d'autre part, que l'utilisation de sérum irradié aux rayons gamma est recommandée.
Références	Actualisation de la liste de références.
Annexes 1 à 6	Ajout de six annexes donnant la justification des épreuves choisies et des notations attribuées dans le Tableau 1.

3.2.4. « Infestation des abeilles mellifères par le petit coléoptère des ruches (*Aethina tumida*) » : la Commission a révisé le chapitre 3.2.4, dont la mise à jour avait été réalisée par les experts du Centre collaborateur de l'OMSA.

Le chapitre 3.2.4 révisé, « Infestation des abeilles mellifères par le petit coléoptère des ruches (*Aethina tumida*) », est présenté à l'annexe 18 pour un premier cycle de commentaires par les Membres.

Les principaux amendements portent sur les points suivants :

Section/paragraphe	Changement
Résumé	Ajout d'une recommandation visant à mettre en place des ruchers sentinelles dans les zones exposées au risque de nouvelles introductions, c'est-à-dire des colonies d'abeilles mellifères parfaitement actives et possédant une reine, afin d'attirer et de piéger les coléoptères adultes.
A. Introduction	Ajout d'informations sur la propagation d'*Aethina tumida* qui n'est plus seulement un ravageur d'*Apis mellifera* mais se maintient et prolifère dans les colonies d'abeilles mellifères de l'espèce asiatique *Apis cerana* et infeste également d'autres pollinisateurs sociaux tels que les bourdons (Hymenoptera : Apidae, Bombini) et les abeilles sans dard (Hymenoptera : Apidae, Meliponini) présents dans les régions tropicales et sous-tropicales des Amériques, d'Afrique et d'Australie.
A.2. Impact de l'infestation	Ajout d'une précision signalant le peu d'informations disponibles sur l'impact du petit coléoptère des ruches sur *Apis cercana*, le caractère encore limité de son impact sur les abeilles sans dard, et l'absence de connaissances quant à ses effets sur les abeilles mellifères et les bourdons sauvages.
Tableau 1. Méthodes d'essai disponibles pour le diagnostic de l'infestation par *Aethina tumida* et emplois	Actualisation du tableau en y ajoutant « Inspection visuelle des colonies ».

Section/paragraphe	Changement
B.1.3. Inspection visuelle des colonies	Ajout d'informations sur l'utilisation de colonies sentinelles pour surveiller la présence et la propagation du petit coléoptère des ruches, et description d'une ruche sentinelle.
B.1.4. Examen d'une colonie au moyen de pièges	Suppression de l'assertion et de la référence suivant lesquelles la terre de diatomées a fait l'objet d'analyses réussies au laboratoire montrant qu'elle constitue un piège efficace dans les conditions d'Amérique du Nord ; ajout d'informations sur les inconvénients des lingettes en microfibres non tissées, en raison du risque de contamination du miel et des abeilles par des microplastiques.
B.2.2. Identification morphologique des adultes et des larves	Procédure d'essai : ajout d'un texte décrivant comment mesurer la longueur des coléoptères adultes.
B.2.2.3. Orientation pour l'identification d'*Aethina tumida*	Forme adulte : ajout d'une liste de caractéristiques morphologiques et d'une nouvelle figure (photographie et dessin) représentant une antenne d'*A. tumida*.
	Forme larvaire : mise à jour de la liste des caractéristiques morphologiques, ajout d'une nouvelle figure avec une représentation détaillée des tubercules dorsaux d'une larve d'*A. tumida*, et recommandation d'effectuer une analyse de confirmation par PCR, compte tenu de la similitude morphologique des larves d'autres espèces de Nitidulidae, dès lors que l'identification est incertaine.
B.2.3. Identification moléculaire	Mise à jour de la section expliquant qu'en raison de l'émergence de variations génétiques due à la propagation mondiale d'*A. tumida*, la méthode recommandée a été adaptée en y ajoutant une amorce sens modifiée afin d'accroître l'échelle de détection de certains spécimens présents dans des pays d'Asie et de l'Océan indien.
B.2.3.1. Préparation des échantillons, équipements et réactifs	Intitulé de la section modifiée en « Préparation des échantillons, équipements et méthode basée sur les réactifs » et ajout d'informations sur la méthode d'extraction d'ADN des échantillons.
Références et lectures complémentaires	Actualisation de la liste de références.
Annexe 1	Annexe ajoutée donnant la justification des épreuves choisies et des notations indiquées dans le Tableau 1.

3.2.5. « Infestation des abeilles mellifères par *Tropilaelaps* spp. » : la Commission a révisé le chapitre 3.2.5, dont la mise à jour avait été réalisée par l'expert du Centre collaborateur de l'OMSA.

Le chapitre 3.2.5 révisé, « Infestation des abeilles mellifères par *Tropilaelaps* spp. », est présenté à l'annexe 19 pour un premier cycle de commentaires par les Membres.

Les principaux amendements portent sur les points suivants :

Section/paragraphe	Changement
Résumé et Introduction	Mise à jour de la taxonomie du genre *Tropilaelaps*.
A. Introduction	Ajout de nouvelles sections : Nature et classification de l'agent pathogène, Potentiel zoonotique et exigences en matière de biosécurité et de biosûreté,

Section/paragraphe	Changement
	et Diagnostic différentiel ; mise à jour des sections sur le cycle évolutif (avec l'ajout d'une nouvelle figure) et sur la description et l'impact de la maladie.
Tableau 1. Méthodes d'essai disponibles pour le diagnostic de l'infestation des abeilles mellifères par *Tropilaelaps* et emplois	Mise à jour du tableau en ajoutant quatre nouvelles méthodes de détection et d'identification.
B.1.1 Collecte d'acariens	Changement de l'intitulé de la section en « Examen des abeilles » et mise à jour des informations.
B.1.2. Examen de la colonie et du couvain	Changement de l'intitulé de la section en « Examen du couvain operculé » et mise à jour des informations.
B.1.3. « Test de résistance au choc (*Bump test*) »	Ajout d'une mise en garde concernant la mortalité élevée que cette méthode pourrait induire dans les couvains et indications sur l'inefficacité dont elle a fait preuve à détecter *Tropilaelaps*.
B.1.4. Examen des plateaux des ruches dotés d'une surface collante	Changement de l'intitulé de la section en « Examen des débris des ruches » et mise à jour des informations.
B.2.2. Identification morphologique de *Tropilaelaps* spp.	Ajout d'informations détaillées sur la procédure d'essai pour évaluer le ratio longueur/largeur du corps de l'acarien.
B.2.3. Identification moléculaire	Mise à jour des informations fournies et ajout de références sur les méthodes moléculaires.
Références	Actualisation de la liste de références.
Annexe 1	Annexe ajoutée donnant la justification des épreuves choisies et des notations attribuées dans le Tableau 1.

3.3.1. « Chlamydiose aviaire » : la Commission a révisé le chapitre 3.3.1, dont la mise à jour avait été réalisée par les experts du Centre collaborateur de l'OMSA.

Le chapitre 3.3.1 révisé, « Chlamydiose aviaire », est présenté à l'annexe 20 pour un premier cycle de commentaires par les Membres.

Les principaux amendements portent sur les points suivants :

Section/paragraphe	Changement
Résumé	Mise à jour de la taxonomie, mention du potentiel zoonotique de *Chlamydia psittaci*, précision concernant la PCR qui est la méthode de diagnostic recommandée.
A.1. Description et impact de la maladie	Mise à jour de la taxonomie et informations sur les différentes espèces de *Chlamydia* ainsi que sur les espèces aviaires chez qui elles ont été détectées ; description des effets sur ces espèces.
A.2. Nature et classification de l'agent pathogène	Ajout de cette nouvelle section.
A.4. Diagnostic différentiel	Ajout de cette nouvelle section.

Section/paragraphe	Changement
Tableau 1. Méthodes d'essai disponibles pour le diagnostic de la chlamydiose aviaire et emplois	Suppression de l'essai par microdamiers d'ADN et inversion de l'ordre des épreuves ELISA et de fixation du complément.
B.1.1. Méthodes moléculaires	Ajout d'une précision soulignant que les techniques PCR ont remplacé l'isolement en tant que méthode recommandée pour la détection de la chlamydiose aviaire ; dans les situations où un test PCR ou immunohistochimique spécifique des Chlamydiaceae est positif et qu'un test spécifique de l'espèce *C. psittaci* est négatif, il conviendra d'envisager qu'il s'agit de l'espèce de *Chlamydia* nouvellement décrite. Il existe des PCR en temps réel spécifiques d'une espèce pour la détection de l'espèce visée ainsi que pour la détection des souches aviaires de *C. abortus*.
B.1.1.1 Amplification en chaîne par polymérase conventionnelle	Ajout d'une précision soulignant qu'aucune épreuve PCR conventionnelle n'a été mise au point pour la détection des nouvelles espèces aviaires qui ont été décrites.
B.1.1.2 PCR en temps réel	Ajout d'un tableau présentant des exemples de tests PCR en temps réel validés et publiés pour le dépistage et l'identification de *C. psittaci*.
B.1.1.3. Microdamiers d'ADN	Suppression de cet essai.
B.1.5. Détection de l'antigène	Suppression des épreuves immuno-enzymatiques qui étaient mentionnées dans cette section.
B.2.1. ELISA utilisant le peptide recombinant de la protéine majeure de la membrane externe (MOMP)	Ajout d'une précision indiquant qu'il n'existe pas de kit ELISA commercial pour la détection d'anticorps dirigés contre *C. psittaci* et d'autres chlamydies.
C. Spécifications applicables aux vaccins	Bien qu'il n'existe pas de vaccins commerciaux, ajout d'informations sur les souches vaccinales candidates, principalement des plasmides ou protéines recombinantes.
Références	Actualisation de la liste de références.
Annexes 1 à 4	Ajout de quatre annexes donnant la justification des épreuves choisies et des notations attribuées dans le Tableau 1.

3.3.2. « Bronchite infectieuse aviaire » : la Commission a révisé le chapitre 3.3.2, dont la mise à jour avait été confiée à des experts de cette maladie.

Le chapitre 3.3.2 révisé, « Bronchite infectieuse aviaire », est présenté à l'annexe 21 pour un premier cycle de commentaires par les Membres.

Les principaux amendements portent sur les points suivants :

Section/paragraphe	Changement
A. Introduction	Ajout d'une recommandation soulignant l'importance de prévoir une approche diagnostique large comportant un diagnostic différentiel, car les infections par le virus de la bronchite infectieuse aviaire ne sont pas spécifiques.
Tableau 1. Méthodes d'essai disponibles pour le diagnostic de	Clarification concernant les deux RT-PCR : la première est une RT-PCR « conventionnelle » (détection de l'ARN viral), tandis que la seconde est une RT-PCR en temps réel (« ciblant un gène conservé ») ; ajout d'une

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la bronchite infectieuse aviaire et emplois	troisième : RT-PCR en temps réel (spécifique d'une lignée ou d'un génotype).
B.1.5. Identification du génotype	Changement de l'intitulé de la section en « Identification de la lignée ou du génotype », en cohérence avec le Tableau 1.
B.1.5.2. Épreuve RT-PCR en temps réel	Ajout d'un tableau présentant les séquences d'amorce/de sonde et les paramètres de cycle pour les RT-PCR en temps réel.
C.2.1.1. Caractéristiques biologiques de la semence virale	Ajout de précisions et d'une référence à des travaux de recherche ayant montré que la protéine S de la souche Beaudette exprimée à partir d'un contexte génomique virulent M41 atténue le virus recombinant et confère une protection contre une inoculation d'épreuve d'une souche M41 virulente, ce qui pourrait indiquer que des passages en nombre n'affectent pas nécessairement l'antigénicité en termes d'induction d'une protection.
C.2.3.2 Critères d'innocuité	Clarification concernant la nécessaire conformité des épreuves de réversion vers la virulence avec la ligne directrice GL41 du VICH (Examen des vaccins vétérinaires vivants chez les animaux cibles pour contrôler l'absence de réversion vers la virulence, 2008).
Références	Actualisation de la liste de références.
Annexes 1 à 6	Ajout d'annexes donnant la justification des épreuves choisies et des notations attribuées dans le Tableau 1.

3.3.4. « Influenza aviaire (y compris l'infection par les virus de l'influenza aviaire hautement pathogènes) » : la Commission a révisé le chapitre 3.3.4, dont la mise à jour avait été réalisée par les experts des Centres collaborateurs de l'OMSA.

Le chapitre 3.3.4 révisé, « Influenza aviaire (y compris l'infection par les virus de l'influenza aviaire hautement pathogènes) », est présenté à l'annexe 22 pour un premier cycle de commentaires par les Membres.

Les principaux amendements portent sur les points suivants :

Section/paragraphe	Changement
Résumé	Mise à jour exhaustive du résumé, y compris la taxonomie, les méthodes de détection et d'identification, et les tests sérologiques.
A. Introduction	Mise à jour de la taxonomie ; clarifications précisant que certaines souches du virus de l'influenza aviaire ont causé des infections zoonotique sporadiques, et que certains sous-types ont été mis en avant en raison de leur potentiel endémique particulier dans l'hypothèse de mutations supplémentaires, ce qui favoriserait une transmission au long cours d'humain à humain ; précisant également qu'une mutation sur le site de clivage de la protéine H risque de faire évoluer les virus H5 ou H7 de faible pathogénicité vers une pathogénicité élevée ; et enfin que dans les régions où la pression infectieuse est forte, des cas ont été décrits de franchissement de la barrière d'espèces vers des espèces non aviaires, y compris de mammifères terrestres et marins.
Tableau 1. Méthodes d'essai disponibles pour le diagnostic de l'influenza aviaire et emplois	Ajout de « (y compris dans les cheptels vaccinés) » dans l'énoncé de l'objectif « Prévalence de l'infection – surveillance » ; relèvement de la notation de la RT-PCR en temps réel de « ++ » à « +++ » pour les emplois « démonstration du statut indemne d'infection d'une population donnée »,

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	« contribution aux politiques d'éradication » et « prévalence de l'infection – surveillance » ; ajout d'une RT-PCR conventionnelle.
B.1.1. Échantillons pour l'isolement viral	Changement de l'intitulé de la section en « Échantillons pour la détection virale » ; ajout d'informations sur l'utilisation des échantillons prélevés de l'environnement pour une surveillance active ; étant donné qu'il a été démontré que certains clades peuvent infecter un grand nombre d'espèces de mammifères, ajout d'informations sur les tissus appropriés à prélever chez ces espèces. Enfin, ajout d'un paragraphe sur l'utilisation de technologies et de réactifs permettant d'accroître la capacité de détection et de diagnostic des infections virales dans des zones reculées, peu dotées en infrastructures de laboratoire et où le maintien de la chaîne du froid est difficile, avec une mise en garde concernant l'inactivation de l'infectivité virale lorsque ces processus sont appliqués, ce qui empêche la caractérisation virale par isolement.
B.1.4. Évaluation de la pathogénicité	Mise à jour des méthodes et critères adoptés par l'OMSA pour déterminer la pathogénicité des virus de l'influenza de type A, y compris les techniques RT-PCR en temps réel qui se révèlent essentielles pour définir rapidement le pathotype viral.
B.1.5. Capture de l'antigène et techniques moléculaires	Ajout d'une présentation schématique des tests de dépistage, des essais de sous-typage et des méthodes de caractérisation virale pour les virus de l'influenza aviaire, ainsi que d'un tableau présentant les séquences d'amorces/sondes et les paramètres des cycles pour une méthode PCR en temps réel et conventionnelle ; les méthodes moléculaires étant privilégiées pour le diagnostic, la détection et la caractérisation des virus de l'influenza de type A, ajout d'une précision soulignant l'importance de se conformer à des protocoles stricts afin de prévenir les contaminations croisées entre différents échantillons cliniques ainsi que la nécessité de valider les méthodologies d'essai pour la détection de l'ARN selon la norme de l'OMSA. Cette section souligne l'importance d'utiliser des épreuves et des méthodes de diagnostic bien conçues, en faisant appel de manière continue à des outils de sous-typage sensibles et spécifiques lorsqu'il est procédé à une actualisation de contrôle ou de routine de la conception des amorces/sondes afin de s'adapter à l'évolution génétique dans les régions du gène ciblées par les essais moléculaires. Lorsque la présence des sous-types H5/H7 dans des échantillons cliniques testés positifs pour l'influenza A a été exclue, les investigations diagnostiques doivent être poursuivies jusqu'à l'identification du sous-type. En raison de ses conséquences économiques pour le secteur avicole et du risque zoonotique dans les zones endémiques, la détection du sous-type H9 est généralement priorisée par rapport à d'autres sous-types. Compte tenu des difficultés à maintenir des outils PCR de sous-typage spécifiques et sensibles, les protocoles basés sur la détection préalable de l'influenza A avant de tenter un sous-typage ont davantage de chances de réussir. Dans les laboratoires faiblement dotés en ressources, la détection du virus de l'influenza aviaire est parfois entravée par la nécessité de maintenir la chaîne du froid pour les réactifs humides et par la difficulté d'accéder aux réactifs adéquats. Dans ce contexte, l'utilisation de réactifs moléculaires lyophilisés conférant une meilleure stabilité thermique devrait être envisagée afin de maximiser les capacités de surveillance.
B.1.5.4. Épidémiologie moléculaire et phylogénétique	Ajout d'informations sur la conception de critères permettant de distinguer les groupes génétiques spécifiques du gène codant l'hémagglutinine (HA) H5 de la lignée GS/GD, y compris un système de nomenclature dynamique pour définir les sous-clades au sein de cette lignée. La circulation continue des virus H5N1 de l'IAHP chez les volailles ou dans l'avifaune a entraîné l'évolution en cours de l'HA H5 avec une émergence de nombreux sous-clades HA. Il est crucial d'accéder intégralement aux séquences génétiques complètes des foyers d'influenza aviaire et aux métadonnées qui leur sont associées, non seulement pour identifier les déterminants de leur transmissibilité et pathogénicité mais aussi pour reconstruire la dynamique

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	de dissémination du virus et suivre son évolution. Compte tenu de l'urgence de rendre publiques les données génomiques lorsqu'une épidémie est en cours, il est recommandé de consulter les Laboratoires de référence de l'OMSA ou de la FAO en cas de difficulté à générer et à analyser les données de séquençage génomique.
B.2.1. Épreuve immuno-enzymatique	Ajout d'une précision indiquant que lors de l'analyse d'échantillons de sérum issus d'animaux afin de déterminer leur exposition aux virus de l'influenza aviaire, un dépistage préliminaire peut être réalisé avec un kit commercial ELISA de compétition multi-espèces ciblant les anticorps spécifiques pour la nucléoprotéine (NP) des virus influenza de type A, en procédant néanmoins à une évaluation préalable de sa validité pour l'espèce considérée. Des tests sérologiques multiplex ont été mis au point qui proposent des solutions pour améliorer la largeur de bande diagnostique, mais il conviendra de poursuivre leur validation pour démontrer qu'ils sont adaptés à l'emploi dans une configuration d'essais de routine au laboratoire.
B.2.3.2. Épreuve d'inhibition de l'hémagglutination	Précision soulignant qu'en cas d'émergence de virus de l'influenza de type A d'origine aviaire chez des espèces autres qu'aviaires, il pourra être nécessaire de détecter la présence d'anticorps dirigés contre l'influenza aviaire chez des mammifères : il sera alors nécessaire de soumettre le sérum à un traitement préalable.
C. Spécifications applicables aux vaccins	Mise à jour des informations contextuelles, les deux décennies écoulées se caractérisant par un changement significatif de la dynamique épidémiologique et écologique des virus de l'IAHP et par l'émergence d'un nombre accru de lignées de l'IAHP chez les volailles.
C.1.1. Principes et utilisation prévue du produit	Changement de l'intitulé de la section en « Principes et utilisation prévue des vaccins » et mise à jour du texte et des référence, y compris la liste des conditions déterminant la réalisation d'évaluations du risque associé à la mise en œuvre de la vaccination contre l'IAHP chez les volailles.
C.2.2.4. Contrôles des lots de produit fini	Activité des lots : ajout d'une précision indiquant qu'en raison de leur aptitude à bloquer l'attachement du virus et son entrée dans les cellules hôtes des virus de l'influenza de type A, les anticorps dirigés contre la protéine HA sont considérés comme les médiateurs les plus puissants de la résistance vis-à-vis de l'infection grippale et le principal corrélat immunitaire de la protection.
C.2.3.2. Critères d'efficacité	Pour la production animale : ajout d'une précision soulignant qu'il est crucial d'utiliser les antigènes appropriés afin d'obtenir une efficacité vaccinale adéquate contre les virus de terrain et de minimiser les échecs de vaccination, quelle que soit la plateforme vaccinale utilisée.
C.3.1. Vaccins disponibles et leurs avantages	Mise à jour de cette section concernant les « Vaccins faisant appel aux biotechnologies » compte tenu des dernières évolutions des technologies vaccinales et des stratégies de vaccination mises en œuvre dans plusieurs Membres de l'OMSA.
C.4. Méthodes de surveillance pour la détection	Changement de l'intitulé de cette section en : « Détection de l'infection dans les cheptels vaccinés et chez les oiseaux vaccinés », et ajout d'un texte soulignant que les méthodes moléculaires sont les outils les plus sensibles pour surveiller les cheptels de volailles vaccinées au regard des virus de l'influenza aviaire présents sur le terrain. Ajout d'une précision signalant l'utilité de la surveillance sérologique basée sur une stratégie DIVA (différenciation des animaux infectés parmi les animaux vaccinés) chez les volailles longévives pour démontrer rétrospectivement l'absence d'infection dans une région, une zone ou un compartiment, puisqu'elle couvre une période antérieure à la vaccination. Néanmoins, l'un des problèmes des tests sérologiques DIVA est la difficulté d'interpréter les résultats dans les scénarios où des virus de l'IAFP circulent en parallèle chez les volailles.

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	Enfin, ajout d'une précision soulignant que toute mise en œuvre d'un plan de vaccination devrait aller de pair avec un système de surveillance renforcée, conçu en tenant compte du scénario épidémiologique, du contexte socio-économique et de l'objectif de la vaccination.
C.5. Évaluation et mise à jour continues des souches de semence pour l'élaboration de vaccins visant à conférer une protection contre les variants émergents des souches virales de terrain	Mention de l'initiative du réseau OFFLU sur l'adéquation des souches vaccinales pour l'influenza aviaire (OFFLU Avian Influenza Matching – AIM) pour améliorer l'information sur les caractéristiques antigéniques des virus de l'influenza aviaire en appui des programmes de vaccination, en particulier pour ce qui concerne la sélection des souches vaccinales. Des mises à jour sur la situation mondiale seront régulièrement publiées sur le site web du réseau OFFLU afin d'apporter des informations sur les caractéristiques antigéniques des virus de l'influenza aviaire en circulation, y compris les souches utilisées pour les inoculations d'épreuve, en appui des décisions relatives à la conception ou à la modification des programmes de vaccination.
Références	Actualisation de la liste de références.
Annexes 2 à 7	Ajout de six annexes donnant la justification des épreuves choisies et des notations attribuées dans le Tableau 1.

3.4.9. « Leucose bovine enzootique » : la Commission a révisé le chapitre 3.4.9, dont la mise à jour avait été réalisée par les experts du Centre collaborateur de l'OMSA.

Le chapitre 3.4.9 révisé, « Leucose bovine enzootique », est présenté à l'annexe 23 pour un premier cycle de commentaires par les Membres.

Les principaux amendements portent sur les points suivants :

Section/paragraphe	Changement
Résumé	Ajout d'une précision clarifiant que l'infection dure toute la vie et se traduit par une réponse en anticorps persistante.
A. Introduction	Création de nouvelles sections : A.1 « Description et impact de la maladie » ; A.2 « Nature et classification de l'agent pathogène » ; A.3 « Potentiel zoonotique et exigences en matière de biosécurité et de biosûreté » ; A.4 « Diagnostic différentiel » ; les informations relatives aux formes sporadiques de la leucose bovine, à la présence du virus de la leucose bovine dans les cellules mononucléées du sang périphérique (PBMC) et aux infections naturelles et expérimentales ont été déplacées vers les nouvelles sections appropriées.
Références	Actualisation de la liste de références.
Annexes 1 et 2	Ajout de deux annexes donnant la justification des épreuves choisies et des notations attribuées dans le Tableau 1.

3.4.13. « Theilériose bovine (infection à *Theileria annulata*, *T. orientalis* et *T. parva*) » : la Commission a révisé le chapitre 3.4.13, dont la mise à jour avait été réalisée par un expert du Centre collaborateur de l'OMSA.

Le chapitre 3.4.13 révisé, « Theilériose bovine (infection à *Theileria annulata*, *T. orientalis* et *T. parva*) », est présenté à l'annexe 24 pour un premier cycle de commentaires par les Membres.

Les principaux amendements portent sur les points suivants :

Section/paragraphe	Changement
Résumé	Ajout d'informations sur *Theileria orientalis*. Bien que le *Code terrestre* précise que le terme *Theileria* recouvre *T. annulata*, *T. orientalis* Ikeda, *T. orientalis* Chitose et *T. parva,* ajout d'une précision clarifiant qu'il n'existe pas encore de test sérologique pour les souches Chitose ou Ikeda de *T. orientalis*.
A. Introduction	Ajout d'une clarification concernant les trois syndromes causés par *T. parva*, qui diffèrent par l'origine du parasite ; suppression de l'information sur *T. lestoquardi* et sur la réponse immunitaire aux parasites Theileriae.
Tableau 1. Méthodes d'essai disponibles pour le diagnostic de la theilériose et emplois	Modification de la notation de la PCR de « + » en « ++ » pour l'emploi «« démonstration du statut indemne d'infection d'une population donnée » et « prévalence de l'infection – surveillance », et de « ++ » en « +++ » pour l'emploi « démontrer l'absence d'infection chez les animaux individuels à des fins de déplacement » en raison de sa sensibilité, spécificité et aptitude à détecter le parasite ; mention dans la note sous le tableau de la nécessité de faire appel en même temps à la détection de l'agent pathogène et à la sérologie pour la certification d'animaux destinés à être déplacés dans le cadre des échanges internationaux, car la parasitémie peut fluctuer en dessous des limites détectables chez les animaux porteurs alors que des anticorps sont encore détectables par la sérologie. Inversement, lors d'une infection précoce ou dans les cas où le porteur n'est pas exposé au vecteur ni à un nouveau contact avec le parasite, le titre d'anticorps peut tomber en dessous de la limite de détection, alors que l'agent lui-même reste décelable.
B.1.2. Méthodes moléculaires	Ajout d'une précision indiquant que les tests PCR en temps réel utilisant des sondes spécifiques de l'espèce ou des sondes à hydrolyse ont largement remplacé les PCR conventionnelles dans les laboratoires de diagnostic en raison de leur facilité d'utilisation sans risque de contamination ; un test PCR multiplex en temps réel a été mis au point qui détecte et différencie les souches buffeli, Ikeda, Chitose et de type 5 ; mise à jour du Tableau 2 « Amorces et sondes PCR pour la détection de *Theileria annulata* ou de *T. parva* » et ajout de *T. orientalis*.
B.2.2. Épreuves immuno-enzymatiques	L'antigène PIM fait apparaître une réactivité croisée entre *T. parva* et les espèces de *Theileria* (chez les buffles) et devrait être considéré comme un facteur de confusion dans les situations où des buffles et des bovins sont en contact.
Références	Actualisation de la liste de références.
Annexe 1	Annexe ajoutée donnant la justification des épreuves choisies et des notations attribuées dans le Tableau 1.

3.4.14. « Trichomonose » : la Commission a révisé le chapitre 3.4.14, dont la mise à jour avait été réalisée par des experts d'un Centre collaborateur de l'OMSA en consultation avec un spécialiste de cette maladie.

Le chapitre 3.4.14 révisé, « Trichomonose », est présenté à l'annexe 25 pour un premier cycle de commentaires par les Membres.

Les principaux amendements portent sur les points suivants :

Section/paragraphe	Changement
Résumé	Ajout d'une précision clarifiant que les épreuves ELISA mises au point pour cibler les IgG, les IgG1 et les IgG2 entiers dans le sérum et l'IgA dans le mucus vaginal sont le plus souvent utilisées pour confirmer le statut immunitaire d'animaux pris individuellement ou de populations (suite à une vaccination). Ajout d'informations clarifiant que l'efficacité de plusieurs vaccins expérimentaux à cellules entières et extraits de *Tritrichomonas fœtus* purifiés sur membrane démontre que la vitesse de clairance de *T. fœtus* est plus courte que chez les génisses non vaccinées, que les vaccins sous-unitaires sont moins efficaces et qu'aucun vaccin recombinant n'a été mis au point à ce jour.
A. Introduction	Création de nouvelles sections : A.1 « Description et impact de la maladie » ; A.2 « Classification taxonomique de l'agent pathogène » ; A.3 « Potentiel zoonotique et exigences en matière de biosécurité et de biosûreté » ; A.4 « Diagnostic différentiel » ; déplacement vers ces nouvelles sections des informations sur les hôtes habituels de *T. fœtus*, sur le risque permanent que représentent les taureaux plus âgés en tant que source d'infection, sur la mise au point d'une protection immunitaire éphémère chez les vaches, et sur les mécanismes de transmission.
B. Techniques de diagnostic	Ajout d'un paragraphe introductif avec des informations sur les critères en faveur d'un diagnostic provisoire à l'échelle du troupeau, sur le diagnostic de confirmation et sur les échantillons et les techniques d'échantillonnage.
Tableau 1. Méthodes d'essai disponibles pour le diagnostic de la trichomonose et emplois	Ajout d'une ligne sur l'ELISA IgG (sérum) et IgA (mucus vaginal) dans le Tableau 1.
B.1.2. Transport	Ajout de cette nouvelle section en soulignant que le choix de l'échantillonnage et des techniques diagnostiques devrait reposer sur la prise en compte de plusieurs facteurs, dont les conditions et possibilités de transport et la durée attendue de celui-ci.
B.1.3.2. Milieux de culture	Ajout des bonnes pratiques : utiliser le milieu avant la date d'expiration, vérifier la qualité de l'eau, réaliser des contrôles de la qualité sur tous les lots du milieu de culture, etc. Mise à jour des informations sur le milieu modifié de Diamond et le milieu *T. fœtus* et suppression de la section sur les caractéristiques de la croissance dans les différents milieux.
B.1.3.3.2. Cryoconservation des cultures	Ajout d'une nouvelle section sur la conservation des cultures de *T. fœtus* au moyen d'une solution de congélation.
B.1.3.4. Détection et identification de *Tritrichomonas fœtus* au microscope	Ajout d'un texte mettant en garde contre le fait que l'examen de routine au microscope peut donner des résultats faux positifs car un certain nombre de microorganismes similaires à *T. fœtus* et infestant également les bovins sont difficiles à distinguer de *T. fœtus* : il est donc recommandé d'utiliser, dans la mesure du possible, une méthode de détection moléculaire parallèlement à la mise en culture.
B.1.4.3. PCR en temps réel	Mise à jour extensive de cette section car il s'agit de la méthode recommandée, et ajout de tableaux sur les séquences d'amorce/sonde et sur les paramètres des cycles.
B.1.4.4. Amplification isotherme à médiation par boucle (LAMP)	Mise à jour de cette section afin de mentionner deux essais LAMP, en précisant que l'un d'eux n'a pas encore été testé sur le terrain et que le deuxième n'a pas encore été testé avec des échantillons positifs provenant de bovins infectés naturellement.
C. Spécifications applicables aux vaccins	Ajout d'une nouvelle section sur les spécifications applicables aux vaccins.

Section/paragraphe	Changement
Références	Actualisation de la liste de références.
Annexes 1 à 5	Ajout de cinq annexes donnant la justification des épreuves choisies et des notations attribuées dans le Tableau 1.

3.6.1. « Peste équine (infection par le virus de la peste équine) » : la Commission a révisé le chapitre 3.6.1, dont la mise à jour avait été réalisée par les experts du Centre collaborateur de l'OMSA.

Le chapitre 3.6.1 révisé, « Peste équine (infection par le virus de la peste équine) », est présenté à l'annexe 26 pour un premier cycle de commentaires par les Membres.

Les principaux amendements portent sur les points suivants :

Section/paragraphe	Changement
Résumé	Détection et identification de l'agent : ajout d'une RT-PCR, en tant que test de diagnostic rapide et présentant une sensibilité élevée. Les isolats viraux peuvent être soumis à un sérotypage par neutralisation virale ou RT-PCR spécifiques de type, ou encore par séquençage. Les méthodes ELISA ne sont pas recommandées en raison de leur sensibilité limitée. Spécifications relatives aux vaccins : ajout d'une précision indiquant que les vaccins utilisés par le passé étaient à virus inactivé et que des vaccins recombinants sont en cours d'évaluation.
A.1. Description et impact de la maladie	Création de cette nouvelle sous-section ; suppression de la description de l'agent étiologique et de ses caractéristiques moléculaires, ainsi que de la section sur le diagnostic différentiel et du paragraphe sur un agent non zoonotique, car ces informations n'ont pas leur place dans cette section.
A.2. Nature et classification de l'agent pathogène	Création de cette nouvelle sous-section qui comporte une description du virus.
A.3. Potentiel zoonotique et exigences en matière de biosécurité et de biosûreté	Création de cette nouvelle sous-section décrivant en détail les mesures de biosécurité applicables à la manipulation d'échantillons contaminés, spécifiques aux maladies potentiellement zoonotiques.
A.4. Diagnostic différentiel	Création de cette nouvelle sous-section détaillant plusieurs méthodes de diagnostic différentiel.
Tableau 1. Méthodes d'essai disponibles pour le diagnostic de la peste équine et emplois	Relèvement de la notation de la RT-PCR en temps réel de « + » à « +++ » pour l'emploi « contribution aux politiques d'éradication » ; relèvement de la notation de la RT-PCR sur gel d'agarose de « – » à « + » pour l'emploi « démontrer l'absence d'infection dans une population donnée », et de « + » à « ++ » pour les emplois « démontrer l'absence d'infection chez les animaux individuels à des fins de déplacement » et « contribution aux politiques d'éradication » ; relèvement de la notation de l'ELISA de « ++ » à « +++ » pour l'emploi « démontrer l'absence d'infection chez les animaux individuels à des fins de déplacement » et abaissement de sa notation de « ++ » à « + » pour l'emploi « confirmation des cas cliniques » ; ajout d'une note au bas du tableau concernant l'emploi « démontrer l'absence d'infection chez les animaux individuels à des fins de déplacement », précisant que les tests sérologiques requièrent l'analyse de deux échantillons comme indiqué à l'article 12.1.7 « Recommandations relatives aux importations en provenance de pays ou de zones infectés par la peste équine » du chapitre 12.1 « Infection par le virus de la peste équine » du *Code terrestre*.

Section/paragraphe	Changement
B. Techniques de diagnostic	Actualisation des réactifs de référence internationaux pour les panels de virus et d'anticorps, ajout de brèves informations sur les essais d'aptitude interlaboratoires ainsi que sur l'importance du choix des échantillons.
B.1.1. Culture cellulaire	Ajout d'informations sur l'interprétation de l'effet cytopathogène et l'importance d'une confirmation rigoureuse au moyen d'une RT-PCR en temps réel.
B.1.2.1. Amplification en chaîne par polymérase couplée à une transcription inverse	Suppression des références obsolètes.
B.1.2.3. Procédure pour une RT-PCR en temps réel (Agüero *et al.*, 2008)	Mise à jour du protocole et suppression de la section sur les caractéristiques diagnostiques.
B.1.2.4. Procédure pour une RT-PCR en temps réel (Guthrie *et al.*, 2013)	Suppression de la section sur les caractéristiques diagnostiques.
Tableau 2. Comparaison des méthodes RT-PCR en temps réel d'Agüero *et al.* (2008) et de Guthrie *et al.* (2013)	Suppression des lignes sur la sensibilité analytique (limite de détection), la spécificité diagnostique et la sensibilité diagnostique.
B.1.3. Typage du virus de la peste équine	Ajout des essais RT-PCR qui sont une méthode diagnostique plus rapide.
B.2. Épreuves sérologiques	Suppression des références datant de plus de vingt ans.
B.2.1.1. Protocole d'essai	Description d'un exemple d'ELISA bloquante.
C.1.1. Principes et utilisation prévue du produit	Ajout d'une précision étayée par une référence, indiquant que la détection du génome viral de la peste équine a été rapportée chez des animaux vaccinés avec des vaccins atténués, jusqu'à 16 semaines après la vaccination ; ajout d'un exemple de vaccins expérimentaux de nouvelle génération.
Références	Actualisation de la liste de références.
Annexes 1 à 6	Ajout de six annexes donnant la justification des épreuves choisies et des notations attribuées dans le Tableau 1.

3.6.5. « Anémie infectieuse des équidés » : la Commission a révisé le chapitre 3.6.5, dont la mise à jour avait été réalisée par les experts du Centre collaborateur de l'OMSA.

Le chapitre 3.6.5 révisé, « Anémie infectieuse des équidés», est présenté à l'annexe 27 pour un premier cycle de commentaires par les Membres.

Les principaux amendements portent sur les points suivants :

Section/paragraphe	Changement
Résumé	Identification de l'agent : révision des critères confirmant une infection en mettant l'accent sur les tests sérologiques et moléculaires et en soulignant l'impossibilité de distinguer le virus de l'anémie infectieuse des équidés d'un certain nombre d'autres agents étiologiques : le diagnostic repose donc exclusivement sur le laboratoire et passe par la mise en évidence d'une

Section/paragraphe	Changement
	réponse spécifique en anticorps, l'isolement viral ou la détection de l'acide nucléique viral.
A.1. Description de la maladie et étiologie	Ajout d'informations concernant l'impact économique et la biodiversité, les signes cliniques et les voies de transmission.
B. Techniques de diagnostic	Ajout d'informations sur les méthodes de diagnostic du virus de l'anémie infectieuse des équidés, en particulier : l'utilisation d'antigènes recombinants afin de prévenir les réactions non spécifiques à l'épreuve d'immunodiffusion en gélose, une méthode ELISA de compétition présentant une spécificité et sensibilité élevées, et un test d'immunochromatographie à l'or colloïdal sur bandes en tant que test rapide sur le lieu d'intervention pour le dépistage primaire des anticorps dirigés contre le virus de l'anémie infectieuse des équidés.
Tableau 1. Méthodes d'essai disponibles pour le diagnostic de l'anémie infectieuse des équidés et emplois	Relèvement de la notation de l'ELISA pour les cinq emplois ; suppression de l'immunoblot qui n'est plus disponible sous forme commerciale.
B.1.1. Isolement viral et identification du virus	Ajout d'informations détaillées sur les échantillons à utiliser pour l'isolement du virus.
B.1.12. Amplification en chaîne par polymérase	Ajout d'informations sur les méthodes PCR en temps réel en précisant qu'il convient d'utiliser les méthodes PCR de détection de l'ARN viral et l'ADN proviral pour accroître la sensibilité du diagnostic.
B.2. Épreuves sérologiques	Ajout d'informations sur l'efficacité d'un algorithme de diagnostic.
B.2.1.1. Préparation de l'antigène	Suppression des informations détaillées sur la méthode d'extraction de l'antigène.
B.2.3. Immunoblot	Ajout d'informations sur les caractéristiques diagnostiques du test, notamment une sensibilité et spécificité élevées, en précisant que ce test n'est pas disponible dans le commerce, d'où sa suppression du Tableau 1, bien qu'il puisse être obtenu auprès du Laboratoire de référence de l'OMSA en Italie.
Test d'immunochromatographie à l'or colloïdal	Ajout d'une nouvelle technique diagnostique de détection des anticorps dirigés contre le virus de l'anémie infectieuse des équidés.
C. Spécifications applicables aux vaccins	Ajout d'une précision clarifiant que les tests de diagnostic ne peuvent pas distinguer les anticorps issus d'animaux vaccinés de ceux issus d'animaux infectés.
Références	Actualisation de la liste de références.
Annexes 1 à 5	Ajout de cinq annexes donnant la justification des épreuves choisies et des notations attribuées dans le Tableau 1.

3.8.2. « Arthrite/encéphalite caprine et maedi-visna » : la Commission a révisé le chapitre 3.8.2, dont la mise à jour avait été réalisée par un expert de cette maladie.

Le chapitre 3.8.2 révisé, « Arthrite/encéphalite caprine et maedi-visna », est présenté à l'annexe 28 pour un premier cycle de commentaires par les Membres

Les principaux amendements portent sur les points suivants :

Section/paragraphe	Changement
Titre	Titre changé en « Lentivirus des petits ruminants (arthrite/encéphalite caprine et maedi-visna) »
A. Introduction	Ajout d'une clarification concernant le fait que si les virus du maedi-visna et de l'arthrite/encéphalite caprine sont classés comme deux espèces virales distinctes, un consensus se dessine pour les considérer comme deux génotypes différents au sein d'un groupe plus large de virus.
Tableau 1. Méthodes d'essai disponibles pour le diagnostic de l'arthrite/encéphalite caprine et du maedi-visna et emplois	Suppression de l'épreuve de fixation du complément (FC), de la neutralisation virale (NV) et de test d'immunofluorescence indirecte pour la détection d'anticorps (IFAT) du Tableau ; relèvement de la notation de la PCR de « ++ » à « +++ » et de l'ELISA de « + » à « ++ » pour l'emploi « confirmation des cas cliniques » ; abaissement de la notation de l'AGID de « +++ » à « ++ » pour les emplois « démontrer l'absence d'infection chez les animaux individuels à des fins de déplacement » et « confirmation des cas cliniques » ; notation de tous les tests abaissée à « – » pour l'emploi « déterminer le statut immunitaire d'animaux pris individuellement ou de populations (après vaccination) », car il n'y a pas de vaccins ; ajout d'une note au-dessous du tableau concernant l'AGID et l'ELISA pour les emplois « démontrer l'absence d'infection chez les animaux individuels à des fins de déplacement » et « contribution aux politiques d'éradication », précisant que les échantillons ne devraient pas être prélevés chez des animaux âgés de moins de 6 mois pour éviter toute interférence avec les anticorps maternels, en particulier si ces animaux ont reçu du colostrum traité à la chaleur à des fins d'éradication.
B.1.2.1. Virus de l'arthrite/encéphalite caprine et virus du maedi-visna	Mise à jour exhaustive de la méthode de collecte d'échantillons.
B.1.3. Méthodes de détection de l'acide nucléique	Ajout de deux tableaux d'amorces pour les PCR en temps réel et conventionnelles.
Références	Actualisation de la liste de références.
Annexes 1 à 5	Ajout de cinq annexes donnant la justification des épreuves choisies et des notations attribuées dans le Tableau 1.

3.8.5. « Avortement enzootique des brebis (chlamydiose ovine) (infection à *Chlamydia abortus*) » : la Commission a révisé le chapitre 3.8.5, dont la mise à jour avait été réalisée par les experts du Centre collaborateur de l'OMSA.

Le chapitre 3.8.5 révisé, « Avortement enzootique des brebis (chlamydiose ovine) (infection à *Chlamydia abortus*)», est présenté à l'annexe 29 pour un premier cycle de commentaires par les Membres

Les principaux amendements portent sur les points suivants :

Section/paragraphe	Changement
A.1. Description et impact de la maladie	Clarification indiquant que les brebis excrètent de grandes quantités de *C. abortus* lors d'avortement ou d'agnelage, ce qui constitue une source d'infection ; et que les brebis n'avortent généralement qu'une fois dans leur vie à cause de cette infection.
A.2. Nature et classification de l'agent pathogène	Remplacement des informations taxonomiques par une description des caractéristiques de la famille des Chlamydiaceae.

Section/paragraphe	Changement
B.2. Épreuves sérologiques	Précision qu'aucune des épreuves sérologiques disponibles ne peut différencier les titres résultant d'une vaccination de ceux résultant d'une infection naturelle (tests DIVA).
B.2.1. ELISA	Ajout d'informations sur les ELISA utilisant des protéines recombinantes spécifiques de *C. abortus*, qui peuvent différencier les animaux infectés avec *C. pecorum*.
Références	Actualisation de la liste de références.
Annexes 1 à 6	Ajout de six annexes donnant la justification des épreuves choisies et des notations attribuées dans le Tableau 1.

3.8.11. « Clavelée et variole caprine » (uniquement la section sur les vaccins) : la Commission a révisé le chapitre 3.8.11, dont la mise à jour avait été réalisée par les experts du Centre collaborateur de l'OMSA en collaboration avec des spécialistes de la maladie et des vaccins.

Le chapitre 3.8.11 révisé, « Clavelée et variole caprine » (section sur les vaccins uniquement), est présenté à l'annexe 30 pour un premier cycle de commentaires par les Membres.

Les principaux amendements portent sur les points suivants :

Mise à jour exhaustive de la section : C. Spécifications applicables aux vaccins
La Section C, « Spécifications applicables aux vaccins », a fait l'objet d'une mise à jour exhaustive réalisée par un Laboratoire de référence de l'OMSA en collaboration avec des concepteurs de vaccins, des experts et des représentants de la communauté scientifique, pour décrire la fabrication de vaccins à la fois purs, puissants, sûrs et efficaces contre la clavelée et la variole caprine, y compris les principaux critères de performance et de qualité attendus de ces vaccins ; en conséquence, la section bibliographique a également été remaniée.

3.9.2. « Peste porcine classique (infection par le virus de la peste porcine classique) » : la Commission a révisé le chapitre 3.9.2, dont la mise à jour avait été réalisée par des experts du Centre collaborateur de l'OMSA.

Le chapitre 3.9.2 révisé, « Peste porcine classique (infection par le virus de la peste porcine classique) », est présenté à l'annexe 31 pour un premier cycle de commentaires par les Membres.

Les principaux amendements portent sur les points suivants :

Section/paragraphe	Changement
C.1. Contexte	Mention soulignant l'importance de disposer de vaccins sûrs et efficaces, qui restent un outil majeur de la lutte et des premières phases d'éradication de la peste porcine classique. Informations sur les vaccins candidats innovants.
C.2. Exposé des normes de fabrication et des normes minimales applicables aux vaccins à virus vivant	Intitulé de la section changé en : « Exposé des exigences de production et des exigences minimales pour les vaccins à virus vivant et les vaccins marqués à virus vivant modifié » et ajout d'un texte introductif qui précise que le chapitre décrit les exigences minimales pour les vaccins vivants, aussi bien les vaccins vivants classiques que les vaccins marqués à virus vivant modifié.
C.2.1.1. Caractéristiques biologiques de la semence virale	Ajout d'informations relatives aux vaccins vivants marqués.

Section/paragraphe	Changement
C.2.2.4. Activité des lots/séries de produit fini	Il est nécessaire de contrôler l'activité des lots/séries de produit fini : le titre approprié pour la libération des lots doit être corrélé avec les résultats des études d'efficacité lors d'expositions d'épreuve, et être suffisamment élevé pour garantir l'efficience du vaccin pendant toute sa durée de conservation.
C.2.3.2 Critères d'innocuité	Clarification concernant la nécessaire conformité des épreuves de réversion vers la virulence avec la ligne directrice GL41 du VICH (Examen des vaccins vétérinaires vivants chez les animaux cibles pour contrôler l'absence de réversion vers la virulence, 2008) ; ajout d'une section sur les exigences particulières applicables aux vaccins marqués vivants.
C.4. Autres vaccins issus des biotechnologies	Ajout d'une nouvelle section.
Références	Actualisation de la liste de références.
Annexes 1 à 5	Ajout de cinq annexes donnant la justification des épreuves choisies et des notations attribuées dans le Tableau 1.

3.9.7. « Maladie vésiculeuse du porc » : la Commission a révisé le chapitre 3.9.7, dont la mise à jour avait été réalisée par des experts du Centre collaborateur de l'OMSA.

Le chapitre 3.9.7 révisé, « Maladie vésiculeuse du porc », est présenté à l'annexe 32 pour un premier cycle de commentaires par les Membres.

Les principaux amendements portent sur les points suivants :

Section/paragraphe	Changement
Résumé	Amendement des critères déterminant un diagnostic positif pour inclure des résultats positifs à la fois au RT-PCR et à l'isolement viral.
A. A. Introduction	Ajout d'informations sur la situation épidémiologique de la maladie.
B.1. Détection et identification de l'agent pathogène	Clarification concernant le fait que l'effet cytopathogène observé dans les cultures inoculées doit être confirmé par ELISA ou RT-PCR pour conclure à la présence du virus de la maladie vésiculeuse du porc.
B1.2. Isolement viral	Mise à jour des informations sur les procédures d'isolement viral.
B.1.4.1. Amplification en chaîne par polymérase couplée à une transcription inverse	Précision indiquant que pour une performance diagnostique optimale, il convient d'intégrer à la RT-PCR en une étape, un format en temps réel avec un colorant fluorescent se liant à l'ADN.
B.1.4.2.1. RT-PCR en une étape	Modification de l'intitulé de cette section en « RT-PCR en temps réel en une étape (avec un colorant fluorescent se liant à l'ADN double brin) » et actualisation du protocole d'essai.
Références	Actualisation de la liste de références.
Annexes 1 à 3	Ajout de deux annexes donnant la justification des épreuves choisies et des notations attribuées dans le Tableau 1.

5.3. Réponse de la Commission aux commentaires formulés par les Membres lors du deuxième cycle de consultations

3.9.1. « Peste porcine africaine (infection par le virus de la peste porcine africaine) » (partie sur les vaccins)

Généralités

Lors de sa réunion de septembre 2023, la Commission a été informée que la partie C, « Spécifications applicables aux vaccins » du chapitre sur la PPA[3], avait fait l'objet d'une mise à jour exhaustive réalisée par un consultant avec la collaboration de concepteurs de vaccins, d'experts et de représentants de la communauté scientifique, des autorités réglementaires et des Laboratoires de référence de l'OMSA, afin de décrire la fabrication de vaccins à la fois purs, puissants, sûrs et efficaces contre la PPA, y compris les principaux critères de performance et de qualité attendus de ces vaccins. Le nouveau projet de section avait été présenté à l'annexe 16 du rapport de cette réunion de septembre pour un premier cycle de consultations. Un document avait également été annexé au projet de chapitre pour information, contenant les résultats de la consultation, les principaux paramètres examinés, les résumés des discussions, etc.

Lors de sa réunion de février 2024, la Commission a noté que ce nouveau projet de section sur les vaccins avait fait l'objet d'un grand nombre de commentaires. Compte tenu du fait que les vaccins à virus vivant modifié sont utilisés dans certains Membres, la Commission a estimé important d'en fournir une norme minimale dans le *Manuel terrestre* de l'OMSA, tout en s'engageant à la réexaminer régulièrement à la lumière des données scientifiques nouvelles qui pourraient être publiées.

Le nouveau projet de section sur les vaccins a été distribué deux fois afin de recueillir les commentaires des membres.

Rapports précédents de la Commission faisant état des discussions sur ce texte

Rapport de septembre 2023 (point 5.1, page 10) ; Rapport de février 2024 (point 5.2, pages 20–28)

Réunion de février 2024

La nouvelle section proposée sur les vaccins avait déjà fait l'objet de deux cycles de commentaires par les Membres mais son adoption prévue en mai 2024 a été reportée en raison du grand nombre de commentaires reçus lors du deuxième cycle. Conformément à la nouvelle politique de transparence de l'OMSA, la Commission a traité l'ensemble de ces commentaires, qui sont présentés à l'annexe 4 du présent rapport, avec les réponses de la Commission.
La section sur les vaccins du chapitre 3.9.1, « Peste porcine africaine (infection par le virus de la peste porcine africaine) » est présentée à l'annexe 33 pour un troisième cycle de commentaires par les Membres.

Chapitres présentés aux Membres dans ce rapport afin de recueillir leurs commentaires, et leurs numéros d'annexe respectifs

	Annexe	N° de Chapitre	Titre du chapitre
1.	5	1.1.3.	Transport de matériels biologiques
2.	6	1.1.7.	Normes pour le séquençage à haut débit, la bioinformatique et la génomique computationnelle
3.	7	1.1.9	Contrôle de la stérilité et de l'absence de contamination des matériels biologiques à usage vétérinaire
4.	8	2.1.1.	Méthodes de laboratoire utilisées pour les essais d'antibiorésistance
5.	9	2.2.1	Mise au point et optimisation des méthodes de détection d'anticorps
6.	10	2.2.2	Mise au point et optimisation des méthodes de détection des antigènes

[3] PPA : peste porcine africaine

	Annexe	N° de Chapitre	Titre du chapitre
7.	11	2.2.3	Mise au point et optimisation des méthodes de détection de l'acide nucléique
8.	12	2.2.5	Méthodes statistiques de validation
9.	13	2.2.8.	Comparabilité des épreuves suite à des changements introduits dans une méthode d'essai validée
10.	14	3.1.2.	Maladie d'Aujeszky (infection par le virus de la maladie d'Aujeszky)
11.	15	3.1.8.	Fièvre aphteuse (infection par le virus de la fièvre aphteuse)
12.	16	3.1.9.	Cowdriose
13.	17	3.1.21.	Peste bovine (infection par le virus de la peste bovine)
14.	18	3.2.4.	Infestation des abeilles mellifères par le petit coléoptère des ruches (*Aethina tumida*)
15.	19	3.2.5.	Infestation des abeilles mellifères par *Tropilaelaps* spp.
16.	20	3.3.1.	Chlamydiose aviaire
17.	21	3.3.2.	Bronchite infectieuse aviaire
18.	22	3.3.4.	Influenza aviaire (y compris l'infection par les virus de l'influenza aviaire hautement pathogènes)
19.	23	3.4.9.	Leucose bovine enzootique
20.	24	3.4.13.	Theilériose bovine (infection à *Theileria annulata*, *T. orientalis* et *T. parva*)
21.	25	3.4.14.	Trichomonose
22.	26	3.6.1.	Peste équine (infection par le virus de la peste équine)
23.	27	3.6.5.	Anémie infectieuse des équidés
24.	28	3.8.2.	Arthrite/encéphalite caprine et maedi-visna
25.	29	3.8.5.	Avortement enzootique des brebis (chlamydiose ovine) (infection à *Chlamydia abortus*)
26.	30	3.8.11.	Clavelée et variole caprine (partie sur les vaccins uniquement)
27.	31	3.9.2.	Peste porcine classique (infection par le virus de la peste porcine classique)
28.	32	3.9.7.	Maladie vésiculeuse du porc
29.	33	3.9.1.	Peste porcine africaine (partie sur les vaccins uniquement)

5.4. Rapport sur une étude interlaboratoires visant à approfondir la validation des tests sérologiques pour le diagnostic de la morve

Le Laboratoire de référence de l'OMSA pour la morve en Allemagne a pris part aux activités organisées au sein d'un réseau international dans le but d'améliorer le diagnostic sérologique de la morve. En 2011, un western blot a été introduit en tant que test de confirmation pour les épreuves de fixation du complément positives ; il bénéficie désormais d'une large reconnaissance internationale bien qu'il s'agisse d'un test complexe et sophistiqué. Des études internationales financées par l'OMSA ont été conduites par la suite pour comparer des méthodes alternatives.

En 2019, une étude a montré que les caractéristiques de performances d'une épreuve ELISA utilisant des protéines recombinantes étaient comparables, voire meilleures, à celles de l'épreuve de fixation du complément (FC) en termes de sensibilité et de spécificité. En 2021, une épreuve ELISA commerciale à double antigène a été validée, ayant fait preuve d'une sensibilité et d'une spécificité supérieures à celles de la FC. Par la rapidité et la simplicité de son protocole, cette épreuve convient pour des analyses à grande échelle au laboratoire de diagnostic et son utilisation a été autorisée en Allemagne en 2020.

En octobre 2021, le chapitre sur la morve mis à jour et mentionnant l'ELISA à double antigène a été distribué aux Membres pour un premier cycle de consultations. Les Membres ont relevé deux sujets de préoccupation : premièrement, l'absence de données de validation concernant les performances de l'ELISA commerciale recombinante à double antigène pour l'analyse de prélèvements de mulets et d'ânes ; deuxièmement, l'absence de sérums de référence positifs disponibles pour réaliser correctement le test et conduire une assurance de la qualité externe. Par conséquent le chapitre a été mis en attente et il a été demandé au Laboratoire de référence d'approfondir

la validation du test en utilisant un éventail plus large d'échantillons couvrant davantage d'espèces d'origines géographiques diverses.

Depuis, le Laboratoire de référence de l'OMSA a organisé une étude comparative interlaboratoires à l'échelle internationale portant sur les deux essais : ELISA et FC. L'étude a confirmé l'aptitude à l'emploi de l'ELISA à double antigène en tant que test fiable pour l'identification des animaux infectés par la morve et pour démontrer l'absence de maladie à des fins de déplacement d'animaux. Néanmoins, l'épreuve ne peut être considérée comme validée que pour une utilisation chez les chevaux.

La Commission a décidé d'amender les notations attribuées à l'ELISA à double antigène dans le Tableau 1, *Méthodes d'essai disponible pour le diagnostic de la morve et emplois* du chapitre 3.6.10 du *Manuel terrestre*, « Morve et mélioïdose », avec une note restreignant son utilisation aux chevaux, et d'ajouter de nouvelles annexes donnant la justification des épreuves choisies et des notations attribuées dans le Tableau 1. Le chapitre fera encore l'objet d'une révision approfondie par les trois Laboratoires de référence puis sera évalué par la Commission lors de la réunion suivantes de février afin d'approuver sa distribution aux Membres pour un second cycle de consultations.

5.5. Demande d'orientations concernant la taxonomie des agents pathogènes et l'utilisation de leurs noms communs

La Commission a examiné une demande d'orientations concernant l'intégration de la nouvelle taxonomie dans le *Manuel terrestre* afin de se conformer à la nomenclature des virus adoptée par l'ICTV. Par exemple, lors de l'adoption de ces noms binomiaux, convient-il de conserver le nom commun, d'usage plus fréquent, à côté du terme scientifique tel qu'actualisé ? La discussion a mis en avant la nécessité d'une utilisation cohérente des dernières classifications taxonomiques, tout en reconnaissant que les noms communs restent largement utilisés chez les scientifiques et les responsables de la réglementation.

La Commission a décidé de s'en tenir à la pratique actuelle, à savoir utiliser la nouvelle taxonomie binomiale tout en gardant les noms communs présentés entre parenthèses pour plus de clarté et accessibilité pour tous les utilisateurs. Ce système a été appliqué au chapitre sur la diarrhée virale bovine adopté en mai 2024, qui classe les virus de la diarrhée virale bovine comme suit : *Pestivirus bovis* (communément appelé BVDV de type 1), *Pestivirus tauri* (BVDV de type 2), et *Pestivirus brazilense* (BVDV de type 3 ou pestivirus de type Hobi).

5.6. Chapitres dédiés à des maladies non listées : les test décrits dans ces chapitres sont-ils toujours considérés comme validés selon les normes de l'OMSA ?

Lors de la réunion de février 2024, la Commission a défini et appliqué les critères du maintien dans le *Manuel terrestre* des chapitres dédiés à des maladies non listées. Suite à la suppression de certains de ces chapitres, un laboratoire qui avait obtenu l'accréditation pour une méthode de détection considérée comme méthode de référence selon la norme de l'OMSA a demandé quel serait désormais le statut de ce test au regard de la validation puisque le chapitre ne figurait plus dans le *Manuel terrestre*. La Commission a indiqué que le laboratoire pourra se référer aux références publiées décrivant les études de validation de cette méthode plutôt qu'au chapitre du *Manuel terrestre*.

Dans ce même ordre d'idées, les versions anciennes du *Manuel terrestre* seront prochainement accessibles pour consultation, sachant que la version actuelle reste la version de référence et contient les informations les plus récentes.

Les éditions antérieures du *Manuel terrestre* peuvent être consultées sur le portail documentaire de l'OMSA (https://doc.woah.org/dyn/portal/listalo.xhtml?dos=71&page=listalo&req=121&menu=) ; la dernière édition imprimée a été publiée en 2018. Les versions antérieures des chapitres adoptés depuis cette date peuvent être obtenues sur demande auprès du Secrétariat de la Commission, à l'adresse : bsc.secretariat@woah.org.

5.7. Statut du *Manuel terrestre* : le point sur les chapitres sélectionnés pour le cycle d'examen 2025/2026

La Commission a encouragé les Laboratoires de référence auxquels sont confiés des chapitres importants à remettre leur texte dans les délais prévus. Les mises à jour programmées pour le cycle d'examen 2025/2026 portent sur les chapitres ci-après (l'année de la dernière adoption est indiquée entre parenthèses) :

1.1.2. Prélèvement, expédition et stockage des échantillons pour le diagnostic (2013)
1.1.4. Biosécurité et biosûreté : norme sur la gestion du risque biologique dans les laboratoires vétérinaires et dans les animaleries (2015)
2.1.2. Progrès de la biotechnologie dans le diagnostic des maladies infectieuses (2021)
2.1.3. Gestion du risque biologique : exemples de stratégies de gestion du risque proportionnelles au risque biologique évalué (2014)

2.2.7 Validation des épreuves diagnostiques pour les maladies infectieuses applicables à la faune sauvage (2014)
2.3.1. Application de la biotechnologie au développement des vaccins à usage vétérinaire (2010)
2.3.3. Exigences minimales pour l'organisation et la gestion d'une installation de production de vaccins (2016)
2.3.5. Exigences minimales pour la production des vaccins en conditions d'asepsie (2016)
3.1.7. Maladie hémorragique épizootique (infection par le virus de la maladie hémorragique épizootique) (2021)
3.1.10. Encéphalite japonaise (2021)
3.1.11. Leishmaniose (2021)
3.1.14. Nagana : infections par des trypanosomoses salivaires (hors *Trypanosoma evansi* et *T. equiperdum*) (2021)
3.1.15. Myiase à *Cochliomyia hominivorax* et myiase à *Chrysomya bezziana* (2019)
3.1.17. Paratuberculose (maladie de Johne) (2021)
3.1.18. Fièvre Q (2018)
3.1.22. Surra chez toutes les espèces (infection à *Trypanosoma evansi*) (2021)
3.1.26. Fièvre de West Nile (2018)
3.3.3. Laryngotrachéite infectieuse aviaire (2021)
3.3.5. Mycoplasmose aviaire (*Mycoplasma gallisepticum*, *M. synoviae*) (2021)
3.3.6. Hépatite virale du canard (2017)
3.3.7. Typhose et pullorose (2018)
3.3.8. Bursite infectieuse (maladie de Gumboro) (2016)
3.3.10. Maladie de Newcastle (infection par le virus de la maladie de Newcastle) (2021)
3.4.2. Babésiose bovine (2021)
3.4.4. Campylobactériose génitale bovine (2021)
3.4.5. Encéphalopathie spongiforme bovine (2021)
3.4.8. Péripneumonie contagieuse bovine (infection à *Mycoplasma mycoides* subsp. *mycoides*) (2021)
3.4.10. Septicémie hémorragique (*Pasteurella multocida* sérotypes 6:b et 6:e) (2021)
3.4.11. Rhinotrachéite infectieuse bovine/vulvovaginite pustuleuse infectieuse (2017)
3.5.1. Variole des camélidés (2021)
3.5.2. Syndrome respiratoire du Moyen-Orient (infection des chameaux/dromadaires par le coronavirus du syndrome respiratoire du Moyen-Orient) (2021)
3.6.6. Grippe équine (infection par le virus de la grippe équine) (2019)
3.6.7. Piroplasmoses équines (2021)
3.6.9. Artérite virale équine (infection par le virus de l'artérite équine) (2013)
3.6.10. Morve et mélioïdose (2018)
3.8.3. Agalaxie contagieuse (2018)
3.8.4. Pleuropneumonie contagieuse caprine (2021)
3.8.7. Épididymite contagieuse ovine (*Brucella ovis*) (2015)
3.8.8. Peste des petits ruminants (infection par le morbillivirus des petits ruminants) (2021)
3.8.10. Tremblante (2022)
3.9.8. Gastro-entérite transmissible (2008)
3.10.2. Cysticercoses (y compris l'infection par *Taenia solium*) (2021)
3.10.4. *Escherichia coli* vérocytotoxinogène (2008)

5.8. Actualisation sur le projet d'outil de navigation en ligne dédié aux normes de l'OMSA

L'outil de navigation en ligne dédié aux normes de l'OMSA est une plateforme conçue pour simplifier l'accès aux normes de l'OMSA. Il s'inscrit dans la stratégie de numérisation de l'OMSA, qui vise à améliorer l'efficience et soutenir une prise de décisions éclairée.

Le projet prévoit la mise en service de trois interfaces essentielles. La première est le site web public de l'OMSA, qui offre une voie d'accès aux normes. La deuxième est l'outil dédié aux Recommandations pour un commerce international sûr, présentées par marchandise, qui permet aux utilisateurs de visualiser les recommandations applicables aux échanges internationaux en actionnant un système de filtres. Cette fonction s'applique actuellement au *Code terrestre* et pourrait s'étendre au *Code aquatique*. La troisième interface est l'outil de navigation et de recherche, qui permet une navigation intuitive dans les *Codes* et *Manuels* terrestres et aquatiques de l'OMSA.

À des fins d'usage interne, une plateforme est en cours de développement pour rendre plus efficaces les activités de gestion et de mise à jour des normes par le personnel de l'OMSA. Le développement des interfaces publiques est désormais achevé ; elles ont été présentées lors de la Session générale de 2024 avec un retour positif. La numérisation et vérification des normes ont été effectuées et les mises à jour sont en cours. Le développement de l'interface à usage interne est dans sa phase finale et l'outil devrait être pleinement opérationnel au deuxième semestre 2024. Une démonstration de l'outil a eu lieu pendant la réunion en mettant en avant ses fonctionnalités et son potentiel pour l'avenir.

6. Centres de référence de l'OMSA

6.1. Examen des candidatures au statut de Centre de référence de l'OMSA

La Commission a recommandé d'accepter les nouvelles candidatures suivantes au statut de Centre de référence de l'OMSA :

Laboratoire de référence de l'OMSA *pour la piroplasmose équine*
ICAR-National Research Centre on Equines (ICAR-NRCE),
(Indian Council of Agricultural Research)
Sirsa Road, Hisar 125 001, Haryana, INDE
Tél. : (+91) 1662-282503 / (+91) 1662-276217
Courriel : Sanjay.nrce@gmail.com; kumar.sanjay@icar.gov.in
Site web : www.nrce.gov.in
Expert désigné : Dr Sanjay Kumar

Centre collaborateur de l'OMSA *pour le commerce d'espèces sauvages et la santé des animaux sauvages*
Centre for Wildlife Forensics, National Parks Board, Animal & Plant Health Centre
6 Perahu Road, SINGAPOUR 718827
Tél. : (+65) 6471-7808
Courriel : CITES@nparks.gov.sg (General Inquiry) / Anna_wong@nparks.gov.sg
Site web : https://www.nparks.gov.sg/CentreforWildlifeForensics
Personne de contact : Dre Anna Wong

Centre collaborateur de l'OMSA *pour la surveillance et l'épidémiologie des maladies de la faune sauvage*
Thailand National Wildlife Health Center (Thailand-NWHC) et Monitoring and Surveillance Center for Zoonotic Diseases in Wildlife and Exotic Animals (MoZWE), Faculty of Veterinary
Science, Mahidol University
999 Phuttamonthon 4 Rd., Salaya, Phuttamonthon, Nakhon Pathom, 73170, THAÏLANDE
Tél. : (+66.2) 441-5242-4
Courriel : witthawat.wir@mahidol.ac.th
Site web : https://vs.mahidol.ac.th/thailand-nwhc/en; https://vs.mahidol.ac.th/mozwe/en/
Personne de contact : Dr Witthawat Wiriyarat

Deux candidatures ont été présentées par des pays de la région Asie-Pacifique pour la désignations de Laboratoires de référence pour la peste porcine africaine (PPA). Bien que le premier demandeur ait démontré qu'il menait des activités de recherche d'un niveau élevé, la Commission a estimé que ses capacités diagnostiques étaient insuffisantes et qu'il manquait dans le dossier des éléments probants sur les activités internationales de l'institution candidate, notamment l'organisation ou participation à des essais d'aptitude interlaboratoires. La Commission a toutefois reconnu que cet institut avait un potentiel intéressant pour le réseau dédié à la PPA et a donc recommandé qu'il soit invité à participer au réseau de l'OMSA sur la PPA en qualité d'observateur. Le deuxième institut demandeur ne remplissait pas les normes requises en termes de capacités de laboratoire et d'expertise sur la PPA. La Commission a estimé que l'information fournie était insuffisante, et imprécise en ce qui concerne les activités portant spécifiquement sur la maladie considérée. En conséquence, la Commission a rejeté cette candidature.

Enfin, la Commission a examiné la candidature présentée par un pays de la région Asie-Pacifique pour la désignation d'un Laboratoire de référence pour la morve. Bien que la Commission ait expressément encouragé les candidatures au statut de Laboratoire de référence pour la morve dans cette région, elle a estimé que l'expertise et l'expérience de l'institution candidate ne satisfaisaient pas aux exigences et conditions attendues d'un Laboratoire de référence de l'OMSA. Le dossier de candidature ne contenait pas d'informations détaillées sur des activités de collaboration et projets récents à l'échelle internationale. Dans l'ensemble, la Commission a estimé que cette candidature au statut de Laboratoire de référence était prématurée et l'a donc rejetée.

6.2. Changements d'experts au sein des Centres de référence de l'OMSA

Les Délégués des Membres concernés ont présenté à l'OMSA des demandes de désignation pour le remplacement des experts des Laboratoires de référence de l'OMSA ci-après. La Commission a recommandé l'approbation de ces désignations :

Maladie d'Aujeszky
Dre Céline Deblanc en remplacement de la Dre Marie-Frédérique Le Potier au Laboratoire de Ploufragan-Plouzané-Niort, ANSES (Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail), FRANCE

Trichinellose
Dr Gianluca Marucci en remplacement de la Dre Maria Angeles Gomez Morales au Département des maladies infectieuses, Istituto Superiore di Sanità, ITALIE

Influenza aviaire et maladie de Newcastle
Dr Ashley Banyard en remplacement du Dr Ian Brown au Laboratoire pour l'influenza aviaire, Animal and Plant Health Agency (APHA), ROYAUME-UNI

Grippe porcine
Dre Helen Everett en remplacement du Dr Ian Brown au Département de virologie, Animal and Plant Health Agency (APHA), ROYAUME-UNI

La Commission a examiné trois autres désignations pour le remplacement d'experts et décidé, à la lumière des informations fournies, que les personnes désignées ne satisfaisaient pas aux conditions attendues d'un expert de l'OMSA.

6.3. Examen des candidatures nouvelles et en instance pour des projets de jumelage entre laboratoires

En septembre 2024, au total 96 projets avaient été menés à bien et 14 autres étaient en cours de réalisation. Trois projets sont en attente de financement avant de pouvoir démarrer. Au total, 14 Laboratoires de référence et 4 Centres collaborateurs de l'OMSA ont été désignés à l'issue de la participation de ces instituts à des projets de jumelage.

L'évaluation du programme de l'OMSA de jumelage entre laboratoires est pratiquement achevée. Dans le cadre de cette évaluation, un séminaire a été organisé les 27 et 28 juin 2024 au siège de l'OMSA afin de recueillir les retours d'information des parties prenantes du programme sur les principaux thèmes ressortant de cette évaluation. La Commission sera informée des conclusions lors de sa réunion de février 2025. Les résultats concrets de ce processus seront l'élaboration d'un nouveau guide de jumelage, la conception d'un outil d'évaluation sur les suites des projets et la production du rapport d'évaluation.

Trois projets de jumelage entre laboratoires ont été présentés à la Commission en vue de leur évaluation :

a. **Italie – Ouzbékistan** pour la Gestion de la qualité des laboratoires et les procédures de biosécurité et de biosûreté appliquée aux maladies à l'interface animaux-humains : la Commission a approuvé le contenu technique de la proposition portée par ce projet.

b. **Italie – Algérie** pour les Systèmes de biosécurité et de biosûreté au laboratoire, la normalisation des performances des laboratoires et le séquençage du génome entier : la Commission a approuvé le contenu technique de la proposition portée par ce projet.

c. **Chine – Kazakhstan** pour l'Anémie infectieuse des équidés : la Commission a approuvé le contenu technique de la proposition portée par ce projet.

6.4. Évaluation des rapports annuels des Centres collaborateurs

La Commission a procédé à l'évaluation des performances de l'ensemble des Centres collaborateurs de l'OMSA en analysant en détail les rapports annuels d'activité soumis en 2023 dans le but de vérifier que chaque Centre remplissait les obligations relevant de son mandat vis-à-vis des Membres de l'OMSA et qu'il ne se trouvait pas dans la situation critique décrite dans l'alinéa iii) de l'article 10 des Procédures de désignation des Centres collaborateurs de l'OMSA au regard des critères de performance.

La Commission a constaté que les activités de trois Centres collaborateurs n'étaient pas conformes aux principaux points relevant de leur mandat. Les Centres collaborateurs concernés seront informés des résultats de l'évaluation et il leur sera demandé de clarifier leur situation et d'expliquer les éventuels motifs de leur inactivité ; le Délégué recevra copie de tout courrier relatif à cette question. La Commission a également recensé 11 Centres collaborateurs présentant un faible niveau d'activités et les a inscrits sur une liste de surveillance afin de procéder à un suivi particulier lors du prochain examen des rapports annuels.

Par manque de temps, il n'a pas été possible à la Commission d'évaluer les rapports annuels des Laboratoires de référence pendant cette réunion. Une réunion virtuelle extraordinaire sera donc organisée en octobre 2024 pour effectuer cet examen. Les conclusions seront présentées dans le rapport de février 2025 de la Commission.

La Commission a remercié les Centres de référence pour leur respect des délais de soumission des rapports annuels et leur adhésion continue aux termes de leur mandat. La Commission a reconnu par ailleurs l'intérêt que revêtent les

contributions de ces Centres aux activités de l'OMSA et encouragé leur participation accrue aussi bien au processus d'établissement des normes qu'à l'intensification du travail en réseau.

6.5. Centres de référence présentant des manquements par rapport aux points essentiels de leur mandat : non-soumission du rapport annuel

Deux Centres de référence n'ont pas soumis leur rapport annuel d'activités pour 2023 car ils n'avaient pas d'expert désigné ni de personne de contact. Le Centre collaborateur sera informé qu'il devra fournir le nom d'une personne de contact s'il souhaite conserver son statut. S'agissant du Laboratoire de référence, il devra proposer un nouvel expert au plus tard à la réunion de février 2025 de la Commission, date marquant le dernier délai pour le faire. D'après le point 9 des Procédures de désignation des Laboratoires de référence de l'OMSA, « Suspension du statut de Laboratoire de référence de l'OMSA », si un Laboratoire de référence se trouve dans l'incapacité temporaire de remplir temporairement son mandat en raison de l'absence d'un expert désigné agréé, le Siège de l'OMSA, en consultation avec la Commission spécialisée concernée, peut décider de suspendre temporairement son statut de Laboratoire de référence de l'OMSA pour une période qui ne pourra pas dépasser deux ans. Si ce laboratoire reste sans expert désigné après la réunion de février 2025, la procédure décrite pourra s'appliquer.

6.6. Le point sur l'avancement de la procédure d'évaluation des Centres à la fin des cinq années de leur mandat

Les Centres collaborateurs sont désignés pour une période de cinq ans au cours de laquelle ils sont tenus de suivre les orientations du programme de travail sur cinq ans qu'ils ont présenté au moment de leur désignation. Pendant sa réunion de février 2024, la Commission des normes biologiques a élaboré un canevas pour l'auto-évaluation par les Centres collaborateurs des activités effectivement menées au cours des cinq années écoulées par rapport à leur programme de travail initial. Ce rapport a pour objet d'apporter une démonstration probante de l'impact et des accomplissements du Centre et de faire le point sur ses contributions. Les Centres étant tenus de soumettre également leur rapport annuel habituel, la Commission procédera pour chaque Centre concerné à l'évaluation des deux documents.

En août 2024, un courrier a été adressé à cet effet aux Centres collaborateurs dont la période de cinq ans s'achèvera en décembre 2024, les informant que les rapports d'auto-évaluation de leur programme de travail sur cinq ans sont attendus au plus tard en novembre 2024.

Les Centres collaborateurs dont les rapports d'auto-évaluation auront été approuvés recevront un courrier leur notifiant leur éligibilité au renouvellement de leur désignation ; à ce stade, il leur sera demandé de soumettre un nouveau programme de travail sur cinq ans. Les Centres collaborateurs dont les performances auront été jugées insatisfaisantes disposeront d'un délai de recours de 6 mois, à l'issue duquel leur désignation sera réévaluée par la Commission lors de sa réunion de septembre. Cette dernière évaluation peut conclure à la révocation de ces Centres collaborateurs.

6.7. Le point sur les propositions des Centres collaborateurs concernant les points à insérer sous le lien « Ce que nous pouvons faire pour vous »

Afin d'accroître la visibilité des Centres collaborateurs, la Commission a proposé en février 2024 de demander aux Centres d'énumérer en trois à cinq points les principaux services qu'ils peuvent offrir. Cette liste de services sera ajoutée au profil de chaque Centre sur la page dédiée du site web de l'OMSA, sous un lien intitulé « Ce que nous pouvons faire pour vous ».

Les Centres atteignant la fin de la période de cinq ans de leur programme de travail pourront donner leur liste de services dans le canevas qui leur est fourni pour la rédaction du rapport final. Les autres Centres seront contactés séparément et invités à soumettre leur liste de services.

6.8. Les besoins en capacités de laboratoire : faire ressortir les besoins à l'échelle mondiale, ne pas fixer de priorités mais documenter et présenter les besoins sur les aspects d'envergure mondiale

La Commission a reconnu la nécessité d'une stratégie claire pour s'assurer que les réseaux de Laboratoires de référence de l'OMSA répondent avec efficacité aux besoins des Membres de l'OMSA. Il ne s'agit pas seulement de veiller à ce que les maladies pour lesquelles ils ont été désignés correspondent aux priorités régionales ; il faut aussi veiller à ce que tous les Centres de référence suivent une stratégie préétablie et coordonnée pour améliorer les efforts à l'échelle mondiale.

La Commission a fait le point sur les maladies listées par l'OMSA et sur les domaines de spécialisation et spécialités particulières pour lesquels la nécessité était apparue de désigner des Centres de référence dans certaines régions

ou à l'échelle mondiale. Les besoins identifiés découlaient des recommandations formulées par des groupes ad hoc de l'OMSA lors de réunions récentes ou avaient été directement constatés par les Membres de l'OMSA ou les agents du siège. La Commission a pris acte de l'importance de mettre en place une stratégie robuste pour faire évoluer et optimiser la structure des réseaux, en veillant à leur efficacité et adéquation aux besoins à l'échelle mondiale, tout en continuant à y intégrer de nouveaux Centres de référence suivant les besoins.

Les Laboratoires de référence de l'OMSA jouent un rôle crucial dans le système des connaissances scientifiques de l'Organisation et à ce titre leur soutien n'a pas de prix. La Commission a donc proposé de réaliser un examen approfondi du statut actuel des Centres collaborateurs et des Laboratoires de référence. Cet examen comportera une analyse exhaustive ainsi qu'un exercice de cartographie visant à comparer les capacités existantes des laboratoires avec l'épidémiologie mondiale des maladies listées par l'OMSA.

Il s'agira de conduire une analyse complète qui identifiera clairement les lacunes du réseau sous sa forme actuelle en fournissant un tableau précis des besoins en capacités de laboratoire. Cette approche contribuera à alimenter la mise en place d'un réseau plus efficient et plus cohérent au plan stratégique pour continuer à répondre aux besoins évolutifs des Membres de l'OMSA.

6.9. Questions en lien avec le déroulement des opérations intéressant les Laboratoires de référence

6.9.1. Déroulement des opérations au sein des Laboratoires de référence de l'OMSA : qui signe l'engagement de confidentialité et la déclaration relative aux conflits potentiels d'intérêt ? L'expert désigné, le directeur de l'institut, ou les deux ?

La Commission a décidé que les formulaires d'engagement de confidentialité et les déclarations relatives aux conflits potentiels d'intérêt devaient être signés par l'expert désigné et par le directeur de l'institut. Cette disposition sera précisée dans les Recommandations pour les candidatures afin d'assurer la conformité et la transparence du processus de candidature.

6.9.2. Que signifie le terme « équivalent » qualifiant un système de gestion de la qualité ?

Les textes fondamentaux adoptés en 2011 comportaient l'exigence d'avoir en place un système de gestion de la qualité (SGQ). Lors de la Conférence mondiale des Centres de référence tenue à Séoul en 2014, il a été annoncé que les Laboratoires de référence de l'OMSA devaient avoir obtenu l'accréditation de leur système de gestion de la qualité selon la norme ISO 17025 ou une norme équivalente avant la fin du mois de décembre 2017 ; depuis lors, cette obligation figure aussi bien dans les procédures de désignation des Laboratoires de référence que dans les Recommandations pour les candidatures au statut de Laboratoire de référence de l'OMSA. Cette obligation fait l'objet d'un suivi lors de l'examen des rapports annuels, les Laboratoires de référence étant tenus d'y indiquer s'ils se sont dotés d'un SGQ et si celui-ci est accrédité.

Lors de l'évaluation des rapports, la Commission a constaté la nécessité de préciser ce qu'il faut entendre par un système « équivalent » de gestion de la qualité. La proposition initiale décrit un système ayant fait l'objet d'un audit externe indépendant, apportant la démonstration documentée (certificats) de l'évaluation du système par rapport à une norme reconnue.

La Commission a estimé que la question devait être approfondie et procédera à une nouvelle révision de la définition et des critères d'équivalence, en collaboration avec la Commission des normes sanitaires pour les animaux aquatiques, lors de la prochaine réunion des Bureaux en février 2025. D'ici là, la Commission examinera avec attention les rapports des Laboratoires de référence qui font appel à un système de gestion de la qualité équivalent, de manière à déterminer les caractéristiques détaillées de ces systèmes.

Enfin, la Commission a examiné le texte décrivant cette condition et décidé de supprimer « dans l'idéal » afin que l'accréditation des laboratoires de référence de l'OMSA couvre spécifiquement la maladie en question. Le texte proposé est donc :

« Fournir des éléments justificatifs (certificats) de l'octroi de l'accréditation suivant la norme ISO 17025 ou une norme équivalente de gestion de la qualité, en précisant~~, dans l'idéal,~~ les tests pertinents pour la maladie considérée couverts par l'accréditation. »

Après la réunion de la Commission des normes biologiques, la Commission pour les animaux aquatiques a également approuvé cette modification apportée aux Recommandations pour les candidatures au statut de Laboratoire de référence de l'OMSA.

6.10. Le point sur les trois réseaux de Laboratoires de référence (PPA, PPR et rage)

Peste porcine africaine

Faute de temps, le réseau sur la PPA n'a pas pu faire le point pour la Commission de l'avancement de ses activités. En outre, le Réseau devant tenir une réunion en Macédoine du Nord la semaine suivante, il a été décidé de reporter cette actualisation à la prochaine réunion de la Commission en février 2025.

Peste des petits ruminants

Le réseau de Laboratoires de référence de l'OMSA pour la PPR met à jour régulièrement son site web et organise des activités visant à soutenir ses membres. La troisième édition de son Bulletin annuel a paru en juillet 2024 et contient des informations actualisées sur les activités récentes et à venir du réseau ainsi que sur les activités de ses membres et d'autres réseaux d'intérêt (Réseau VETLAB de la FAO/AIEA[4]).

Le réseau a organisé en décembre 2023 un séminaire virtuel pour ses membres, ainsi qu'un cours de formation sur le diagnostic moléculaire et sérologique de la PPR, conjointement avec la FAO, l'AIEA, le Secrétariat du Programme mondial d'éradication de la PPR de la FAO et le Bureau national de la FAO au Nigéria.

Les principales activités d'ici à la fin de l'année 2024 sont notamment un cours de formation aux techniques de diagnostic de la PPR organisé au CIRAD[5] en novembre, un webinaire visant à discuter du rôle des laboratoires de diagnostic dans la mise en œuvre d'une approche épisystémique de la PPR afin d'atteindre les objectifs du Programme mondial d'éradication de la PPR, un exercice comparatif interlaboratoires sur le diagnostic de la PPR organisé conjointement avec le réseau VETLAB de la FAO/AIEA, et la tenue du séminaire annuel du réseau qui se déroulera en décembre 2024 sous un format hybride.

Enfin, la désignation d'un nouveau Laboratoire de référence de l'OMSA pour la PPR en Inde a été adoptée lors de la Session générale de mai 2024, portant leur nombre à quatre et non plus trois.

Rage

Le Réseau des Laboratoires de référence de l'OMSA pour la rage (RABLAB) est constitué de 14 laboratoires, après l'adoption lors de la Session générale de l'OMSA de mai 2024 de la désignation de deux nouveaux laboratoires, l'un en Italie et l'autre au Taipei chinois. Ce groupe a maintenu le rythme bimensuel de ses réunions afin de partager des informations et d'harmoniser les activités des différents Laboratoires de référence ; un nouvel onglet sur la page du site web de l'OMSA dédiée aux Laboratoires de référence a été ajouté afin de donner plus de visibilité au RABLAB.

Le réseau RABLAB soutient les Membres de l'OMSA dans la mise en place de capacités de surveillance, de diagnostic et de laboratoires en proposant des formations et des programmes de jumelage entre laboratoires ; il soutient également l'établissement de réseaux de laboratoires pour la rage en Asie du Sud et en Afrique méridionale, et prévoit des activités en 2025 pour contribuer à établir un réseau de laboratoires pour la rage en Afrique du Nord. Le réseau RABLAB continue également à aider les Membres de l'OMSA à élaborer leurs plans nationaux stratégiques et à concevoir des stratégies de vaccination en masse des chiens.

En réponse aux préoccupations suscitées par l'utilisation dans certains pays de vaccins contre la rage canine non conformes, le réseau RABLAB a publié une déclaration soulignant l'importance d'utiliser des vaccins antirabiques approuvés et de bonne qualité, qui répondent aux normes internationales en termes d'efficacité, de sécurité et de stérilité.

Le RABLAB a également pris note du problème rencontré par des pays qui recourent à la surveillance sérologique pour le suivi des campagnes de vaccination antirabique des chiens, alors que les ressources dont ils disposent sont insuffisantes pour acquérir les vaccins ou pour intensifier la vaccination. Le RABLAB rappelle aux Membres de l'OMSA qu'il n'est pas recommandé de recourir à la surveillance sérologique pour le suivi des programmes de vaccination des chiens car il s'agit d'un outil coûteux et peu fiable (Troisième rapport du Comité OMS[6] d'experts sur la rage).

Le réseau RABLAB continue de jouer un rôle crucial d'appui au forum Tous unis contre la rage, notamment en pilotant le programme de celui-ci en faveur des Partenariats entre pays, en améliorant la coordination et la transparence des activités en lien avec la rage par le biais de la Carte des partenariats Tous unis contre la rage, et en contribuant à

[4] AIEA : Agence internationale de l'énergie atomique
[5] CIRAD: Centre de coopération internationale en recherche agronomique pour le développement (France)
[6] OMS : Organisation mondiale de la santé

une plus ample diffusion des connaissances scientifiques à travers le Bulletin de veille scientifique sur la rage et les webinaires trimestriels Tous unis contre la rage.

6.11. Examen de l'intitulé du Laboratoire de référence pour la nagana : infections par des trypanosomes salivaires et trypanosomose (transmise par la mouche tsé-tsé)

La Commission a pris en compte la demande qui lui avait été faite de réviser et de simplifier l'intitulé du Laboratoire de référence pour la nagana : infections par des trypanosomes salivaires et trypanosomose (transmise par la mouche tsé-tsé).

Après délibérations, la Commission a décidé de modifier l'intitulé comme suit : « Nagana (trypanosomose animale africaine transmise par la mouche tsé-tsé) », pour plus de clarté et en cohérence avec la terminologie établie pour cette maladie.

La Commission a saisi cette occasion de rappeler aux Laboratoires de référence que l'une des activités prévues dans leur mandat consiste à établir et animer un réseau avec les autres Laboratoires de référence de l'OMSA désignés pour le même agent pathogène ou la même maladie, et à organiser régulièrement des essais interlaboratoires pour garantir la comparabilité des résultats.

7. Réactifs internationaux de référence approuvés par l'OMSA

Lors de sa réunion de février 2024, la Commission avait examiné les lignes directrices minimales élaborées par le réseau dédié à la PPR pour la préparation et la validation des matériels de référence destinés aux méthodes de diagnostic de la PPR ; après y avoir introduit quelques amendements visant à les rendre plus génériques, elle avait proposé que le document soit transmis aux autres réseaux dédiés à des maladies afin de recueillir leurs commentaires.

La Commission a examiné les commentaires soumis par les réseaux dédiés à la rage et à la PPA, et les a acceptés. Le Secrétariat transmettra le document amendé au réseau dédié à la fièvre aphteuse pour un dernier cycle de consultations avant son approbation et publication sur le site de l'OMSA. La Commission espère que ces lignes directrices encourageront davantage de laboratoires à soumettre leurs propres réactifs en vue de les faire approuver par l'OMSA en tant que réactifs de référence.

8. Suites données à la Session générale

La Commission a été informée du très bon accueil réservé par les Délégués à sa présentation à l'Assemblée lors de la Session générale de mai 2024, le Président ayant répondu à toutes leurs questions lors de son intervention. Il n'y a eu aucune question devant faire l'objet d'un suivi particulier après la Session générale.

9. Conférences, ateliers, réunions

9.1. Le point sur le Séminaire de l'OMSA qui se tiendra en marge du Symposium de la WAVLD à Calgary (Canada) en 2025

Le prochain symposium international de l'Association mondiale des spécialistes des laboratoires de diagnostic vétérinaire (WAVLD) se tiendra à Calgary (Canada) du 12 au 14 juin 2025 ; la Commission des normes biologiques organisera son séminaire d'une journée en marge de ce Symposium, le 13 juin 2025. Lors de sa réunion de février 2024, la Commission avait demandé au secrétariat de préparer un ordre du jour provisoire et de contacter les experts pressentis pour présenter des exposés sur les technologies de pointe dans le domaine du diagnostic des maladies, couvrant notamment des thèmes sur la PPA, la PPR, la rage, la fièvre aphteuse et l'influenza aviaire pouvant intéresser les réseaux de l'OMSA dédiés à ces maladies. Le secrétariat a fait le point pour la Commission sur les propositions soumises par les experts, notamment des présentations sur les technologies de séquençage de nouvelle génération pour le diagnostic de l'influenza aviaire, la validation des tests à flux latéral rapide pour le diagnostic de la rage, les techniques les plus récentes pour le diagnostic de terrain et au laboratoire de la PPA, l'approche Une seule santé pour contenir la propagation de l'encéphalite japonaise en Australie et enfin un examen de la méthode des tableaux de bord pour le partage d'informations et la collaboration en matière de lutte contre la fièvre aphteuse. La Commission a approuvé ces propositions thématiques et suggéré de consacrer une séance aux réseaux dédiés à des maladies afin qu'ils puissent partager leur expérience de la collaboration et présenter les résultats obtenus grâce au travail en réseau. Le secrétariat contactera des experts supplémentaires afin de traiter d'autres sujets d'actualité et proposera l'ordre du jour complet lors de la réunion de février 2025 de la Commission pour discussion et approbation.

10. Informations diverses pertinentes

10.1. Le point sur la peste bovine

La Commission a été informée de l'évolution des activités post-éradication en matière de peste bovine. L'OMSA continue à travailler en partenariat avec la FAO à la réduction du nombre d'établissements détenant du matériel contenant le virus de la peste bovine (MCVPB) dans le monde, hors matériel de diagnostic et vaccins. Cet effort permettra de réduire le nombre d'établissements de catégorie A habilités par la FAO et l'OMSA à détenir du MCVPB, en plus de réduire le MCVPB détenu par des Membres de l'OMSA dans des établissements non habilités. À ce jour, cinq pays détiennent du MCVPB dans des établissements non habilités à cette fin ; en outre, sept établissements de catégorie A et B ont été désignés dans six Membres. Parmi ces derniers, trois établissements de catégorie B détiennent des souches vaccinales pour une utilisation mondiale. Malheureusement, aucune avancée n'a été enregistrée en matière de séquestration et de destruction du MCVPB dans les cinq Membres détenant ce matériel en dehors des établissements habilités par la FAO-OMSA, malgré les discussions directes sur le sujet lors de la Session générale de l'OMSA en mai 2024.

La nouvelle composition du Comité consultatif mixte (JAC) FAO-OMSA pour la peste bovine a été confirmée en mars 2024. La 19e réunion du JAC s'est ensuite tenue sous forme virtuelle le 26 avril 2024. La première partie de la réunion a été consacrée à faire le point et à s'assurer que les nouveaux membres du JAC sont bien informés des activités menées par la FAO et l'OMSA depuis 2012 et des priorités de la phase de post-éradication. Pendant cette réunion, le JAC a approuvé deux propositions de recherche destinées à des projets de « séquençage puis destruction », présentées respectivement par l'Institut national de la santé animale (Japon) et le CIRAD (France). Le Comité a discuté le concept de MCVPB « essentiel » et fera connaître son opinion officielle sur cette question. Les participants à la réunion ont commencé à réviser la liste de vérification utilisée par les experts lors des inspections des établissements habilités à détenir du MCVPB. Le processus de révision a été alimenté par une consultation organisée avec l'OMS en octobre 2023. Les actions à mener par le Comité et par le Secrétariat pour la peste bovine ont été définies et une évaluation des progrès sera réalisée lors d'une activité de suivi prévue le 23 septembre 2024. Enfin, la discussion a abordé la préparation des réinspections qui seront organisées au dernier trimestre 2024 dans les établissements habilités à détenir du MCVPB au sein du CIRAD (France) et de l'IVDC (Rép. pop. de Chine) et la primo-inspection du ICAR-NIHSAD (Inde) prévue au premier semestre 2025.

10.2. Le point sur le projet de biobanque virtuelle

L'expert qui dirige le Centre collaborateur pour les biobanques virtuelles a fait le point pour la Commission sur le projet de biobanque virtuelle de l'OMSA. Le premier objectif d'une biobanque virtuelle est de faciliter l'accès aux ressources biologiques collectées et conservées par les Centres de référence de l'OMSA et les Laboratoires de référence nationaux. Ces ressources sont essentielles pour concevoir des tests de diagnostic et les normaliser, et pour lutter contre les maladies listées par l'OMSA.

Approuvé dans un premier temps à la fin de l'année 2019, le projet a été mis en attente en raison de la pandémie de COVID-19 ; il a été relancé officiellement en avril 2023 après une année de procédure d'appel d'offres visant à sélectionner une entreprise de technologies de l'information pour l'exécution du projet. La direction du projet est assurée par l'IZSLER, Centre collaborateur pour une biobanque de produits biologiques vétérinaires, avec le soutien du réseau des Istituti Zooprofilattici Sperimentali en Italie. Le déploiement de la phase pilote du projet est prévu début 2025.

L'expert a demandé à la Commission de fournir des orientations sur un certain nombre de questions, comme suit :

Éligibilité des fournisseurs : la Commission a recommandé que pendant la phase initiale du projet, les fournisseurs principaux de la biobanque virtuelle soient exclusivement des Laboratoires de référence de l'OMSA et des laboratoires de référence nationaux répondant à des normes de qualité établies. À un stade ultérieur, Il pourra être envisagé d'inclure d'autres institutions dotées de systèmes solides d'assurance qualité, par exemple des centres de recherche gouvernementaux.

Restrictions concernant les types de matériel : La Commission a examiné les types de matériel susceptibles de figurer dans le catalogue et proposé d'appliquer des restrictions sur les agents pathogènes à haut risque. En raison des risques élevés associés à ces agents pathogènes, il conviendra d'imposer des conditions strictes de documentation et d'autorisation afin de s'assurer que tous les fournisseurs appliquent des normes rigoureuses de qualité en conformité avec un cadre juridique strict. L'inclusion de matériels associés à des maladies non listées par l'OMSA a également été envisagée.

Normes de qualité pour les laboratoires : les premières discussions en 2017 ont recommandé que les laboratoires soient dotés de certifications suivant les normes ISO 9001 et ISO 20387 ; néanmoins, l'expert a fait observer qu'à ce jour le nombre de laboratoires dotés d'une certification ISO 20387 reste très limité. La Commission a estimé qu'il

conviendrait sans doute de prévoir des lignes directrices supplémentaires sur la qualité et la caractérisation des matériels. Ces lignes directrices pourraient être rédigées en collaboration avec les institutions compétentes afin de s'assurer de la conformité des laboratoires participants avec les normes appropriées.

Documentation requise : la Commission a examiné la question de savoir s'il convient d'établir des exigences visant à normaliser la documentation requise dans le cadre du projet, notamment pour ce qui concerne les autorisations de manipuler des agents pathogènes soumis à restrictions, l'utilisation d'OGM, et la déclaration des recherches à double usage préoccupantes. Actuellement, seul l'Accord de transfert de matériel (MTA) est normalisé. Il a été convenu que la responsabilité légale de la qualité du matériel incombe au fournisseur et à l'utilisateur. Les fournisseurs sont tenus de garantir la conformité du matériel avec les normes requises et d'informer le destinataire des mesures de contrôle de la qualité en place. L'utilisateur destinataire doit garantir que le matériel est manipulé conformément aux réglementations nationales et internationales.

Conformité au protocole de Nagoya : l'expert a clarifié qu'il n'y aura pas d'échanges physiques de matériel biologique via la biobanque virtuelle ; les échanges auront lieu directement entre laboratoires, la plateforme servant uniquement de catalogue virtuel afin de faciliter les contacts entre fournisseurs et utilisateurs. La conformité au protocole de Nagoya est essentielle et les laboratoires parties prenantes de l'échange de matériel sont responsables de cette conformité.

Taxonomie vérifiée des parasites et champignons : la Commission a souligné l'importance d'une taxonomie vérifiée pour les parasites et les champignons. La participation des Laboratoires de référence à la mise en place d'une expertise dans ce domaine permettrait de garantir l'exactitude des données et leur cohérence au sein de la biobanque virtuelle.

Lignes directrices sur la qualité du matériel : la Commission a estimé qu'il était essentiel d'évaluer l'identité, la concentration, la pureté et la stabilité du matériel afin de préserver une qualité élevée. Il sera probablement nécessaire de prévoir des lignes directrices sur la qualité et la caractérisation du matériel afin de mettre en place une norme minimale pour tout matériel référencé dans la biobanque virtuelle.

Globalement, la Commission a estimé qu'il fallait continuer à développer la biobanque virtuelle et souligné la nécessité de poursuivre le dialogue afin de traiter les aspects légaux, techniques et stratégiques du projet.

10.3. Produits vétérinaires non conformes et falsifiés

L'OMSA a constaté des lacunes dans les capacités de certains Membres à gérer la qualité des produits vétérinaires une fois l'enregistrement effectué, ainsi que des insuffisances en matière de prévention et de détection des produits vétérinaires non conformes et falsifiés et dans les capacités d'intervention lorsque la présence de ces produits est avérée. Or, si certains chapitres du *Code terrestre* mentionnent les produits vétérinaires non conformes et falsifiés, il n'y a pas d'orientations spécifiques sur le sujet.

Afin de traiter ce problème, l'OMSA a mis en œuvre un nouveau programme d'appui à la prévention, détection et réponse aux produits vétérinaires non conformes et falsifiés, basé sur une méthode intersectorielle et transnationale. L'activité la plus avancée dans le cadre de ce programme est l'élaboration d'un système de suivi et de surveillance vétérinaire des produits vétérinaires non conformes et falsifiés (V-SAFE), conformément à la recommandation formulée par les Délégués lors de la deuxième Conférence mondiale de l'OMSA sur la résistance aux agents antimicrobiens en 2018. V-SAFE dans sa phase pilote permet aux Membres de l'OMSA de notifier les suspicions de produits vétérinaires non conformes ou falsifiés telles que relevées principalement par les systèmes de pharmacovigilance, de surveillance passive, de suivi de la qualité des vaccins, etc.

La Commission a examiné la question de savoir si cette initiative devait être intégrée dans le *Manuel terrestre*, le *Code terrestre*, ou les deux. La Commission a d'abord estimé que la portée du programme de suivi et de surveillance des produits vétérinaires non conformes et falsifiés dépassait le cade du *Manuel terrestre*, celui-ci traitant principalement des méthodes de diagnostic et des vaccins. La Commission a souligné que les recommandations du *Manuel terrestre* portent sur la qualité des vaccins tandis que le programme V-SAFE a pour objet de vérifier que les vaccins utilisés sur le terrain conservent cette qualité élevée et continuent à présenter l'efficacité pour laquelle ils ont été approuvés, par exemple à travers une surveillance active après l'autorisation de mise sur le marché.

En réponse à ces préoccupations, il a été proposé d'amender le chapitre 1.1.8 du *Manuel terrestre*, « Principes de production des vaccins vétérinaires » afin de mentionner, dans la section sur la mise sur le marché, la surveillance active consécutive aux autorisations de mise sur le marché. La Commission a souscrit à cette proposition et suggéré de reporter à plus tard la décision d'étoffer cet amendement pour couvrir d'autres produits pharmaceutiques, tels que les agents antiparasitaires et antimicrobiens.

La Commission a manifesté son intérêt pour le processus d'élaboration de ce programme et a pris note que la révision des documents en préparation devrait commencer dans un avenir proche. La Commission a encouragé les discussions à se poursuivre afin de réussir une intégration effective du programme V-SAFE dans les normes et lignes directrices de l'OMSA.

La Commission a pris note du fait que les autres Commissions spécialisées sont tenues informées de l'évolution et que la Commission scientifique pour les maladies animales a d'ores et déjà indiqué qu'elle recommandait d'ajouter dans le *Code terrestre* des définitions pour les termes « non conformes » et « falsifiés ». Cette recommandation a également été approuvée par la Commission des normes biologiques.

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…/annexes

Annexe 1. Ordre du jour adopté

RÉUNION DE LA COMMISSION DES NORMES BIOLOGIQUES DE L'OMSA
Paris, 9–13 septembre 2024

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1. Mots de bienvenue des directrices

1.1. Directrice générale
1.2. Directrice générale adjointe, Normes internationales et science

2. Adoption de l'ordre du jour

3. Relations avec les autres Commissions spécialisées

3.1. Questions transversales intéressant les Commissions spécialisées

3.1.1. Travaux relatifs aux animaux hôtes pris en compte dans les normes de l'OMSA pour une maladie listée des animaux terrestres

3.2. Commission scientifique pour les maladies animales

3.2.1. Définitions d'un cas : paratuberculose, arthrite/encéphalite caprine et maedi-visna
3.2.2. Évaluation de la maladie des moutons de Nairobi au regard des critères d'inclusion dans la liste
3.2.3. Recommandations du Groupe ad hoc sur la tremblante

3.3. Commission des normes sanitaires pour les animaux terrestres

3.3.1. Réunion des Bureaux

3.4. Commission des normes sanitaires pour les animaux aquatiques

4. Programme de travail

5. Manuel des tests de diagnostic et des vaccins pour les animaux terrestres

5.1. Format du rapport et système de soumission des commentaires
5.2. Examen et approbation des projets de chapitre destinés à être distribués aux Membres pour un premier cycle de consultations

n°	n° de chapitre	Titre du chapitre (année de la dernière adoption)
1.	1.1.3.	Transport de matériel biologique (2018)
2.	1.1.7.	Normes pour le séquençage à haut débit, la bioinformatique et la génomique computationnelle (2016)
3.	1.1.9	Contrôle de la stérilité et de l'absence de contamination des matériels biologiques à usage vétérinaire (2024)
4.	2.1.1.	Méthodes de laboratoire utilisées pour les tests de sensibilité des bactéries aux antimicrobiens (2019)
5.	2.2.1	Mise au point et optimisation des méthodes de détection des anticorps (2014)
6.	2.2.2	Mise au point et optimisation des méthodes de détection des antigènes (2014)
7.	2.2.3	Mise au point et optimisation des méthodes de détection de l'acide nucléique (2014)
8.	2.2.5	Méthodes statistiques de validation (2014)
9.	2.2.8.	Comparabilité des épreuves suite à des changements introduits dans une méthode d'essai validée (2016)
10.	2.3.2.	Rôle des autorités officielles dans la réglementation internationale des produits biologiques à usage vétérinaire (2018)
11.	3.1.2.	Maladie d'Aujeszky (infection par le virus de la maladie d'Aujeszky) (2018)
12.	3.1.8.	Fièvre aphteuse (infection par le virus de la fièvre aphteuse) (2021)
13.	3.1.9.	Cowdriose (2018)
14.	3.1.21.	Peste bovine (infection par le virus de la peste bovine) (2018)
15.	3.2.4.	Infestation des abeilles mellifères par le petit coléoptère des ruches (*Aethina tumida*) (2018)
16.	3.2.5.	Infestation des abeilles mellifères par *Tropilaelaps* spp. (2018)
17.	3.3.1.	Chlamydiose aviaire (2018)
18.	3.3.2.	Bronchite infectieuse aviaire (2018)

n°	n° de chapitre	Titre du chapitre (année de la dernière adoption)
19.	3.3.4.	Influenza aviaire (y compris l'infection par les virus de l'influenza aviaire hautement pathogènes) (2021)
20.	3.4.9.	Leucose bovine enzootique (2018)
21.	3.4.13.	Theilériose bovine (infection à *Theileria annulata*, *T. orientalis* et *T. parva*) (2018)
22.	3.4.14.	Trichomonose (2018)
23.	3.6.1.	Peste équine (infection par le virus de la peste équine) (2019)
24.	3.6.5.	Anémie infectieuse équine (2019)
25.	3.8.2.	Arthrite/encéphalite caprine et maedi-visna (2017)
26.	3.8.5.	Avortement enzootique des brebis (chlamydiose ovine) (infection à *Chlamydia abortus*) (2018)
27.	3.8.11.	Clavelée et variole caprine (2024) : partie sur les vaccins
28.	3.9.1.	Peste porcine africaine (2021) : partie sur les vaccins
29.	3.9.2.	Peste porcine classique (infection par le virus de la peste porcine classique) (2022)
30.	3.9.7.	Virus de la maladie vésiculeuse du porc (2018)

5.3. Réponse de la Commission aux commentaires formulés par les Membres lors du deuxième cycle de consultations
5.4. Rapport sur une étude interlaboratoires visant à approfondir la validation des tests sérologiques pour le diagnostic de la morve
5.5. Demande d'orientations concernant la taxonomie des agents pathogènes et l'utilisation de leurs noms communs
5.6. Chapitres dédiés à des maladies non listées : les test décrits dans ces chapitres sont-ils toujours considérés comme validés selon les normes de l'OMSA ?
5.7. Statut du *Manuel terrestre* : le point sur les chapitres sélectionnés pour le cycle d'examen 2025/2026
5.8. Actualisation sur le projet d'outil de navigation en ligne dédié aux normes de l'OMSA

6. Centres de référence de l'OMSA

6.1. Examen des candidatures au statut de Centre de référence de l'OMSA
6.2. Changements d'experts au sein des Centres de référence de l'OMSA
6.3. Examen des candidatures nouvelles et en instance pour des projets de jumelage entre laboratoires
6.4. Évaluation des rapports annuels des Centres collaborateurs
6.5. Centres de référence présentant des manquements par rapport aux points essentiels de leur mandat : non-soumission du rapport annuel
6.6. Le point sur l'avancement de la procédure d'évaluation des Centres à la fin des cinq années de leur mandat
6.7. Le point sur les propositions des Centres collaborateurs concernant les points à insérer sous le lien « Ce que nous pouvons faire pour vous »
6.8. Les besoins en capacités de laboratoire : faire ressortir les besoins à l'échelle mondiale, ne pas fixer de priorités mais documenter et présenter les besoins sur les aspects d'envergure mondiale
6.9. Questions en lien avec le déroulement des opérations intéressant les Laboratoires de référence

6.9.1. Déroulement des opérations au sein des Laboratoires de référence de l'OMSA : qui signe l'engagement de confidentialité et la déclaration relative aux conflits potentiels d'intérêt ? L'expert désigné, le directeur de l'institut, ou les deux ?

6.9.2. Que signifie le terme « équivalent » qualifiant un système de gestion de la qualité ?

6.10. Le point sur les trois réseaux de Laboratoires de référence (PPA, PPR et rage)
6.11. Examen de l'intitulé du Laboratoire de référence pour la nagana : infections par des trypanosomes salivaires et trypanosomose (transmise par la mouche tsé-tsé)

7. Normalisation et harmonisation internationales

Liste de réactifs – Lignes directrices génériques pour la préparation et la validation du matériel de référence destiné aux méthodes de diagnostic

8. Suites données à la Session générale

Extraits du Rapport final

9. Conférences, ateliers, réunions

9.1. Le point sur le Séminaire de l'OMSA qui se tiendra en marge du Symposium de la WAVLD à Calgary (Canada) en 2025

10. Informations diverses pertinentes

10.1. Le point sur la peste bovine
10.2. Le point sur le projet de biobanque virtuelle
10.3. Produits vétérinaires non conformes et falsifiés

11. Synthèse de la réunion

Annexe 2. Liste des participants

RÉUNION DE LA COMMISSION DES NORMES BIOLOGIQUES
Paris, 9–13 septembre 2024

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MEMBRES DE LA COMMISSION

Prof. Emmanuel Couacy-Hymann
(Président)
Professeur de virologie
CNRA/LIRED
Abidjan
CÔTE D'IVOIRE

Prof. Ann Cullinane
(Vice-Présidente)
Cheffe de l'Unité de virologie
Irish Equine Centre
Naas
IRLANDE

Prof. Chris Oura
(Vice-Président)
Professeur de virologie vétérinaire, The University of the West Indies, St-Augustine
TRINIDAD-ET-TOBAGO

Dr Joseph S. O'Keefe
(Membre)
Directeur du Laboratoire de santé animale
Ministry for Primary Industries
Upper Hutt
NOUVELLE-ZÉLANDE

Dr Satoko Kawaji
(Membre)
Chercheur principal, Institut national de santé animale, Naro
JAPON

Dr Donald King
(Membre)
Directeur du Groupe de Laboratoires de référence pour la maladie vésiculeuse
The Pirbright Institute
ROYAUME-UNI

CONSULTANT RÉDACTEUR DU *MANUEL TERRESTRE*

Dr John Passick
Consultant rédacteur
CANADA

SIÈGE DE L'OMSA

Dr Gregorio Torres
Chef de service
Service scientifique

Sara Linnane
Responsable scientifique senior
Service scientifique

Dre Mariana Delgado
Responsable du Secrétariat scientifique
Service scientifique

Dre Charmaine Chng
Adjointe du chef du Service
Service scientifique

Annexe 3. Programme de travail de la Commission des normes biologiques de l'OMSA

RÉUNION DE LA COMMISSION DES NORMES BIOLOGIQUES
Paris, 9–13 septembre 2024

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Sujet	Questions à examiner	État d'avancement et mesures à prendre
Mise à jour du *Manuel terrestre*	1) Distribuer aux Membres les chapitres approuvés par la Commission pour un premier cycle de consultations	Pour février 2025
	2) Relancer les auteurs concernant les chapitres précédemment définis comme étant à réviser mais qui n'ont pas encore été reçus, et adresser une invitation aux auteurs des chapitres dont la révision vient d'être décidée	En cours
	3) Transférer et rendre publique la base de données intégrant les rapports de validation à publier sur le site web de l'OMSA pour les tests recommandés dans le *Manuel terrestre*, et prévenir les experts des Laboratoires de référence	Terminé
	4) Présenter sous forme d'annexes à la fin des chapitres consacrés à des maladies particulières les tableaux justificatifs des notations des tests dans le Tableau 1, *Méthodes d'essai disponibles et emplois*. Ajouter les liens vers les rapports de validation des tests dès que ceux-ci sont disponibles (voir le point 3 ci-dessus)	Terminé
	5) Demander aux Centres de référence de fournir les liens vers des vidéos didactiques afin de les insérer à la fin des chapitres consacrés à des maladies particulières. La Commission révisera les vidéos proposées lors de l'inscription du chapitre dans le cycle de révision	En cours
	6) Définir les critères justifiant la suppression des chapitres dédiés à des maladies non listées, et évaluer les chapitres au regard de ces critères	Terminé
	7) Examiner les nouvelles évolutions des maladies ayant un impact important au niveau mondial (par ex., influenza aviaire, peste porcine africaine) et prioriser ces chapitres	En cours
	8) Commencer le processus visant à permettre l'accès aux versions antérieures et modifiées du *Manuel terrestre*, comme cela a été fait pour le *Code terrestre*, afin de répondre aux demandes en ce sens	En cours
	9) Ajouter un modèle de tableau relatif aux PCR dans les Instructions destinées aux contributeurs (harmonisation avec le *Manuel aquatique*)	En cours
Centres collaborateurs	1) Mise en œuvre des procédures de désignation adoptées :	
	a) Préparer un canevas destiné aux Centres collaborateurs pour le rapport d'évaluation de leurs performances au cours des cinq années écoulées au regard de leur programme initial d'activités sur cinq ans	Terminé
	b) Adresser aux Centres collaborateurs concernés le formulaire d'évaluation de leur programme de travail sur cinq ans	Terminé

Sujet	Questions à examiner	État d'avancement et mesures à prendre
	2) Évaluer le retour d'informations émanant des Centres arrivés au terme des cinq ans de leur mandat et vérifier la pertinence actuelle de leur domaine d'activités en vue du renouvellement de leur mandat	Pour février 2025
	3) Améliorer la visibilité des Centres actuels : leur demander de soumettre une liste de 5 points maximum qui sera ajoutée à l'entrée qui leur correspond sur le site web, sous l'intitulé « Ce que nous pouvons faire pour vous »	En cours
	4) Réfléchir à de nouveaux mécanismes permettant d'améliorer la collaboration en réunissant les Centres ayant le même domaine de spécialisation (actuellement six Centres) : participation du secteur privé ou d'autres partenaires pour le financement	En cours
	5) Élaborer un questionnaire pour recueillir des informations auprès des Centres collaborateurs sur leur expérience en tant que Centres de collaborateurs de l'OMSA, comme cela a été fait avec les Laboratoires de référence	Septembre 2025
Laboratoires de référence	1) Inscrire les laboratoires présentant un déficit de performances sur une liste de surveillance et assurer un suivi	En cours
	2) Mettre en œuvre le nouveau système d'évaluation des rapports annuels et répartir les rapports à évaluer entre les membres de la Commission	Terminé
	3) Évaluation des rapports annuels	23 octobre 2024
	4) Obtenir un retour d'information des Laboratoires de référence concernant le questionnaire	Terminé
	5) Étudier les améliorations pouvant être apportées au processus de soumission des rapports annuels : possibilité de remplir le modèle tout au long de l'année	Terminé
Réseaux de Centres de référence	1) Suivi des trois réseaux de Laboratoires de référence (PPA, PPR et rage) et potentiellement, PPC et fièvre aphteuse, OFFLU)	En cours
Normalisation/ harmonisation	1) Projet visant à étoffer la liste des réactifs de référence approuvés par l'OMSA	
	a) Demander aux autres réseaux s'ils approuvent le document de normes minimales préparé par le réseau sur la PPR. Une fois finalisé, télécharger le document pour les besoins de la mise en œuvre	Pour février 2025
	2) Projet d'élaboration d'un étalon international de substitution pour le test à la tuberculine bovine et aviaire : finaliser le rapport et le présenter en vue de son adoption	En cours
Groupes ad hoc	1) Contribuer à la révision du chapitre 4.7 du *Code terrestre*, « Collecte et traitement de la semence de bovins, de petits ruminants et de verrats »	En cours
	2) Contribuer au Groupe ad hoc sur les maladies émergentes et les facteurs d'émergence des maladies animales	En cours
Projets	1) Biobanque vétérinaire (projet)	On-going
	2) BIO-PREVAIL : Une initiative de sécurité sanitaire pour des laboratoires durables	En cours

Sujet	Questions à examiner	État d'avancement et mesures à prendre
Participation de membres de la Commission à des conférences, ateliers ou réunions	1) Feuille de route sur la recherche en matière de sécurité biologique	Terminé
	2) Séminaire de l'OMSA en marge du Symposium de la WAVLD en juin 2025 au Canada : définir le thème et préparer le programme et la liste d'orateurs	Juin 2025
Performances	1) Échanger avec les Laboratoires de référence sur le processus en cours concernant les problèmes de performances	En cours
Normes de laboratoire pour les maladies émergentes	1) Examiner le chapitre du *Code terrestre* après son adoption et envisager d'introduire un chapitre équivalent dans le *Manuel terrestre*	Après mai 2025
Définitions d'un cas	1) Assurer un suivi de l'application des procédures normalisées pour la définition d'un cas	En cours

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Annexe 4. Point 5.3 – Réponses de la Commission aux commentaires reçus des Membres lors du deuxième cycle de consultations sur le chapitre 3.9.1, « Peste porcine africaine (infection par le virus de la peste porcine africaine) » qui avait été annexé au rapport de février 2024 avant la Session générale (NB : cette annexe est présentée pour information uniquement et non pour recueillir des commentaires. La version comportant toutes les mises à jour de ce chapitre est présentée à l'annexe 33 pour un troisième cycle de consultations)

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SECTION 3.9.

SUIDAE

CHAPTER 3.9.1.

AFRICAN SWINE FEVER (INFECTION WITH AFRICAN SWINE FEVER VIRUS)

Reference	Comment	Biological Standards Commission response
3.9.1_1	**Category:** general Our country thanks the Committee for seriously considering and adopting some of our recommendations. And our country appreciates WOAH for promoting the development of safe and effective African swine fever vaccines to enhance members' comprehensive prevention and control capabilities against African swine fever. However, we should pay attention to the complex epidemic situation of African swine fever around the world, and the challenges faced by vaccine research and development. In particular, the safety issue of live attenuated vaccines has not been effectively solved, and the data on vaccine field use are insufficient. Under such circumstances, it's hasty to support the use of African swine fever vaccine, which is likely to bring serious risks to the global pig industry. In order to reduce the risk of vaccine and improve vaccine safety, our country suggests that this chapter be revised as follows. **First of all,** we suppose the safety of the vaccine for sows should be emphasised in the draft. As we know the vaccine will be used in large quantities and may be in wide range after approval. Even if it does not directly immunise sows, it is very likely to infect sows through horizontal transmission. Considering that, our country suggests to add "sow safety" to the	**EN:** First point: agreed, due to possible risks from horizontal transmission, to add an extra section on safety testing in pregnant sows similar to the classical swine fever chapter, as the population that is likely to be the most sensitive indicator of any adverse effects (one single dose for pregnant sows). Safety tests for other growth stages could be undertaken as preferred. Second point: Without a better understanding of the frequency of recombination, this kind of recombination evaluation testing would be difficult to introduce. Identifying and monitoring recombination under field conditions is not a trivial undertaking and there is currently insufficient information to expand the section on strain recombination evaluation testing in the field. This should be included in separate guidelines on post-vaccination monitoring that are being developed. Conditional licensing under controlled conditions could be a solution to obtain field data for final decisions on the use of the vaccine to complement national control measures. Prevalence of circulating virus strains should be evaluated prior to commencing vaccination campaigns. Third point: see later in the chapter. **FR**: Premier point : compte tenu du risque posé par la probabilité d'une transmission horizontale, la Commission accepte la proposition d'ajouter un paragraphe supplémentaire dédié aux essais d'innocuité chez les truies gestantes, similaire à

Reference	Comment	Biological Standards Commission response
	minimum requirements for vaccines, and supplement the safety evaluation indicators for sows. **Secondly,** it might be necessary to add vaccine recombination evaluation test. In view of the pathogenic characteristics of African swine fever, strain recombination is an important factor affecting the safety of live attenuated African swine fever vaccine, therefore recombination evaluation should be run through the whole process from R & D to field use. At current, the revised draft has clarified the risk of strain recombination and ask for post-vaccination surveillance for recombination. On this basis, our country suggests to further supplement the strain recombination evaluation test, specifically including laboratory evaluation and field evaluation. **Thirdly,** we suppose to improve another two technical indicators (in text).	celui qui existe dans le chapitre sur la peste porcine classique : en effet la sensibilité de cette catégorie d'animaux à d'éventuels effets indésirables en fait un bon indicateur (dose unique pour les truies gestantes). Des essais d'innocuité spécifiques pour d'autres stades de croissance peuvent être effectués suivant les préférences. Deuxième point : tant que la fréquence des recombinaisons n'est pas mieux comprise, il paraît difficile d'introduire des tests de ce type pour déterminer l'existence d'une recombinaison. L'identification et le suivi des recombinaisons dans des conditions de terrain ne sont pas des entreprises anodines et les informations actuellement disponibles sont insuffisantes pour proposer un texte sur les tests visant à déterminer l'existence de recombinaisons entre souches sur le terrain. Cet aspect devrait être traité dans les lignes directrices sur le suivi post-vaccinal qui sont en préparation. La délivrance d'une licence provisoire dans des conditions contrôlées pourrait être une solution afin d'obtenir des données de terrain pour une décision définitive sur l'utilisation du vaccin, en complément des mesures nationales de lutte contre la maladie. La prévalence des souches virales présentes sur le terrain devrait faire l'objet d'une évaluation avant de démarrer les campagnes de vaccination. Troisième point : voir plus loin dans le chapitre.
		ESP: Primer punto: se acuerda, debido a los posibles riesgos de transmisión horizontal, añadir una sección adicional sobre pruebas de seguridad en cerdas gestantes similar al capítulo sobre peste porcina clásica, ya que es la población que probablemente sea el indicador más sensible de cualquier efecto adverso (una sola dosis para cerdas gestantes). Si se prefiere, podrían realizarse pruebas de seguridad para otras fases de crecimiento. Segundo punto: Sin un mejor conocimiento de la frecuencia de la recombinación, sería difícil introducir este tipo de pruebas de evaluación de la recombinación. La identificación y el seguimiento de la recombinación en condiciones de campo no es una tarea trivial y actualmente no hay información suficiente para ampliar la sección sobre las pruebas de campo de evaluación de la recombinación de cepas. Esto debería incluirse en directrices separadas sobre el seguimiento post-vacunación que se están desarrollando. La concesión condicional de licencias en condiciones controladas podría ser una solución para obtener datos de campo para las decisiones finales sobre el uso de la vacuna como complemento de las medidas nacionales de control. La prevalencia de las cepas de virus circulantes debería evaluarse antes de iniciar las campañas de vacunación. Tercer punto: véase más adelante en el capítulo.

Reference	Comment	Biological Standards Commission response
3.9.1_2	**Category:** general We thank the WOAH Biological Standards Commission for this work being moved forward. We can in general support this revised chapter. It appears that there are still several points that need to be clarified and we invite the Commission to review them. As a general point, we note that throughout the text reference is sometimes made to either "licensing", "registration" or "authorisation". We would recommend to rather use the term "regulatory approval", which works in all jurisdictions across the world, and has been used systematically in other recently adopted Manual chapters. This would include the title of Section 2.3. "Requirements for authorisation/registration/ licensing" (Line 297), that should be replaced with "Requirements for regulatory approval".	**EN**: Agreed, text amended in multiple places for consistency. **FR**: La Commission a souscrit au commentaire ; le texte est modifié à plusieurs endroits pour des raisons de cohérence. **SP**: Aceptado, el texto se modificó en múltiples lugares por coherencia.
3.9.1_3	**Category:** general This section needs significant proofreading. There are many structural issues that make the ideas hard to follow or the requirements of the chapter unclear. There are many instances of requirements worded as either suggestions (often using the word "should"), or as technical methods (e.g. To obtain individual and group mean baseline temperatures…). These need to be revised for clarity. There is unnecessary repetition and redundancy in the writing. The structure of some sentences/paragraphs makes the intent hard to understand (I have given the first instance of these issues specific comments). There are technical instructions for how to perform tests in the requirements for test compliance, and requirements for test compliance in the instructions for how to perform the tests. These should be kept to their sections.	**EN**: Agreed, editorial amendments have been made. Some of the repetition is necessary to enable different sections of the text to be read in isolation, but much of the repetition has been removed. **FR**: La Commission a souscrit à ce commentaire et introduit quelques amendements de nature éditoriale. Certaines des répétitions invoquées sont nécessaires car chaque section du texte doit pouvoir être lue de manière autonome ; les répétitions inutiles ont été supprimées. **SP**: De acuerdo, se han introducido modificaciones de redacción. Algunas de las repeticiones son necesarias para permitir la lectura aislada de las diferentes secciones del texto, pero se ha suprimido gran parte de las repeticiones.

SUMMARY

African swine fever (*ASF*) *is an infectious disease of domestic and wild pigs of all breeds and ages, caused by ASF virus* (*ASFV*). *The clinical syndromes vary from peracute, acute, subacute to chronic, depending on the virulence of the virus. Acute disease is characterised by high fever, haemorrhages in the reticuloendothelial system, and a high mortality rate. Soft ticks of the* Ornithodoros *genus, especially* O. moubata *and* O. erraticus*, have been shown to be both reservoirs and transmission vectors of ASFV. The virus is present in tick salivary glands and passed to new hosts* (*domestic or wild suids*) *when feeding. It can be transmitted sexually between ticks, transovarially to the eggs, or transtadially throughout the tick's life.*

ASFV is the only member of the Asfarviridae *family, genus* Asfivirus.

Laboratory diagnostic procedures for ASF fall into two groups: detection of the virus and serology. The selection of the tests to be carried out depends on the disease situation and laboratory diagnostic capacity in the area or country.

Identification of the agent: *Laboratory diagnosis must be directed towards isolation of the virus by inoculation of pig leukocyte or bone marrow cultures, the detection of antigen in smears or cryostat sections of tissues by fluorescent antibody test and/or the detection of genomic DNA by the polymerase chain reaction (PCR) or real-time PCR. The PCRs are excellent, highly sensitive, specific and rapid techniques for ASFV detection and are very useful under a wide range of circumstances. They are especially useful if the tissues are unsuitable for virus isolation and antigen detection. In doubtful cases, the material is passaged in leukocyte cell cultures and the procedures described above are repeated.*

Serological tests: *Pigs that survive natural infection usually develop antibodies against ASFV from 7–10 days post-infection and these antibodies persist for long periods of time. Where the disease is endemic, or where a primary outbreak is caused by a strain of low or moderate virulence, the investigation of new outbreaks should include the detection of specific antibodies in serum or extracts of the tissues submitted. A variety of methods such as the enzyme-linked immunosorbent assay (ELISA), the indirect fluorescent antibody test (IFAT), the indirect immunoperoxidase test (IPT), and the immunoblotting test (IBT) is available for antibody detection.*

Requirements for vaccines*: ~~At present, there is no vaccine for ASF.~~ Commercially produced modified live virus vaccines are ~~available and licenced~~ under field evaluation in some countries.*

Reference	Comment	Biological Standards Commission response
3.9.1_SUMMARY_1	**Category:** change Lines 34–35: This text should be updated and aligned with the information given in lines 163–164.	**EN**: Agreed.
		FR: La Commission a souscrit au commentaire.
		SP: Aceptado.

A. INTRODUCTION

The current distribution of African swine fever (ASF) extends across more than 50 countries in three continents (Africa, Asia and Europe). Several incursions of ASF out of Africa were reported between the 1960s and 1970s. In 2007, ASF was introduced into Georgia, from where it spread to neighbouring countries including the Russian Federation. From there ASF spread to eastern European countries extending westwards and reaching the European Union in 2014. Further westward and southern spread in Europe has occurred since that time. In all these countries, both hosts – domestic pig and wild boar – were affected by the disease. In August 2018, the People's Republic of China reported its first outbreak of ASF and further spread in Asia has occurred. ASF was identified on the island of Hispaniola (Haiti and the Dominican Republic) in 2021. See WAHIS (https://wahis.woah.org/#/home) for recent information on distribution at the country level.

ASF virus (ASFV) is a complex large, enveloped DNA virus with icosahedral morphology. It is currently classified as the only member of the *Asfaviridae* family, genus *Asfivirus* (Dixon *et al.*, 2005). More than 60 structural proteins have been identified in intracellular virus particles (200 nm) (Alejo *et al.,* 2018). More than a hundred infection-associated proteins have been identified in infected porcine macrophages, and at least 50 of them react with sera from infected or recovered pigs (Sánchez-Vizcaíno & Arias, 2012). The ASFV double-stranded linear DNA genome comprises between 170 and 193 kilobases (kb) and contains between 150 and 167 open reading frames with a conserved central region of about 125 kb and variable ends. These variable regions encode five multigene families that contribute to the variability of the virus genome. The complete genomes of several ASFV strains have been sequenced (Bishop *et al.,* 2015; Chapman *et al.,* 2011; de Villiers *et al.,* 2010; Portugal *et al.,* 2015). Different strains of ASFV vary in their ability to cause disease, but at present there is only one recognised serotype of the virus detectable by antibody tests.

The molecular epidemiology of the disease is investigated by sequencing of the 3' terminal end of the B646L open reading frame encoding the p72 protein major capsid protein, which differentiates up to 24 distinct genotypes (Achenbach *et al.*, 2017; Boshoff *et al.,* 2007; Quembo *et al.* 2018). To distinguish subgroups among closely related ASFV, sequence analysis of the tandem repeat sequences (TRS), located in the central variable region (CVR) within the B602L gene (Gallardo *et al.,* 2009; Lubisi *et al.,* 2005; Nix *et al.,* 2006) and in the intergenic region between the I73R and I329L genes, at the right end of the genome (Gallardo *et al.,* 2014), is undertaken. Several

other gene regions such as the E183L encoding p54 protein, the CP204L encoding p30 protein, and the protein encoded by the EP402R gene (CD2v), have been proved as useful tools to analyse ASFVs from different locations and hence track virus spread.

ASF viruses produce a range of syndromes varying from peracute, acute to chronic disease and subclinical infections. Pigs are the only domestic animal species that is naturally infected by ASFV. European wild boar and feral pigs are also susceptible to the disease, exhibiting clinical signs and mortality rates similar to those observed in domestic pigs. In contrast African wild pigs such as warthogs (*Phacochoerus aethiopicus*), bush pigs (*Potamochoerus porcus*) and giant forest hogs (*Hylochoerus meinertzhageni*) are resistant to the disease and show few or no clinical signs. These species of wild pig act as reservoir hosts of ASFV in Africa (Costard *et al.,* 2013; Sánchez-Vizcaíno *et al.,* 2015).

The incubation period is usually 4–19 days. The more virulent strains produce peracute or acute haemorrhagic disease characterised by high fever, loss of appetite, haemorrhages in the skin and internal organs, and death in 4–10 days, sometimes even before the first clinical signs are observed. Case fatality rates may be as high as 100%. Less virulent strains produce mild clinical signs – slight fever, reduced appetite and depression – which can be readily confused with many other conditions in pigs and may not lead to suspicion of ASF. Moderately virulent strains are recognised that induce variable disease forms, ranging from acute to subacute. Low virulence, non-haemadsorbing strains can produce subclinical non-haemorrhagic infection and seroconversion, but some animals may develop discrete lesions in the lungs or on the skin in areas over bony protrusions and other areas subject to trauma. Animals that have recovered from either acute, subacute or chronic infections may potentially become persistently infected, acting as virus carriers. The biological basis for the persistence of ASFV is still not well understood, nor it is clear what role persistence plays in the epidemiology of the disease.

ASF cannot be differentiated from classical swine fever (CSF) by either clinical or post-mortem examination, and both diseases should be considered in the differential diagnosis of any acute febrile haemorrhagic syndrome of pigs. Bacterial septicaemias may also be confused with ASF and CSF. Laboratory tests are essential to distinguish between these diseases.

In countries free from ASF but suspecting its presence, the laboratory diagnosis must be directed towards isolation of the virus by the inoculation of pig leukocyte or bone marrow cultures, the detection of genomic DNA by polymerase chain reaction (PCR) or the detection of antigen in smears or cryostat sections of tissues by direct fluorescent antibody test (FAT). Currently the PCR is the most sensitive technique and can detect ASFV DNA from a very early stage of infection in tissues, ethylene diamine tetra-acetic acid (EDTA)-blood and serum samples. The PCR is particularly useful if samples submitted are unsuitable for virus isolation and antigen detection because they have undergone putrefaction. Pigs that have recovered from acute, subacute or chronic infections usually exhibit a viraemia for several weeks making the PCR test a very useful tool for the detection of ASFV DNA in pigs infected with low or moderately virulent strains. Virus isolation by the inoculation of pig leukocyte or bone marrow cultures and identification by haemadsorption tests (HAD) are recommended as a confirmatory test when ASF is positive by other methods, particularly in the event of a primary outbreak or a case of ASF.

~~As no vaccine is available, the presence of ASFV antibodies is indicative of previous infection and, as antibodies are produced from the first week of infection and persist for long periods, they are a good marker for the diagnosis of the disease, particularly in subacute and chronic forms.~~

Vaccines should be prepared in accordance with Chapter 1.1.8 *Principles of veterinary vaccine production*. Current ASF modified live virus (MLVs) vaccines are based on the live virus that have been naturally attenuated or attenuated by targeted genetic recombination through cell cultures (Gladue & Borca, 2022). MLV production is based on a seed-lot system consistent with the *European Pharmacopoeia* (11th edition) and that has been validated with respect to virus identity, sterility, purity, potency, stability, safety and immunogenicity (including spread), ~~non-transmissibility, stability and immunogenicity~~. ASF MLV first generation vaccines ~~– defined as those for which peer-reviewed publications are in the public domain –~~ should meet or exceed the minimum standards as described below. ~~Paramount~~ Demonstration of acceptable safety and efficacy against the epidemiologically relevant ASFV field strain(s) circulating in areas where the vaccine is intended for use ~~are~~ is required. At the present time, a variety of mutants (Forth *et al.,* 2023) and recombinants (Zhao *et al.,* 2023) have emerged globally, and the prevalence of these strains is increasing. In addition, there is a risk that vaccine strains will recombine with circulating strains. These conditions should be taken into account in vaccine development. ~~acceptable efficacy should be shown against the B646L (p72) genotype II pandemic virus lineage currently circulating widely in domestic pigs and wild boar.~~

ASF MLV first generation vaccines allowing the differentiation of infected animals from vaccinated animals (DIVA) by suitable methods (e.g. serology-based tests) are preferred. Demonstration of MLV safety and efficacy in pigs at different growth stages (suckling piglets, nursery pigs, fattening pigs), the safety in breeding-age boars, gilts and pregnant sows, ~~and onset and duration of protective immunity,~~ are also preferred but are not required to meet the minimum standard. Additional data will likely be required by Regulatory Authorities if these categories are included in the indications for the vaccine. Details of the onset of immunity (the interval of time elapsed between vaccination

and challenge if protection is confirmed) and the duration of immunity (the time point at which vaccine-induced immunity begins to decline and provides less protection) are also required to meet minimum standards.

Reference	Comment	Biological Standards Commission response
3.9.1_INTRO_3	**Category**: change ASF MLV first generation vaccines allowing the differentiation of infected animals from vaccinated animals (DIVA) by suitable methods (e.g. serology-based tests) are preferred. Demonstration of MLV safety and efficacy in pigs at target age and safety at different growth stages (suckling piglets, nursery pigs, fattening pigs), ~~the safety in~~ breeding-age boars, gilts and pregnant sows, ~~and onset and duration of protective immunity,~~ are also preferred but are not required to meet the minimum standard. **Reason**: The efficacy in pigs at different growth stages, breeding-age boars, gilts and pregnant sows can be preferred, but not required. Efficacy is required for the targeted pig groups because the vaccine will be used for the animals in this groups. However, the efficacy for non-targeted groups will be benefit for further improvement of the vaccine, but it is not a required condition because the vaccine does not claim to be used for the animals other than the targeted animals. But the safety in pigs at different growth stages, breeding-age boars, gilts and pregnant sows should be a required condition. Due to the evidence of horizontal spread of MLV, it is essential to demonstrate the safety of a MLV in pigs at different growth stages (suckling piglets, nursery pigs, fattening pigs), in breeding-age boars, gilts and pregnant sows, because horizontal transmission could happen from vaccinated pigs to those non-target pigs.	**EN**: Agreed in part. A section on safety testing in pregnant sows, as the population that is likely to be the most sensitive indicator of any adverse effects (one single dose for pregnant sows) has been added as a minimum standard. Safety tests for other growth stages could be undertaken as preferred. **FR**: Commentaire accepté en partie. Un paragraphe sur les essais d'innocuité chez les truies gestantes a été ajouté en tant que norme minimale, cette population étant, par sa sensibilité à d'éventuels effets indésirables, un bon indicateur (dose unique pour les truies gestantes). Des essais d'innocuité pour d'autres stades de croissance peuvent être effectués suivant les préférences. **SP**: Aceptado en parte. Se ha añadido como norma mínima una sección sobre pruebas de seguridad en cerdas gestantes, ya que es la población que probablemente sea el indicador más sensible de cualquier efecto adverso (una sola dosis para cerdas gestantes). Podrían realizarse pruebas de seguridad para otras etapas de crecimiento según se prefiera
3.9.1_INTRO_4	**Category**: deletion "Demonstration of MLV safety and efficacy in pigs at different growth stages (suckling piglets, nursery pigs, fattening pigs), the safety in breeding-age boars… " **Comment**: We do not think this is necessary and it is confusing (as tests are recommended in 4-10 week-old piglets, therefore categories mentioned in the brackets could be deleted)	**EN**: Agreed. **FR**: La Commission a souscrit au commentaire. **SP**: Aceptado.
3.9.1_INTRO_5	**Category**: Editorial *….if ~~these~~ specific categories are included….*	**EN**: Agreed. **FR**: La Commission a souscrit au commentaire. **SP**: Aceptado.
3.9.1_INTRO_6	**Category**: change *"….(the time point at which vaccine-induced immunity begins to decline and provides less protection)…."* **comment**: In our opinion this sentence is not accurate, and we propose to change as follow:	**EN**: Agreed. **FR**: La Commission a souscrit au commentaire. **SP**: Aceptado.

Reference	Comment	Biological Standards Commission response
	"Last time-point at which vaccine-induced immunity ~~begins to decline and provides less protection~~ has been demonstrated."	

ASF epidemiology is complex with different epidemiological patterns of infection occurring in Africa and Europe. ASF occurs through transmission cycles involving domestic pigs, wild boar, wild African suids, and soft ticks (Sánchez-Vizcaíno *et al.,* 2015). In regions where *Ornithodoros* ~~soft bodied~~ ticks are present, the detection of ASFV in these reservoirs of infection contributes to a better understanding of the epidemiology of the disease. This is of major importance in establishing effective control and eradication programmes (Costard *et al*., 2013).

ASF is not a zoonotic disease and does not affect public health (Sánchez-Vizcaíno *et al*., 2009).

ASFV should be handled with an appropriate level of bio-containment, determined by risk analysis in accordance with Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*.

...

C. REQUIREMENTS FOR VACCINES ~~-~~ [UNDER REVIEW]

~~At present there is no commercially available vaccine for ASF.~~ Commercially produced modified live virus vaccines are being evaluated and licensed for field use.

3.9.1_C_1	**Category**: editorial **Lines 135-136**: This text should be aligned with the information given in lines 162-163 about currently authorised vaccines and others under development.	**EN**: Comment not clear and no rationale provided. Changes have been made that we hope resolve this.
		FR: Ce commentaire manque de clarté et aucune justification n'est fournie. Des modifications ont été introduites par la Commission dans l'espoir de résoudre la question.
		SP: Comentario poco claro y sin justificación. Se han introducido cambios que esperamos resuelvan este problema.

1. Background

The ASF p72 genotype II strains (ASFV Georgia 2007/1 lineage) (NCBI, 2020) are recognised to be the current highest global threat for domestic pig production worldwide (Penrith *et al.*, 2022). However, genotype I attenuated strains and genotype I/II recombinant strains have been reported to be circulating. In Africa, multiple genotypes are circulating.

Reference	Comment	Biological Standards Commission response
3.9.1_C1_1	**Category**: change However, genotype I low virulent ~~attenuated~~ strains and genotype I/II recombinant strains have been reported to be circulating. In Africa, multiple genotypes are circulating. **Reason**: The term "attenuated" better applies to a laboratory procedure that weakens a pathogen. The term "low virulent" is better used when referring to a natural product.	**EN**: Agreed. **FR**: La Commission a souscrit au commentaire. **SP**: Aceptado.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of Veterinary Vaccine Production*. Varying additional requirements relating to quality (including purity and potency), safety, and efficacy will apply in particular countries or regions for manufacturers to comply with local regulatory requirements.

Wherever live, virulent ASFV or ASF MLVs are stored, handled and disposed, the appropriate biosecurity level, procedures and practices should be used. The ASF MLV vaccine production facility should meet the requirements for containment outlined in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*.

An optimal ASF MLV first generation vaccine for the target host should have the following general characteristics (minimum standards):

- Safe: demonstrate absence of persistent fever as defined below (see Section 2.3.2) and clinical signs of acute or chronic ASF in vaccinated and in-contact animals, minimal and ideally no vaccine virus transmission, and absence of an increase in virulence (genetic and phenotypic stability);
- Efficacious: protects against mortality, reduces acute disease (fever accompanied by the appearance of clinical signs caused by ASF) and ~~reduces vertical (boar semen and placental) and~~ horizontal disease transmission;

Reference	Comment	Biological Standards Commission response
3.9.1_C1_2	**Category**: addition and ~~reduces vertical (boar semen and placental) and~~ reduces vertical (boar semen and placental) and horizontal disease transmission. **Reason**: The term “attenuated” better applies to a laboratory p This is an important consideration for field use, especially if vaccine virus is transmitted to wild suids. Certain strains of vaccine virus have been shown to revert to virulence and be transmissible to non-vaccinates. If used in a domestic swine population, vaccine virus could theoretically transmit to wild swine populations, revert to virulence, and cause disease where none was present before. In regard to vertical transmission in wild suids, this could present a long-term problem due only to vaccine virus usage. This is a major concern when considering vaccination before an outbreak vs vaccination in response to a natural outbreak. Procedure that weakens a pathogen. The term “low virulent” is better used when referring to a natural product.	**EN**: Agreed. **FR**: La Commission a souscrit au commentaire. **SP**: Aceptado.

- Quality – purity: free from wild-type ASFV and extraneous microorganisms that could adversely affect the safety, potency or efficacy of the product;

Reference	Comment	Biological Standards Commission response
3.9.1_C1_3	**Category**: deletion ….~~*that could adversely affect the safety, potency or efficacy of the product:*~~ **Comment**: This text is not needed and can be deleted. If left, please delete ‘potency’ because this word is mostly linked to efficacy of the vaccine so using both is redundant.	**EN**: Agreed to remove “potency”, but maintain the rest of the text because the presence of extraneous microorganisms or wildtype ASFV can impact the efficacy and safety **FR**: La Commission a accepté la suppression de « *potency* » (activité), mais le reste de la phrase est maintenu car la présence de micro-organismes adventices ou de souches sauvages du virus de la PPA peut avoir une incidence sur l’efficacité et la sécurité. **SP**: Se acepta suprimir «potencia», pero mantener el resto del texto, ya que la presencia de microorganismos extraños o de virus de la peste porcina africana de tipo salvaje puede afectar a la eficacia y la seguridad.

- Quality – ~~potent~~ stability: the ~~log10~~ virus titre maintained throughout the vaccine shelf life that guarantees the efficacy demonstrated by the established minimum immunising (protective) dose.
- ~~Identity~~ Vaccine matching: based on the capacity to protect against the ASFV B646L (p72) genotype II pandemic strain or other p72 genotypes of recognised epidemiologic importance.

Vaccine production should be carried out using a validated, controlled and consistent manufacturing process.

ASF MLV first generation vaccines must be safe (i.e. an acceptable safety profile) for non-target species and the environment in general.

Ideally, ASF MLV first generation vaccines that meet the minimum standards should also fulfil the following additional general characteristics: i) prevents acute and persistent ~~(carrier state)~~ disease; ii) prevents horizontal and vertical disease transmission; iii) induces rapid protective immunity (e.g. < 2 weeks); and iv) confers stable, life-long immunity.

Furthermore, ASF MLV second and future generation vaccines should meet the minimum safety and efficacy standards as ASF MLV first generation vaccines, and ideally provide additional product profile benefits, including but not limited to: i) contain a negative marker allowing the differentiation of infected from vaccinated animals (DIVA) by reliable discriminatory tests such as serology-based tests; and ii) confer broad range of protection against other p72 genotype field strains of varying virulence (low, moderate, and high).

The majority of ASF global vaccine research groups and companies are currently focused on ASF MLV first generation vaccine candidates that are safe and efficacious against ASF viruses belonging to the ASFV p72 genotype II pandemic strain (ASFV Georgia 2007/1 lineage) (NCBI, 2020). More research is needed to determine whether these genotype II-specific MLVs can effectively protect against newly circulating variants of genotype II and recombinant strains.

Reference	Comment	Biological Standards Commission response
3.9.1_C1_4	**Category**: deletion The majority of ASF global vaccine research groups and companies are currently focused on ASF MLV first generation vaccine candidates ~~that are safe and efficacious~~ against ASF viruses belonging to the ASFV p72 genotype II pandemic strain (ASFV Georgia 2007/1 lineage) (NCBI, 2020). **Reason**: Certain strains of vaccine virus have been shown to revert to virulence and be transmissible to non-vaccinates. The "current focus" is on a strain that was shown to revert to virulence in certain conditions. The deletion makes this chapter more adaptable as further scientific data is generated/published.	**EN**: Agreed. **FR**: La Commission a souscrit au commentaire. **SP**: Aceptado.

Currently, two recombinant gene deleted MLV ~~recombinant~~ vaccines (ASFV-G-ΔI177L and ASFV-G-ΔMGF) have been licenced ~~for field use~~ in Vietnam for use in domestic pigs ~~following supervised field testing to evaluate the safety and effectiveness of several vaccine batches~~.

There are numerous, promising ASF MLV vaccine candidates targeting the p72 genotype II pandemic strain under development, including:

- A naturally attenuated field strain (Lv17/WB/Rei1) (Barasona *et al.*, 2019) being developed as an oral bait vaccine for wild boar~~s~~;
- A laboratory thermo-attenuated field strain (ASFV-989) (Bourry *et al.*, 2022);
- Single gene-deleted, recombinant viruses (e.g. SY18ΔI226R, ASFV-G-ΔA137R) (Gladue *et al.*, 2021; Zhang *et al.*, 2021);
- Double gene-deleted, recombinant viruses (e.g. ASFV-G-Δ9GL/ΔUK; ASFV-SY18-ΔCD2v/UK; Arm-ΔCD2v-ΔA238L) (O'Donnell *et al.*, 2016; Pérez-Núñez *et al.*, 2022; Teklue *et al.*, 2020);

- Multiple gene-deleted, recombinant viruses (ASFV-G-ΔI177L/ΔLVR; ASFV-G-ΔMGF; ~~BA71ΔCD2;~~ HLJ/18-7GD; ASFVGZΔI177LΔCD2vΔMGF) (Borca *et al.*, 2021; Chen *et al.*, 2020; Kitamura *et al.*, 2023; Liu *et al.*, 2023; Monteagudo *et al.*, 2017; O'Donnell *et al.*, 2015).

Reference	Comment	Biological Standards Commission response
3.9.1_C1_5	**Category**: addition Multiple gene-deleted, recombinant viruses (ASFV-G-ΔI177L/ΔLVR; ASFV-G-ΔMGF; ~~BA71ΔCD2;~~ HLJ/18-7GD; ASFVGZΔI177LΔCD2vΔMGF; Arm07ΔMGF) (Borca *et al.,* 2021; Chen *et al.,* 2020; Kitamura et al., 2023; Liu *et al.*, 2023; Monteagudo *et al.*, 2017; O'Donnell *et al.,* 2015) **Reason**: We request the inclusion of the strain name, which was mistakenly not included in the latest draft despite BSC's previous agreement to include it. Our country's research has led to the development of a vaccine candidate strain with a larger gene deletion than those referenced in the current draft. This strain has shown strong viremia suppression, suggesting higher safety levels than previous strains. We propose adding this strain as an example because the original paper is already referenced. **Reference:** https://pubmed.ncbi.nlm.nih.gov/36851524/#full-view-affiliation-1	**EN**: Agreed. **FR**: La Commission a souscrit au commentaire. **SP**: Aceptado.

Information regarding many of these MLV vaccine candidates can be found in a recent review publication (Brake, 2022).

Different DIVA strategies using serological methods (e.g. ELISA) or genome detection methods (e.g. differential real-time PCR) are not widely available for these ASF MLV first generation vaccine candidates. Therefore, there is still room for improvement with respect to marker vaccines and their companion diagnostic tests.

Inactivated (non-replicating) whole virus vaccines are not presently available and may be difficult to develop to meet minimum efficacy standards. Recombinant vectored, subunit vaccine candidates that can be produced in scalable vaccine platform expression systems and mRNA-based ASF vaccines are being evaluated in ongoing laboratory research, testing and evaluation in experimental challenge models. The publicly available *Center of Excellence for African Swine Fever Genomics* (ASFV Genomics, 2022[1]) that provides the structural protein predictions for all 193 ASFV proteins may help accelerate ASF first and second generation vaccine research and development.

Reference	Comment	Biological Standards Commission response
3.9.1_C1_6	**Category**: addition As most Members have ratified the Cartagena Protocol on Biosafety, they are required to adhere to its regulations concerning genetically modified organisms (GMOs) handling. Therefore, we request this paragraph include a reference to Chapter 1.1.8 section 7.2.3.2. to highlight that the field use of genetically modified ASFV strains with marker genes, regardless of the presence or degree of its virulence, should be compliant with the Cartagena Protocol's regulations for conserving biodiversity. **Rationale:** In this section, it is indicated that specific attenuated strains of ASFV, which are genetically modified for use in vaccines, contain inserted exogenous genes coding for fluorescent proteins or enzymes. These genes serve as markers during production and purification of the ASFV strains. It is	**EN**: Agreed. **FR**: La Commission a souscrit au commentaire. **SP**: Aceptado.

[1] http://asfvgenomics.com. Accessed 4/4/2023.

	crucial to emphasise that deploying such GMOs in the field without assessing their environmental impact contravenes the Cartagena Protocol on Biosafety, which is designed to protect biodiversity in the environment. Thus, this issue should be highlighted as a significant point to be noted.	

Any future use of vaccine candidates should be based on a thorough risk–benefit assessment considering all safety and efficacy features, as well as the potential vaccination scenario. Fit-for-purpose vaccine use scenarios matched to the intended use in a domestic pig-specific type of production system may require different vaccine product profiles or may influence the focus of essential versus ideal vaccine requirements. Prudent use of ASF MLVs as part of strict, controlled vaccination programmes, especially in the areas where ASF is not prevalent, should be implemented.

Reference	Comment	Biological Standards Commission response
3.9.1_C1_7	**Category**: addition Prudent use of ASF MLVs as part of strict, controlled vaccination programmes, especially in the areas where ASF is not prevalent, should be implemented. Transmission of vaccine virus to non-vaccinates (domestic or wildlife) is particularly problematic in areas where ASF is not present. **Reason**: Certain strains of vaccine virus have been shown to revert to virulence and be transmissible to non-vaccinates. If used in a domestic swine population, vaccine virus could theoretically transmit to wild swine populations, revert to virulence, and cause disease where none was present before. This is a major concern when considering vaccination before an outbreak vs. vaccination in response to a natural outbreak.	**EN**: Agreed, but changed "is" to "could be" **FR**: Commentaire accepté, mais en remplaçant « *is* » (est) par « *could be* » (pourrait être). **SP**: Aceptado, pero se ha cambiado «es» por «podría ser».

It is important to know what genotypes of ASFV are circulating in a population before vaccination is introduced. Due to the potential risk of recombination events between circulating low and high virulent field strains with future licensed vaccine strains, and the possibility of reversion to virulence of vaccine strains, strict pharmacovigilance post-vaccination is essential. Field pharmacovigilance data should be collected and analysed during vaccination campaigns using ASF MLV first generation vaccines post-licensing. Active post-vaccination surveillance programmes for the detection of new ASF viruses that may arise from MLV vaccine strains and naturally circulating wild-type virus recombination, as well as revertant vaccine strains, should be implemented. It is also recommended that vaccine manufacturers carry out laboratory experiments to further evaluate the risk of vaccine virus recombination with field and vaccine strains.

Reference	Comment	Biological Standards Commission response
3.9.1_C1_8	**Category**: addition Due to the potential risk of recombination events between circulating low and high virulent field strains with future licensed vaccine strains, and the possibility of reversion to virulence of vaccine strains, strict pharmacovigilance using standard reporting criteria (e.g. any fever is reportable – in contrast to the safety testing criteria below of 3 days of fever) post-vaccination is essential. Field pharmacovigilance data should be collected and analysed during vaccination campaigns using ASF MLV first generation vaccines post-licensing. Active post-vaccination surveillance programmes for the detection of new ASF viruses that may arise from MLV vaccine strains and naturally circulating wild-type virus recombination, as well as revertant vaccine strains, should be implemented. It is also recommended that vaccine manufacturers carry out laboratory experiments to further evaluate the risk of	**EN**: Agreed. **FR**: La Commission a souscrit au commentaire. **SP**: Aceptado.

Reference	Comment	Biological Standards Commission response
	vaccine virus reversion to virulence and/or recombination with field and vaccine strains. **Reason**: Given the looser criteria proposed in other sections of this document for adverse event reporting in licensure studies – e.g. the safety testing criteria – it is worthwhile to note criteria for "strict" pharmacovigilance that is noted as essential to avoid confusion in reporting.	

As with any ~~MLV~~ vaccine, all ASF ~~MLV~~ vaccines should be used according to the label instructions, under the strict control of the country's Regulatory Authority.

The minimum standards given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national, regional, and veterinary international medicinal product harmonised requirements. Minimum data requirements for an authorisation in exceptional circumstances (e.g. unexpected introduction of the virus, sudden outbreaks of the disease) should be considered where applicable.

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed virus

2.1.1. Biological characteristics of the master seed virus

ASF MLVs are generally produced from ASFV field strains derived from naturally attenuated field isolates or using DNA homologous (genetically targeted) recombination techniques in cell cultures to delete one or more ASFV genes or gene families. These molecular techniques typically involve replacement of the targeted ASFV gene(s) with one or more positive, marker fluorescent (e.g. BFP, eGFP, mCherry) or enzyme-based (e.g. β-glucuronidase) ASFV promoter-reporter gene systems that allow the use of imaging microscopy or flow cytometry to visualise, select, and clone gene-deleted, recombinant, ASF MLVs. MLV production is carried out in cell cultures based on a seed-lot system.

Master seed viruses (MSVs) for MLVs should be selected and produced based on their ease of growth in cell culture, virus yield (log_{10} infectious titre) and genetic stability over multiple cell passages. Preferably, a continuous well-characterised cell line (e.g. ZMAC-4; PIPEC; IPKM) (Borca *et al.*, 2021; Masujin *et al.*, 2021; Portugal *et al.*, 2020) is used to produce a master cell bank (MCB) on which the MSV and MSV-derived working seed virus (WSV) can be produced. The exact source of the underlying ASFV isolate, the whole genome sequence, and the passage history must be recorded.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

Only MSVs that have been established as sterile, pure (free of wild-type parental virus and free of extraneous agents as described in Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*, and those listed by the appropriate licensing authorities) and immunogenic, should be used as the vaccine virus (WSV and vaccine batch production). ~~Live vaccines must be shown not to cause disease or other adverse effects in target animals in accordance with chapter 1.1.8, Section 7.1 *Safety tests* (for live attenuated MSVs), that includes target animal safety tests, increase in virulence tests, assessing the risk to the environment) and if possible, no transmission to other animals.~~

Identity of the MSV must be confirmed using appropriate methods (e.g. through the use of vaccine strain-specific whole genome detection methods such as next generation sequencing).

Demonstration of MSV stability over several cell passages is necessary, typically through at least five passages (e.g. MSV+5). For those MLV vaccines for which attenuation is linked to specific characteristics (gene deletion, gene mutations, etc.), genetic stability of attenuation throughout the production process should be confirmed by full genome sequencing and confirmation of the vaccine phenotype, for example, by confirming the virus titre obtained by growth in the cell line used for production ~~using suitable methods. Suitable techniques to demonstrate genetic stability may include but are not limited to: genome sequencing, biochemical, proteomic, genotypic (e.g. detection of genetic markers) and phenotypic strain characterisation~~. If final product yields (infectious titres) are relatively low, as is typically the case with ASFV, demonstration of stability

is required for the maximum passage for use in the final product manufacturing as defined by the producer~~ genetic stability at a minimum of MSV+10 should be demonstrated to allow more flexibility in the outline of production. For example, if MSV+8 is the maximum passage for use in final product manufacturing, demonstration of genetic stability to at least MSV+10 is warranted~~.

2.1.3. Validation as a vaccine strain

The vaccine derived from the MSV must be shown to be satisfactory with respect to safety and efficacy.

Reference	Comment	Biological Standards Commission response
3.9.1_C2.1.3_1	**Category**: addition The vaccine derived from the MSV must be shown to be satisfactory with respect to safety and efficacy. Live vaccines must be shown not to cause disease or other adverse effects in target animals in accordance with chapter 1.1.8, Section 7.1 *Safety tests* (for live attenuated MSVs), that includes target animal safety tests, increase in virulence tests, assessing the risk to the environment) and if possible, no transmission to other animals. **Reason**: This text deleted above is appropriate here.	**EN**: Agreed. **FR**: La Commission a souscrit au commentaire. **SP**: Aceptado.

Even if pigs are not known for susceptibility to transmissible spongiform encephalopathy (TSE) agents, consideration should also be given to minimising the risk of TSE transmission by ensuring that animal origin materials from TSE-relevant species, if no alternatives exist for vaccine virus propagation, comply with the measures on minimising the risk of transmission of TSE.

Ideally, the vaccine virus in the final product should generally not differ by more than five passages from the master seed lot.

ASF vaccines should be presented in a suitable pharmaceutical form (e.g. lyophilisate or liquid form).

2.2. Method of manufacture

2.2.1. Procedure

The MLV ~~virus~~ is used to infect swine primary cell cultures obtained from specific-pathogen free pigs, the requirements for which are defined in specific monographs (Chapter 2.3.3 *Minimum requirements for the organisation and management of a vaccine manufacturing facility*, Section 2.4.2). Compared with primary cell cultures, use of a continuous cell line generally allows for more consistency, higher serial volumes in manufacturing and aligns better with a seed lot system. Thus, preferably a master cell bank based on an established, continuous cell line shown to support genetically stable ASFV replication and acceptable titres over several passages should be used.

Reference	Comment	Biological Standards Commission response
3.9.1_C2.2.1_1	**Category**: addition The MLV ~~virus~~ is used to infect swine primary cell cultures obtained from specific-pathogen free pigs, the requirements for which are defined in specific monographs (Chapter 2.3.3 *Minimum requirements for the organisation and management of a vaccine manufacturing facility*, Section 2.4.2). It should be noted that each donor pig should be considered a different "master cell stock" and be tested for purity and extraneous agents to account for the risk of contamination during cell collection and processing. Similar considerations should apply to collections over	**EN**: Partly agreed: accepted first sentence and modified second sentence for clarity **FR**: Commentaire retenu en partie : première phrase acceptée, deuxième phrase modifiée pour plus de clarté. **SP**: Parcialmente aceptado: aceptada la primera frase y

	time at the same time that herd health is closely monitored. Compared... **Reason**: Good to note limitations of primary cell culture production methods.	modificada la segunda para mayor claridad.

Cell cultures shall comply with the requirements for cell cultures for production of veterinary vaccines in chapter 1.1.8. Regardless of the production method, the substrate should be harvested under aseptic conditions and may be subjected to appropriate methods to release cell-associated virus (e.g. freeze–thaw cycles, detergent lysis). The harvest can be further processed by filtration and other purification methods. A stabiliser or other excipients may be added as appropriate. The vaccine is homogenised to ensure a uniform batch/serial.

2.2.2. Requirements for ingredients

All ingredients used for vaccine production should be in line with requirements in chapter 1.1.8.

2.2.3. In-process controls

In-process controls will depend on the protocol of production; they include virus titration of bulk antigen and sterility tests.

Reference	Comment	Biological Standards Commission response
3.9.1_C2.2.3_1	**Category**: addition A description of the required biosafety level for handling ASF vaccine strains during in-process quality control should be included in this chapter. **Rationale:** There is currently no description of the biosafety level in the production process in which the viruses are handled. Given varying national regulations, it may be difficult to specify the biosafety level applicable to all Members. Hence, at least this chapter should provide a guidance on biosafety requirements.	**EN**: Partly agreed, WOAH no longer uses biosafety levels. Amended the text to refer to the outcome of biosafety risk assessments in line with WOAH Standards. Please also see lines 130–132 and 145–148 above.
		FR: Commentaire accepté en partie, car l'OMSA ne se réfère plus aux niveaux de biosécurité. Le texte a été amendé afin de renvoyer aux résultats des évaluations des risques de biosécurité, conformément aux normes de l'OMSA. Voir également les lignes 130–132 et 145–148 ci-dessus.
		SP: Parcialmente aceptado, la OMSA ya no utiliza niveles de bioseguridad. Se ha modificado el texto para hacer referencia al resultado de las evaluaciones de riesgos de bioseguridad de acuerdo con las normas de la OMSA. Véanse también las líneas 130–132 y 145–148.

2.2.4. Final product batch tests

i) Sterility

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9.

ii) Identity

Appropriate methods such as specific genome detection methods (e.g. specific differential real-time PCR) should be used for confirmation of the identity of the vaccine virus and differentiation from the parent strain of the virus as a potential contaminant.

iii) Purity

Appropriate methods should be used to ensure that the final product batch does not contain any residual wild-type ASFV.

iv) Safety

Batch safety testing is to be carried out unless consistent safety of the product is demonstrated and approved in the registration dossier and the production process is approved for consistency in accordance with the standard requirements referred to in chapter 1.1.8.

v) Batch/serial potency

Virus titration is a reliable indicator of vaccine potency once a relationship has been established between the vaccine minimum immunising dose (MID) (minimum protective dose) and titre of the modified live vaccine in vitro. In the absence of a demonstrated correlation between the virus titre and protection, an efficacy test will be necessary (Section C.2.3.3 *Efficacy requirements*, below).

vi) Residual humidity/residual moisture

The test should be carried out consistent with VICH[2] GL26 (*Biologicals: Testing of Residual Moisture*, 2003[3]). Required for MLV vaccines presented as lyophilisates ~~for suspension for injection~~.

2.3. Requirements for authorisation/registration/licensing

2.3.1. Manufacturing process

For regulatory approval of a vaccine, all relevant details concerning history of the pre-MSV, preparation of MSV, manufacture of the vaccine and quality control testing (Sections C.2.1 *Characteristics of the seed* and C.2.2 *Method of manufacture*) should be submitted to the authorities.

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.1_1	**Category**: deletion ~~For regulatory approval of a vaccine,~~ All relevant details... **Reason**: WOAH chapters should not define acceptance criteria for different regulatory authorities all over the world.	**EN**: Agreed. **FR**: La Commission a souscrit au commentaire. **SP**: Aceptado.

Information shall be provided from three preferably consecutive vaccine batches originating from the same MSV and representative of routine production~~, with a volume not less than 1/10, and more preferably with a volume not less than 1/3 of the typical industrial batch volume~~. The in-process controls are part of the manufacturing process.

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.1_2	**Category**: addition Suggest reversing the deletion above as this is a good general guideline. If modification is needed, suggest adding "if volumetric standards cannot be met, explain how scale up will be addressed in production."	**EN**: Disagreed to this addition as it is not in line with the VICH guidelines. **FR**: La Commission n'a pas souscrit à cette proposition d'ajout qui n'est pas conforme aux lignes directrices du VICH. **SP**: No aceptado con este añadido ya que no está en consonancia con las directrices de la VICH.

[2] VICH: International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medical Products
[3] https://www.ema.europa.eu/en/documents/scientific-guideline/vich-gl26-biologicals-testing-residual-moisture-step-7_en.pdf

2.3.2. Safety requirements

For the purpose of gaining regulatory approval, the following safety tests should be performed satisfactorily.

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.2_1	**Category**: deletion For the purpose of gaining regulatory approval, the following safety tests should be performed ~~satisfactorily~~. This is tacit and stating that it must be satisfactory weakens the directive to perform the tests.	**EN**: Agreed. **FR**: La Commission a souscrit au commentaire. **SP**: Aceptado.

As a minimum standard, vaccines should be tested for any pathogenic effects on healthy domestic pigs of the target age intended for use. Additional demonstration of MLV safety in breeding age gilts and pregnant sows is preferred ~~but not required as a minimum standard~~. If in the future a vaccine intended for use in breeding animals is developed, an evaluation of the impact of the vaccine on reproductive performance will be a standard safety requirement.

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.2_2	**Category**: additions **Lines 239-241:** This is not clear. Our general requirements (Ph. Eur. 0062) for example are that vaccine production is not undertaken using a virus more than 5 passages from the master seed lot, unless otherwise justified. In any case, demonstration of genetic stability is always required to the maximum passage level used for vaccine production'; therefore, the requirement explained here is a general requirement rather than a special requirement. An alternative wording is proposed: "Genetic stability of attenuation throughout the production process (i.e. to the maximum passage level to be used for vaccine production) should be confirmed by full genome sequencing and confirmation of virus phenotype (e.g. virus yield in cell line used for production)".	**EN**: Agreed. **FR**: La Commission a souscrit au commentaire. **SP**: Aceptado.
3.9.1_C2.3.2_3	**Lines 239-241:** *"Additional demonstration……..safety requirement"*. New proposed text: the text below would be preferable for clarity. Also, the existing vaccines in Vietnam could be authorised for use in breeding animals in the future, if relevant supporting safety data are presented to the authorities i.e. use in breeding animals not only for newly developed vaccines) Proposed text: "Additional demonstration of MLV safety in breeding age gilts and pregnant sows is preferred. When the vaccine is recommended for use or may be used ~~but not required as a minimum standard. If in the future a vaccine intended for use~~ in breeding animals ~~is developed~~, an evaluation of the impact of the vaccine on reproductive performance will be a standard safety requirement.	**EN**: Agreed. **FR**: La Commission a souscrit au commentaire. **SP**: Aceptado.

i) Safety in young animals

Carry out the test by each recommended route of administration using, in each case, piglets a minimum of ~~6~~ 4 weeks old and not older than 10-weeks old.

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.2_4	**Category**: change **"….***piglets a minimum of 4 weeks old or not older than 10 weeks old"* Our requirements for example are for general safety tests to be conducted in the most sensitive category of animals for which the vaccine is recommended, usually animals of the youngest age. Is there any reason as to why a prescriptive age range (4–10 weeks of age) has been defined?	**EN**: It is stated above that 'as a minimum standard, vaccines should be tested for any pathogenic effects on healthy domestic pigs of the target age intended for use'. During the consultation process it was agreed by experts and regulatory authorities to provide details here for safety testing in young pigs as this would be the age-group of pigs that would most likely be vaccinated.
		FR: Le texte précise ci-dessus que dans la norme minimale, les vaccins devraient être testés afin de rechercher l'apparition d'un effet pathogène chez des porcs sains appartenant à la classe d'âge visée. Lors du processus de consultation, les experts et les autorités réglementaires ont estimé nécessaire de fournir des informations détaillées concernant les tests d'innocuité chez les porcelets, cette classe d'âge étant celle qui aurait la plus grande probabilité d'être vaccinée.
		SP: Más arriba se afirma que como norma mínima, las vacunas deben probarse para detectar todo posible efecto patógeno en cerdos domésticos sanos de la edad prevista para su uso». Durante el proceso de consulta, los expertos y las autoridades reguladoras acordaron proporcionar detalles para las pruebas de seguridad en cerdos de corta edad, ya que este sería el grupo de edad de los cerdos que con mayor probabilidad serían vacunados.

The test is conducted using no fewer than eight healthy piglets, and preferably no fewer than ten healthy piglets.

Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine.

Administer to each piglet a quantity of the vaccine virus equivalent to not less than ten times the maximum virus titre (e.g. 50% haemadsorption dose [HAD_{50}], 50% tissue culture infective dose [$TCID_{50}$]~~, quantitative PCR, etc.~~) (maximum release dose) likely to be contained in one dose of the vaccine.

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.2_5	**Category**: addition Suggest reversing this deletion as some jurisdictions may choose to use qPCR for potency testing.	**EN**: Disagreed, quantitative PCR does not equate to infectious virus. It is important to look for the presence live virus.
		FR: Le Commission n'a pas souscrit à ce commentaire car une PCR quantitative ne signifie pas virus infectieux. Il est important de rechercher la présence du virus vivant.
		SP: No aceptado, la PCR cuantitativa no equivale a virus infeccioso. Es importante buscar la presencia de virus vivo.
3.9.1_C2.3.2_6	**Category**: change This sentence is hard to parse. The phrase "equivalent to not less than" is confusing and having two parenthetical statements in the middle of the sentence fragments the ideas it's trying to relate. We suggest rewording to: "Administer to each piglet a quantity of the vaccine virus ~~equivalent to~~ not less than ten times the maximum virus titre ~~(e.g. 50%~~	**EN**: Agreed.
		FR: La Commission a souscrit au commentaire.
		SP: Aceptado.

	~~haemadsorption dose [HAD$_{50}$], 50% tissue culture infective dose [TCID$_{50}$], quantitative PCR, etc.) (maximum release dose)~~ likely to be contained in one dose of the vaccine." Also suggest either moving the parentheses to the end of the sentence, moving the information in the parentheses into a second sentence, or removing the parenthetical statements altogether.	

To obtain individual and group mean baseline temperatures, the body temperature of each ~~vaccinated~~ piglet is measured on at least the 3 consecutive days preceding administration of the vaccine.

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.2_7	**Category**: change This doesn't flow from the previous sentence at all - there has been no mention of taking temperatures before this point. As written, it is unclear if taking temperature is required, only that if they are taken it must be measured in the prescribed way.	**EN**: Agreed, amended for clarity and moved the sentence.
		FR: Commentaire accepté ; le texte a été amendé pour plus de clarté et la phrase a été déplacée.
		SP: Aceptado, modificado para mayor claridad y la frase se ha trasladado.

To confirm the presence or absence of fever accompanied by acute and chronic disease, observe the piglets 4 hours after vaccination and then at least once daily for at least 45 days, preferably 60 days post-vaccination. Carry out the daily observations for signs of acute and chronic disease using a quantitative clinical scoring system adding the values for multiple clinical signs (e.g. Gallardo *et al.*, 2015a). These clinical signs should include fever, anorexia, recumbency, skin haemorrhage or cyanosis, joint swelling and necrotic lesions around the joints, respiratory distress and digestive findings).

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.2_8	**Category**: change To confirm the presence or absence of fever accompanied by acute and chronic disease, observe the piglets 4 hours after vaccination and then at least once daily for at least 45 days, preferably 60 days post-vaccination. Carry out the daily observations for signs of acute and chronic disease using a quantitative clinical scoring system adding the values for multiple... This is redundant with the start of the next paragraph (which dictates when the piglets should be euthanised). As this paragraph is longer we suggest moving the instruction for the length of observation to the next paragraph.	**EN**: Disagreed, the two paragraphs refer to two different aspects, the first refers to what timepoints the pigs should be monitored for signs of disease/clinical signs using the clinical scoring system. The second paragraph describes when the pigs should be euthanised and checked for pathology.
		FR: La Commission n'a pas souscrit à cette proposition car les deux paragraphes se réfèrent à deux aspects différents : le premier concerne les moments auxquels il convient d'examiner les porcs pour rechercher d'éventuels signes de maladie/signes cliniques en appliquant le système d'évaluation clinique. Le deuxième paragraphe décrit le moment auquel les porcs devraient être euthanasiés et soumis à un examen anatomo-pathologique.
		SP: No aceptado, los dos párrafos se refieren a dos aspectos diferentes, el primero se refiere a qué puntos de tiempo los cerdos deben ser monitoreados para detectar signos de enfermedad/signos clínicos utilizando el sistema de puntuación clínica. El segundo párrafo describe cuándo se debe practicar la eutanasia a los cerdos y comprobar si presentan signos anatomopatológicos.

At a minimum of 45 days post-vaccination, humanely euthanise all vaccinated piglets. Conduct gross pathology on spleen, lung, tonsil, and kidney tissue samples and at least three different lymph nodes (which should include lymph node closest to site of inoculation, gastrohepatic and submandibular nodes).

The vaccine complies with the test if:

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.2_9	**Category**: change The vaccine complies with this manual, not the test, reword: "The vaccine is compliant if:" Or "The vaccine is acceptable if:"	**EN**: Agreed, amended the proposal for clarity. **FR:** Commentaire accepté ; le texte proposé a été amendé pour plus de clarté. **SP**: Aceptado, se ha modificado la propuesta para mayor claridad.

- No piglet shows abnormal (local or systemic) reactions or notable signs of disease, or reaches the pre-determined humane endpoint defined in the clinical scoring system or dies from causes attributable to the vaccine;

- ~~The average body temperature increase for all vaccinated piglets (group mean) for the observation period does not exceed 1.5°C above baseline; and no individual piglet shows a temperature rise above baseline greater than 2.5°C for a period exceeding 3 days.~~

- On each day during the observation period, the maximum increase in body temperature above the baseline observed for each pig will be used to calculate the daily group mean temperature rise. This mean value should not exceed 1.5°C and no individual pig should show a rise in temperature above baseline greater than 1.5°C for a period exceeding 3 consecutive days.

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.2_10	**Category**: deletion • On each day during the observation period, the maximum increase in body temperature above the baseline observed for each pig will be recorded. ~~used to calculate the daily group mean temperature rise~~. ~~This mean value should not exceed 1.5°C and~~ No individual pig should show a rise in temperature above baseline greater than 1.5°C for a period exceeding ~~3~~ 2 consecutive days. **Reason**: These criteria are set to be acceptable for the current, most developed vaccine strain and are not objectively developed. Additionally, taking the average temperature of the group could mask individual reactions. Minimal temperature rise of 1 day was seen in safety studies - 3 days seems long based on current data. Tran *et al.,* 2022.	**EN**: Agreed. **FR**: La Commission a souscrit au commentaire. **SP**: Aceptado.
3.9.1_C2.3.2_11	**Category**: change This is an instruction that should either be included in the previous section that outlined how to collect the temperature data or rephrased to be similar to the struck-through bullet point above.	**EN**: Agreed. **FR**: La Commission a souscrit au commentaire. **SP**: Aceptado.

- No vaccinated pigs show notable signs of disease by gross pathology

ii) Safety test in pregnant sows and test for transplacental transmission

There is limited ~~currently an absence of~~ published information on ASFV pathogenesis in breeding-age gilts and in pregnant sows associated with ASFV transplacental infection and fetus abortion/stillbirth. If a label claim is pursued for use in breeding age gilts and sows, then a safety study in line with VICH GL44 (*Guidelines on Target Animal Safety for Veterinary Live and Inactivated Vaccines, Section 2.2. Reproductive Safety Test*, 2009[4]) should be completed.

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.2_12	**Category**: addition ...VICH GL44 (*Guidelines on Target Animal Safety for Veterinary Live and Inactivated Vaccines, Section 2.2. Reproductive Safety Test*, 2009) should be completed. The precedent seen with the ASFV-G-Δ9GL single-deletion mutant should be considered when planning and evaluating these studies. **Reason**: Doses of the single deletion mutant similar to that proposed for the current vaccine strains under consideration (10E4 HAD^{50}) produced a virulent phenotype in inoculated pigs. O'Donnell et al. 2015 doi:10.1128/JVI.00969-15.	**EN**: Disagreed, the Commission did not believe that the addition was essential. **FR**: La Commission n'a pas retenu ce commentaire, estimant que l'ajout proposé ne revêtait pas un caractère essentiel. **SP**: No aceptado, la Comisión no cree que el añadido sea esencial.
3.9.1_C2.3.2_13	**Category**: change "*If a label claim……..should be completed*" It is suggested to revert the order of the sentences i.e. first the general statement on requirements (if a label claim…) and then the specific info on existing published info.	**EN**: Agreed. **FR**: La Commission a souscrit au commentaire. **SP**: Aceptado.

iii) Horizontal transmission

The test is conducted using no fewer than 12 healthy piglets, a minimum of ~~6~~ 4-weeks old and not older than 10-weeks old and of the same origin, that do not have antibodies against ASFV, and blood samples are negative on real-time PCR. ~~All piglets are housed together from day 0 and the number of vaccinated animals is the same as the number of naïve, contact animals.~~ Co-mingle equal numbers of vaccinated and naïve, contact piglets from day 0 in the same pen or room.

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.2_14	**Category**: change The test is conducted using ~~no fewer than 12~~ a sufficient number of healthy piglets, a minimum of 4-weeks old and not older than 10-weeks old ~~and of the same origin~~, that do not have antibodies against ASFV, and blood samples are negative on real-time PCR. **Rationale:** Request to change "no fewer than 12" to "a sufficient number of" as there is no scientific basis for using 12 piglets. The decision to not agree with our proposal for the reason of consistency with CSF chapter in the 2024 February BSC report cannot be justified as a specific number of animals in the efficacy test is not stipulated in many other chapters (Swine Influenza, FMD, PRRS, West Nile Fever etc.). In addition, there is no specification in the VICH	**EN**: Did not agree to remove the detail of number of piglets as of use to Members, but did agree to remove "of the same origin" **FR**: La Commission n'a pas retenu la proposition de supprimer l'indication du nombre de porcelets à utiliser mais a accepté de supprimer l'indication suivant laquelle ces porcelets devaient être « de même origine ». **SP**: No se ha aceptado suprimir el detalle del número de lechones por ser de utilidad para los Miembros, pero sí «del mismo origen».

[4] https://www.ema.europa.eu/en/documents/scientific-guideline/vich-gl44-target-animal-safety-veterinary-live-inactived-vaccines-step-7_en.pdf.

Reference	Comment	Biological Standards Commission response
	guidelines regarding test setting for horizontal transmission, such as number of pigs kept, age, consistency of origin, composition. Furthermore, request to remove the term "the same origin" as its definition is unclear. If intended to mean "same farm," it is not realistic because animals for experiment would need to be sourced from different farms to have required number of animals. A definition should be provided if the term is used to mean other specific characteristics.	
3.9.1_C2.3.2_15	**Category**: change The test is conducted using no fewer than 12 healthy piglets, a minimum of 4-weeks old and not older than 10-weeks old and of the same origin, that do not have antibodies against ASFV, and blood samples are negative on real-time PCR. This sentence is saying too much and should be split into multiple sentences or a bullet list.	**EN**: Agreed. The sentence has been split up and "of the same origin" has been removed. **FR**: Commentaire accepté. La phrase a été scindée et l'indication « *of the same origin* » (de même origine) a été supprimée. **SP**: Aceptado. Se ha dividido la frase y se ha eliminado «del mismo origen».
3.9.1_C2.3.2_16	**Category**: change Co-mingle equal numbers of vaccinated and naïve, contact piglets from day 0 in the same pen or room. The order that these requirements are listed makes it confusing. At this point it's not clear if equal numbers of vaccinated and naive piglets – if no fewer than 12 piglets means 12 of each or 12 total (6 of each). The instruction in the next paragraph that no fewer than 6 piglets be vaccinated should be before this statement so that the reader can understand it on the first reading.	**EN**: Agreed and amended the sentence for clarity. **FR**: Commentaire accepté ; la phrase a été amendée pour plus de clarté. **SP**: Aceptado y se ha modificado la frase para mayor claridad.

Use vaccine virus at the least attenuated passage level that will be present between the master seed lot and a batch of the vaccine. Administer by each recommended route of administration to no fewer than six piglets a quantity of the vaccine virus equivalent to not less than the maximum virus titre (maximum release dose) likely to be contained in 1 dose of the vaccine.

To obtain individual and group mean baseline temperatures, the body temperature of each naïve, contact piglet is measured on at least the 3 consecutive days preceding co-mingling with vaccinated piglets. The body temperature of each naïve, contact piglet is then measured daily for at least 45 days, preferably 60 days.

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.2_17	**Category**: deletion ~~To obtain individual and group mean baseline temperatures,~~ The body temperature of each naïve, contact piglet is measured on at least the 3 consecutive days preceding co-mingling with vaccinated piglets. **Reason**: These criteria are set to be acceptable for the current, most developed vaccine strain and are not objectively developed. Additionally, taking the average temperature of the group could mask individual	**EN**: Agreed, amended for clarity and moved the sentence. **FR**: Commentaire accepté ; la texte a été amendé pour plus de clarté et la phrase a été déplacée. **SP**: Aceptado, se ha modificado para mayor claridad y se ha trasladado la frase.

Reference	Comment	Biological Standards Commission response
	reactions. Minimal temperature rise of 1 day was seen in safety studies - 3 days seems long based on current data. Tran *et al.* 2022.	
3.9.1_C2.3.2_18	**Category**: change We ask to rationalise the sentence *"….contact piglet is measured on at least the 3 consecutive days preceding co-mingling with vaccinated piglets. The body temperature of each naïve, contact piglet is then measured daily for at least 45 days, preferably 60 days*".	**EN**: This has been clarified in the revised text. Temperatures are measured for 3 consecutive days prior to co-mingling with the vaccinated pigs to obtain a baseline temperature for each 'contact' pig. This baseline temperature is used to assess temperature rises in each contact pig post-mingling – see below.
		FR: Ceci est présenté de manière plus claire dans le texte révisé. Un relevé des températures est réalisé pendant trois jours consécutifs avant que les porcs ne soient mélangés aux porcs vaccinés, ce qui permet d'obtenir une température de référence pour chaque porc « contact » (c'est-à-dire exposé). Cette température de référence sert à mesurer toute augmentation de la température chez les porcs exposés après regroupement – voir ci-dessous.
		SP: Esto se ha aclarado en el texto revisado. Las temperaturas se miden durante 3 días consecutivos antes de la mezcla con los cerdos vacunados para obtener una temperatura de referencia para cada cerdo «de contacto». Esta temperatura de referencia se utiliza para evaluar los aumentos de temperatura en cada cerdo de contacto después de la mezcla (véase más abajo).

To confirm the presence or absence of fever accompanied by disease, observe the naïve, contact piglets daily for at least 45 days, preferably 60 days. On each day during the observation period the maximum increase in body temperature above the baseline observed for each pig will be used to calculate the daily group mean temperature rise. This mean value should not exceed 1.5°C and no individual pig should show a rise in temperature above baseline greater than 1.5°C for a period exceeding 3 consecutive days. Carry out the daily observations for signs of acute and chronic clinical disease using a quantitative clinical scoring system adding the values for multiple clinical signs (e.g. Gallardo *et al.*, 2015a). These clinical signs should include fever, anorexia, recumbency, skin haemorrhage or cyanosis, joint swelling and necrotic lesions around the joints, respiratory distress and digestive findings.

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.2_19	**Category**: change On each day during the observation period the maximum increase in body temperature above the baseline observed for each pig will be recorded. ~~used to calculate the daily group mean temperature rise. This mean value should not exceed 1.5°C and~~ No individual pig should show a rise in temperature above baseline greater than 1.5°C for a period exceeding ~~3~~ 2 consecutive days. **Reason**: These criteria are set to be acceptable for the current, most developed vaccine strain and are not objectively developed. Additionally, taking the average temperature of the group could mask individual reactions. Minimal temperature rise of 1 day was seen in safety studies - 3 days seems long based on current data. Tran *et al.,* 2022.	**EN**: Agreed (see comment 3.9.1_C2.3.2_10above). **FR**: Commentaire accepté (voir commentaire 3.9.1_C2.3.2_10 ci-dessus). **SP**: Aceptado (véase el comentario 3.9.1_C2.3.2_10 anterior).
3.9.1_C2.3.2_20	**Category**: deletion ~~This mean value should not exceed 1.5°C and no individual pig should show a rise in temperature above baseline greater than 1.5°C for a period exceeding 3 consecutive days.~~ This is in the section below, it does not need to be written twice and this is not the appropriate location for it.	**EN**: Agreed. **FR**: La Commission a souscrit au commentaire. **SP**: Aceptado.

~~In addition,~~ Blood should be taken from the naïve contact piglets at least twice a week for the first 21 days post-vaccination and then on a weekly basis. From the blood samples, determine vaccine virus titres by quantitative virus isolation (HAD_{50}/ml, $TCID_{50}$/ml or other methods, e.g. titration using IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration as described above ~~infectious virus titres by quantitative virus isolation (e.g. HAD_{50}/ml or $TCID_{50}$/ml) and using a real-time PCR test.~~

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.2_21	Please rationalise the changes to the following sentence: "*Quantitative PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration as described above*"	**EN**: Although PCR can be used to detect positive samples, it is important to confirm PCR results by infectious virus titration to confirm the presence of actual virus in the samples. **FR**: Bien que la PCR puisse être utilisée pour détecter les échantillons positifs, il est important de confirmer les résultats obtenus par PCR en procédant à un titrage du virus infectieux, ce qui confirme la présence effective du virus dans l'échantillon. **SP**: Aunque la PCR puede utilizarse para detectar muestras positivas, es importante confirmar los resultados de la PCR mediante una valoración del virus infeccioso para confirmar la presencia de virus real en las muestras.

~~If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test only may be used.~~

Collect blood (serum) samples from the naïve contact pigs at least at day 21 and day 28 days and carry out an appropriate test to detect vaccine virus antibodies. At a minimum of 45 days, humanely euthanise all naïve, contact piglets. Conduct gross pathology on spleen, lung, tonsil, and kidney tissue samples and at least three different lymph nodes. Determine virus titres in all collected samples by quantitative virus isolation (HAD_{50}/mg or $TCID_{50}$/mg) or other appropriate methods (e.g. IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration as described above ~~and real-time(RT) PCR (see Section B.1. Identification of the agent). If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test or other appropriate method (e.g. titration using IPT or FAT detection) may be used~~.

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.2_22	**Category**: change Collect blood (serum) and oronasal and fecal swabs ~~samples~~ from the naïve contact pigs at least at day 21 and day 28 days and carry out an appropriate test to detect vaccine virus antibodies. **Reason**: Relevant sample types to evaluate transmission by all possible routes inserted.	**EN**: Disagreed, this change is not necessary as the text is about looking for evidence of horizontal transmission of vaccine virus between vaccinated and the naïve contact pigs, not onward transmission from the naïve contact pigs.
		FR: La Commission n'a pas souscrit à ce commentaire : le changement proposé ne s'impose pas car la recherche dont il s'agit est celle d'une transmission horizontale du virus vaccinal entre les porcs vaccinés et les porcs naïfs exposés, et non d'une transmission ultérieure à partir des porcs naïfs exposés.
		SP: No aceptado, este cambio no es necesario ya que el texto trata sobre la búsqueda de pruebas de transmisión horizontal del virus vacunal entre los cerdos vacunados y los cerdos de contacto nunca antes expuestos, no de la transmisión hacia adelante desde los cerdos de contacto nunca antes expuestos.
3.9.1_C2.3.2_23	**Category**: change Please rationalise the following sentence: "Collect blood (serum) samples from the naïve contact pigs at least at day 21 and day 28 days and carry out an appropriate test to detect vaccine virus antibodies"	**EN:** Agreed: this instruction is to look for evidence of horizontal transmission of vaccine virus from the vaccinated pigs to the naïve contact pigs. If the contact pigs are infected, antibodies should appear at around 7–10 days post-infection. The peak of vaccine virus excretion is likely to be around 3–7 days post-vaccination, so timepoints of 21 and 28 days post-mingling with the vaccinated pigs should detect any horizontal transmission in the naïve contact pigs.
		FR: Commentaire accepté : cette consigne vise à démontrer l'existence d'une transmission horizontale du virus vaccinal des porcs vaccinés aux porcs naïfs exposés. En cas d'infection des porcs exposés, des anticorps devraient apparaître chez ces derniers dans les 7 à 10 jours suivant l'infection. Le niveau maximal de l'excrétion du virus vaccinal se situant entre le 3e et le 7e jour après la vaccination, les échéances de 21 jours et de 28 jours après le regroupement des porcs naïfs avec les porcs vaccinés devraient permettre de détecter toute transmission horizontale chez les porcs naïfs exposés.
		SP: Aceptado: esta instrucción es para buscar evidencia de transmisión horizontal del virus vacunal de los cerdos vacunados a los cerdos de contacto nunca antes expuestos. Si los cerdos de contacto están infectados, los anticuerpos deberían aparecer alrededor de los 7–10 días post-infección. El pico de excreción del virus de la vacuna es probable que sea

Reference	Comment	Biological Standards Commission response
		alrededor de 3–7 días después de la vacunación, por lo que los puntos de tiempo de 21 y 28 días después de la mezcla con los cerdos vacunados deben detectar toda posible transmisión horizontal en los cerdos de contacto nunca antes expuestos.
3.9.1_C2.3.2_24	**Category**: deletion "samples by quantitative virus isolation (HAD_{50}/mg or $TCID_{50}$/mg) or other appropriate methods (e.g. IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration as described above" This is repeated many times throughout the document word for word, sometimes (as in this case) in two paragraphs one after another. This makes the document very repetitive to read. Consider if this could be defined once early on then referred back to	**EN:** Agreed – but it is important to repeat this in separate sections as sections of the document may be read in isolation. When the same information is repeated in the same section, we have referred to it 'above', so it is not repeated. **FR**: Commentaire accepté – mais il est important que l'information figure dans toutes les sections pertinentes du document, lesquelles doivent pouvoir être consultées séparément. Lorsque la même information est fournie plusieurs fois dans une même section, la formulation « *above* » (comme ci-dessous) y renvoie, afin d'éviter la répétition. **SP**: Aceptado, pero es importante repetirlo en secciones separadas, ya que las secciones del documento pueden leerse de forma aislada. Cuando se repite la misma información en la misma sección, nos hemos referido a ella «más arriba», para que no se repita.

The vaccine complies with the test if:

- No vaccinated or naïve contact piglet shows abnormal (local or systemic) reactions or notable signs of disease, reaches the predetermined humane endpoint defined in the clinical scoring system or dies from causes attributable to the vaccine;

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.2_25	**Category**: addition Please note that whilst quantitative PCR is mentioned above as optional, then one of the compliance criteria is based on it. Perhaps a more neutral wording could be used here as the suitable testing methods are described above: *"No or a low percentage of naïve, contact piglets test positive to the vaccine virus and/or to antibodies against the vaccine virus"*	**EN**: Agreed. **FR**: La Commission a souscrit au commentaire. **SP**: Aceptado.

- On each day during the observation period the maximum increase in body temperature above the baseline observed for each pig will be used to calculate the daily group mean temperature rise. This mean value should not exceed 1.5°C and no individual pig should show a rise in temperature above baseline greater than 1.5°C for a period exceeding 3 consecutive days ~~The average body temperature increase for all naïve, contact piglets (group mean) for the observation period does not exceed 1.5°C; above baseline; and no individual piglet shows a temperature rise above baseline greater than 2.5°C for a period exceeding 3 days;~~

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.2_26	**Category**: deletion On each day during the observation period the maximum increase in body temperature above the baseline observed for each pig ~~will be used to calculate the daily group mean temperature rise. This mean value should not exceed 1.5°C and no individual pig~~ should show a rise in temperature above baseline greater than 1.5°C for a period exceeding ~~3~~ 2 consecutive days **Reason**: These criteria are set to be acceptable for the current, most developed vaccine strain and are not objectively developed. Additionally, taking the average temperature of the group could mask individual reactions. Minimal temperature rise of 1 day was seen in safety studies - 3 days seems long based on current data. Tran et al. 2022	**EN**: Agreed. **FR**: La Commission a souscrit au commentaire. **SP**: Aceptado.
3.9.1_C2.3.2_27	**Category**: change On each day during the observation period the maximum increase in body temperature above the baseline observed for each pig will be used to calculate the daily group mean temperature rise. As with the safety in young animals section, this should be in the instructions above, not in the requirements for compliance.	**EN**: Agreed. **FR**: La Commission a souscrit au commentaire. **SP**: Aceptado.

- No naïve, contact piglet shows notable signs of disease by gross pathology ~~and no virus is detected in their blood or tissue samples~~;
- No or a low percentage of contact piglets test both real-time PCR positive and seropositive ~~No naïve contact pigs test positive for antibodies to the vaccine virus~~.

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.2_28	**Category**: deletion No ~~or a low percentage of~~ contact piglets test both real-time PCR positive and seropositive. ~~No naïve contact pigs test positive for antibodies to the vaccine virus.~~ **Reason**: If one piglet positive, it indicates there must be horizontal transmission for the vaccine virus.	**EN**: Disagreed, as these vaccines are MLVs, some level of horizontal transmission is likely and it is up to regulators to decide whether a vaccine is safe.
		FR: La Commission est en désaccord avec ce commentaire ; il s'agit de vaccins à virus vivant modifié, de sorte qu'un certain niveau de transmission horizontale est probable. Il revient aux autorités de réglementation de décider si un vaccin est sûr.
		SP: No aceptado, ya que estas vacunas son MLV, es probable cierto nivel de transmisión horizontal y corresponde a los reguladores decidir si una vacuna es Segura.
3.9.1_C2.3.2_29	**Category**: deletion No ~~or a low percentage of~~ contact piglets test both real-time PCR positive and seropositive. **Reason**: This should not be set to meet pre-determined criteria. The test for horizontal transmission should be reported - whether the data are acceptable to individual regulatory authorities is a separate decision.	**EN** Disagreed, as these vaccines are MLVs, some level of horizontal transmission is likely and it is up to regulators to decide whether the vaccine is safe.
		FR: La Commission est en désaccord avec ce commentaire ; il s'agit de vaccins à virus vivant modifié, de sorte qu'un certain niveau de transmission horizontale est probable. Il revient aux autorités de réglementation de décider si le vaccin est sûr.
		SP: No aceptado, ya que estas vacunas son MLV, es probable cierto nivel de transmisión horizontal y corresponde a los reguladores decidir si la vacuna es segura.
3.9.1_C2.3.2_30	**Category**: change No or a low percentage of contact piglets test both real-time PCR positive and seropositive This should be more clearly defined. If there are 6 contact piglets and one tests positive, 16.7% of the piglets are positive - is that a low percentage?	**EN** Disagreed, some level of horizontal transmission is likely and it is up to regulators to decide whether the vaccine is safe.
		FR: La Commission est en désaccord avec ce commentaire ; un certain niveau de transmission horizontale est probable et il revient aux autorités de réglementation de décider si le vaccin est sûr.
		SP: No aceptado, es probable cierto nivel de transmisión horizontal y corresponde a los reguladores decidir si la vacuna es segura.

iv) Post-vaccination kinetics of viral replication (MLV blood and tissue dissemination) study

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.2_31	**Category**: change **Line 459**: A general, more neutral heading would be preferrable as the details are then given in the actual text. '*Dissemination in the vaccinated animal*'	**EN**: Agreed, but slightly modified the proposal
		FR: Commentaire accepté et proposition légèrement amendée.
		SP: Aceptado, pero modificando ligeramente la propuesta.

Prior to the reversion to virulence study (Section C2.3.2.v. below), a minimum of one study should be performed to determine the post-vaccination kinetics of virus replication in the blood (viremia), tissues and viral shedding.

The test consists of the administration of the vaccine virus from the master seed lot to no fewer than eight healthy piglets, and preferably ten healthy piglets, a minimum of ~~6~~ 4-weeks old and not older than 10-weeks old and of the same origin, that do not have antibodies against ASFV, and blood samples are negative on real-time PCR.

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.2_32	**Category**: change Again, this sentence is too long and says too much, significantly reducing readability. Break it into multiple sentences or bullet points.	**EN**: Agreed. The sentence has been split up and "of the same origin" has been removed.
		FR: Commentaire accepté. La phrase a été scindée et les mots « *of the same origin* » (de la même origine) ont été supprimés.
		SP: Aceptado. Se ha dividido la frase y se ha eliminado «del mismo origen».

Administer to each piglet, using the recommended route of administration most likely to result in spread (such as the intramuscular route or intranasal route), a quantity of the master seed vaccine virus equivalent to not less than the maximum virus titre (maximum release dose) likely to be contained in 1 dose of the final product of the vaccine.

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.2_33	**Category**: change Please substitute "~~*final product of the vaccine*~~" with "commercial vaccine".	**EN**: Disagreed, the vaccine may not be commercial
		FR: La Commission n'a pas retenu ce commentaire, car il peut s'agir de vaccins non commerciaux.
		SP: No aceptado, la vacuna puede no ser commercial.

Record daily body temperatures and observe inoculated animals daily for clinical disease for at least 45 days, preferably 60 days.

Carry out the daily observations for signs of acute and chronic clinical disease using a quantitative clinical scoring system adding the values for multiple clinical signs (e.g. Gallardo *et al.* (2015a). These clinical signs should include fever, anorexia, recumbency, skin haemorrhage or cyanosis, joint swelling and necrotic lesions around the joints, respiratory distress and digestive findings.

Collect blood samples from all the piglets at least two times per week from 3 days post-vaccination for the first 2 weeks, then weekly for the duration of the test. Determine vaccine virus titres by quantitative virus isolation (HAD_{50}/ml or $TCID_{50}$/ml) or other appropriate methods (e.g. titration using IPT or FAT detection). Quantitative PCR may be used to detect positive samples but results should be confirmed by infectious virus titration as described above ~~and using a real-time PCR test. If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test only may be used~~.

Determine which blood timepoint(s) should be used in the design of the reversion to virulence study (Section C2.3.2.v. below), for example, specific blood sample(s) at specific timepoints that show the highest titres should be considered for selection and use in the reversion to virulence study.

Collect oral, nasal and faecal swab samples (preferably devoid of blood to minimise assay interference) at least two times per week from 3-days post-vaccination for the first 2 weeks, then weekly for the duration of the test. Test the swabs for the presence of vaccine virus. Determine virus titres in all collected samples by quantitative virus isolation (HAD_{50}/ml or $TCID_{50}$/ml) or other appropriate methods (e.g. titration using IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration as described above ~~and using a real-time PCR test. If the vaccine~~

~~virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test or other appropriate method (e.g. titration using IPT or FAT detection) may be used.~~

Euthanise at least two piglets on days 5, 7, 14, 21, and preferably on day 28 (±2 days at each timepoint) and collect spleen, lung, tonsil, kidney tissue samples and at least three different lymph nodes (which should include lymph node closest to site of inoculation, gastrohepatic and submandibular nodes). Determine virus titres in all collected samples by quantitative virus isolation (HAD_{50}/mg or $TCID_{50}$/mg) or other appropriate methods (e.g. titration using IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration as described above ~~and using real-time PCR test. If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test or other appropriate method (e.g. titration using IPT or FAT detection) may be used.~~

Determine which tissue(s) and timepoint(s) should be used to aid in the design of the reversion to virulence study (Section C.2.3.2.v), for example, specific tissues at specific timepoints which show the highest titres should be considered for selection and use in the reversion to virulence study.

v) Reversion to virulence

The test carried out should be consistent with VICH GL41 (Examination of live veterinary vaccines in target animals for absence of reversion to virulence, 2008[5]).

The test for increase in virulence consists of the administration of the vaccine master seed virus to healthy piglets of an age (e.g. between ~~6~~ 4 weeks and 10 weeks old) suitable for recovery of the strain and of the same origin, that do not have antibodies against ASFV, and blood samples that are negative on real-time PCR. This protocol is typically repeated five times.

First passage (p1)

Administer to no fewer than two piglets, and preferably no fewer than four piglets using the intended route of administration for the final product, a quantity of the master seed vaccine virus equivalent to not less than the maximum virus titre (maximum release dose) likely to be contained in 1 dose of the final product of the vaccine. Observe inoculated animals daily for the appearance of ~~at least two and preferably at least three~~ clinical signs ~~and record daily body temperatures~~ using a quantitative clinical scoring system adding the values for multiple clinical signs (e.g. Gallardo *et al.*, 2015a) and record daily body temperatures.

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.2_34	**Category**: change Administer to no fewer than two piglets, and preferably no fewer than four piglets using the intended route of administration for the final product, a quantity of the master seed vaccine virus equivalent to not less than the maximum virus titre (maximum release dose) likely to be contained in 1 dose of the master seed of the ~~final product of the~~ vaccine. **Reason**: The International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) defines worldwide standards for veterinary biologics. Guideline 41 specified master seed for this study design as passages of the master seed may be more attenuated, and this evaluation should be on the virus most likely to revert to virulence	**EN**: Disagreed, proposal not clear and original text conforms to VICH guidelines. It states that the master seed virus should be used equivalent to not less than the maximum virus titre (maximum release dose) likely to be contained in 1 dose of the final product of the vaccine. **FR**: La Commission n'a pas retenu ce commentaire, car la proposition manque de clarté et le texte original est conforme aux lignes directrices du VICH. Celles-ci indiquent que la semence primaire de virus utilisée doit correspondre à une quantité qui ne peut être inférieure à la titration virale maximale (dose de libération maximale) susceptible

[5] https://www.ema.europa.eu/en/documents/scientific-guideline/vich-gl41-target-animal-safety-examination-live-veterinary-vaccines-target-animals-absence-reversion_en.pdf

Reference	Comment	Biological Standards Commission response
	https://www.vichsec.org/en/index.php?option=com_attachments&view=attachments&task=download&id=321	d'être contenue dans une dose du produit final du vaccin. **SP**: No aceptado, la propuesta no es clara y el texto original se ajusta a las directrices de la VICH. Establece que el inóculo vírico primario debe utilizarse equivalente a no menos del título máximo del virus (dosis máxima de liberación) que probablemente contenga 1 dosis del producto final de la vacuna.

Based on results from at least one completed post-vaccination kinetics of viral replication (MLV ~~vaccine shed and spread (virus~~ blood and tissue dissemination study (Section C.2.3.2.iv above), collect an appropriate quantity of blood from each piglet on the predetermined ~~single~~ timepoint(s) (day ~~5–3~~–13). Determine virus titres in individual blood samples by quantitative virus isolation (HAD_{50}/ml or $TCID_{50}$/ml) or other appropriate methods (e.g. titration using IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration as described above ~~and by real-time PCR. If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test or other appropriate method (e.g. titration using IPT or FAT detection) may be used~~. Identify the individual blood sample(s) with the highest infectious titre and reserve for the subsequent *in-vivo* passage (second pass, p2).

Based on results from at least one completed ~~vaccine virus~~ MLV blood and tissue ~~distribution~~ dissemination study (Section C.2.3.2.iv above), euthanise piglets on the predetermined timepoint (i.e. day 5, 7, 14, 21, or 28). Determine infectious virus titres in individual tissue samples by quantitative virus isolation (HAD_{50}/ml or $TCID_{50}$/ml) or other appropriate methods (e.g. titration using IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration as described above. ~~If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test or other appropriate method (e.g. titration using IPT or FAT detection) may be used.~~ Identify individual tissue-sample type(s) with the highest infectious titre. Pool the tissues with the highest titres from different organs from ~~all~~ each animal~~s with the highest titres~~ and prepare ~~at least~~ a ~~10%~~ virus suspension ~~to obtain a virus titre within the range used for inoculation~~ in PBS, pH 7.2 kept at 4°C or at –70°C for longer storage.

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.2_35	**Category**: deletion Pool the tissues with the highest titres from different organs from ~~all~~ each animal~~s with the highest titres~~ and prepare ~~at least~~ a ~~10%~~ virus suspension ~~to obtain a virus titre within the range used for inoculation~~ in PBS, pH 7.2 kept at 4°C or at –70°C for longer storage. **Reason**: The International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) defines worldwide standards for veterinary biologics. Guideline 41 specifies "The initial administration and subsequent passages shall be carried out using a recommended route of administration or natural route of infection that is the most likely to lead to reversion to or increase in virulence and result in recovery of the organism following replication in the animal." Diluting the virus to the range used in the initial inoculum instead of passing all recovered virus could decrease the likelihood of detecting a reversion to virulence.	**EN**: Agreed. **FR**: La Commission a souscrit au commentaire. **SP**: Aceptado.

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.2_36	**Category:** change There is no need to pool tissue samples. Samples with the highest infectious titres should be used for further passage. If appropriate, some tissue samples with the highest infectious titres may be pooled to prepare inoculum for further passages. **Rationale:** Regarding infectivity, there is only one type of "tissue with the highest infectivity" (except for using more than one types of tissues with identical infectivity) and mixing it with other samples would rather decrease its potency. In other words, it should be made clear that there is no need to pool multiple samples to prepare inoculum for further passages.	**EN**: Agreed that there is no need to pool. Text has been changed accordingly. **FR**: La Commission est convenue qu'il n'était pas nécessaire d'assembler les échantillons. Le texte a été modifié en conséquence. **SP**: Aceptado que no es necesario llevar a cabo una combinación. El texto se ha modificado en consecuencia.

Test each blood and tissue sample pool used for inoculation by PCR to confirm the absence of potential viral agent contaminants (i.e. CSFV, FMDV, PRRS, PCV2). Blood and pooled tissue (p1) are used to inoculate 2 ml of positive material diluted to the maximum release dose likely to be contained in 1 dose of the vaccine using the intended route of administration for the final product to each of at least two and ideally at least four further pigs of the same age and origin.

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.2_37	**Category**: deletion Blood and pooled tissue (p1) are used to inoculate 2 ml of positive material ~~diluted to the maximum release dose likely to be contained in 1 dose of the vaccine~~ using the intended route of administration for the final product to each of at least two and ideally at least four further pigs of the same age and origin. **Reason**: These should not be diluted or manipulated to increase the chance of detecting reversion to virulence.	**EN**: Agreed. **FR**: La Commission a souscrit au commentaire. **SP**: Aceptado.
3.9.1_C2.3.2_38	**Category**: deletion Propose to delete the text regarding dilution of samples. **Rationale:** Samples should not be diluted. The sample with the highest infectious titre should be used as a 10% tissue	**EN**: Agreed. **FR**: La Commission a souscrit au commentaire. **SP**: Aceptado.
3.9.1_C2.3.2_39	**Category**: change "….release dose…". Please change to '*maximum release ~~dose~~ virus titre*'. Dose is mentioned later in the sentence referring to vaccine dose, so it is confusing. After "*1 dose of vaccine*", please add: if needed. Indeed, often the titre of the virus recovered after passage is lower than the original virus titre inoculated.	**EN**: Agreed. **FR**: La Commission a souscrit au commentaire. **SP**: Aceptado.

~~*Second pass (p2)*~~

If no virus is found at passage 1 (p1), repeat the administration by the intended route ~~once~~ again with the same pooled material (blood and pooled tissue, p1) in another ten healthy piglets of the same age and origin. If no virus is found at this point ~~during this second passage (p2) at this point~~, end the process here.

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.2_40	**Category**: change This instruction is confusing. The first sentence seems to say that if no virus is found to move on to passage 2, then the second sentence says that if no virus is found to end the process. Based on the following section it appears that p2 is only required if there is virus so the first sentence doesn't make sense. We suggest a change to make it clearer, but we cannot confidently determine what the intent is to offer suggested wording.	**EN**: Agreed. **FR**: La Commission a souscrit au commentaire. **SP**: Aceptado.

Second passage (p2)

If ~~however~~ virus is found in p1, carry out a second series of passages by administering 2 ml of positive material diluted to the maximum release dose likely to be contained in 1 dose of the vaccine using the intended route of administration for the final product to each of no fewer than two piglets, and preferably no fewer than four piglets of the same age and origin. Observe inoculated animals daily for the appearance of ~~at least two and preferably at least three~~ clinical signs using a quantitative clinical scoring system adding the values for multiple clinical signs (e.g. Gallardo *et al.*, 2015a), ~~and~~ record daily body temperatures and determine infectious virus titres in individual blood and tissue samples as described for p1 above.

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.2_41	**Category**: deletion If ~~however~~ virus is found in p1, carry out a second series of passages by administering 2 ml of positive material ~~diluted to the maximum release dose likely to be contained in 1 dose of the vaccine~~ using the intended route of administration for the final product to each of no fewer than two piglets,... **Reason**: These should not be diluted or manipulated to increase the chance of detecting reversion to virulence.	**EN**: Agreed (see 3.9.1_C2.3.2_37) **FR**: Commentaire accepté (voir 3.9.1_C2.3.2_37) **SP**: Aceptado (véase 3.9.1_C2.3.2_37)

~~*Third and fourth pass (p3 and p4)*~~

If no virus is found at ~~in~~ (p2~~)~~, repeat the ~~intramuscular~~ administration by the intended route ~~once again~~ with the same pooled material (blood and pooled tissue, p2) in another eight healthy piglets of the same age and origin. If no virus is found at this point, end the process here.

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.2_42	**Category**: change This has the same issue as the instruction at the end of passage 1. We think the intent is that you repeat passage 2 using the passage 1 material, but this seems to suggest that you used the p2 material, making it essentially a third passage.	**EN**: Agreed – changes have been made to the text to clarify the use of passage 1 material here. **FR**: Proposition acceptée – texte modifié afin de clarifier que le matériel utilisé est celui du premier passage. **SP**: Aceptado - se han introducido cambios en el texto para aclarar el uso del material del pasaje 1 aquí.

Third and fourth passage (p3 and p4)

If, ~~however,~~ virus is found on p2, carry out this passage operation no fewer than two additional times (p3 and p4) (to each of no fewer than two piglets, and preferably no fewer than four piglets of the same age and origin) and verifying the presence of the virus at each passage in blood and tissues. Observe inoculated animals daily for the appearance of ~~at least two and preferably at least three~~ clinical signs using a quantitative clinical scoring system adding the values for multiple clinical signs (e.g. Gallardo *et al.*, 2015a) and record daily body temperatures.

Fifth passage (*p5*)

Administer 2 ml of the blood and pooled tissue (p4) to each of at least eight healthy piglets of the same age and origin. Observe inoculated animals daily for at least 28 days post-inoculation for the appearance of ~~at least two and preferably at least three~~ clinical signs using a quantitative clinical scoring system adding the values for multiple clinical signs (e.g. Gallardo *et al.*, 2015a), ~~and~~ record daily body temperature and determine infectious virus titres in individual blood and tissue samples as described above.

The vaccine virus complies with the test if:

- No piglet shows abnormal (local or systemic) reactions, ~~or notable signs of disease, or~~ reaches the pre-determined humane endpoint defined in the clinical scoring system or dies from causes attributable to the vaccine; and

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.2_43	**Category**: addition Suggest reversing deletion above given recent data on U.S. select agent exclusion recission. We don't know what clinical signs will be caused by revertant vaccine strains – this consideration should be broad and inclusive (local or systemic reactions and/or notable signs of disease).	**EN**: Agreed. **FR**: La Commission a souscrit au commentaire. **SP**: Aceptado.

- There is no indication of increasing virulence (as monitored by daily body temperature accompanied by clinical sign observations) of the maximally passaged virus compared with the master seed virus.

At a minimum, a safe MLV vaccine shall demonstrate ALL the following features (minimal standards):

- Absence of fever (on each day during the observation period, the maximum increase in body temperature above the baseline observed for each pig will be used to calculate the daily group mean temperature rise. This mean value should not exceed 1.5°C and no individual pig should show a rise in temperature above baseline greater than 1.5°C ~~(defined as average body temperature increase for all vaccinated piglets (group mean) for the observation period does not exceed 1.5°C above baseline; and no individual piglet shows a temperature rise above baseline greater than 2.5°C~~ for a period exceeding 3 days);

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.2_44	**Category**: change Absence of fever (on each day during the observation period, the maximum increase in body temperature above the baseline observed for each pig will be recorded, ~~will be used to calculate the daily group mean temperature rise. This mean value should not exceed 1.5°C~~ and no individual pig should show a rise in temperature above baseline greater than 1.5°C ~~defined as average body temperature increase for all vaccinated piglets (group mean) for the observation period does not exceed 1.5°C above baseline; and no individual piglet shows a temperature rise above baseline greater than 2.5°C~~ for a period exceeding ~~3~~ 2 days);	**EN**: Agree (see comment 3.9.1_C2.3.2_10 above) **FR**: Proposition acceptée (voir plus haut le commentaire 3.9.1_C2.3.2_10) **SP**: Aceptado (véase el comentario 3.9.1_C2.3.2_10 anterior)

	Reason: These criteria are set to be acceptable for the current, most developed vaccine strain and are not objectively developed. Additionally, taking the average temperature of the group could mask individual reactions. Minimal temperature rise of 1 day was seen in safety studies - 3 days seems long based on current data. Tran et al. 2022.	
3.9.1_C2.3.2_45	**Category**: addition In line with the compliance criteria of the safety tests described above the following minimum requirement is missing and should also be added: "Absence of abnormal (local or systemic) reactions" It is understood that the absence of acute/chronic clinical signs/gross pathology are related to ASF. Local/systemic reactions refer to more general observations (injection site reactions, anaphylactic reactions, etc.).	**EN**: Agreed. **FR**: La Commission a souscrit au commentaire. **SP**: Aceptado.

- Absence of chronic and acute clinical signs and gross pathology over the entire test period or ~~minimal chronic~~ mild clinical signs (~~defined as~~ e.g. mild swollen joints with a low clinical score that resolve within 1 week).
- Minimal (defined as no naïve, contact piglet shows notable signs of disease by clinical signs and gross pathology and no or a low percentage of contact piglets test both real-time PCR positive and seropositive) or no vaccine virus transmission (defined as no naïve, contact piglet shows notable signs of disease by clinical signs and gross pathology and no contact piglets test both real-time PCR positive and seropositive) over the entire test period;

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.2_46	**Category**: change This needs to be clarified. As it reads there must be absence of mild clinical signs. Is this the intention? Is it necessary this part? **"***both real-time PCR positive and seropositive..."* Please see the previous comment on this point.	**EN**: Agreed. **FR**: La Commission a souscrit au commentaire. **SP**: Aceptado.

- Absence of an increase in virulence (genetic and phenotypic stability) (complies with the reversion to virulence test).

In addition, for regulatory approval, ASF MLV ~~the~~ vaccines ~~in their commercial presentation before being authorised for general use~~ should be tested for safety ~~in the~~ under field conditions (see chapter 1.1.8 Section 7.2.3). ~~Additional~~ Field safety studies generally ~~evaluation studies~~ may include measurement of body temperatures, observation of local or systemic reactions and, where appropriate, performance measurements ~~but are not limited to: environmental persistence (e.g. determination of virus recovery from bedding or other surfaces), assessment of immunosuppression, and negative impacts on performance~~.

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.2_47	**Category**: addition Field safety studies generally ~~evaluation studies~~ may include measurement of body temperatures, observation of local or systemic reactions and, where appropriate, performance measurements such as: environmental persistence (e.g. determination of virus recovery from bedding or other surfaces), assessment of	**EN**: Agreed. **FR**: La Commission a souscrit au commentaire. **SP**: Aceptado.

Reference	Comment	Biological Standards Commission response
	immunosuppression, and negative impacts on performance. **Reason**: Suggest reversing deletion above. Environmental persistence is a very relevant criterion for evaluation given recent data our country select agent exclusion recission and the potential for reversion to virulence and spread to wild suids. Other criteria are also valid given the unknown clinical signs that might be seen with revertant viruses and are listed as examples for incorporation.	
3.9.1_C2.3.2_48	**Category**: deletion ~~Additional~~ Field safety studies generally evaluation studies... "generally may include..." is redundant.	**EN**: Agreed. **FR**: La Commission a souscrit au commentaire. **SP**: Aceptado.

2.3.3. Efficacy requirements

i) Protective dose

Vaccine efficacy is estimated in immunised animals directly, by evaluating their resistance to live virus challenge. The test consists of a vaccination/challenge trial in piglets a minimum of ~~6~~ 4 weeks old and not more than 10-weeks old, free of antibodies to ASFV, and negative blood samples by real-time PCR. The test is conducted using no fewer than 15 and preferably no fewer than 24 vaccinated pigs, and no fewer than five non-vaccinated control piglets.

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.3_1	**Category**: addition *"…..by evaluating ~~their resistance~~ protection against live virus challenge…."*	**EN**: Agreed. **FR**: La Commission a souscrit au commentaire. **SP**: Aceptado.

The test is conducted to determine the minimal immunising dose (MID) (also referred to as the minimal protective dose [MPD] or protective fraction); using at least three groups of no fewer than five and preferably not fewer than eight vaccinated piglets per group, and one additional group of no fewer than five non-vaccinated piglets of the same age and origin as controls. Use vaccine containing virus at the highest passage level that will be present in a batch of vaccine.

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.3_2	**Category**: change The test is conducted to determine the minimal immunising dose (MID) (also referred to as the minimal protective dose [MPD] or protective fraction)~~; using at least three groups of no fewer than five and preferably not fewer than eight vaccinated piglets per group, and one additional group of no fewer than five non-vaccinated piglets of the same age and origin as controls. Use vaccine containing virus at the highest passage level that will be present in a batch of vaccine~~. It may be possible to combine the data obtained by independent experiments	**EN**: Disagreed: more robust as written originally **FR**: La Commission n'a pas retenu ce commentaire car le texte est plus solide dans sa rédaction initiale. **SP**: No aceptado: más robusto tal y como estaba redactado originalmente.

Reference	Comment	Biological Standards Commission response
	separately conducted under the same condition due to the limitation of capacity of the facility. **Rationale:** Propose to delete the text that is not in line with the VICH guidelines and to add more feasible recommendation. There is no specification in the VICH guidelines regarding test setting for protective dose such as number of pigs kept, age, consistency of origin, composition. In addition, this description should clearly define "protective dose" (preferably based on PD_{50}) or how to determine the minimum release dose (titre) per vaccine vial. The step described should be performed with MSV but with not each product batches. Hence, the sentence should be deleted at this step.	

Each group of piglets, except the control group, is immunised with a different vaccine virus content in the same vaccine volume. In at least one vaccinated group, piglets are immunised with a vaccine dose containing not more than the minimum virus titre (minimum release dose) likely to be contained in one dose of the vaccine as stated on the label.

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.3_3	**Category**: deletion Please delete ~~(*minimum release dose*)~~. Please note that the minimum release titre may be higher than the minimum efficacious titre, to compensate for the potential losses in titre during shelf life (overage).	**EN**: Agreed. **FR**: La Commission a souscrit au commentaire. **SP**: Aceptado.

Twenty-eight days (±2 days) after the single ~~injection~~ dose of vaccine (or if using two ~~injections~~ doses of the vaccine then 28 days [±2 days] following the second ~~injection~~ dose), challenge all the piglets by the intramuscular route. If previous studies have demonstrated acceptable efficacy using IM challenge, then a different challenge route (e.g. direct contact, oral or oronasal) may be used. Challenged, vaccinated piglets may be housed in one or more separate pens in the same room or in different rooms. Challenged, naïve controls can be housed in one or more rooms that are separate from challenged, vaccinated piglets.

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.3_4	**Category**: addition A suitable challenge model should be developed based on anticipated field usage of the vaccine. As a baseline protocol, 28 days... **Reason**: National Veterinary Joint Stock Company (NAVETCO) updated label claims to allow a single vaccine dose. Suggest this modification to allow different regulatory authorities flexibility to pursue their own criteria for protection.	**EN**: Agreed. **FR**: La Commission a souscrit au commentaire. **SP**: Aceptado.

Carry out the test using an ASFV representative strain of the epidemiologically relevant field strain(s) where the vaccine is intended for use (e.g. ASFV B646L [p72] genotype II pandemic strain and other p72 virulent genotype of recognised epidemiologic importance). For gene deleted, recombinant MLV viruses, if neither challenge virus type is available, then carry out the test with the parental, virulent virus used to generate the MLV recombinant virus. Use a

10e3–10e4 HAD_{50} (or $TCID_{50}$ for non-HAD viruses) challenge dose sufficient to cause death or meet the humane endpoint in 100% of the nonvaccinated piglets in less than 21 days. Higher or lower challenge doses can be considered if appropriately justified.

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.3_5	**Category**: change Propose to amend "~~HAD_{50} (or $TCID_{50}$ for non-HAD viruses)~~" to "HAD_{50} or $TCID_{50}$." **Rationale:** Regardless of whether the virus is a hemadsorption (HAD) strain or a non-hemadsorption (non-HAD) strain, it is possible to apply HAD_{50} or $TCID_{50}$ as units of virus titre as appropriate. Specifically, the titre of non-HAD strains can only be expressed in $TCID_{50}$, but if it is a HAD strain, it can be expressed in either HAD_{50} or $TCID_{50}$, and the values are almost synonymous.	**EN**: Agreed. **FR**: La Commission a souscrit au commentaire. **SP**: Aceptado.

The rectal temperature of each vaccinated piglet is measured on at least the 3 days preceding administration of the challenge virus, at the time of challenge, 4 hours after challenge, and then daily for the observation period of at least ~~28~~ ~~45~~ days, preferably ~~35~~ 60 days. ~~Observe the piglets at least daily for at least 28 days, preferably 35 days.~~ Carry out the daily observations for signs of acute and chronic clinical disease using a quantitative clinical scoring system adding the values for multiple clinical signs (e.g. Gallardo *et al.*, 2015). These clinical signs should include fever, anorexia, recumbency, skin haemorrhage or cyanosis, joint swelling and necrotic lesions around the joints, respiratory distress and digestive findings.

Collect oral, nasal, anal and blood samples from the vaccinated challenged piglets at least two times ~~once~~ per week from 3 days post-challenge for at least ~~28~~ 14 days, then weekly up to 35 days post-challenge and then every 14 days up to the end of the observation period ~~preferably 35 days~~. From the blood samples, determine infectious virus titres by quantitative virus isolation (HAD_{50}/ml or $TCID_{50}$/ml) or other appropriate methods (e.g. titration using IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration as described above ~~and using a real-time PCR test. If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test only may be used~~.

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.3_6	**Category**: addition Collect oral, nasal, and faecal ~~anal~~ swabs, and blood samples... **Reason**: Relevant sample types to evaluate transmission by all possible routes inserted.	**EN**: Agreed. **FR**: La Commission a souscrit au commentaire. **SP**: Aceptado.
3.9.1_C2.3.3_7	**Category**: change "Collect oral, nasal, anal and blood samples from the vaccinated..." Oral, nasal, and anal samples is open to interpretation - this should specify swabs. Also, there are no clear instructions to use these samples later on in the procedure, so their purpose should be clarified.	**EN**: Agreed. **FR**: La Commission a souscrit au commentaire. **SP**: Aceptado.

At the end of the test period, humanely euthanise all vaccinated challenged piglets. Conduct gross pathology (and histopathology if considered necessary) on spleen, lung, tonsil, and kidney tissue samples and at least three different lymph nodes (which should include lymph node closest to site of inoculation, gastrohepatic and submandibular nodes). Determine virus titres in all collected samples by quantitative virus isolation (HAD_{50}/mg or $TCID_{50}$/mg) or other appropriate methods (e.g. titration using IPT or FAT detection). Quantitative PCR

may be used to detect positive samples, but results should be confirmed by infectious virus titration as described above ~~and real-time PCR (see Section B.1. Identification of the agent). If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test or other appropriate method (e.g. titration using IPT or FAT detection) may be used~~.

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.3_8	**Category**: change The aim of the validity criterion is to validate the suitability of the challenge. It needs to be reminded that one of the key minimum standards for efficacy has been set for protection against mortality. Therefore, it would be expected that the challenge model used is suitable to evaluate such efficacy endpoint. The proposed validity criterion is not considered appropriate: 1) the number of vaccinated piglets dying or reaching the humane endpoint should always be 0 (to fulfil the compliance criterion); 2) it might be challenging to prove statistical significance with the minimum number of animals that can be included per group; 3) even if statistical significance is proven, it may not be meaningful from a biological point of view e.g. 0% mortality in vaccinated vs. 15% mortality in controls might render a statistically significant difference, but it is not considered appropriate to validate a challenge aimed at demonstrating protection against mortality (low severity of the challenge). The severity of the challenge should be suitable to evaluate the efficacy endpoints that are then required as minimum standards. Ideally, a minimum percentage of control piglets dying/reaching humane endpoint should be defined (e.g. minimum 80%, meaning that 1 out of 5 controls may not die or reached the humane endpoint). The experts on the disease may provide advice on which would be a realistic percentage (representative of field conditions). Please also note text in lines 668–670 where validity criterion is also set at 100% mortality in control piglets.	**EN**: Agreed. **FR**: La Commission a souscrit au commentaire. **SP**: Aceptado.
3.9.1_C2.3.3_9	**Category**: change Considering that it would be nearly impossible to develop a vaccine with 100% protection, this sentence is proposing an excessive high standard in which even a single vaccinated challenged piglet should not die. This type of unfeasible standards should not be included in the *Manual*. In addition, the current draft text "No vaccinated challenged piglet dies or..." contradicts the earlier statement "The test is invalid if the difference between the number of unvaccinated control piglets infected with the live challenge virus and the number of vaccinated/ challenged piglets that die or reach a humane endpoint is not statistically significant", thus Japan requests to amend the text to align it with the first statement.	**EN**: Agreed. The text has been modified. and now refers to challenge with a highly virulent ASFV virus strain, where experts agree that one would expect all the pigs in the unvaccinated control group to die or reach humane endpoints when infected with the live challenge virus for the test to be considered valid. **FR**: Commentaire accepté. Le texte a été modifié et se réfère désormais à une inoculation d'épreuve avec une souche du virus de la PPA hautement virulente, dont les experts auraient déterminé, afin de fonder la validité du test, que l'inoculation d'épreuve entraînerait chez les porcs non vaccinés du groupe de contrôle soit la mort, soit un état correspondant au point limite imposant d'euthanasier l'animal. **SP**: Aceptado. El texto ha sido modificado y ahora se refiere al desafío con una cepa altamente

		virulenta del virus de la peste porcina africana, donde los expertos están de acuerdo en que se esperaría que todos los cerdos del grupo de control no vacunado murieran o alcanzaran puntos finales humanitarios cuando se infectaran por el virus vivo de desafío para que la prueba se considerara válida.

The test is invalid if ~~fewer than 100%~~ the difference between ~~in~~ the number of unvaccinated control piglets infected with the live challenge virus and the number of vaccinated/ challenged piglets ~~vaccinated with the minimum release dose~~ that die or reach a humane endpoint is not statistically significant.

The vaccine (or a specific vaccine virus dose if conducting a vaccine dose titration study) complies with the test if:

- No vaccinated challenged piglet dies or ~~shows abnormal (local or systemic) reactions,~~ reaches the humane endpoint ~~or dies~~ from causes attributable to ASF;

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.3_10	**Category**: change Suggest reversing deletion above given recent data our country select agent exclusion recission. We don't know what clinical signs will be caused by revertant vaccine strains – this consideration should be broad and inclusive (local or systemic reactions and/or notable signs of disease)	**EN**: Agreed. **FR**: La Commission a souscrit au commentaire. **SP**: Aceptado.

- On each day during the observation period the maximum increase in body temperature above the baseline observed for each pig will be used to calculate the daily group mean. This mean value should not exceed 1.5°C and no individual pig should show a rise in temperature above baseline greater than 2.0°C for a period exceeding 2 consecutive days ~~The average body temperature increase for all vaccinated challenged piglets (group mean) for the observation period does not exceed 2.0°C above baseline; and no individual piglet shows a temperature rise above baseline greater than 2.0°C;~~

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.3_11	Category: change On each day during the observation period the maximum increase in body temperature above the baseline observed for each pig will be recorded. ~~used to calculate the daily group mean. This mean value should not exceed 1.5°C and~~ No individual pig should show a rise in temperature above baseline greater than ~~2.0~~ 1.5°C for a period exceeding 2 consecutive days **Reason**: These criteria a re set to be acceptable for the current, most developed vaccine strain and are not objectively developed. Additionally, taking the average temperature of the group could mask individual reactions. Minimal temperature rise of 1 day was seen in safety studies - 3 days seems long based on current data. Tran et al. 2022.	**EN**: Agreed – changes made (see comments: 3.9.1_C2.3.2_10 3.9.1_C2.3.2_19 3.9.1_C2.3.2_44 **FR**: Proposition acceptée – les changements ont été introduits. (voir les commentaires : 3.9.1_C2.3.2_10 3.9.1_C2.3.2_19 3.9.1_C2.3.2_44) **SP**: Aceptado - cambios realizados (ver comentarios: 3.9.1_C2.3.2_10 3.9.1_C2.3.2_19 3.9.1_C2.3.2_44

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.3_12	**Category**: clarification Request to provide the rationale to change the average body temperature increase (group mean) from 2.0°C to 1.5°C? **Rationale:** For clarity of the scientific grounds.	**EN**: Group means are no longer measured, so this comment is no longer valid **FR**: Les moyennes du groupe ne sont plus mesurées, ce commentaire n'est donc plus valable **SP**: Ya no se miden las medias de grupo, así que este comentario ya no es válido.
3.9.1_C2.3.3_13	**Category**: change "On each day during the observation period the maximum increase in body temperature above the baseline observed for each pig will be used to calculate the daily group mean." As previously, this should be in the instructions for the test, not the compliance requirements.	**EN**: Agreed. **FR**: La Commission a souscrit au commentaire. **SP**: Aceptado.

- The vaccinated challenged piglets display a reduction or absence of typical acute clinical signs of disease and gross pathology and a reduction or absence of challenge virus levels in blood and tissues.

ii) Assessment for horizontal transmission (challenge virus shed and spread study)

The ASF basic reproduction number, R0, can be defined as the average number of secondary ASF disease cases caused by a single ASFV infectious pig during its entire infectious period in a fully susceptible population (Hayes *et al.*, 2021). In general, if the ASFV effective reproduction number Re=R0 × (S/N) (S= susceptible pigs; N= total number of pigs in a given population) is greater than 1.0, disease is predicted to spread. Ideally, ASF vaccination should reduce Re to less than 1.0 by reducing the number of susceptible, naïve, contact pigs exposed to vaccinated, infected pigs.

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.3_14	**Category**: deletion ~~The ASF basic reproduction number, R0, can be defined as the average number of secondary ASF disease cases caused by a single ASFV infectious pig during its entire infectious period in a fully susceptible population (Hayes *et al.*, 2021). In general, if the ASFV effective reproduction number Re=R0 × (S/N) (S= susceptible pigs; N= total number of pigs in a given population) is greater than 1.0, disease is predicted to spread. Ideally, ASF vaccination should reduce Re to less than 1.0 by reducing the number of susceptible, naïve, contact pigs exposed to vaccinated, infected pigs.~~ **Reason**: These are population level statistics and are not valid when calculated with small sample sizes in defined settings.	**EN**: Agreed. **FR**: La Commission a souscrit au commentaire. **SP**: Aceptado.
3.9.1_C2.3.3_15	**Category:** change We query why this provision on assessment of horizontal transmission (challenge virus shed and spread study) is included in this chapter. Generally, there are no established protocols for evaluating the inhibitory effect on virus shedding in vaccinated and challenged animals for other veterinary vaccines, and this kind evaluation is not normally	**EN**: Agreed to delete the section, though the information is useful, it is not required for vaccine registration **FR**: La Commission a accepté de supprimer ce paragraphe ; l'information dont il s'agit, bien

	a part of requirements for authorisation/registration/licensing.	qu'utile, n'est pas nécessaire pour l'enregistrement du vaccin.
		SP: Se acuerda suprimir la sección, aunque la información es útil, no es necesaria para el registro de vacunas.

To evaluate ASF vaccine impact on ASF disease transmission, the test consists of a vaccination/challenge trial in piglets a minimum of ~~6~~ 4-weeks old and not older than 10-weeks old, free of antibodies to ASFV, and negative blood samples by real-time PCR.

The test is conducted using no fewer than 15 healthy piglets at a ratio comprising twice the number of vaccinated piglets to naïve piglets (e.g. ten vaccinated and five naïve). Use vaccine containing virus at the highest passage level that will be present in a batch of the vaccine.

The quantity of vaccine virus administered to each pig is equivalent to be not less than the minimum virus titre (minimum dose) likely to be contained in one dose of the vaccine as stated on the label. Following immunisation, vaccinated and naïve piglets should continue to be co-mingled.

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.3_16	**Category**: deletion Please delete ~~(minimum dose)~~. Not necessary and it might be confusing as reference to "dose" is made in the same sentence with a different meaning.	**EN**: Comment obsolete as the entire Section has been deleted
		FR: Commentaire obsolète car toute la section a été supprimée
		SP: Comentario obsoleto, ya que se ha suprimido toda la sección.

Twenty-eight days (±2 days) after the single ~~injection~~ dose of vaccine (or if using two ~~injections~~ doses of the vaccine then 28 days [±2 days] following the second ~~injection~~ dose), temporarily separate [into different pen(s) or room(s)] all vaccinated piglets from naïve piglets. Challenge all vaccinated piglets by the intramuscular or other previously verified route. Carry out the challenge using an ASFV representative strain of the epidemiologically relevant field strain(s) where the vaccine is intended for use (e.g. ASFV B646L [p72] genotype II pandemic strain and other p72 virulent genotype of recognised epidemiological importance). For gene deleted, recombinant MLV viruses, if neither challenge virus type is available, then carry out the test with the parental, virulent virus used to generate the MLV recombinant virus. Use a 10e3–10e4 HAD_{50} (or $TCID_{50}$ for non-HAD viruses challenge dose sufficient to cause death or met the humane endpoint in 100% of the nonvaccinated piglets in less than 21 days. Higher or lower challenge doses can be considered if appropriately justified.

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.3_17	**Category**: change *"….for TCID…….less than 21 days"*. This text will need to be revised to align with the final decision on the validity criterion.	**EN**: Comment obsolete as the entire Section has been deleted
		FR: Commentaire obsolète car toute la section a été supprimée
		SP: Comentario obsoleto, ya que se ha suprimido toda la sección.

Approximately 18–24 hours later, re-introduce naïve piglets to vaccinated, challenged piglets and allow for direct nose to nose contact exposure with vaccinated, challenged piglets. Allow for continuous contact exposure by co-mingling both groups through the end

of the study. If more than one pen or room is used for co-housing, following reintroduction initially maintain a ratio of 2:1 of challenged, vaccinated piglets to contact exposed, naïve piglets.

The rectal temperature of each contact piglet is measured on at least the 3 days preceding administration of the challenge virus to vaccinated pigs, immediately prior to direct contact exposure, 4 hours post-contact exposure, and then daily for at least 28~~, preferably 35~~ days and twice a week for at least 60 days. Observe all contact exposed piglets ~~at least~~ daily for at least 28 days, and then twice a week for at least 60 days ~~preferably for at least 35 days~~.

Carry out the ~~daily~~ observations in each contact piglet for signs of acute and chronic clinical disease using a quantitative clinical scoring system adding the values for multiple clinical signs (e.g. Gallardo *et al.*, 2015a). These clinical signs should include fever, anorexia, recumbency, skin haemorrhage or cyanosis, joint swelling and necrotic lesions around the joints, respiratory distress and digestive findings.

In addition, ~~blood should be taken from the naïve contact piglets at least twice a week from 3 days post-contact exposure for the duration~~ collect blood samples from the contact piglets at least two times per week from 3 days post-contact for at least 14 days, then weekly up to 35 days post-contact exposure and then every 14 days up to the end of the test period. Determine virus titres in all collected samples by quantitative virus isolation (HAD_{50}/mg or $TCID_{50}$/mg) or other appropriate methods (e.g. titration using IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration as described above ~~From the blood samples, determine infectious challenge virus titres by quantitative virus isolation (HAD_{50}/ml or $TCID_{50}$/ml) and using a real-time PCR test. If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test only may be used~~.

Collect blood (serum) samples from the naïve contact pigs at least at day 21 and day 28 (±2 days), and at the end of the test period, and carry out an appropriate test to detect vaccine virus antibodies.

Collect oral, nasal and faecal swab samples (preferably devoid of blood to minimise assay interference) from all contact-exposed naïve piglets at least two times per week from 3-days post-contact exposure for the first 2 weeks, then weekly for the duration of the test and test swabs for the presence of challenge virus. Determine virus titres in all collected samples by quantitative virus isolation (HAD_{50}/mg or $TCID_{50}$/mg) or other appropriate methods (e.g. titration using IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration as described above ~~Determine virus titres in all collected samples by quantitative virus isolation (HAD_{50}/ml or $TCID_{50}$/ml) and using a real-time PCR test. If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test or other appropriate method (e.g. titration using IPT or FAT detection) may be used~~.

At the end of the test period, humanely euthanise all contact piglets. Conduct gross pathology on spleen, lung, tonsil, and kidney tissue samples and at least three different lymph nodes. (which should include lymph node closest to site of inoculation, gastrohepatic and submandibular nodes). Determine virus titres in all collected samples by quantitative virus isolation (HAD_{50}/mg or $TCID_{50}$/mg) or other appropriate methods (e.g. titration using IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration as described above ~~Determine virus titres in all collected samples by quantitative virus isolation (HAD_{50}/mg or $TCID_{50}$/mg) and real-time PCR (see Section B.1. Identification of the agent). If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test or other appropriate method (e.g. titration using IPT or FAT detection) may be used~~.

The test is invalid if the vaccine fails to comply with the compliance criteria described for the protected dose test in vaccinated pigs (Section C.2.3.3.i above).

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.3_18	**Category**: addition The test is invalid if the vaccine fails to comply with the compliance criteria described for the protected dose test in vaccinated pigs (Section C.2.3.3.i above) or evidence of disease is seen in non-vaccinated pigs. **Reason**: This section is evaluation of horizontal transmission and did not include evidence of disease in non-vaccinates as a criteria as the reference is to an efficacy evaluation.	**EN**: Comment obsolete as the entire Section has been deleted **FR**: Commentaire obsolète car toute la section a été supprimée **SP**: Comentario obsoleto, ya que se ha suprimido toda la sección.

If the manufacturer claims that the vaccine induces sterilising immunity, the vaccine complies with the test for a reduction in horizontal disease transmission if all the following conditions are satisfied:

- No naïve, contact exposed piglet shows abnormal (local or systemic) reactions, reaches the defined humane endpoint or dies from causes attributable to ASF;

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.3_19	**Category**: deletion "~~shows abnormal (local or systemic) reactions~~". Please delete: Reference to abnormal (local or systemic) reactions is normally used relating safety observations after vaccine administration which is not the case. The point (clinical signs of disease) is covered in the next bullet point.	**EN**: Comment obsolete as the entire Section has been deleted **FR**: Commentaire obsolète car toute la section a été supprimée **SP**: Comentario obsoleto, ya que se ha suprimido toda la sección.

- No naïve, contact exposed piglet displays fever accompanied by typical signs of disease, including gross pathology.
- Naïve contact pigs show an absence of challenge virus in blood and tissues.
- No naïve contact pigs test positive for antibodies to the challenge virus.

Otherwise, the vaccine complies with the test for a reduction in horizontal disease transmission if:

- Naïve contact pigs show a reduction or absence of challenge virus levels in blood and tissues.
- None of or a reduced number of naïve contact exposed pigs test positive for antibodies to the challenge virus.

At a minimum, an efficacious MLV vaccine shall demonstrate ALL the following features (minimal standards):

- Protects against mortality;
- Reduces acute disease (fever accompanied by a reduction of typical clinical and pathological signs of acute disease)
- Reduces levels of viral shedding and viraemia.
- Reduces horizontal disease transmission (~~no~~ none of or a reduced number of naïve, contact exposed piglets shows abnormal [local or systemic] reactions, reaches the humane endpoint or dies from causes attributable to ASF, ~~and~~ displays fever accompanied by typical acute disease signs caused by ASF) and test positive for antibodies to the challenge virus.

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.3_20	**Category**: deletion "~~(none of or a reduced number of naïve, contact exposed piglets shows abnormal [local or systemic] reactions, reaches the humane endpoint or dies from causes attributable to ASF, displays fever accompanied by typical acute disease signs caused by ASF) and test positive for antibodies to the challenge virus.~~" We would suggest deleting this part. The key compliance criteria for transmission are already given above and specifically in lines 731-733, so the info would appear redundant and makes reading more difficult.	**EN**: Comment obsolete as the entire Section has been deleted **FR**: Commentaire obsolète car toute la section a été supprimée **SP**: Comentario obsoleto, ya que se ha suprimido toda la sección.

~~• Reduces levels of viral shedding and viraemia.~~

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.3_21	**Category**: change In Section C.2.3.3 of efficacy requirement, it is suggested to add "at least 80% of pigs in the immune group shall have no clinical signs and no viral shedding in mouth, nose and anus" to the minimum requirements for attenuated live vaccine.	**EN**: Comment obsolete as the entire Section has been deleted **FR**: Commentaire obsolète car toute la section a été supprimée **SP**: Comentario obsoleto, ya que se ha suprimido toda la sección.

In general, for regulatory approval, ASF MLV ~~addition, the~~ vaccines ~~in their commercial presentation before being authorised for general use~~ should be tested for efficacy ~~in the~~ under field conditions (see chapter 1.1.8 Section 7.2.3). ~~Additional~~ Field efficacy ~~evaluation~~ studies ~~may~~ generally include ~~but are not limited to: onset of immunity, duration of immunity, and impact on disease transmission~~ measurement of relevant efficacy parameters including but limited to mortality, clinical signs, impact on disease transmission, performance parameters.

2.3.4. Duration of immunity

Although not included in the guidance for ASF MLV first generation vaccines, manufacturers are ~~encouraged~~ required, as part of the authorisation procedure, to define and demonstrate the duration of immunity of a given vaccine ~~by evaluation of potency at the end of the claimed period of protection~~.

2.3.5. Stability

Stability of the vaccine should be demonstrated over the shelf life recommended for the product. Although not included in the standards for first generation MLV ASF vaccines, manufacturers are ~~encouraged~~ required, as part of the authorisation procedure, to generate data supporting the retention of immunogenicity over a defined period of ~~validity~~ time of a lyophilised or other pharmaceutical form of the ASF vaccine as part of the authorisation procedure.

Reference	Comment	Biological Standards Commission response
3.9.1_C2.3.5_1	**Category**: deletion Suggest reversal of all these changes - this should not be required for an initial approval.	**EN**: Partly agreed, the text has been amended to state that manufacturers are "in general" required… **FR**: Commentaire accepté en partie – le texte a été amendé et indique désormais qu'il est « généralement » demandé aux fabricants etc.

		SP: En parte aceptado, el texto se ha modificado para indicar que «en general» se exige a los fabricantes..
3.9.1_C2.3.5_2	**Category**: addition *"….are required…."*. Perhaps to cater for possible differences in different regulatory jurisdictions, "in general" could be added: "Manufacturers are in general required to (…)" Change proposed: The text can be simplified and rationalised as: "Although not included in the standards for first generation MLV ASF vaccines, manufacturers are required, as part of the authorisation procedure, to generate data (including potency) showing that the vaccine remains stable over the shelf life recommended for the product ~~supporting the retention of immunogenicity over a defined period of validity time of a lyophilised or other pharmaceutical form of the ASF vaccine as part of the authorisation procedure~~."	**EN**: Agreed. **FR**: La Commission a souscrit au commentaire. **SP**: Aceptado.

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NB: There are WOAH Reference Laboratories for African swine fever
(please consult the WOAH Web site:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on
diagnostic tests and reagents for African swine fever

NB: FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2021.

Annexe 5. Chapitre 1.1.3. Transport de materiel biologique

RÉUNION DE LA COMMISSION DES NORMES BIOLOGIQUES
Paris, 9–13 septembre 2024

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CHAPTER 1.1.3.

TRANSPORT OF BIOLOGICAL MATERIALS

INTRODUCTION

The transport of biological materials, including infectious substances, is covered by international, regional or national regulations that are updated on a regular basis and are widely accessible via the internet, or through commercial and regulatory transportation affiliates. The transport of biological materials within a country and between countries will be explained in this chapter.

The international regulations for the transport of infectious substances by any mode of transport are based upon the Recommendations on the Transport of Dangerous Goods made by the Subcommittee of Experts on the Transport of Dangerous Goods (UN SCETDG), a subcommittee of the United Nations Economic and Social Council. The Recommendations are presented in the form of Model Regulations covering air, rail, road, sea and also include international mail. The World Health Organization (WHO) guidance document on "Transport of Infectious Substances" summarising the different transport regulations is regularly updated. Countries, other international organisations, international treaties and conventions such as the International Air Transport Association (IATA), the World Customs Organization (WCO), the Convention on International Trade in Endangered Species (CITES), and the Convention on Biodiversity (CBD), especially the Nagoya Protocol, provide additional guidance and regulations that should be considered in planning the transportation of biological materials.

In the interest of animal and human health, biological materials collected from animals must be transported safely, efficiently and legally from the place where they are collected to the place where they are analysed, studied or used. The collection of specimens from animals is covered in Chapter 1.1.2 Collection, submission and storage of diagnostic specimens.

For the purpose of this chapter, animals are defined as all members of the Kingdom Animalia except humans, and biological materials include specimens or samples from animals, cell cultures, zoonotic and animal microorganisms and genetically modified or synthetic organisms, and biological products such as vaccines and reagents.

All reasonable precautions have been taken to verify the information contained in this chapter, which provides guidance on proper transport of biological materials. However, this text does not replace local or global regulations. In no event is WOAH liable for violations that arise from its use. Persons should always consult current local and global regulations at the time of transport.

A. RESPONSIBILITIES

All personnel involved in the packaging, labelling and shipping of biological materials must be appropriately trained, certified, competent and knowledgeable of the relevant national, regional and international regulations.

Biological materials should be transported to ensure a rapid and reliable system for delivery to the recipient using individuals such as professional logistics service providers that are trained and competent in the shipping and transportation process.

The efficient transport and transfer of biological materials requires co-ordination between the sender (shipper, consignor), the logistic providers, the carrier and the recipient (consignee) to ensure safe transport and arrival on time and in proper condition.

The sender (shipper, consignor) is responsible for providing the applicable documentation (e.g. certifications, permits) required by the national authorities of the countries of export, transhipment and import as well as ensuring that the shipment also complies with all other applicable regulations, such as:

i) Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from their Utilization to the Convention on Biological Diversity (CBD): Biological material containing genetic resources as defined under the CBD may be subject to Access and Benefit-Sharing legislation in both the country where it is sourced and the country where it is sent.

ii) CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora): All import, export, re-export and introduction from the sea of species covered by the Convention has to be authorised through a licensing system. Resolution Conf. 12.3. (Rev.CoP17) on Permits and Certificates, contains a section XII, regarding the use of simplified procedures to issue permits and certificates (https://cites-tsp.org/sites/default/files/resources_files/2022-12/E-Res-12-03-R17-min.pdf).

Procedures for incidents such as spills or theft of materials during transportation and any other realistic and foreseeable emergencies should be part of a risk management system in order to respond adequately to emergencies (for basic principles see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

1. The sender (shipper, consignor)

i) Before any shipment of biological materials, the sender must be able to:

   a) Identify and classify, pack (including temperature control), ensure quantity limits, mark and label the package of biological materials,

   b) Ensure the correct documentation of all biological materials intended for transport,

   c) Complete and produce a Shipper's Declaration for Dangerous Goods (DGD), when required,

   d) Ensure biological materials are not forbidden for transport;

   e) Ensure that the arrival date of the shipment does not fall on a week-end or day off;

   f) In the event that samples need to be sent under controlled temperature conditions, measures to maintain a cold chain need to be carefully considered throughout the entire shipment (including accommodating any possible delays that may occur).

ii) Prepares necessary documentation, including permits, dispatch and shipping documents if necessary;

iii) Notifies the recipient of transportation arrangements once these have been made, well in advance of the expected arrival time;

iv) The air way bill (AWB) is the standard shipping document for shipping goods by air. While it is common practice for the air carrier or freight forwarder to complete the air waybill, the sender may be required to provide it;

v) Makes advance arrangements with the recipient including investigating the need for import/export permits;

vi) Makes advance arrangements with the carrier to ensure:

   a) that the shipment will be accepted for appropriate transport;

   b) that the shipment is undertaken by the most direct routing, as appropriate.

2. The carrier/courier

i) The following measures must be taken by the carrier:

   a) Routing: appropriate routing must be ensured, such as by the shortest or most secure route.

   b) Transhipment: when transfers are necessary, precautions must be taken to assure special care, expeditious handling and monitoring of the substances in transit for both safety and security purposes.

ii) For air transport, the carrier is required by the regulations to use, when applicable, an acceptance checklist to verify that the shipment complies with:

   a) marking and labelling requirements; and

   b) documentation requirements.

iii) Provide advice to the sender and assistance regarding the necessary shipping documents and instructions for their completion as well as correct packaging

iv) Assists the sender in arranging the most appropriate routing and then confirms the routing and provides, if possible, ways to track the shipment;

v) Maintains and archives documentation for shipment and transport.

3. The recipient (consignee)

i) Obtains the necessary authorisation(s) from national authorities for the importation of the material;

ii) Provides the sender with the required import permit(s), letter(s) of authorisation, or other document(s) required by the national authorities;

iii) Arranges for the most timely and efficient collection on arrival;

iv) Should acknowledge receipt to the sender.

Shipments should not be dispatched until all the necessary arrangements between the sender, carrier and recipient have been made.

The transportation chain involves many more stakeholders with specific roles and responsibilities. These are explained in more details in the framework of aviation security in a joint ICAO (International Civil Aviation Organization) and WCO (World Customs Organization) brochure that can be accessed using the following link: http://www.wcoomd.org/en/topics/facilitation/instrument-and-tools/tools/~/media/4B167884A3064E78BCF5D29E29F4E57E.ashx.

In addition, Material Transfer Agreements (MTA) should be considered because they:

i) Protect the interests of all involved parties in relation to;

   a) Intellectual property

   b) Potential alternative uses

   c) Commercial aspects

   d) Liability to third parties

   e) Potential further transfers/uses

ii) Help to avoid misunderstandings around the use of materials

iii) Clarify ownership of property

With reference to Article 4 of the “Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from their Utilization (ABS) to the Convention on Biological Diversity”, the MTA is intended to establish a platform for the open exchange of materials among laboratories and to constitute Prior Informed Consent (PIC) on Mutually Agreeable Terms, while avoiding as much as possible the potential adverse impacts of the Nagoya requirements.

Appendix 3 is provided as a generic template for use in transfer of materials. It is intended to be modified and adapted as required to meet individual circumstances.

Appendix 3 does not constitute legal advice and users are responsible to ensure it meets their objectives and fulfils requirements of local legislation. Users are encouraged to consult their own legal professionals for advice on use of this template.

B. CLASSIFICATION AND CATEGORISATION

When transporting biological materials, the certified/ trained sender must determine whether the material should be classified as dangerous goods or not. Dangerous goods (hazardous materials, HAZMAT) are materials that can harm humans, animals and other living organisms, property, or the environment, and their transport is regulated by United Nations (UN) regulations[1]. Dangerous goods are assigned a *UN number* and *proper shipping name* based on the classification of the dangerous goods. The transport regulations assign a packing instruction against the UN number and proper shipping name, to specify the packaging/packing method to ensure that the dangerous goods do not pose a hazard in transport. Of the biological materials that are discussed in this Chapter, infectious

[1] http://www.unece.org/trans/danger/publi/unrec/rev13/13nature_e.html; http://www.unece.org/trans/danger/publi/unrec/rev20/20files_e.html

substances are classified as dangerous goods and are assigned to UN 2814, UN 2900, UN 3373, or UN 3291, as appropriate. In addition, Genetically Modified Microorganisms (GMMOs) and Genetically Modified Organisms (GMOs) are classified as Class 9 and assigned to UN 3245 if they are not classified as Category A or Category B.

Table 1. Summary of classification, categorisation, identification and packaging of infectious substances.

Dangerous goods classifications	Categorisation	Proper shipping name[2]	UN number[2]	Packing instruction/ packaging requirements
Class 6, Division 6.2	Category A	Infectious substance, affecting humans	UN 2814	P620
		Infectious substance, affecting animals	UN 2900	
Class 6, Division 6.2	Category B	Biological substance, Category B	UN 3373	P650
Class 6, Division 6.2	Exempt human/animal specimens	Exempt human/animal specimens	N/A	Triple packaging to include a leakproof primary container. A leakproof secondary container with absorbent material and outer rigid packaging
not subject to dangerous goods regulations	Biological materials not subject to dangerous goods regulations	N/A	N/A	N/A
Class 9	GMMOs and GMOs that are not classified as Category A or B infectious substances	Genetically modified microorganisms; Genetically modified organisms	UN 3245	P904 (ICAO/IATA PI 959), IBC99

If it is likely that microorganisms that are present in the biological materials can cause harm to humans or animals then they must be assigned either to Category A or B.

The proper shipping name (see Table 1) must be supplemented with the technical name (scientific name of the pathogen) in parenthesis on the transport document, but not on the outer packaging. When the identity of the infectious substances to be transported are unknown, but are suspected of meeting the criteria for inclusion in category A, the words “suspected category A infectious substance” must be shown, in parenthesis, following the proper shipping name on the transport document.

1. Category A

A Category A substance is an infectious substance which is transported in a form that, when exposure to it occurs, is capable of causing permanent disability, life-threatening or fatal disease in otherwise healthy humans or animals. Assignment to UN 2814 or UN 2900 (see Table 1) must be based on the known medical history of the animal(s), signs and individual circumstances of the specimen source, and endemic local disease conditions, or professional judgement concerning individual circumstances of the source, human or animal.

Some organisms are considered Category A only when in culture form (e.g. *Bacillus anthracis*, foot and mouth disease virus). Indicative examples of substances that meet these criteria are given in the Table 3. The table is not exhaustive. Infectious substances, including new or emerging pathogens, that do not appear in the Table but that meet the same criteria must be assigned to Category A. In addition, if there is doubt as to whether or not a substance meets the criteria it must be assigned to Category A.

Some infectious substances may have a high economic or trade impact on specific countries should there be release to the environment. Therefore, other infectious substances may be added to the list by individual countries (e.g. cultures of Newcastle disease virus where the virus is exotic to the country or region).

Medical or clinical waste containing Category A infectious substances shall be assigned to UN 2814 or UN 2900 as appropriate. **Solid** medical waste containing Category A infectious substances generated from the medical

[2] Dangerous goods are assigned UN numbers and proper shipping names according to their hazard classification and condition under the Dangerous Goods Regulations. See the Dangerous Goods List at pages 191–304 of the UN Model Regulations http://www.unece.org/fileadmin/DAM/trans/danger/publi/unrec/rev20/Rev20e_Vol1.pdf

treatment of humans or veterinary treatment of animals may be assigned to UN 3549. It should be noted that Medical or clinical waste from bioresearch or liquid waste must not be assigned to UN3549.

2. Category B

Biological materials containing pathogens that do not meet the criteria for Category A (i.e. do not cause life-threatening disease to humans or animals) shall be assigned to Category B (UN 3373).

Typically, a specimen with a high likelihood to contain pathogenic organisms shipped for disease diagnosis (e.g. confirmatory diagnosis of suspected or clinical cases, specimens for differential diagnosis, such as blood samples for classical swine fever or sheep pox diagnostics or throat samples from chickens for avian influenza) can be assigned to Category B.

It is important to note that unlike cultures, *patient specimens* which may contain infectious microorganisms listed as 'cultures only' in Table 3 (Category A infectious substances) do not require Category A transport practices. For these specimens Category B transport practice should be applied. In this case, although directly collected specimens (e.g. serum) can be shipped as Category B, pure cultures of the same pathogens must follow the requirements of Category A due to the characteristics of the specific organism. Some examples are classical swine fever virus isolates or sheep pox virus isolates (see Table 3). Specimens from animals intentionally infected with Category A pathogens must be sent as Category A, even if they are assigned to Category A (cultures only).

Shipments of cultures of non-category A agents can be assigned to Category B.

Medical or clinical waste containing Category B infectious substances shall be assigned to UN 3291.

3. Exempt specimens

Animal specimens for which there is minimal likelihood that pathogens are present can be transported as Exempt Specimens. Examples of specimens in the veterinary field which may be transported as exempt include specimens from surveillance studies, export controls of healthy animals (e.g. certification of freedom from classical swine fever) or determination of immune status of individual animals or populations (post-vaccination).

These specimens are not subject to dangerous goods regulations if the specimen is transported in a packaging that will prevent any leakage and that is marked appropriately (triple packaging principle, see Section C. *Packaging* and Figure ~~3~~ 6 of Appendix 1.1.3.2).

4. Biological materials not subject to Dangerous Goods Regulations

Based on the known medical history of the animal(s), signs and individual circumstances of the source of the biological materials, and endemic local disease conditions, the following *are not subject to dangerous goods regulations, unless they meet the criteria for inclusion in another class* (*such as Class 9*):

i) biological materials ~~that do~~ known to not contain infectious substances

ii) biological materials containing microorganisms that are non-pathogenic to humans or animals;

iii) biological materials in a form in which any pathogens present have been neutralised or inactivated such that they no longer pose a health risk;

iv) Environmental specimens (including food and water specimens) that are not considered to pose a significant risk of infection;

v) Dried blood spots, collected by applying a drop of blood onto absorbent material.

Note: Materials not subject to dangerous goods regulations may need to be risk assessed before proceeding with their shipment. There may be specific regulations in place in some countries for the shipment, export or import of nucleic acids.

5. Contaminated items

These listed below are also included in infectious substances in the international regulations on transport of dangerous goods, however the details are not discussed in this chapter. For more information see UN Model Regulations, paragraphs 2.6.3.2.3.3 and .9 respectively.

207 Table 2. Summary of classification, categorisation, identification and packaging of contaminated items with
208 infectious substances.

Dangerous goods classifications	Categorisation	Proper shipping name[3]	UN number[3]	Package
Class 6, Division 6.2	Category A	Medical* devices or equipment contaminated with or containing infectious substances in Category A	UN2814, UN2900 as appropriate	Must be marked "Used Medical Device" or "Used Medical Equipment"
Class 6, Division 6.2	Exemption when condition is met	Medical* devices, medical equipment	N/A	See UN Model Regulations 2.6.3.2.3.9 and IATA Dangerous Goods Regulations (DGR) 3.6.2.2.3.9
Class 6, Division 6.2	Category A	Medical* waste, Category A, affecting humans, **solid**; Medical waste, Category A, affecting animals only, **solid**	UN 3549	P622, LP622
Class 6, Division 6.2	Category B	Clinical waste, Unspecified, n.o.s. (not otherwise specified); (Bio) medical waste, n.o.s.; Regulated medical waste, n.o.s.	UN3291	P621 (PI622), IBC620, LP621

209 *including veterinary use

210 6. Infectious substances included in Category A

211 Table 3. Indicative examples of infectious substances included in Category A.

UN number and proper shipping name	Microorganism
UN 2814 Infectious substance, affecting humans	*Bacillus anthracis* (cultures only)
	Brucella abortus (cultures only)
	Brucella melitensis (cultures only)
	Brucella suis (cultures only)
	Burkholderia mallei – Pseudomonas mallei – Glanders (cultures only)
	Burkholderia pseudomallei – Pseudomonas pseudomallei (cultures only)
	Chlamydia psittaci – avian strains (cultures only)
	Clostridium botulinum (cultures only)
	Coccidioides immitis (cultures only)
	Coxiella burnetii (cultures only)
	Crimean–Congo haemorrhagic fever virus
	Dengue virus (cultures only)
	Eastern equine encephalomyelitis virus (cultures only)
	Escherichia coli, verotoxigenic (cultures only)[4]

[3] Dangerous goods are assigned UN numbers and proper shipping names according to their hazard classification and condition under the Dangerous Goods Regulations. See the Dangerous Goods List at pages 191–304 of the UN Model Regulations http://www.unece.org/fileadmin/DAM/trans/danger/publi/unrec/rev20/Rev20e_Vol1.pdf

[4] For surface transport (ADR) nevertheless, when the cultures are intended for diagnostic or clinical purposes, they may be classified as infectious substances of Category B.

UN number and proper shipping name	Microorganism
	Ebola virus
	Flexal virus
	Francisella tularensis (cultures only)
	Guanarito virus
	Hantaan virus
	Hantaviruses causing haemorrhagic fever with renal syndrome
	Hendra virus
	Hepatitis B virus (cultures only)
	Herpes B virus (cultures only)
	Human immunodeficiency virus (cultures only)
	Highly pathogenic avian influenza virus (cultures only)
	Japanese encephalitis virus (cultures only)
	Junin virus
	Kyasanur Forest disease virus
	Lassa virus
	Machupo virus
	Marburg virus
	Monkeypox virus
	Mycobacterium tuberculosis (cultures only)[1]
	Nipah virus
	Omsk haemorrhagic fever virus
	Poliovirus (cultures only)
	Rabies virus (cultures only)
	Rickettsia prowazekii (cultures only)
	Rickettsia rickettsii (cultures only)
	Rift Valley fever virus (cultures only)
	Russian spring–summer encephalitis virus (cultures only)
	Sabia virus
	Shigella dysenteriae type 1 (cultures only)
	Tick-borne encephalitis virus (cultures only)
	Variola virus
	Venezuelan equine encephalitis virus (cultures only)
	West Nile virus (cultures only)
	Yellow fever virus (cultures only)
	Yersinia pestis (cultures only)
UN 2900 Infectious substance, affecting animals only	African swine fever virus (cultures only)
	Avian paramyxovirus Type 1 – Velogenic Newcastle disease virus (cultures only)
	Classical swine fever virus (cultures only)
	Foot and mouth disease virus (cultures only)
	Goatpox virus (cultures only)

UN number and proper shipping name	Microorganism
	Lumpy skin disease virus (cultures only)
	Mycoplasma mycoides – contagious bovine pleuropneumonia (cultures only)
	Peste des petits ruminants virus (cultures only)
	Rinderpest virus (cultures only[5])
	Sheep-pox virus (cultures only)
	~~Goatpox virus (cultures only)~~
	Swine vesicular disease virus (cultures only)
	Vesicular stomatitis virus (cultures only)

C. PACKAGING

1. Principles

All biological materials should be packaged and transported in accordance with local, national and international regulations. The procedures should minimise the risk of exposure for those engaged in transportation and should protect the environment and susceptible animal populations from potential exposures. Additionally, ineffective packaging that does not protect specimens or preservatives (e.g. ice) from damage or prevent leakage will likely delay the delivery of the shipment to the laboratory, delaying or preventing critical laboratory analyses from being performed. Biological materials should always be packaged and transported to protect the integrity of the specimens, as well as to avoid cross-contaminating other specimens and environmental contamination. Minimum requirements for the transport of specimens follow the principle of triple packaging, consisting of three layers as described below:

i) a primary leak-proof receptacle;

ii) a secondary leak-proof packaging with absorbent material;

iii) ~~a~~n rigid outer packaging~~;~~

~~of which either the secondary or the outer packaging must be rigid~~.

1.1. Primary receptacle

A primary receptacle, leak-proof for liquids or sift-proof for solids containing the specimen. Primary receptacle(s) must be packed into the secondary packaging with enough absorbent material (e.g. cellulose wadding, paper towels, household paper, cotton balls) to absorb all fluid in case of breakage. Even though the regulations do not prohibit glass, primary receptacles should preferably be non-breakable. In addition, they must not contain any sharps (e.g. vacutainer with needle), particularly when using soft secondary or outer containers. If screw cap vials are used, they shall be secured by e.g. tape. A flip-top vial must not be used.

1.2. Secondary packaging

A second durable, leak-proof packaging to enclose and protect the primary receptacle(s) (e.g. sealed plastic bag, plastic container, screwcap can).

For transport by air, the primary receptacle or the secondary packaging shall be capable of withstanding, without leakage, an internal pressure of 95 kPa (0.95 bar) in the range of –40°C to +55°C (–40°F to +130°F).

[5] Subject to prior approval by the FAO-WOAH rinderpest secretariat

1.3. Outer packaging

Secondary packaging is placed in a rigid outer shipping packaging (e.g. sturdy insulated fibre board box) with suitable cushioning material. Outer packaging protects the contents from outside influences, such as physical damage, while in transit.

2. Category A

Due to the highly hazardous nature of the Category A samples the packaging must meet special requirements. The principle of triple packaging applies here, and the transport containers and outer packaging must meet the criteria defined in the relevant regulations. Category A must only be transported in packaging that meets the United Nations class 6.2 specifications, complies with Packing Instruction P620 and have passed specific tests and with UN specification marking as P620. This ensures that strict performance criteria are met; tests for compliance with these criteria include a 9-metre (29.5 feet) drop test, a puncture test, a pressure test and a stacking test. The packages are labelled to provide information about the contents of the package, the nature of the hazard and the packaging standards applied.

Marking and labelling is as follows (see Figure 3 of Appendix 1.1.3.2):

i) *The delivery address* (*consignee*) *and sender's details* (*shipper*), *as well as 24/7 emergency contact details including named persons with telephone numbers to guarantee safe delivery.*

ii) *The proper shipping name and the UN number.*

Proper shipping name	*UN number*
INFECTIOUS SUBSTANCE, AFFECTING HUMANS	*UN2814*
INFECTIOUS SUBSTANCE, AFFECTING ANIMALS only	*UN2900*

iii) *The Infectious Substance label* (*Figure 1*).

NB: This label is only for Category A. This label must not be used when shipping Category B.

Fig. 1. Infectious Substance label for the transport of Category A.

iv) *UN specification marking for P620 packaging (printed on the box*).

v) *Orientation label, Cargo only label, if required* (*depending on the Net Weight* [*kg*] *of the infectious substance in a P620 box*).

The exact details can be found in P620 Packing Instruction[6].

For air transport:

i) The primary receptacle or secondary packaging must be capable of withstanding, without leakage, an internal pressure of 95 kPa. The primary receptacle or secondary packaging must also be capable of withstanding temperatures in the range of –40°C to +55°C;

ii) For liquids: the net quantity of infectious substances per one P620 box shall not exceed 50 ml for transport in cargo space of a passenger aircraft; and must not contain more than 4 litres (contain multiple primary receptacles totalling more than 4 litres) for transport on a cargo only aircraft;

iii) For solids: the net quantity of infectious substances per one P620 box shall not exceed 50 g for transport in cargo space of a passenger aircraft, and must not contain more than 4 kg (even if containing multiple primary receptacles totalling more than 4 kg) for transport on a cargo only aircraft. This quantity limit doesn't apply for animal parts, organs and whole carcasses.

iv) The three triple packaging principle has to be adopted accordingly using appropriate packaging systems;

v) The entire package must have been tested and complies with Packing Instruction P620.

For further information on marking and labelling of the Category A shipment package, see P620 Packing Instruction for UN Nos 2814 and 2900[6]

3. Category B

Category B must be transported in a packaging that complies with the requirements of packing instruction P650. The approval of the box by the government is not required, thus UN specification marking is not required.

Marking is as follows:

i) Packages should be clearly labelled with the delivery address and sender's details to guarantee safe delivery in time at the correct destination.

[6] Page 81 at: http://www.unece.org/fileadmin/DAM/trans/danger/publi/unrec/rev20/Rev20e_Vol2.pdf

ii) Label with the *proper shipping name* in letters at least 6 mm high: “BIOLOGICAL SUBSTANCE, CATEGORY B” (Figure 2)

iii) In addition to the proper shipping name, the mark shown below (UN3373 in diamond) is used for shipments of Category B substances. The UN3373 mark must always be visible on the outer packaging.

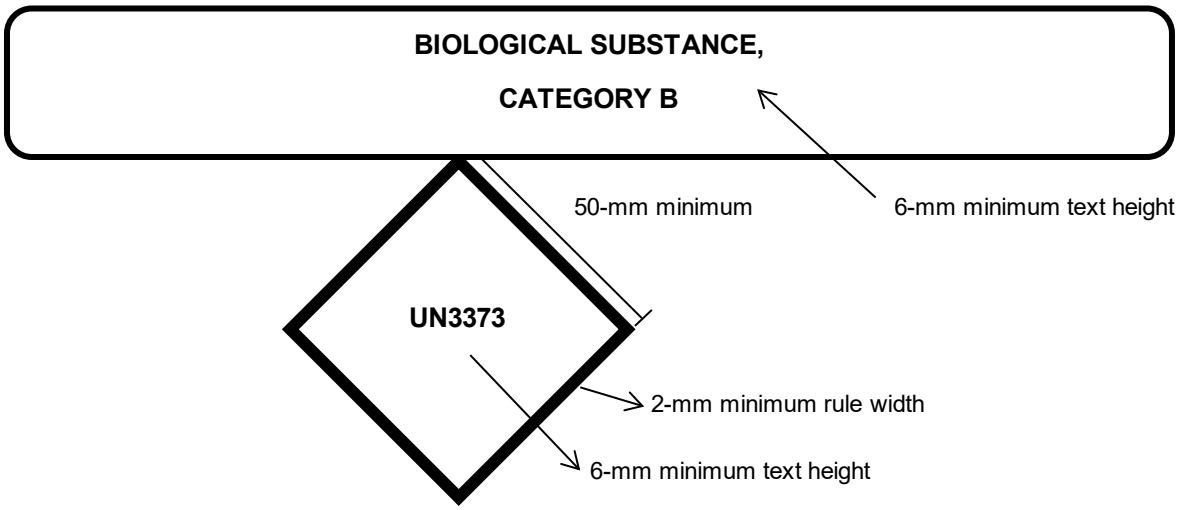


Fig. 2. UN3373 mark for the transport of Category B substances.

Additional requirements do apply as for category A for international shipment and air transport. One of the main differences between P650 and P620 is the reduced drop-test to 1.2 metres (4 feet).

For air transport:

i) The primary receptacle or secondary packaging must be capable of withstanding, without leakage, an internal pressure of 95 kPa. The primary receptacle or secondary packaging must also be capable of withstanding temperatures in the range of –40°C to +55°C;

ii) For liquids: no primary receptacle shall exceed 1 litre and the outer packaging must not contain more than 4 litres (contain multiple primary receptacles totalling more than 4 litres);

iii) For solids: the outer packaging must not contain more than 4 kg. This restriction doesn’t apply for animal parts, organs and whole carcasses.

The exact details can be found in P650 Packing Instruction for UN No. 3373[7].

4. Exempt specimens

Biological materials for which there is a minimal likelihood that pathogens are present are not subject to regulation if the specimen is carried in a packaging which will prevent any leakage and which is marked with the words “Exempt animal specimens”, as appropriate. The triple packaging system must still be applied.

5. Biological materials not subject to Dangerous Goods Regulations

This exemption refers to biological materials that do not contain infectious substances and are therefore not subject to dangerous goods regulations (such as class 6.2) and any packaging requirements*, unless they meet the criteria for inclusion in another class* (*such as class 9*).

Note: There may be specific regulations in place in some countries for the shipment, export or import of nucleic acids.

6. Overpack

“Overpack” is the term used when one or more packages are combined to form one unit and sent to the same destination by a single shipper. When refrigerants are used to protect contents, the overpacks may comprise insulated vessels or flasks. Whenever an overpack is used, the required marks and labels shown on the outer

[7] Page 82 at: http://www.unece.org/fileadmin/DAM/trans/danger/publi/unrec/rev20/Rev20e_Vol2.pdf

packaging must be repeated on the outermost layer of the overpack, except for the UN specification marking on P620. This requirement applies to all infectious substances including Categories A and B. Overpacks are also required to be marked with the word “overpack”.

Combining different categories of infectious substance in a same overpack is permissible however in this case outer labelling should indicate the highest category included in the package.

7. Cold chain transportation

Refrigerants may be used to stabilise specimens during transport.

Ice, ice packs or dry ice shall be placed outside the secondary receptacle. Wet ice shall be placed in a leak-proof container; the outer packaging or overpack shall also be leak-proof.

Dry ice (solid carbon dioxide) must not be placed inside the primary or secondary receptacle because of the risk of explosion. A specially designed insulated packaging may be used to contain dry ice, typically a polystyrene or waxed-treated cardboard box to prevent leakage and maintain temperature. The packaging must permit the release of carbon dioxide gas if dry ice is used and the package (the outer packaging or the overpack) shall be marked “UN 1845” and “Carbon dioxide, solid as coolant” or “Dry ice as coolant” and the weight of the dry ice in Kilograms should also be indicated on the labelling. The package must also bear the Class 9 – Miscellaneous hazard label.

The secondary receptacle shall be secured within the outer package to maintain the original orientation of the inner packages after the refrigerant has melted or dissipated.

If liquid nitrogen is used as a refrigerant, additional requirements have to be followed according to the relevant regulations for dangerous goods (Division 2.2, UN 1977). Information on Dry Shipper is available in p19 of WHO Guidance on regulations for the transport of infectious substances 2017–2018[8].

D. ADDITIONAL CONSIDERATIONS

In addition to the transport regulations described above, other international agreements might be applicable.

1. CITES

The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) is an international agreement between governments with the aim to ensure that international trade in specimens of wild animals and plants does not threaten their survival. The Convention is in effect in 183 Parties (including one economic integration organisation, the European Union).

Some specimens to be transported from one country to another may be derived from species covered by CITES (roughly 5,600 animal species and 30,000 plant species). Depending on the classification of the species in one of the three Appendices of the Convention and the movement involved, a CITES export permit, both an export and import permit, or re-export certificates may be required. The appropriate documents must be obtained from National CITES Management Authorities. Simplified procedures for the issuance of permits and certificates exist to facilitate and expedite trade in biological specimens from CITES-listed species.

There may be some variation from one country to another in their CITES trade requirements (some countries take stricter domestic measures and some countries have added additional species requirements for permits in addition to the CITES lists), therefore It is always advisable to check the national legislation that applies.

Further information on CITES: https://cites.org

2. Nagoya protocol and access and benefit-sharing

“Fair and equitable sharing of benefits arising from the utilisation of genetic resources” (Access and Benefit Sharing, or ABS) is one of the three objectives of the Convention on Biodiveristy (CBD). The CBD confirms that States have sovereign rights over their genetic resources, including animals, plants, fungi and microorganisms. Consequently, States may choose to regulate access to these, requiring researchers both within and outside their borders to seek permission. Permission (*Prior Informed Consent* or PIC) will be the responsibility of the State and maybe other

[8] http://www.who.int/ihr/publications/WHO-WHE-CPI-2017.8/en/

stakeholders, and the conditions for sharing benefits (*Mutually Agreed Terms* or MAT) may be agreed with a range of actors, including laboratories.

In 2014 the *Nagoya Protocol on Access and Benefit Sharing* (NP) was agreed by the Parties of the CBD. Nagoya Protocol requirements have been implemented in a large number of countries. This obliges the Parties to take compliance measures and monitor researchers and others utilising genetic resources (accessed from other Parties) within their jurisdiction. Biological material containing genetic resources as defined under the CBD may be subject to national Access and Benefit-Sharing (ABS) legislation in both the country where it is sourced and the country where it is sent. This may include compliance with the NP if both countries are Party to the NP. To discover whether this is the case, consult the ABS Clearing House (https://absch.cbd.int/), and ask the National Focal Point of both countries (contact details on ABS Clearing House). If the source country has ABS legislation it may be necessary to seek a permit (Prior Informed Consent and Mutually Agreed Terms) prior to international transport of the material.

Benefits may be monetary or non-monetary. "Utilisation of genetic resources" is defined by the CBD as "means to conduct research and development of the genetic and/or biochemical composition of genetic resources, including through the application of biotechnology as defined in Article 2 of the Convention". Consequently, many activities carried out by WOAH Members and their constituents may be classified as ABS.

Compliance will require researchers to produce documentary evidence that genetic resources were accessed with appropriate PIC and MAT, and declaring the type of utilisation being undertaken. This information will be transmitted to the provider country to check if the information accords with their records. There is no exemption for organisms of veterinary importance from ABS provisions in the NP, but countries where they are being accessed may choose to make this distinction and exemption.

Article 8 of the Nagoya Protocol states "*In the development and implementation of its access and benefit-sharing legislation or regulatory requirements, each Party shall: …* (*b*) *Pay due regard to cases of present or imminent emergencies that threaten or damage human, animal or plant health, as determined nationally or internationally. Parties may take into consideration the need for expeditious access to genetic resources and expeditious fair and equitable sharing of benefits arising out of the use of such genetic resources, including access to affordable treatments by those in need, especially in developing countries*;". However, this is not required and countries may choose to take no action. In addition, there is a 90-day grace period allowed to complete required documents after transport of the biological material.

If the research or diagnostic work on biological material of veterinary concern is considered 'utilisation' by the country (Party) in which it takes place the researcher will be required to provide information, including:

i) The Internationally Recognised Certificate of Compliance (number) from the ABS Clearing House, if available; or
   a) ABS Permit reference
   b) Evidence of Prior Informed Consent
   c) Evidence of Mutually Agreed Terms
   d) The entity to whom PIC and MAT was granted
ii) The Provider Country
iii) Date and place of access
iv) Description of the Genetic Resources

This information should be included in the documents with the transported biological material.

Although this documentation is only required if both providing country and recipient country are Party to the Nagoya Protocol the provider may have Access and Benefit-Sharing legislation even if not a Party. This should be respected and any documentation required, including permits, Prior Informed Consent and Mutually Agreed Terms, acquired and dispatched with the material.

Further information can be found on:

i) The ABS Clearing House – https://absch.cbd.int/
ii) The CBD Website – https://www.cbd.int/abs/default.shtml

E. REFERENCES AND FURTHER READING

WHO Guidance on regulations for the transport of infectious substances 2017–2018; applicable as of 1 January 2017, covering transport regulations on national and international and air transport by different means: ~~http://www.who.int/ihr/publications/WHO-WHE-CPI-2017.8/en/~~ https://iris.who.int/handle/10665/254788

Swiss Expert Committee on Biosafety on Transport of biological substances~~, import and export of substances consisting of or containing pathogenic or genetically modified (micro)organisms~~"; practical explanation on how to transport biological substances according to the specific dangerous goods transport regulations:

~~http://www.efbs.admin.ch/en/transport/index.html~~ https://www.efbs.admin.ch/index.php?id=462&L=3

IATA: http://www.iata.org/whatwedo/cargo/dgr/Documents/infectious-substance-classification-DGR56-en.pdf

F. ADDITIONAL INFORMATION ON THE UNITED NATIONS SYSTEM FOR THE TRANSPORT OF DANGEROUS GOODS

The United Nations dangerous goods web site provides comprehensive detail concerning the United Nations Recommendations on the Transport of Dangerous Goods. It also provides links to the modal agencies: http://www.unece.org/trans/danger/danger.html

The site below provides the full text of the United Nations Recommendations on the Transport of Dangerous Goods – Model Regulations, which can be downloaded in PDF format. Readers wishing to see the text relating to the transport of infectious substances should download Part 2, Part 4 and Part 5 of the Recommendations: http://www.unece.org/trans/danger/publi/unrec/rev20/20files_e.html

The site below provides the full text of the European Agreement concerning the International Carriage of Dangerous Goods by Road (ADR) of 2017, which entered into force on 1 January 2017, which can be downloaded in PDF format. Readers wishing to study the text relating to the transport of infectious substances should download Part 2 (2.2.62), Part 4 (search P620, P650) and Part 5: http://www.unece.org/trans/danger/publi/adr/adr2017/17contentse0.html and http://www.unece.org/trans/danger/publi/adn/adn2017/17files_e0.html

Contracting parties to the various conventions for the transport of dangerous goods can be found on a number of web sites:

Air	ICAO: http://www.icao.int/Pages/default.aspx and https://www.icao.int/safety/DangerousGoods/Pages/StateVariationPage.aspx
	IATA: http://www.iata.org/whatwedo/cargo/dgr/Documents/infectious-substance-classification-DGR56-en.pdf
Rail	RID (Intergovernmental Organisation for International Carriage by Rail): http://www.otif.org/. RID is primarily for the countries of Europe, North Africa and the Middle East. There are a number of countries (mainly Eastern Europe and Asia that apply RID through the Organization for Cooperation of Railways (OSJD); details of RID membership can be found at http://www.otif.org/en
Road	ADR: ~~http://www.unece.org/trans/danger/publi/adr/country-info_e.htm~~ https://unece.org/about-adr (lists competent authorities)
Sea	IMO (International Maritime Organization): http://www.imo.org
Post	UPU (Universal Postal Union): http://www.upu.int/

NB: FIRST ADOPTED IN 1992 AS SAMPLING METHODS.
MOST RECENT UPDATES ADOPTED IN 2018.

APPENDIX 1.1.3.1.

DEFINITIONS

The following definitions are for the purposes of this chapter only. For general definitions please see the Glossary.

- **Biological products**

Biological products are those products derived from living organisms which are manufactured and distributed in accordance with the requirements of appropriate national authorities, which may have special licensing requirements, and are used either for prevention, treatment, or diagnosis of disease in humans or animals, or for development, experimental or investigational purposes related thereto. They include, but are not limited to, finished or unfinished products such as vaccines.

- **Cultures**

Cultures are the result of a process by which pathogens are intentionally propagated. This definition does not include human or animal patient specimens as defined below.

- **Infectious substances**

For the purposes of transport, infectious substances are defined as substances which are known or are reasonably expected to contain pathogens. Pathogens are defined as microorganisms (including bacteria, viruses, parasites, fungi) and other agents such as prions, which can cause disease in humans or animals. Infectious substances are further classified according to risk into two categories.

- **Genetically modified microorganisms (GMMOs) and organisms (GMOs)**

Genetically modified microorganisms not meeting the definition of infectious substance are classified in Class 9 (Miscellaneous dangerous substances and articles, including environmentally hazardous substances). GMMOs and GMOs are not subject to dangerous goods regulations when authorised for use by the competent authorities of the countries of origin, transit and destination. Genetically modified live animals shall be transported under terms and conditions of the competent authorities of the countries of origin and destination. DNA, RNA or plasmids are not considered as GMMO and not subject to dangerous goods regulations.

- **Medical or clinical wastes**

Medical or clinical wastes are wastes derived from the veterinary treatment of animals, the medical treatment of humans or from bio-research.

- **Patient specimens**

Patient specimens are those, collected directly from humans or animals, including, but not limited to, excreta, secreta, blood and its components, tissue and tissue fluid swabs, and body parts being transported for purposes such as research, diagnosis, investigational activities, disease treatment and prevention.

*
* *

APPENDIX 1.1.3.2.

EXAMPLE OF THE TRIPLE PACKAGING SYSTEM (IATA RECOMMENDATIONS) FOR THE PACKING AND LABELLING OF DIFFERENT TYPES OF BIOLOGICAL MATERIALS

Fig. 3. Example of triple packaging system for the packaging and labelling of Category A, UN2814 and UN2900 infectious substances (Figure kindly provided by IATA, Montreal, Canada).

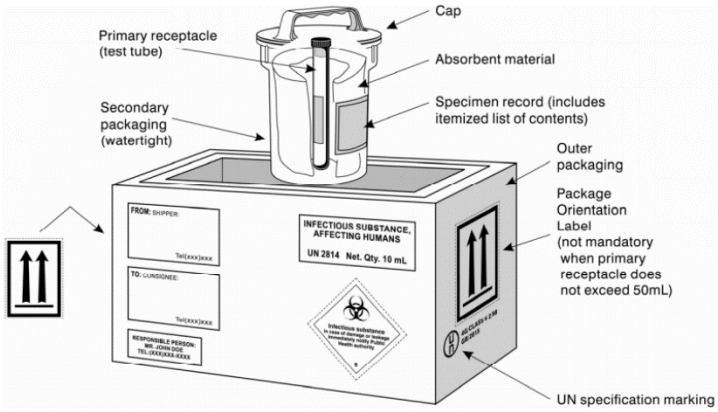


Fig. 4. Example of the triple packaging system for the packing and labelling of Category B, UN3373 infectious substances (Figure kindly provided by IATA, Montreal, Canada).

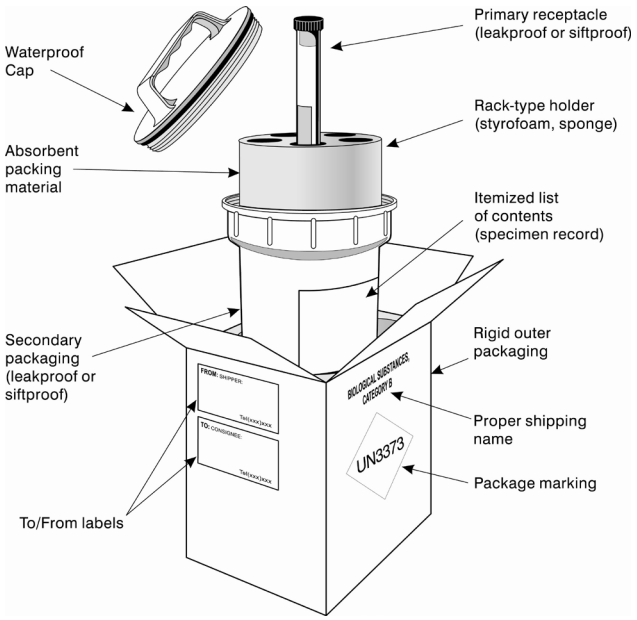


Fig. 5. Example of the triple packaging system for the packing and labelling of Category B, UN3373 infectious substances with non-rigid leakproof secondary packaging (Figure kindly provided by IATA, Montreal, Canada).

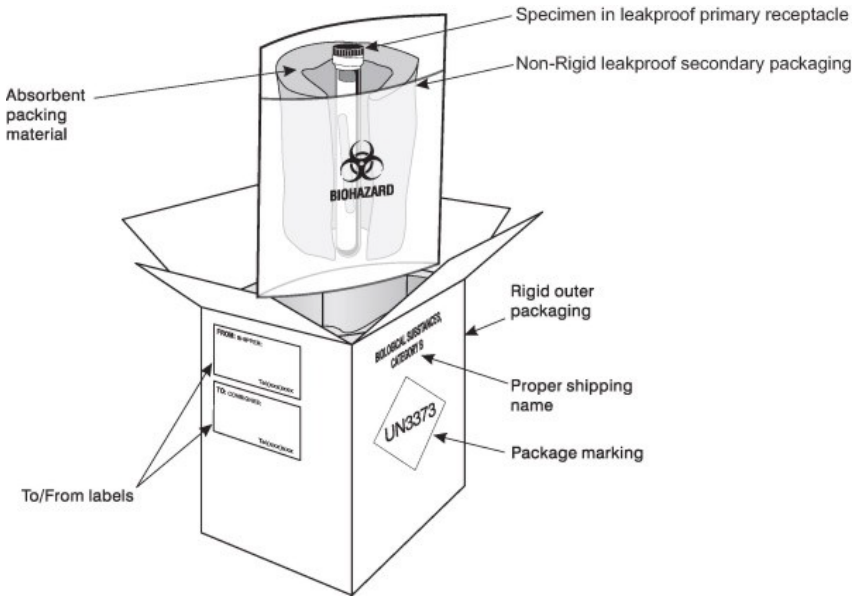
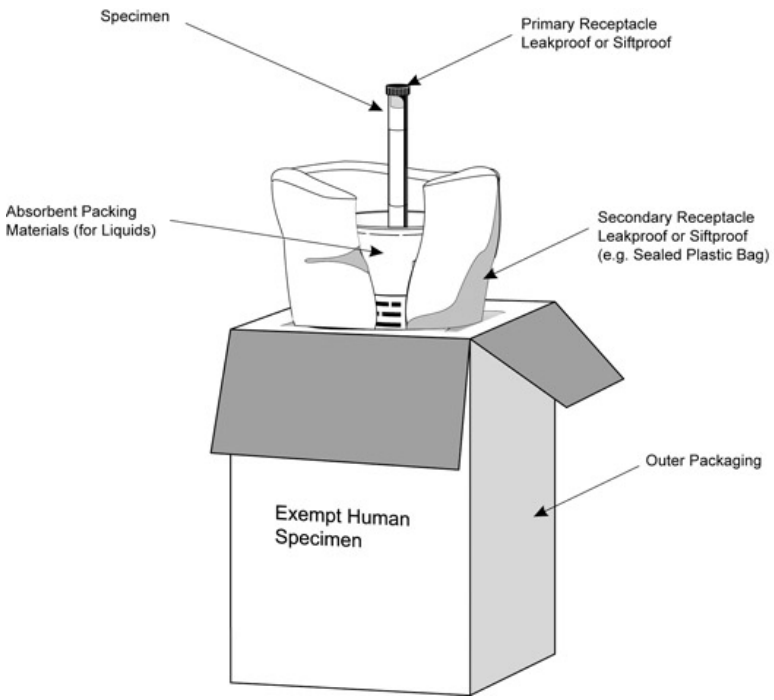


Fig. 6. Example of the triple packaging system for the packing and labelling of Exempt specimen (Figure kindly provided by IATA, Montreal, Canada).



APPENDIX 1.1.3.3.

MATERIAL TRANSFER AGREEMENT

MATERIAL TRANSFER AGREEMENT

BETWEEN

PROVIDER

Organisation:
Address:
Country:

PROVIDER SCIENTIST

Title and name:
Organisation:
Address:

AND

RECIPIENT

Organisation:
Address:
Country:

RECIPIENT SCIENTIST

Title and name:
Organisation:
Address:

ORIGINAL MATERIAL

Description of the material being transferred

SHIPPING ADDRESS

Title and name:
Address:

I. OBJECTIVE AND PURPOSE

[Insert a short statement about the objectives and intended purpose of the agreement and background to the parties to the agreement.]

II. DEFINITIONS

Provider
Organisation providing the *original material*. The name and address of this party will be specified in the first page of this MTA.

Provider scientist
The name and address of this party will be specified in an implementing letter.

Recipient
Organisation receiving the *original material*. The name and address of this party will be specified in an implementing letter.

Recipient scientist
The name and address of this party will be specified in an implementing letter.

Original material
The description of the material being transferred will be specified in an implementing letter.

Material
Original material, *progeny*, and *unmodified derivatives*. The *material* shall not include: (a) *modifications*, or (b) other substances created by the *recipient* through the use of the *material* which are not *modifications*, *progeny*, or *unmodified derivatives*.

Progeny
Unmodified descendant from the *material*, such as virus from virus, cell from cell, or organism from organism.

Unmodified derivatives
Substances created by the *recipient* which constitute an unmodified functional subunit or product expressed by the *original material*. Some examples include: subclones of unmodified cell lines, purified or fractionated subsets of the *original material*, proteins expressed by DNA/RNA supplied by the *provider*, or monoclonal antibodies secreted by a hybridoma cell line.

Modifications
Substances created by the *recipient* which contain/incorporate the *material*.

Commercial purposes
The sale, lease, license, or other transfer of the *material* or *modifications* to a for-profit organisation. *Commercial purposes* shall also include uses of the *material* or *modifications* by any organisation, including *recipient*, to perform contract research, to screen compound libraries, to produce or manufacture products for general sale, or to conduct research activities that result in any sale, lease, license, or transfer of the *material* or *modifications* to a for-profit organisation. However, industrially sponsored academic research shall not be considered a use of the *material* or *modifications* for *commercial purposes* per se, unless any of the above conditions of this definition are met.

Non-profit organisation(*s*)
A university or other institution of higher education or an organisation exempt from taxation or any nonprofit scientific or educational organisation qualified under a state nonprofit organisation statute. As used herein, the term also includes government agencies.

Agreement
Material Transfer Agreement (MTA)

III. TERMS AND CONDITIONS OF THIS AGREEMENT

Ownership
The *provider* retains ownership of the *material*, including any *material* contained or incorporated in *modifications*.

The *recipient* retains ownership of:

(a) *modifications* (except that, the *provider* retains ownership rights to the *material* included therein);

(b) those substances created through the use of the *material* or *modifications*, but which are not *progeny*, *unmodified derivatives* or *modifications* (i.e., do not contain the *original material*, *progeny*, *unmodified derivatives*). If either 2 (a) or 2 (b) results from the collaborative efforts of the *provider* and the *recipient*, joint ownership may be negotiated.

Non-commercial use
The *recipient* and the *recipient scientist* agree that the *material* is to be used solely for either teaching, non-commercial research or academic research purposes. In this Agreement, non-commercial research purpose and academic research purpose mean that the *material* cannot be used for *commercial purposes*, and the *recipient* may not exploit commercially the results, inventions, discoveries or know-how which incorporates the *materials* for its own benefit nor for a third party, without the consent of the *provider*.

Ownership of the results, inventions, discoveries or know-how generated by the *recipient* using the *material* shall rest with the *recipient.* Nevertheless, any results, inventions, discoveries or know-how which contain or incorporate the *material*, generated by the *recipient* using the *material* ("*modifications*") shall be jointly owned by the *provider* and the *recipient*. However, both the *provider* and the *recipient* agree that should the *recipient* having completed work under this MTA wish to use the *material* or *modifications* for commercial purposes it will be necessary for the *recipient* to negotiate the terms of a license Agreement with the *provider*, No right are given, implied or intended by this Agreement or the material transfer other than those explicitly stated in this Agreement.

Distribution to third parties
This *material* should be considered a property of the *provider*. The *recipient* therefore agrees to retain control over this material, and further agrees not to transfer the *material* to third parties or to personnel of the *recipient* not working under the supervision of the *recipient scientist*. The *recipient* agrees to refer to the *provider* any request for the *material* from anyone other than those persons working under the *recipient scientist*'s direct supervision. The *provider* reserves the right to distribute the *material* to others and to use it for its own purposes.

The *recipient* shall have the right, without restrictions, to distribute substances created by the *recipient* through the use of the *original material* only if those substances are not *progeny*, *unmodified derivatives*, or *modifications*.

Under a separate agreement at least as protective of the *provider*'s rights, the *recipient* may distribute *modifications* to *non-profit organisation(s)* for non-commercial research purposes and academic research purposes only, subject to prior written notice to the *provider*.

Confidentiality
The *recipient* agrees to treat the *materials* as it would treat its own confidential and proprietary information and at least no less than a reasonable degree of care, and to take all reasonable precautions to prevent unauthorised disclosure to any third party of the *material* which it receives hereunder. The *provider* agrees to keep confidential that the *recipient* is using the *material*.

Publications
This Agreement shall not be interpreted to prevent or delay publication of research findings resulting from the use of the *material* or the *modifications*. The *recipient scientist* agrees to provide appropriate acknowledgement of the source of the *material* in all publications.

Material use liability
The *material* is provided as a service to the research community at large. It is provided without warranty of merchantability or fitness for a particular purpose or any other warranty, express or implied. No indemnification for any damages is intended or provided under this Agreement. Each party should accept liability for their own actions. The parties make no express or implied warranty as to any matter whatsoever, including the conditions of the research or any invention or product, whether tangible or intangible, made, or developed under this Agreement, or the ownership, merchantability, or fitness for a particular purpose of the research or any invention or product. The parties further make no warranty that the use of any invention or other intellectual property or product contributed, made or developed under this Agreement will not infringe any patent or other intellectual property right. In no event, will any party be liable to any other party for compensatory, punitive, exemplary or consequential damages.

Misuse, dual use and biosafety
The *recipient* accepts full responsibility for the safety of the research and warrants that the research will be performed in accordance with all local or national laws, rules and regulations. In particular, this *material* will only be used for research purposes by the *recipient* in its laboratory under suitable containment conditions.

Under the terms of this Agreement, the *material* may not be used in human beings.

Termination of the Agreement
The term of this Agreement is 2 years as of the effective date of termination, unless an extension is mutually agreed by the *provider* and the *recipient*. At the end of this term, the Agreement shall automatically terminate. Upon the effective date of termination, or if requested, the deferred effective date of termination, the *recipient* will discontinue its use of the *material* and will, upon direction of the *provider*, return or destroy any remaining *material*. The cost of return or destruction will be taken by the *recipient*.

Dispute
Any dispute arising under this Agreement instituted against the *recipient* by the *provider* shall be brought in the court of the *recipient*'s country of domicile. Any claims and proceedings against the *provider* by the *recipient* shall be brought in the courts of the *provider*'s country of domicile.

Modification of the Agreement and signatures
This agreement may not be modified, in whole or in part, except by the written consent of both parties. If any provision of this Agreement may be signed in counterpart, and by the parties hereto or separate counterparts, each of which shall be deemed an original.

This Agreement is effective when signed by all parties. The parties executing it certify that their respective organisations have accepted the terms of this Agreement, and further agree to be bound by the terms, for the transfer specified above.

Recipient responsibility
The Recipient undertakes to use the Material in full compliance with any national and international applicable law, including any disposition and guidelines regarding health and scientific research. In particular, the Material having intrinsic health risk shall be handled in full respect of the specific law and in compliance with all the necessary precautions.

The Recipient represents that within its laboratories:

- Access to the Material, Progeny and Modification will be restricted to personnel capable and qualified to safely handle those substances, using appropriate containment;
- Recipient shall use the utmost precaution to minimise any risk of harm to persons and property and to safeguard them from theft or misuse.

The Recipient also acknowledges that in no event the Material applies directly or indirectly to humans.

The Recipient assumes all liability for any and all third party damages and claims arising out of or relating to this Agreement, including the receipt, use, handling, storage, conservation of the Material. To the extent permitted by applicable law, the Recipient agrees to indemnify, defend and hold harmless the provider against all third party claims, losses, expenses and damages, including reasonable attorney's fees.

The provider shall have no liability towards the Recipient or its employees in the event that the Material and/or Derivatives infringe any intellectual property rights of third parties. The provider makes no warranties for the absence of any third party industrial property rights on the Material.

IV. PAGE OF SIGNATURES

AUTHORISED SIGNATURE OF THE PROVIDER SCIENTIST

Signature:
Title and print name:
Date:

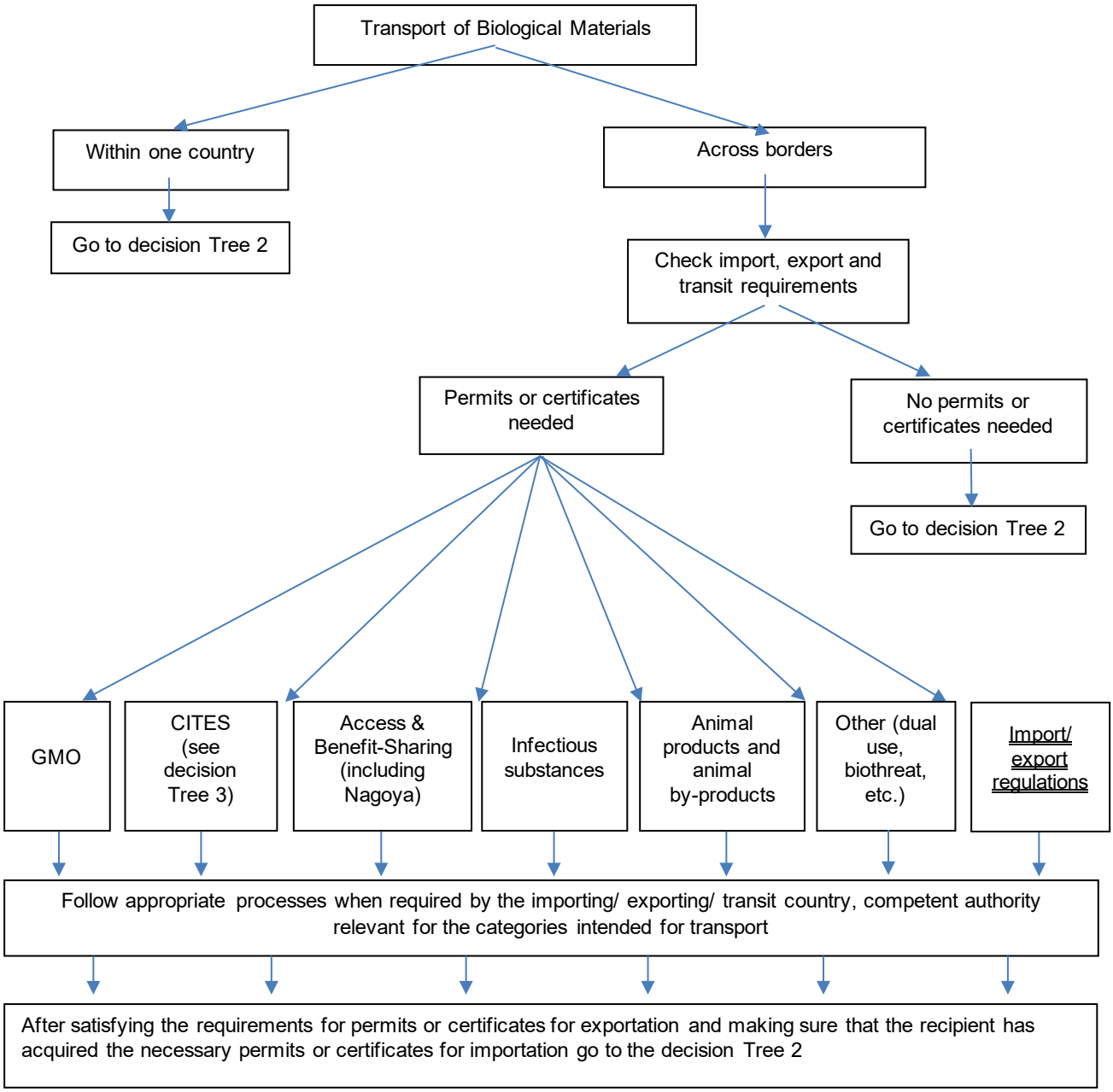
AUTHORISED SIGNATURE OF THE RECIPIENT SCIENTIST

Signature:
Title and print name:
Date:

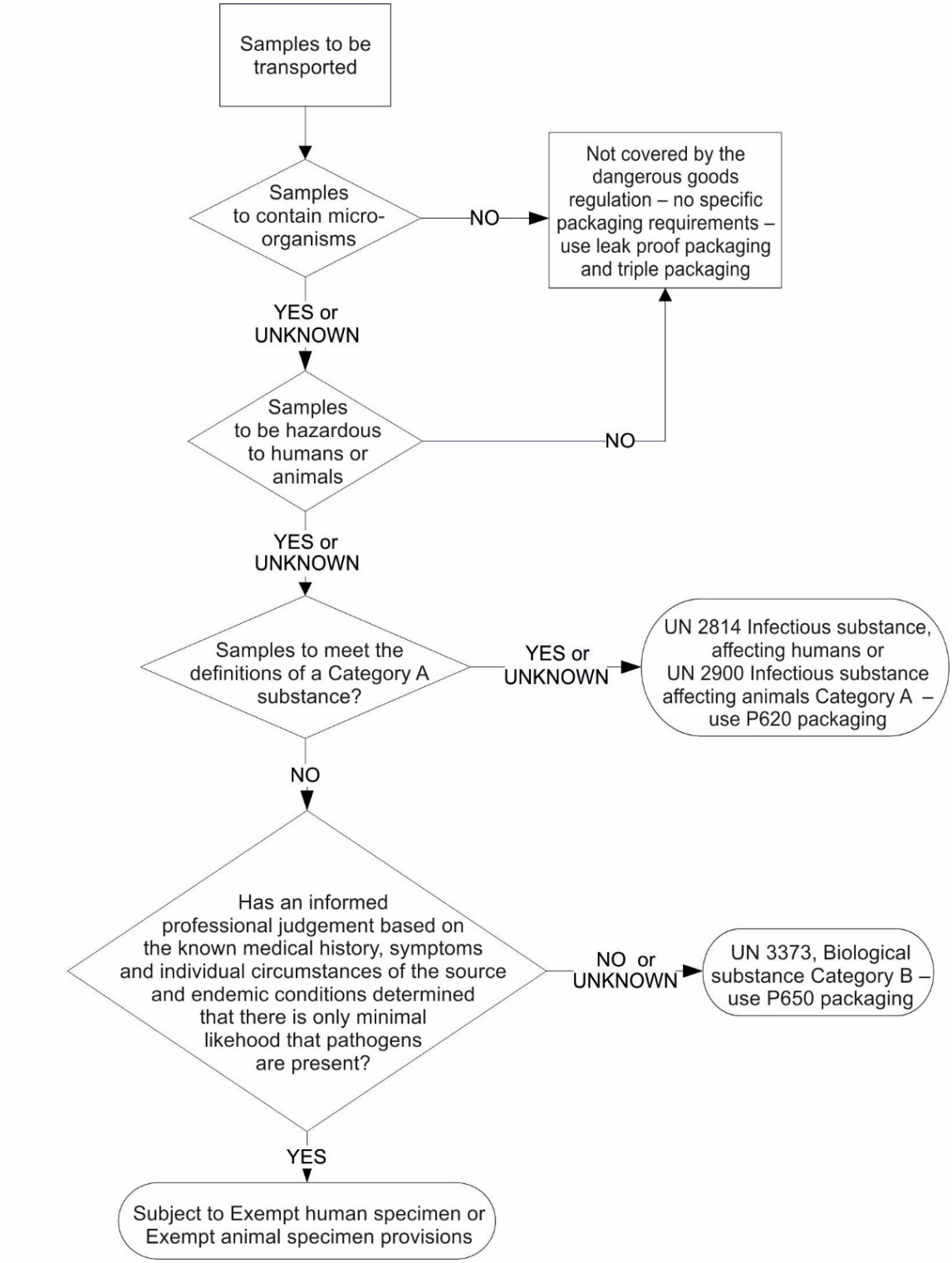
APPENDIX 1.1.3.4.

DECISION TREES FOR THE TRANSPORT REQUIREMENTS OF BIOLOGICAL MATERIALS

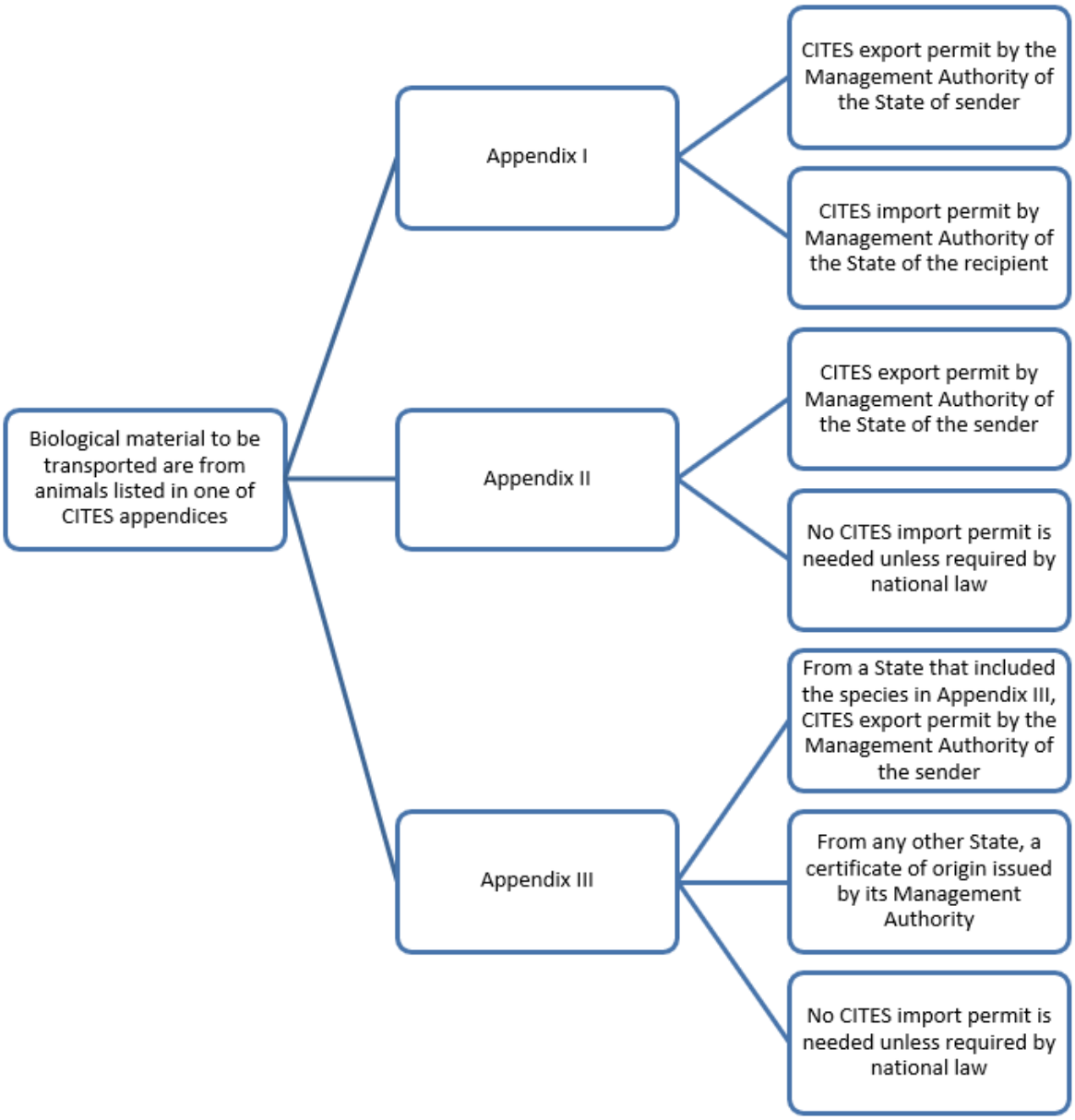
Decision Tree 1:



Decision Tree 2



Decision Tree 3



Annexe 6. Chapitre 1.1.7. Normes pour le séquençage à haut débit, la bioinformatique et la génomique computationnelle

RÉUNION DE LA COMMISSION DES NORMES BIOLOGIQUES
Paris, 9–13 septembre 2024

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CHAPTER 1.1.7.

STANDARDS FOR HIGH THROUGHPUT SEQUENCING, BIOINFORMATICS AND COMPUTATIONAL GENOMICS

INTRODUCTION

High throughput sequencing, bioinformatics and computational genomics (*HTS-BCG*) *in animal health and food safety investigations should be used in accordance with standards for laboratory testing, just as any other laboratory tool or procedure. As HTS-BCG is a relatively new procedure, compared with other standard diagnostic assays, the purpose of this chapter is to assist laboratories by defining standards that will allow inclusion of the capability into a laboratory's scope of operations in a way in which the users of the results can have confidence.*

A. GENERAL CONSIDERATIONS

Sequence information is playing an increasingly important role in the diagnosis and management of microbial infections, including in the characterisation of infectious agents, their possible phenotypic characteristics and their epidemiology. Consequently, it is incumbent on laboratories to adopt policies and practices for generating, analysing and managing genomic sequence data that are based on accurate information and rigorously interpreted.

Increasingly, for full identification and characterisation of a microorganism, there is an expectation that essential features of its genome should be described. For viruses, this may be the whole genome, while for bacteria and parasites, it may be only partial sequences. However as sequencing technology is continually developing ~~so rapidly~~, ~~within a short time~~ whole genome sequences for ~~these~~ larger microorganisms may soon also be routinely generated ~~after suitable bioinformatics procedures have been developed~~.

The standards described here apply to the generation of genomic sequence data during investigations of infections of single animals, animal populations and their environment. They also apply to the ~~generation,~~ management and use of such data within the accepted practices of veterinary investigations and within a laboratory's quality assurance system.

B. THE CONDUCT OF VETERINARY INVESTIGATIONS INCORPORATING HTS-BCG

Sequence data of microorganisms, such as is generated by high throughput sequencing (HTS) or metagenomics approaches, is only a tool, although a powerful one, to assist in the investigation of ~~issues regarding~~ animal health and food safety. Appropriate experts should perform the processing and analysis of sequence data. The interpretation of ~~that~~ data in relation to the disease investigation should be led by suitably qualified ~~veterinarians~~ individuals in close collaboration with data analyses experts, consistent with the standard requirements for diagnosis of animal disease.

The sequence and sequence analysis of infections associated with cases, outbreaks and investigations of animal disease and food safety by laboratories should be recorded and analysed together with all other information relating to the reporting and recording of such cases and outbreaks. These data should be considered a necessary part of such reports and records.

HTS-BCG (bioinformatics and computational genomics) can be deployed for a range of purposes in the detection of infectious agents and their characterisation, either in biological material such as diagnostic or surveillance specimens or propagated in cultures or as isolates. For primary diagnostic applications, the users of the technology should consider the purposes of their testing in relation to the normal purposes of testing as defined in Chapter 1.1.6 *Validation Of diagnostic assays for infectious diseases of terrestrial animals*. HTS-BCG may also be applied as a confirmatory assay for organisms detected in some other primary assay, or to provide additional characterisation of such organisms.

Further to these general purposes of testing, HTS-BCG offers specific opportunities for:

i) The detection, identification and characterisation of previously unidentified microorganisms;

ii) The improved diagnosis of known diseases;

iii) The improved diagnosis of emerging or re-emerging diseases with known or unknown aetiology;

iv) The development of single 'universal' diagnostic assays, able to identify any potential pathogen;

v) The simultaneous and quick detection of multiple agents in diseases with multifactorial aetiologies;

vi) The increased capability to study the evolutionary dynamics of pathogens at the farm, local, national and global levels;

vii) The deeper understanding of the epidemiology of infectious diseases and the phylogeography of infectious agents;

viii) The enhanced traceability of infectious diseases and modes of pathogen transmission including applications in forensic epidemiology;

ix) More extensive characterisation of 'populations' of known pathogens (e.g. relevant minority strains, escape mutants) that in turn facilitates the design of better vaccines, antivirals, etc.;

x) Better links between pathogen genotype and phenotypes enabled through full genome sequence of multiple strains (including reference strains) of a single agent.

C. STANDARDS FOR THE USE OF HTS-BCG

1. Selection of a technology platform or service

Laboratories may choose to establish a HTS-BCG capability in-house, contract commercial suppliers of services or submit specimens to designated Reference Centres.

Where the laboratory establishes its own capability there are a number of commercially available sequencing platforms for the purpose of generating sequence information from test samples. The choice of platform should be based on a consideration of the intended purpose or combination of purposes as outlined in Section B above.

Of primary concern is that the technology selected is fit for the intended purpose, that it is appropriate for the production of sequence information from the types of genome intended for study. Other considerations may take into account the time required to conduct a sequencing run, including sample preparation; inherent error rate of the technology; ancillary equipment needed in addition to the actual sequencing device; the capital cost of the purchase and set up of all necessary equipment and the cost of annual licences or service agreements, including manufacturer's recommended maintenance schedule; costs of implementation of technology/workflows, following instrument acquisition; the availability of supporting expertise from the supplier; the cost of reagents for a sequencing run and the likely availability of reagents in the country concerned; the expected sample throughput and consequent cost impact; the requirement of a laboratory infrastructure compatible with best practices for sequencing workflows; staff requirements and training required to operate the equipment and to conduct the associated bioinformatic analyses; access to well maintained server for data storage and analyses; ~~and the~~ data management requirements. Currently available systems have been reviewed (~~Belák *et al.*, 2013;~~ Granberg *et al.*,

2016; ~~Marston *et al.*, 2013;~~ Momoi & Matsuu, 2021; Satam *et al.*, 2023; Suminda *et al.*, 2022), but new ~~models and~~ technologies and computational approaches can be expected to become available frequently and applied to animal health.

Where a laboratory or veterinary service contracts an external provider to supply HTS-BCG services, they should ensure that the service provider meets the standards defined in this chapter.

2. Sampling and reporting

HTS-BCG is a relatively new technological tool in the management of diseases of animals and its use should be adopted within the context of tried and accepted processes for the management of animal health and food safety including clinical or epidemiological field investigations and the sampling of animals, animal populations or other epidemiologically relevant situations. The use of the technology should be appropriate to the purpose of the investigation. ~~and~~ The sampling strategy and the specimens taken should be appropriate for that investigation, based on an understanding of the pathogenesis and epidemiology of the infection under study or the likely pathogenesis and epidemiology of any novel infectious agent suspected. ~~Such investigations should be under the supervision of appropriately qualified veterinarians.~~

In laboratories where HTS-BCG is used it should be managed within the context of the laboratory's quality assurance system. Hence the results of HTS-BCG must be interpreted in the context of the pathogenesis and epidemiology of the infection in the animal species under study. Results should be reported by appropriately qualified veterinary investigators with the authority to make diagnoses of animal diseases under the laboratory's quality assurance system and in the jurisdiction where the investigation is conducted.

Laboratories should have clear guidelines to avoid sampling biases for each study type, as well as which sample/veterinary clinical metadata to record.

3. Specimens and sample preparation

Specimens should be collected and submitted to the testing laboratory in accordance with the standards communicated in Chapter 1.1.2 *Collection, submission and storage of diagnostic specimens.* The normal comprehensive information regarding the individual animal, the case or reason for sampling and the relevant epidemiological information should be recorded in the laboratory's accessions processes, as for any submission to the laboratory.

As with other laboratory processes, ensuring the integrity of the specimen and the samples to be tested is critical. Nucleic acids, either DNA or RNA, need to be extracted from the samples. In some cases, host depletion or targeted enrichment strategies may be applied during extraction or library preparation to increase the ratio of pathogen to host nucleic acids ~~can be used to~~ and maximise the sensitivity of the technique. However, care must be taken to avoid biasing the outcome in the context of the intended purpose. Precautions to ensure the integrity and quality of nucleic acids must be followed similarly to any other molecular technique (e.g. polymerase chain reaction [PCR]) as already described in Chapter 2.1.2 *Biotechnology advances in the diagnosis of infectious diseases*. Those precautions are particularly relevant in HTS applications as they rely on nucleic acid fragment length and microorganism genome coverage for effective pathogen detection and identification, as opposed to PCR assays that amplify only a short fragment of nucleic acid. Once nucleic acids are extracted from the samples, they need to be further manipulated (e.g. reverse transcription of RNA into complementary DNA) in order to be used in HTS. Different technological platforms require specific sets of reagents in order to generate the final material ("libraries") ready for sequencing. Commercial kits are available for this purpose.

HTS is an extremely sensitive technology and even few molecules of nucleic acid ~~could~~ can be detected. Hence, precautions to avoid cross-contamination~~s~~ must be followed as in the case of ~~many~~ other molecular techniques used to detect nucleic acids (e.g. PCR). Separation of work areas and decontamination of working spaces is an essential requirement to avoid cross contamination with nucleic acid ~~from other molecular investigations. Separation of work areas from the possibility of cross contamination with nucleic acid from other molecular investigations is an essential requirement~~. In addition, HTS very frequently involves "multiplexing" of several samples in a single reaction. Individual samples are "tagged" during one of the stages of sample preparation by the use of short index sequences linked to nucleic acid molecules. Best practices should include index usage rotation in benches and sequencers. Index sequences must be of sufficient quality and design to be relied on as a signature for the tagged library for HTS use in order to avoid artefacts during bioinformatics analysis of sequencing data obtained. Furthermore, index misassignment metrics including index hopping should be determined for each workflow and instrument, as well as instrument carryover, when applicable. When available, the amount of the target sequence should be determined by prior analyses to inform sample-batch processing.

Every application of HTS-BCG technology should include positive and negative controls appropriate to the investigation and that have been incorporated through the sample preparation processes of the sequencing run as

well as the actual run on the technology platform. The use of unique molecules spiked in each sample is highly advisable to monitor intra-batch contamination, mainly in workflows using enrichment methods. Appropriate controls should be used to verify each step of the procedure including nucleic acid quality, library preparation, cross-contamination (including multiplexing) sensitivity and reproducibility.

As with any other diagnostic method, confirmation of results ~~would~~ may require resampling of the original specimen, which therefore has to be protected from cross-contamination and be stored appropriately. Validation of HTS results could also be achieved using a different diagnostic method.

4. Generation of sequence data

While HTS platforms differ widely in ~~their details~~ chemistry and protocols, basic principles of quality control relevant to the technology can be followed, and generic recommendations for acceptable quality metrics can be made. Suitable control measures might include the use of positive, negative and no-template controls run in replicates of the test, iteratively covering different locations in the multiwell plate, and a quality scoring system. Sequencing quality metrics provide suitable parameters for the validation and monitoring of platform performance. Most platforms offer the possibility to spike controls in reagents and to use the control's QC metrics to monitor platform and reagent performance. Additional technology specific performance metrics can be used to monitor platform performance and to identify aberrant sequencing runs. Equally, maintenance of sample handling log files facilitates investigations in case of suspected contaminations that may lead to false positives.

Quality metrics for the evaluation of the analytical performance of HTS-based tests, include:

i) Depth of coverage. This indicates the number of sequence reads providing information about a given nucleotide position. When ongoing quality monitoring shows that the coverage depth at a given nucleotide is below the validated minimum coverage, confirmation should be provided using alternate methods (e.g. Sanger sequencing) or additional sequencing.

ii) Uniformity of coverage. This parameter describes how the depth of coverage is distributed over the test's target region(s). Coverage across a pathogen genome is rarely uniform. However, deviations of uniformity of coverage from the validated range can potentially indicate errors in the testing process.

iii) GC bias. The GC content (relative abundance of G and C nucleotides) of a target region affects the efficiency of sequencing reactions and will affect the uniformity of coverage. ~~Where possible, the amount of GC bias in the test's target region(s) should be determined during validation and monitored to evaluate test performance.~~

iv) Low complexity regions. Homopolymer regions and microsatellites in a target region (or close to a target region) may compromise accuracy. This should be determined during validation and if not possible to avoid, carefully assess performance.

v) Base call quality scores. These are platform-derived reflections of the signal-to noise ratio and reflect the probability that the base call was correct. An acceptable raw base call quality threshold should be established during validation, and incorporated in bioinformatics filters to eliminate poor quality data during analysis.

vi) Decline in signal intensity or read length. Depending on the exact application, HTS platform and chemistry, sequence reads have a typical distribution of read length and signal intensity. The expected signal intensity across reads (or read length distribution) should be established during validation and monitored for each run. Deviations in the distribution of read lengths may indicate problematic datasets. "Reads "trimming" is usually applied by removing low-quality bases or adapter sequences from raw reads, aiming therefore to balance read retention and read accuracy. The choice on the trimming threshold depends on the diagnostic question and the required read accuracy. For example, identifying a co-infection with two similar viral strains or detecting drug resistance at a sub-consensus level requires higher data quality.

vii) Mapping quality. This is a measure of uncertainty that a read is mapped properly to a genomic position within the target region. Acceptable values (e.g. proportion of reads mapping to the target) should be established during validation of bioinformatics workflows and the proportion of reads not mapping to the target can be monitored during each run.

viii) Internal controls. Most platforms offer the possibility to spike an internal control at very low frequency during the sequencing run. The quality metrics of those reads can be compared to previously reported quality metrics.

5. Bioinformatics

An absolute requirement for any laboratory intending to establish a HTS-BCG capability is the employment (or partnership) of specialised staff with bioinformatics skills. Even if platforms with supporting software for specific analyses in defined clinical situations ~~were to become~~ may be available, the use of such packages would not remove the responsibility of the laboratory to be able to competently analyse its own data.

The bioinformatic analysis assembling the pathogen genomic sequence from the raw data and the subsequent secondary analysis are the critical elements in HTS-BCG. Hence the approaches used must be transparent, with clear guidelines on how to record bioinformatic metadata, including a declaration of the software packages, software versions, and reference databases or sequences used should be a component of every report of sequence analysis. Software programs used for these analyses must be readily available (commercially or open access) in order to be evaluated by the international community.

As with any laboratory procedure, attention must be given to quality assurance. The test method should include criteria for acceptance or rejection of each run based on the satisfactory analyses of the controls and adequate reference material. Sequencing data must be documented to have satisfied minimum quality scores and coverage for each nucleotide of the assembled final consensus sequence obtained.

The appropriateness of chosen bioinformatics software for particular analyses can be evaluated through testing its performance against standard data sets containing data relating to agents expected to be present in the specimens to be tested and by comparing and benchmarking different bioinformatic tools.

6. Data management

The data generated from HTS-BCG operations are essential to reach the diagnosis or other scientific purpose of the investigation, such as agent characterisation, and are an integral component of the process. As such it is an essential requirement of laboratories to have policies, processes and supporting systems to curate, manage and store the data generated.

Different HTS technology platforms produce raw data in different formats and stage of pre-analysis, so it is necessary for laboratories to have policies and processes specific to the technology platform in use. Data management systems will include aspects of which data to keep, ~~and~~ the length of time for which they will be kept, and the back-up strategies to protect against accidental loss or deliberate erasure, and submission to national or international archives or databases. Metadata describing the generation and analysis of the sequence data is essential, so that the process itself can be analysed or repeated.

Where a sequence analysis leads to an output of animal health significance, especially one of trade or international significance, it is an absolute requirement that the data on which the analysis was performed be kept available for audit or confirmatory analysis for a period of time commensurate with the significance of the animal health finding. This is particularly important where the finding may be disputed. Failure to be able to produce the required data for independent analysis could be taken to invalidate the finding.

Sequence data should be stored in a manner in which there is a clear link to the metadata associated with the specimen that was the subject of the analysis. As is standard practice in laboratory investigations, such metadata includes information regarding the animal sampled, its ownership and location, and accompanying clinical and epidemiological information regarding the animal population.

Careful consideration should be given to platforms compatible with integration of data from different sources namely sample, clinical and sequencing metadata.

7. Validation of test systems for designated purposes

The concepts of test validation as stated in chapter 1.1.6 are broadly applicable to HTS-BCG (Van Borm *et al.,* 2016). All procedures including sample processing (nucleic acid extraction, library preparation, tagging, target enrichment), sequencing, bioinformatics and reporting should be documented in SOPs before validation can start. Stage 1 validation data must be developed to confirm the analytic sensitivity (Se) and specificity (Sp) of the technique, and its repeatability. For sequencing based tests, analytic sensitivity can be defined as the likelihood that the assay will detect the targeted sequence variations, if present, at a given probability (e.g. 95% confidence), while analytical specificity can be defined as the probability that an assay will not detect a sequence variation when none are present at a given probability. Furthermore, each type of specimens has its own characteristics that have to be considered, e.g. nasal swab, sera or faeces. Well described samples with known concentrations of target analyte or non-target analytes and matrix components can be used to assess the analytical performance. This should include, as a minimum, serial dilutions of each type of specimen containing defined organisms to document the limits of detection of designated whole genomes or genetic sequences representative of the type for which the HTS-BCG capability will be used in the laboratory. Care should be used to dilute samples in the same sample matrix containing the normal amount of non-target nucleic acids as detection sensitivity often depends on the ratio of target v. non-target molecules. For viral disease investigations, test specimens could be prepared to contain representative viruses of the full range of viral families from which agents may be present in test specimens of the type to be investigated in routine operations. Documentation of the laboratory's HTS-BCG system to detect these viruses will be established. The same principles apply to genetic markers, bacteria or other organisms for which the HTS-BCG capability will be used in planned routine operations. In all these runs designed to establish sensitivity

and specificity, the sample preparation steps should be part of each assessment as these steps are likely to be critical to all aspects of overall test performance.

Several factors complicate the validation of NGS tests as primary diagnostic assays including:

i) The weight of the analytical and diagnostic validation required (chapter 1.1.6);

ii) The operational cost of the technology;

iii) The challenges of validation of a data analysis workflow;

iv) The high need for investment in hardware and expertise;

v) The time taken to obtain a result ~~(currently days~~ compared with ~~hours for specific~~ other molecular diagnostics such as real-time PCR~~)~~.

Confirmatory adjunct or secondary diagnostic assays need to be validated only for their analytical performance, e.g. analytical sensitivity and specificity, and repeatability and initial reproducibility (stage 1) and not to the full diagnostic extent (diagnostic sensitivity and specificity, stage 2).

It is recognised that it may not always be practical to produce large data sets on test performance such as would normally allow calculation of test diagnostic sensitivity and specificity, but other aspects of validation such as demonstration of test reproducibility among laboratories conducting similar investigations should be undertaken.

8. Quality assurance

Testing using HTS-BCG for the purposes of investigations of animal health and food safety should be conducted in accordance with the requirements of the laboratory's quality assurance system, the features of which will meet the standards listed in Chapter 1.1.5 *Quality management in veterinary testing laboratories*. Where the laboratory is accredited, the testing ~~should~~ could be part of the laboratory's scope of accreditation.

Standard data sets against which the usefulness of bioinformatics software packages can be verified have been developed. Laboratories using HTS-BCG should ensure that their software packages for bioinformatics meet expected performance criteria against data standards.

Where proficiency testing strategies have been developed, laboratories using HTS-BCG should participate.

9. Interpretation of results

HTS-BCG can be used for a variety of purposes ranging from pathogen discovery to diagnosis or in-depth characterisation of known infectious agents. Consequently, the interpretation of the results obtained will be in the context of the specific clinical and epidemiological situation, reassured by satisfactory performance against all specified controls and quality assurance parameters. As with any other laboratory tests, these considerations are one among a number of parameters to be taken into account.

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NB: There is a WOAH Collaborating Centre for Viral Genomics and Bioinformatics (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/collaborating-centres/#ui-id-3 Please contact the WOAH Collaborating Centre for any further information on HTS-BCG.

NB: FIRST ADOPTED IN 2016.

Annexe 7. Chapitre 1.1.9. Contrôle de la stérilité et de l'absence de contamination des matériels biologiques à usage vétérinaire

RÉUNION DE LA COMMISSION DES NORMES BIOLOGIQUES
Paris, 9–13 septembre 2024

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CHAPTER 1.1.9.

TESTS FOR STERILITY AND FREEDOM FROM CONTAMINATION OF BIOLOGICAL MATERIALS INTENDED FOR VETERINARY USE

INTRODUCTION

The international trade-related movements of biological materials intended for veterinary use are subject to restrictions imposed to minimise the spread of animal and human pathogens. Countries may impose requirements for proof-of-freedom testing before allowing the regulated importation of materials of animal derivation and substances containing such derivatives. Where chemical or physical treatments are inappropriate or inefficient, or where evidence of the effectiveness of the treatment is lacking, there may be general or specific testing requirements imposed by authorities of countries receiving such materials. This chapter provides guidance on the approach to regulated testing, particularly as might be applied to the movement of vaccine master seed and master cell stocks, and to related biological materials used in manufacturing processes. While the onus for ensuring safety of a product remains with the manufacturer and may be regulated by therapeutic guidelines, this chapter provides procedures that are designed to minimise the risk of undetected contaminants in veterinary therapeutics and biological reagents causing the cross-border spread of agents of concern to importing countries. Farsang & Kulcsar (2012) and WHO (2015) describe case studies of veterinary and human vaccines contaminated with extraneous agents and findings support the need of accurate and validated amplification and detection methods as key elements for effective detection and control. Further examples are given in Section ~~G~~ H. Protocol examples *below. Control of contamination with transmissible spongiform encephalopathy (TSE) agents is not covered in this chapter because standard testing and physical treatments cannot be used to ensure freedom from these agents. Detection methods are described in Chapter 3.4.5.* Bovine spongiform encephalopathy.

Sterility is defined as the absence of viable microorganisms, which for the purpose of this chapter, includes viruses. It should be achieved using aseptic techniques and validated sterilisation methods, including heating, filtration, chemical treatments, and irradiation that fits the intended purpose. Freedom from contamination is defined as the absence of specified viable microorganisms. This may be achieved by selecting materials from sources shown to be free from specified microorganisms and by conducting subsequent procedures aseptically. Adequate assurance of sterility and freedom from contaminating microorganisms can only be achieved by proper control of the primary materials used and their subsequent processing. Tests on intermediate products are necessary throughout the production process to check that this control has been achieved.

Biological materials subject to contamination that cannot be sterilised before or during use in vaccine production, such as ingredients of animal origin, e.g. serum and trypsin, primary and continuous cell lines, and viral, ~~or~~ bacterial or parasitic seed stocks, etc., should be tested for viable extraneous agents before use. Assays to detect viral contaminants, if present, can be achieved by various culture methods, including use of embryonated eggs, which are supported by cytopathic effects (CPE) detection/embryo death, fluorescent antibody techniques and suitable (fit for purpose), methods such

as polymerase chain reaction (*PCR*) *and antigen detection ELISA* (*enzyme-linked immunosorbent assay*). *As is explained in more detail in this chapter care must be taken when using PCR and ELISA techniques for detection as such tests do not distinguish viable from non-viable agent detection. Specific assays to detect other contaminants, such as fungi, protozoa and bacteria* (*including rickettsia and mycoplasma*) *are also described.*

Testing procedures should be validated and found to be "fit for purpose" following Chapter 1.1.6. Validation of diagnostic assays for infectious diseases of terrestrial animals*, where possible.*

It is a requirement of many regulators, that a laboratory testing report notes the use of validated procedures and describes the validated procedures in detail including acceptance criteria. This gives the regulator transparency in the procedures used in a testing laboratory.

The validation assessment of an amplification process in cell culture should include documentation of the history of permissive cell lines used, reference positive controls and culture media products used in the process of excluding adventitious agents, to ensure the process is sound and is not compromised. The validation assessment should give information (*published or in-house*) *of the limitations that may affect test outcomes and an assessment of performance characteristics such as analytical specificity and sensitivity of each cell culture system, using well characterised, reference positive controls.*

It is the responsibility of the submitter to ensure a representative selection and number of items to be tested. Appendix 1.1.2.1 Epidemiological approaches to sampling: sample size calculations *of Chapter 1.1.2* Collection, submission and storage of diagnostic specimens *describes the principles to be applied. Chapter 1.1.2 and Chapter 1.1.3* Transport of biological materials *describe transportation requirements*.

A. AN OVERVIEW OF TESTING APPROACHES

Although testing is seen as a key component of biosafety in biological products intended for veterinary use, testing is not enough to ensure a given product is free of viable infectious contaminants, and so a holistic, multifaceted approach must be taken. Such an approach includes risk assessment, risk mitigation and management strategies (Barone *et al.,* 2020). In general:

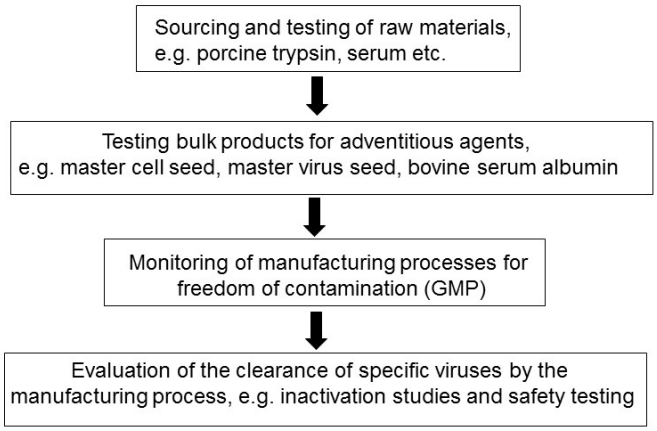
- Primary materials must be collected from sources shown to be free from contamination and handled in such a way as to minimise contamination and the opportunities for any contaminants to multiply (Figure 1).

- Materials that are not sterilised and those that are to be processed further after sterilisation must be handled aseptically. Such materials will require further assessment of freedom of contaminants at certain stages of production to assure freedom of adventitious agents.

- Materials that can be sterilised without their biological activities being affected unduly must be sterilised by a method effective for the pathogens of concern. The method must reduce the level of contamination to be undetectable, as determined by an appropriate sterility test study. If a sterilisation process is used, it shall be validated to demonstrate that it is fit for purpose. Suitable controls will be included in each sterilisation process to monitor efficiency.

- The environment in which any aseptic handling is carried out must be maintained in a clean state, protected from external sources of contamination, and controlled to prevent internal contamination. Rules governing aseptic preparation of vaccines are documented in Chapter 2.3.3 *Minimum requirements for the organisation and management of a vaccine manufacturing facility*.

Fig. 1. Risk assessment flowchart for vaccine production.



Some procedures have been properly validated and found to be fit for purpose, whilst others may have undergone only limited validation studies. For example, methods for bacterial and fungal sterility may have not been formally validated although they have been used for many years. In particular, *in-vivo* and *in-vitro* methods have essentially unknown sensitivity and specificity (Sheets *et al*., 2012) though there is an accepted theoretical sensitivity, regarding cell culture of 1 plaque-forming unit (PFU). For example, an evaluation of methods to detect bovine and porcine viruses in serum and trypsin based on United States (of America) Code of Federal Regulations, Title 9 (9CFR) revealed gaps in sensitivity, even within virus families (Marcus-Secura *et al*., 2011). It is therefore important to interpret, and report results in the light of specific conditions of cultures employed and considering sensitivity and specificity of detection systems.

Newer, more sensitive methods such as molecular assays may afford the ability to detect contaminants, which may not be successfully amplified in traditional culturing systems. The detection range can be broadened by using family specific primers and probes if designed appropriately. However, most, if not all molecular-based tests are also able to detect evidence for non-infectious contaminants, such as traces of nucleic acid from inactivated contaminants. Note: molecular assays if not designed as fit for purpose may miss detection of contaminating agents or lack sensitivity to do so (Hodinka, 2013).

More recently metagenomic high throughput sequencing (HTS) workflows have shown potential for quality control of biological products (van Borm *et al*., 2013) and vaccines (Baylis *et al.,* 2011; Farsang & Kulcsar, 2012; Neverov & Chumakov, 2010; Onions & Kolman, 2010; Victoria *et al*., 2010) in particular for the identification and characterisation of unexpected highly divergent pathogen variants (Miller *et al*., 2010; Rosseel *et al*., 2011) that may remain undetected using targeted diagnostic tests. Nevertheless, targeted assays, e.g. amplification in cell culture followed by polymerase chain reaction (PCR) may be superior to HTS for specific agent detection (Wang *et al*., 2014) due to lack of sensitivity of HTS at this time. Chapter 1.1.7. gives an overview of the standards for high throughput sequencing, bioinformatics and computational genomics. Similarly, recent improvements in protein and peptide separation efficiencies and highly accurate mass spectrometry have promoted the identification and quantification of proteins in a given sample. Most of these new technologies are broad screening tools, limited by the fact that they cannot distinguish between viable and non-viable organisms.

Given the availability of new technologies, there will be future opportunities and challenges to determine presence of extraneous agents in biologicals intended for veterinary use for industry and regulators. Problems can arise when the presence of genome positive results are interpretated as evidence for the presence of contamination (Mackay & Kriz, 2010). When using molecular technologies, it is important to understand the correlation between genome detection and detection of live agent. It cannot be assumed that detection of genome corresponds to the presence of an infectious agent.

B. LIVING VIRAL VACCINES FOR ADMINISTRATION BY INJECTION, OR THROUGH DRINKING WATER, SPRAY, OR SKIN SCARIFICATION

1. Materials of animal origin should be sterilised and obtained from healthy animals that, in so far as is possible, should be shown to be free from pathogens that can be transmitted from the species of origin to the species to be vaccinated, or any species in contact with them by means of extraneous agents testing.

2. Seed lots of virus, any continuous cell line and biologicals used for virus growth should be shown to be free from bacteria, fungi, mycoplasmas, protozoa, rickettsia, and extraneous viruses that can be transmitted from the species of origin to the species to be vaccinated or any species in contact with them.

For production of vaccines in embryonated chicken eggs and the quality control procedures for these vaccines, it is recommended that eggs from specific pathogen-free birds should be used.

3. Each batch of vaccine should pass tests for freedom from extraneous agents that are consistent with the importing country's requirements for accepting the vaccine for use. Some examples of published methods that document acceptable testing processes in various countries include:
   - Code of Federal Regulations, Title 9 (9CFR) (of the United States of America) (2015).
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   - European Pharmacopoeia, 11th Edition (2023).
   - World Health Organization (WHO) (1998; 2013).

4. Tests for freedom of contamination should be appropriate to prove that the vaccine is free from viable extraneous viruses, bacteria including rickettsia and mycoplasmas, fungi, and protozoa. Each country will have requirements as to what agents should be tested for and by which procedures. Such tests will include amplification of extraneous agents using cell culture that is susceptible to known viruses of the species of concern, tests in embryonated eggs, bacterial, mycoplasma and fungal culturing techniques and, where there is no alternative, animal inoculation. Use of antisera to 'inactivate' the living virus seed or vaccine prior to exclusion of extraneous agents is recommended to ensure testing in permissive amplification systems (including culture) is sensitive. PCR, fluorescence antibody test (FAT), presence of colonies or cytopathic effects (CPE) and antigen detection ELISA can be used for detection purposes after amplification using culturing techniques to improve specificity and sensitivity. If *in-vitro* or *in-vivo* amplification of the target agent is not possible, direct PCR may be useful if validated for this purpose.

C. INACTIVATED VIRAL, ~~AND~~ BACTERIAL AND PARASITIC VACCINES

1. Each batch of vaccine shall pass a test for inactivation of the vaccinal seed and should include inactivation studies on representative extraneous agents if the virus, ~~or~~ bacterial or parasitic seed has not already been tested and shown to be free from extraneous agents. An example of a simple *in-vitro* inactivation study could include ~~assessment of the titre of live vaccine before and after inactivation and~~ assessing the $\log_{10}$ drop in titre ~~during~~ in cell culture before and after the inactivation process for a virus vaccine. This would give an indication of the efficacy of the inactivation process. There is evidence that titration tests may not have sufficient sensitivity to ensure complete inactivation. In these circumstances, a specific innocuity test would need to be developed and validated to be fit for increased sensitivity. To increase sensitivity more than one passage would be required depending on the virus, ~~or~~ bacteria or parasite of concern. An example of this approach can be found at:
   https://www.aphis.usda.gov/animal_health/vet_biologics/publications/memo_800_117.pdf (accessed 20/09/2024).

2. If studies on representative extraneous agents are required, then spiking inactivated vaccine with live representative agents and following the example of an inactivation study could be useful. The inactivation process and the tests used to detect live agent after inactivation must be validated and shown to be suitable for their intended purpose.

In addition, each country may have its own requirements for sourcing or tests for sterility as detailed in Section B above.

D. LIVING BACTERIAL VACCINES

1. See Section B.

2. Seed lots of bacteria shall be shown to be free from other bacteria as well as fungi and mycoplasmas, protozoa, rickettsia, and extraneous viruses. Agents required for exclusion will be dependent on the country accepting the vaccine for use. Use of antibiotics to 'inactivate' the living bacterial seed or vaccine prior to exclusion of ~~viruses and fungi~~ extraneous agents is recommended to ensure testing in permissive amplification systems (including culture) is sensitive. Due to the difficulties and reduced sensitivity in exclusion of extraneous bacteria and some mycoplasma, protozoa, and rickettsia from high-titred seed lots of bacteria, the use of narrow-range antibiotics aimed specifically at reducing seed lot bacteria is useful if antibiotics do not affect the growth of bacteria being excluded. The optimal concentration of antibiotics can be determined in a dilution experiment such as documented in 9CFR Section 113.25(d). Other methods of exclusion of extraneous bacteria from bacterial seeds may include filtering for size exclusion such as removing bacteria

seed to look for mycoplasma contamination and use of selective culturing media. Such processes would require verification to ensure the process does not affect the sensitivity of exclusion of extraneous agents of concern.

3. Sonication of a living bacterial seed may be useful when excluding specific viral agents. Once again, the inactivation procedure would require a verification process to ensure the adventitious virus being excluded is not affected by the treatment. Use of a suitable reference virus control during the exclusion process would be required.

4. Direct PCR techniques may be useful when culturing processes fail to be successful in detecting extraneous bacteria from live bacterial seeds or vaccines.

E. LIVING PARASITIC VACCINES

1. See Section B.

2. Seed lots of live parasites shall be shown to be free from other parasites as well as bacteria, fungi and mycoplasmas, protozoa, rickettsia and extraneous viruses. Agents required for exclusion will be dependent on the country accepting the vaccine for use. Use of antiparasitic agents to 'inactivate' the living parasite seed or vaccine prior to exclusion of extraneous agents is recommended to ensure testing in permissive amplification systems are sensitive (See Section H.5.5. *Test 5. Test for chicken anaemia virus* [*CAV*] for an example of a Coccidiosis vaccine).

~~E.~~F. SERA, PLASMA AND DIAGNOSTIC AGENTS FOR ADMINISTRATION TO ANIMALS

Some countries require quarantine, health certification, and tests for specific diseases to be completed for all serum and plasma donor animals, for example, 9CFR (2015) and Australian Quarantine Policy and Requirements for the Importation of Live and Novel Veterinary Bulk and Finished Vaccines (1999). For some diseases, for example equine infectious anaemia, the product (plasma) must be stored until the seroconversion period has been exceeded and the donors tested negative.

~~F.~~G. EMBRYOS, OVA, SEMEN

Special precautions must be taken with relation to the use of embryos, ova, semen (Hare, 1985). Most countries will have regulatory guidelines for import of these biologicals for veterinary use. Such guidelines can be found at various websites such as the European Commission (2015), FAO and Department of Agriculture Forest and Fisheries (2021a; 2021b), though some guidelines may give more detail in regard to the food safety aspect.

~~G.~~H. PROTOCOL EXAMPLES

1. Introduction

This section provides some examples to illustrate scope and limitations of testing protocols. It is not intended to be prescriptive or exhaustive. Examples are based on standards and published methods to increase the sensitivity for exclusion of live adventitious agents, using general and specific techniques.

In principle, proposed testing represents attempted isolation of viable agents in culturing systems normally considered supportive of the growth of each specified agent or group of general agents. After amplification, potential pathogens can be detected further, by sensitive and specific diagnostic tests such as FAT or PCR as required. General detection systems can include haemabsorbance and CPE by immunohistochemistry staining methods. The example procedures for detection of contamination testing and general detection of virus, fungi, protozoa and bacteria (including rickettsia and mycoplasma) described below are derived from standards such as the 9CFR (2015), European Pharmacopoeia, 11th Edition (2023), European Medicines Agency Sciences Medicines Health (2016), Department of Agriculture, Forest and Fisheries (Australia) (2013) and World Health Organization (1998; 2012).

Individual countries or regions should adopt a holistic, risk-based approach to determine the appropriate testing protocols based on their animal health status. As well as applying general testing procedures documented in

national or regional standards as mentioned above, it may be necessary to apply rigorous exclusion testing for specific agents that are exotic to the country or region of concern.

General procedures do not necessarily detect all extraneous agents that may be present in biological material; however, they are useful as screening tests. Some examples of agents that may require specific methods for detection in biologicals refer to Table 1 below. Procedures documented in the Review of Published Tests to Detect Pathogens in Veterinary Vaccines Intended for Importation into Australia (2013) available from the Department of Agriculture, Forest and Fisheries are able to address such agents in offering sensitive testing approaches based on reputable publications. A reflection paper published by the Committee of Veterinary Medicinal Products (CVMP) in (2016), lists specific test method approaches for a number of agents that cannot be excluded using general test procedures (Table 1).

Exclusion of specific agents requires procedures that maximise sensitivity by providing ideal amplification and detection of the pathogen in question. Extraneous agents, for example, Maedi Visna virus (and other retroviruses), *Trypanosoma evansi* and porcine respiratory coronavirus are difficult to culture even using the most sensitive approaches. In these circumstances, application of molecular assays directly to the biological material, assessing for the presence of nucleic acid from adventitious agents offers an alternative, though detection of the presence of non-viable and host associated agents is also possible.

Table 1 gives examples of causative infectious agents that may be present in animal biologicals intended for veterinary use, for example PCV-1 in a rotavirus vaccine (WHO, 2015). BVDV is well known for its presence in many bovine associated biologicals, including cell culture. More recently, non-CPE pestivirus, BVD type 3 (HoBi-like) are found in foetal calf serum and cell culture. Classical Swine fever has contaminated various porcine cell lines used for African swine fever and FMDV diagnosis, and thus the potential for contamination of porcine based vaccines. PEDV is linked to spray-dried porcine plasma used for feed. This is not an exhaustive list of agents of concern or by any means required for exclusion by every country based on risk, they are examples of infectious agents that are not culturable using general culturing procedures and require use of specialised culturing processes and specific detection processes. Notably, some subtypes of an agent type may be detectable by general methods, and some may require specialised testing for detection. For example, bovine adenovirus subgroup 1 (serotypes 1, 2, 3 and 9) can be readily isolated using general methods (Vero cells) however bovine adenovirus subgroup 2 (serotypes 4, 5, 6, 7, 8 and 10) are not readily isolated and required specialised methods for isolation.

Table 1. Examples of infectious agents of veterinary importance that require specialised culturing and detection techniques

Rotaviruses	**Pestiviruses (non-CPE)**	**Turkey rhinotracheitis**
Porcine epidemic diarrhoea virus	Bluetongue virus	*Brucella abortus* (see Section ~~G~~ H.2.3)
Porcine circoviruses	Swine pox virus	Rickettsias
Swine/equine influenza, some strains	Some adenoviruses	Protozoa
Bovine respiratory syncytial virus	Rhabdoviruses (e.g. rabies virus)	Some fungi (e.g. *Histoplasma*)

2. Example of detection of bacteria and fungi

2.1. General procedure for assessing the sterility of viable bacteria and fungi

Standard tests for detecting extraneous bacteria and fungi (sterility testing) in raw materials, master cell stocks, or final product are the membrane filtration test or the direct inoculation sterility test.

For the membrane filtration technique, a filter having a nominal pore size not greater than 0.45 µm and a diameter of at least 47 mm should be used. Cellulose nitrate filters should be used if the material is aqueous or oily; cellulose acetate filters should be used if the material is strongly alcoholic, oily or oil-adjuvanted. Immediately before the contents of the container or containers to be tested are filtered, the filter is moistened with 20–25 ml of Diluent A or B.

2.1.1. Diluent A

Diluent A is for aqueous products or materials. Dissolve 1 g peptic digest of animal tissue in water to make 1 litre, filter, or centrifuge to clarify, adjust the pH to 7.1 ± 0.2, dispense into containers in 100 ml quantities, and sterilise by steam.

2.1.2. Diluent B

Diluent B is for oil-adjuvanted products or materials: Add 1 ml polysorbate 80 to 1 litre Diluent A, adjust the pH to 7.1 ± 0.2, dispense into containers in 100 ml quantities, and sterilise by steam.

If the biological being tested has antimicrobial properties, the membrane is washed three times after sample application with approximately 100 ml of the appropriate diluent (A or B). The membrane is then transferred to culture media, aseptically cut into equal parts and placed in media, or the media is transferred to the membrane in the filter apparatus. If the test sample contains merthiolate as a preservative, FTM (fluid thioglycollate medium) is used in test vessels incubated at both 30–35°C and 20–25°C. Growth should be clearly visible after an appropriate incubation time (see Section H.2.1.3 *Example of growth promotion and test interference*). If the test sample is a killed biological without merthiolate, or a live bacterial biological, FTM is used at 30–35°C and SCDM (soyabean casein digest medium) at 20–25°C. If the sample tested is a live viral biological, SCDM is used at both incubation temperatures. It has been suggested that sulfite-polymyxin-sulfadiazine agar be used to enhance the detection of *Clostridium* spp. when the membrane filtration technique is used (Tellez *et al*., 2005).

If direct inoculation of culture media is chosen, a sterile pipette or syringe and needle are used to aseptically transfer the biological material directly into liquid media. If the biological being tested has antimicrobial properties, the ratio of the inoculum to the volume of culture medium must be determined before the test is started, for example as explained in 9CFR 113.25(d) and detailed testing procedures can be found for example in supplemental assay method USDA SAM 903 https://www.aphis.usda.gov/animal_health/vet_biologics/publications/sam903.pdf (accessed 20/09/2024). To determine the correct medium volume to negate antimicrobial activity, 100 CFU of the control microorganisms listed in Table 2 are used. If the test sample contains merthiolate as a preservative, or if it is a killed biological without merthiolate or a live bacterial biological, see paragraph above for recommendations. If the test sample is a live viral biological, SCDM is used at both incubation temperatures. If the inactivated bacterial vaccine is a clostridial biological, or contains a clostridial component, the use of FTM with 0.5% added beef extract (FTMB) in place of FTM is preferred. It may also be desirable to use both FTM and SCDM for all tests.

Table 2. Some American Type Culture Collection [1] (ATCC) strains with their respective medium and incubation conditions

Medium	**Test microorganism**	**Incubation**	
		Temperature (°C)	Conditions
FTM	*Bacillus subtilis* ATCC # 6633	30–35	Aerobic
FTM	*Candida krusei* ATCC # 6258	20–25	Aerobic
SCDM	*Bacillus subtilis* ATCC # 6633	30–35	Aerobic
SCDM	*Candida krusei* ATCC # 6258	20–25	Aerobic
FTMB	*Clostridium sporogenes* ATCC # 11437	30–35	Anaerobic
FTMB	*Staphylococcus aureus* ATCC #6538	30–35	Aerobic
PDA [2]	Aspergillus brasiliensis ATCC #16404	20–25	Aerobic
Nutrient agar, nutrient broth	*Pseudomonas aeruginosa* ATCC #9027	30–35	Aerobic

For both membrane filtration and direct inoculation sterility tests, all media are incubated for no fewer than 14 days. At intervals during incubation, and after 14 days' incubation, the test vessels are examined for evidence of microbial growth. Microbial growth should be confirmed by subculture and Gram stain.

2.1.3. Example of growth promotion and test interference

The sterility of the media should be confirmed by incubating representative containers at the appropriate temperature for the length of time specified for each test.

The ability of the culture media to support growth in the presence and absence of product, product components, cells, seeds, or other test material should be validated for each product to be tested, and for each new batch or lot of culture media for example as outlined in 9CFR 113.25(b). Detailed testing procedures can be found for example in USDA SAMs 900-902, See USDA APHIS |Supplemental Assay Methods – 900 Series (accessed 4 July 2022).

[1] American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia 20110-2209, USA.
[2] PDA: potato dextrose agar

To test for ability to support growth in the absence of the test material, media should be inoculated with 10–100 viable control organisms of the suggested ATCC strains listed in Table 2 and incubated according to the conditions specified.

To test for ability of the culture media to support growth in the presence of the test material, containers should be inoculated simultaneously with both the test material and 10–100 viable control organisms. The number of containers used should be at least one-half the number used to test the product or product component. The test media are satisfactory if clear evidence of growth of the control organisms appears in all inoculated media containers within 7 days. In the event that growth is evident, the organism should be identified to confirm that it is the organism originally added to the medium. The sterility test is considered invalid if any of the media show inadequate growth response, or if the organism recovered, is not the organism used to inoculate the material.

If the material being tested renders the medium turbid so that the presence or absence of microbial growth cannot be readily determined by visual examination, 14 days after the beginning of incubation transfer portions (each not less than 1 ml) of the medium to fresh vessels of the same medium and then incubate the original and transfer vessels for not less than 4 days.

2.2. Example of general procedure for testing seed lots of bacteria and live bacterial biologicals for purity

Each seed lot of bacteria or batch of live bacterial biological should be tested for purity by inoculation of SCDM, which is incubated at 20–25°C for 14 days, and FTM, which is incubated at 30–35°C for 14 days. Using good practices in sterile technique to avoid laboratory contamination, a sterile pipette or syringe and needle is used to aseptically transfer the quantity of biological directly into the two types of culture medium. The minimum ratio of inoculum to culture medium is 1/15. Both positive and negative controls are set up as well.

If the inoculum or growth of the bacterial vaccine renders the medium turbid so that the absence of atypical microbial growth cannot be determined by visual examination, subcultures should be made from all turbid tubes on day 3 through until day 11. Subculturing is done by transferring 0.1–1.0 ml to differential broths and agar and incubating for the balance of the 14-day period. Microscopic examination by Gram stain should also be done.

If no atypical growth is found in any of the test vessels when compared with a positive control included in the test, the lot of biological may be considered satisfactory for purity. If atypical growth is found but it can be demonstrated by a negative control that the media or technique were faulty, then the first test should be repeated. If atypical growth is found but there is no evidence invalidating the test, then a retest should be conducted. Twice the number of biological containers and test vessels of the first test are used in the retest. If no atypical growth is found in the retest, the biological could be considered to be satisfactory for purity but the results from both the initial and retest should be reported for assessment by the individual countries relevant regulatory agency if the laboratory is sure that the first test result was not due to in-laboratory contamination. If atypical growth is found in any of the retest vessels, the biological is considered to be unsatisfactory for purity. If, however, it can be demonstrated by controls that the media or technique of the retest were faulty, then the retest should be repeated.

2.3. Example of a specific test procedure for exclusion of *Brucella sp.* including *B. abortus* (where general testing is not sufficient)

It should be confirmed that each batch of culture medium supports the growth of *B. abortus* by inoculating plates and flasks of biphasic medium with a known number of cells (around 100) of the fastidious *B. abortus* biovar 2. If the media supports the growth of this biotype it will support all other biovars.

Inoculate 1.0 ml of prepared master or working live agent or cell seed material (not containing antibiotics) by inoculating 50 µl of the test product into each of 10 flasks containing biphasic medium. At the same time 10 plates of serum dextrose agar (SDA) are inoculated with 50 µl of inoculum and spread with a sterile bent glass Pasteur pipette or hockey stick. An un-inoculated serum dextrose agar plate and a biphasic flask are also set up at the same time as negative controls.

For assessment of inhibitory substances 50 µl of previously prepared master or working ~~viral~~ live agent or cell seed material and 10–100 CFU of *B. abortus* are inoculated on to duplicate SDA plates. Positive controls are prepared by inoculating 10–100 CFU of *B. abortus* on to duplicate SDA plates.

All plates and flasks are incubated at 37°C in a 5–10% CO_2 environment. Plates are incubated with the agar uppermost and flasks with the agar slope vertical. Flasks are incubated with the cap loose.

Plates are checked for growth of colonies at days 4 and 8 of incubation. The biphasic medium is examined every 4 to 7 days for 28 days. After each examination of the flasks, they are tilted so that the liquid phase runs over the solid phase, then righted and returned to the incubator.

During the incubation period, SDA plates with positive control and test material are visually compared with plates with the positive control only and if there is no inhibition of growth of the organism in the presence of the test material, the interference testing test is successful, and testing can be assured to be sensitive.

Any signs of growth of suspicious contaminating microorganisms on SDA plates, cloudiness or colonies in biphasic flasks require follow-up testing by PCR to confirm whether *B. abortus* is present.

2.4. Example of a general procedure for detection of *Salmonella*

Each batch of biological reagents made in eggs should be free from contamination with *Salmonella*. This testing must be done before bacteriostatic or bactericidal agents are added. Five samples of each batch should be tested; 5 ml or one-half of the container contents, whichever is the lesser, of the sample should be used to inoculate 100 ml of tryptose broth and tetrathionate broth. The inoculated broths should be incubated for 18–24 hours at 35–37°C. Transfers from these broths should be made on to MacConkey and *Salmonella–Shigella* agar, incubated for 18–24 hours, and examined. If no growth typical of *Salmonella* is noted, the agar plates should be incubated an additional 18–24 hours and again examined. If colonies typical of *Salmonella* are observed, further subculture on to suitable differential media should be made for positive identification. Sensitive PCR tests are available for the detection of *Salmonella* serovars in cultured material. If *Salmonella* is detected, the batch is determined to be unsatisfactory.

3. Example of detection of *Mycoplasma*

3.1. An example of a specific procedure for exclusion of *Mycoplasma mycoides* subsp. *mycoides* (where general testing is not sufficient)

Prior to beginning testing it is necessary to determine that each batch of media promotes the growth of *M. mycoides* subsp. *mycoides* (*Mmm*) type strain PG1. General mycoplasma broth and agar are used but contain porcine serum as a supplement. Each batch of broth and agar is inoculated with 10–100 CFU of *Mmm*. The solid medium is suitable if adequate growth of *Mmm* is found after 3–7 days' incubation at 37°C in 5–10% CO_2. The liquid medium is suitable if the growth on the agar plates subcultured from the broth is found by at least the first subculture. If reduced growth occurs another batch of media should be obtained and retested.

1 ml of cell or virus seed to be tested is inoculated into 9 ml of the liquid medium and 100 µl on to solid mycoplasma agar. The volume of the product is inoculated so that it is not more than 10% of the volume of the medium. The liquid medium is incubated at 37°C in 5–10% CO_2 and 100 µl of broth is subcultured on to agar at days 7, 14 and 21. The agar plates are incubated at 37°C in 5–10% CO_2 for no fewer than 14 days, except those corresponding to day 21 subculture, which are incubated for 7 days. An un-inoculated mycoplasma broth and agar plate are incubated as negative controls. For assessment of inhibitory substances, inoculate 1 ml of sample to be tested into 9 ml of the liquid medium and 100 µl on to solid medium and add 10–100 CFU of *Mmm* to each. Prepare positive control by inoculating 9 ml of mycoplasma broth and a mycoplasma agar plate with 10–100 CFU of *Mmm*. Incubate as for samples and negative controls.

During incubation time, visually compare the broth of the positive control with sample present with the positive control broth and, if there is no inhibition of the organism either the product possesses no antimicrobial activity under the conditions of the test, or such activity has been satisfactorily eliminated by dilution. If no growth or reduced growth of *Mmm* is seen in the liquid and solid medium with test sample when compared with the positive control, the product possesses antimicrobial activity, and the test is not satisfactory. Modifications of the conditions to eliminate the antimicrobial activity and repeat test are required.

If antimicrobial activity is present it is necessary to dilute the test product further. Repeat the test above using 1.0 ml of sample in 39 ml of mycoplasma broth and then inoculate with 10–100 CFU of *Mmm* and incubate as above. All broths and plates are examined for obvious evidence of growth. Evidence of growth can be determined by comparing the test culture with the negative control, the positive control, and the inhibition control.

If evidence of microbial growth is found in the test samples the contaminating bacterium will be identified and confirmed as *Mmm* by specific PCR assay.

3.2 General testing for exclusion of *Mycoplasma* spp.

General testing for exclusion of *Mycoplasma* spp. that are less fastidious may require up to 28 days in culture, using general mycoplasma media. Some mycoplasmas cannot be cultivated, in which case the live biological sample will have to be tested using an indicator cell line such as Vero cells, DNA staining, or PCR methods.

Further detailed procedures can be found in Veterinary Medicinal Products, VICH GL34: https://www.ema.europa.eu/en/vich-gl34-biologicals-testing-detection-mycoplasma-contamination-scientific-guideline

and

USDA SAM 910: https://www.aphis.usda.gov/animal_health/vet_biologics/publications/910.pdf, (both accessed 20/09/2024).

4. Example of detection of rickettsia and protozoa

There are no general test procedures for exclusion of rickettsia or protozoa. Procedures to exclude specific agents of concern such as *Coxiella burnetii* (Q fever), *Ehrlichia canis, Trypanosoma evansi* and *Babesia caballi* can be found for example, in the Review of Published Tests to detect pathogens in veterinary vaccines Intended for Importation into Australia (Australian Government Department of Agriculture, Forest and Fisheries (2013]). The review is based on the reading and interpretation of applicable published papers from reputable journals and are regarded as examples of sensitive methods for detection of specified agents.

4.1. Example of a specific test protocol based on published methods for exclusion of *Babesia caballi* and *Theileria equi*

Babesia caballi and *Theileria equi* can be cultured *in vitro* in 10% equine red blood cells (RBC) in supportive medium supplemented with 40% horse serum and in a micro-aerophilic environment. Culture isolation of *T. equi* is more sensitive than for *B. caballi*. Giemsa-stained blood smears are prepared from cultures daily for 7 days (Avarzed *et al*., 1997; Ikadai *et al*., 2001). *Babesia caballi* is characterised by paired merozoites connected at one end. *Theileria equi* is characterised by a tetrad formation of merozoites or 'Maltese cross'. Confirmation of the diagnosis is by PCR (see Chapter 2.5.8 *Equine piroplasmosis*). Molecular diagnosis is recommended for the testing of biological products that do not contain whole blood or organs. Molecular diagnosis by PCR or loop-mediated isothermal amplification (LAMP) assay are the most sensitive and specific testing methods for detection of the pathogens of equine *piroplasmosis* (Alhassan *et al*., 2007).

5. Example of a procedure for extraneous testing of a live parasitic vaccine

Live parasitic vaccines can be assessed using testing procedures much the same as those for living viral/bacterial vaccines. The following is an example to exclude avian extraneous agents from a live *Eimeria* spp. vaccine, produced in live chickens and intended for chicken immunisation. Coccidiosis vaccine (live) for chickens is a preparation of sporulated oocysts of a suitable line or lines of species of coccidial parasites (*Eimeria* spp.) and vaccine stocks are produced in specific pathogen free (SPF) chickens. Manufacture and assessment of the vaccine is well described in European Pharmacopoeia (2023). For the exclusion of extraneous agents in live vaccine, reference is made in the specific Monograph *Coccidiosis vaccine (Live) for Chickens* to Monograph 2.6.24: *Avian viral vaccines: test for extraneous agents in seed lots*, with the instruction to follow Tests 1 to 6. In using the tests prescribed, the master seed lot must use organisms that are not more than five passages from the master seed lot at the start of the tests.

For procedures noted in European Pharmacopoeia Chapter 2.6.24, general provisions a) to d), f) and h) also apply to the assessment of Coccidiosis vaccine. To summarise, chickens, chicken material and cell cultures used for testing must be free from specified pathogens; cell cultures used must be well characterised, a minimum of two replicates for cell culture, although more may be prescribed; test substance must contain at least 10 doses of vaccine in inoculum; reagents used in the exclusion/neutralisation of vaccine seed process must be free of inhibitory substances and extraneous antibodies that may affect exclusion of avian extraneous agents. Nucleic acid amplification techniques can be useful in specific detection of various agents.

5.1. Test 1. Test for extraneous agents using embryonated hens' eggs

Testing material must be diluted and neutralised accordingly to contain at least 10 doses of vaccine in 0.2 ml inoculum. 0.2 ml of neutralised seed is then inoculated into:

Pass 1

i) 10 × 9- to 11-day-old embryonated eggs into the allantoic cavity, check viability for 7 days

ii) 10 × 9- to 11-day-old embryonated eggs onto the chorio-allantoic membrane (CAM), check viability for 7 days

iii) 10 × 5- to 6-day-old embryonated eggs into the yolk sac, check viability for 12 days.

Any deaths in the first 24 hours are discarded as nonspecific. For validity, at least six of ten embryos must survive past 24 hours. Check macroscopically for abnormalities of all embryos and CAMs that die after 24 hours and survivors at the end of the incubation period. Test all allantoic fluid harvests for the presence of haemagglutinating agents.

Pool live embryo material and dead embryo material separately for a further pass in eggs following Pass 1 procedure.

Seed lot complies if test embryos/CAMs show no macroscopic abnormalities, embryos do not die from toxicity or test material and HA testing of allantoic fluids are negative.

5.2. Test 2. Test in chicken kidney cells

Inoculate 0.1 ml test material (prepared as for Test 1) onto 5 × 25 cm^2 just confluent chicken kidney monolayers. 2 × 25 cm^2 monolayers are kept as negative control and treated the same way, but with no test material. Incubate for 1 hour to adsorb prior to addition of maintenance media. Incubate for 7 days with 24- to 48-hour checks for CPE. Freeze–thaw at –80°C once or twice between Pass 1 to 2 to break up cells. Subculture cells into Pass 3 for detection assessment. Monolayers required for detection assessment (from each of the five monolayers) include at least a 10 cm^2 monolayer for Giemsa or haematoxylin and eosin staining, 25 cm^2 monolayers for haemadsorption of chicken red blood cells and tissue culture supernatant from each flask for haemagglutination (HA) assessment, using chicken red blood cells.

Test validity is based on no signs of extraneous agents in negative cultures. The batch complies if there is no evidence of extraneous agents.

5.3. Test 3. Test for avian leukosis viruses (ALV)

Inoculate 0.1 ml test material (prepared as for Test 1) onto 5 × 25 cm^2, replicate just confluent DF-1 cells. Each replicate is to have a total of 50 cm^2 area = 10 × 25 cm^2 total. If available, primary/secondary chicken embryo fibroblast cells can be used but must be produced from the tissues of 9- to 11-day-old SPF embryos that are known to be genetically susceptible to subgroups A, B and J of ALV and support growth of only exogenous ALV: Cells from C/E strain of chickens are suitable. Extra 2 × 25 cm^2 replicate (× 4) monolayers are used as negative control. Extra 2 × 25 cm^2 replicate monolayers are infected, each, with a suitable strain of ALV subgroup A, B and J (not more than 10 median cell culture infectious doses [$CCID_{50}$] in 0.1 ml) for use as positive controls (total of 12 positive control flasks.). Incubate for 1 hour to adsorb prior to addition maintenance media. Incubate for a total of at least 9 days, subculturing at 3- to 4-day intervals.

Retain cells from each passage and harvest at the end of the incubation period. Pooled cells from each set of replicate flasks are washed and resuspended at a count of 10^7 cells/ml in barbital-buffered saline. Cells are freeze–thawed three times to release any bound antigen and then tested by a complement fixation test for ALV (COFAL) or by antigen-detection ELISA.

The test is valid if group specific antigen is detected in at least five of the six positive control replicate monolayers, negative test monolayers are negative for detection of antigen. If more than one of the test monolayers are inconclusive, further testing of cell lysates are required until they are negative.

Batch complies if there is no evidence of ALV antigen.

5.4. Test 4. Test for avian reticuloendotheliosis virus (REV)

Inoculate 0.1 ml test material (prepared as for Test 1) onto 5 × 25 cm^2 just confluent chicken embryo fibroblast monolayers (produced from the tissues of 9- to 11-day-old SPF embryos). Extra 2 × 25 cm^2 monolayers are used as negative controls and extra 4 × 25cm^2 monolayers are infected with a suitable strain of REV (not more than 10 $CCID_{50}$ in 0.1 ml) for use as positive controls. Incubate for 1 hour to adsorb prior to addition maintenance media. Incubate for a total of 10 days, subculturing at 3- to 4-day intervals.

The test is not valid if fewer than four of five test monolayers, fewer than three of the four positive controls or neither of the two negative control monolayers survive after any passage.

For the last subculture, subculture each of the 11 × flasks into suitable substrates to obtain at least 10 cm^2 monolayer for each flask. Look for the presence of REV by using immunostaining techniques (FA) for detection of the specific agent.

The test is valid if at least three of the four positive controls are positive for REV by FA test. Negative controls and test flasks must not be positive by FA to REV.

Batch complies if there is no evidence of REV.

5.5. Test 5. Test for chicken anaemia virus (CAV)

Inoculate 0.1 ml test material (prepared as for Test 1) into 5 × 20 ml of MDCC-MSBI cell suspensions at 5 × 10^5 cell/ml, each suspension is placed in a single 25 cm^2 flask. Extra 2 × cell suspensions in 25 cm^2 flasks are used as negative controls and 4 × cell suspensions in 25 cm^2 flasks are infected with a suitable strain of CAV (not more than 10 $CCID_{50}$ in 0.1 ml) for use as positive controls. Incubate for a total of 24 days, subculturing eight times at 3- to 4-day intervals. During the subculture process, indication of CAV can be seen by metabolic colour changes. Media will become red compared with negative control flasks. CPE will also be detected.

With the indication of a positive test result or at the end of the incubation period, harvest cells from each flask, centrifuge at low speed to pellet and resuspend cells at around 1 × 10^6/ml. Dry 25 µl cell suspension on suitable glass slides and fix ready for immunostaining to detect for CAV.

The test is valid if at least three of the four positive controls are positive for CAV. Negative controls and test flasks must not be positive by FA to CAV.

Batch complies if there is no evidence of CAV.

5.6. Test 6. Test for extraneous agents using chicks

Inoculate at least 10 × 2-week-old chicks with equivalent of 100 doses of vaccine by the intramuscular route and with the equivalent of 10 does by eye drop. If the dosage used is pathogenic to chickens at this age, then older chickens may be used, if required and can be justified. Repeat the inoculations 2 weeks later.

Chicks are observed for 5 weeks from the day of inoculation. Antimicrobials and are not to be administered during the test period.

Test is valid if more than 80% of chicks survive to the end of the testing period.

Collect serum from all chicks at the end of testing and test for serum antibodies selecting a test type from each agent listed in Table 3.

Clinical disease other than that attributed to the test material, and detection of antibodies of any agent listed in Table 3 gives evidence of presence of extraneous agent(s).

Table 3. Detection of antibodies of the following agents by the corresponding tests provides evidence of the presence of extraneous agent(s)

Agent	**Type of test**
Avian adenoviruses group 1	VNT, ELISA, AGID
Avian encephalomyelitis virus	ELISA, AGID
Avian infectious bronchitis virus	ELISA, HI
Avian infectious laryngotracheitis virus	VNT, ELISA, IS
Avian leukosis viruses	VNT, ELISA
Avian nephritis virus	IS
Avian orthoreoviruses	IS, ELISA
Avian reticuloendotheliosis virus	AGID, IA, ELISA
Chicken anaemia virus	IS, ELISA VNT
Egg drop syndrome	IS, ELISA, VNT
Avian infectious bursal disease virus	Serotype 1: AGID, ELISA, VNT
	Serotype 2: VNT
Influenza A virus	AGID, ELISA, HI
Marek's disease virus	AGID
Newcastle disease virus	HI, ELISA
Avian metapneumovirus	ELISA
Salmonella pullorum	SAT

AGID: agar gel immunodiffusion; ELISA: enzyme-linked immunosorbent assay; HI: haemagglutination inhibition; IS: immunostaining (including FA: fluorescent assay); SAT: serum agglutination test; VNT: virus neutralisation test.

~~5.~~6. Example of detection of virus

In brief, general testing usually includes the use of continuous and primary cell lines of the source species; cells of known susceptibility to likely viral contaminants, which are inoculated for usually a period of 3–4 weeks with weekly subcultures. Virus seeds also require testing on a primary cell line of the species in which the final product is intended. At Day 21 or 28, assessment of the monolayers is done using appropriate histology staining procedures to assess CPE, and haemadsorption with guinea-pig and chicken RBC to assess the presence of haemadsorbing agents. Note that general testing is useful as a screening tool though not sufficiently sensitive enough to detect all viruses of concern to all countries.

Specific testing requires test material to be inoculated on to sensitive, susceptible cells lines for the virus to be excluded; the amplification process in cell culture is usually up to 28 days but depending on the virus, may require longer culturing times. Detection of specific viral contaminants is by recognition of CPE in conjunction with more sensitive antigen detection or molecular tests such as FAT and PCR and ELISA after the amplification process in cell culture is completed.

All testing using cell lines to amplify for target viruses is contingent on the sensitivity of the cells for the target agent and the ability to recognise the presence of the agent in the cells. The quality, characteristics, and virus permissibility profile of cell lines in use should be determined as fit for purpose and appropriately maintained.

If a virus seed is known to cause cytopathic effect (CPE) in a permissive cell line, the effect must be specifically neutralised without affecting the likelihood of isolation of the target agent. The serum must be shown to be free from antibodies against any agents for which the test is intended to detect. Antiserum should be tested for nonspecific inhibiting affects. For a general test, this can be difficult to ascertain. Serum should be of sufficiently high titre to neutralise the seed virus effectively with the use of an approximately equal volume or less of serum. A microplate block titration is useful to determine the amount of the antiserum required to neutralise a known amount of CPE causing virus seed. This is done in the normal conditions required of each test system (e.g. time, temperature, cell type etc.).

If a virus seed is known to be high-titred or difficult to neutralise, antiserum can be added to the growth medium in a test system at a final concentration of 1–2%.

Cell seed stocks do not require a neutralisation process.

~~5~~6.1. Example of general testing procedures for the exclusion of viruses from virus and cell seed stocks used in production of veterinary vaccines

~~5~~6.1.1 Example of amplification in cell culture

Continuous and primary, 75 cm^2 area monolayers of the source species (and intended species as applicable) are infected with 1 ml of seed stocks and passaged weekly for up to 21–28-days. Depending on the procedure followed, monolayers can be subcultured between passes or freeze/thawed to disrupt cells. Negative and positive controls should be also set up at each pass using the same cell population. Certain relevant viruses may be selected as indicators for sensitivity and interference (positive controls) but these will not provide validation for the broader range of agents targeted in general testing. The final culture is examined for cytopathology and haemadsorption.

~~5~~6.1.2 Example of general detection procedures: cytopathology

May–Grünwald–Giemsa or H&E staining procedures are used to assess for cytopathological changes associated with virus growth. Monolayers must have a surface area of at least 6 cm^2 and can be prepared on appropriate chambered tissue culture slides and incubated for 7 days. The plastic wells of the slides are removed leaving the rubber gasket attached to the slide. The slides are rinsed in Dulbecco's phosphate buffered saline (PBS), fixed in acetone, methanol or formalin depending on the stain used and placed on a staining rack. For May–Grünwald–Giemsa staining: the slides are stained for 15 minutes at room temperature with May–Grünwald stain diluted 1/5 with absolute methanol. The May–Grünwald stain is removed by inverting the slides. The slides are then stained for 20 minutes with Giemsa stain diluted 1/15 in deionised water. The Giemsa stain is removed by inverting the slides and rinsing them in deionised water for 10–20 seconds. The slides are air-dried and mounted with a coverslip using paraffin oil. The May–Grünwald–Giemsa stain differentially stains ribonucleoprotein (RNP); DNA RNP stains red-purple, while RNA RNP stains blue. The monolayers are examined with a conventional microscope for the presence of inclusion bodies, an abnormal number of giant cells, or other cytopathology attributable to a viral contaminant of the test product. The inoculated monolayers are compared with suitable control non-inoculated monolayers. If specific cytopathology attributable to an extraneous virus is found, results are reported, and additional specific testing may be conducted.

~~5~~6.1.3 Example of general detection procedures: haemadsorption

Testing for haemadsorption requires the use of 75 cm^2 area monolayers established in tissue culture flasks after the 28-day passage period described above. Guinea-pig, chicken, and any other blood for use in this assay is collected in an equal volume of Alsever's solution and may be stored at 4°C for up to 7 days. Immediately prior to use, the stored erythrocytes are again washed by adding 5 ml of blood in Alsever's solution to 45 ml of calcium and magnesium-free PBS (PBSA) and centrifuging in a 50 ml centrifuge tube at 500 ***g*** for 10 minutes. The supernatant is aspirated, and the erythrocytes are suspended in PBSA and re-centrifuged. This washing procedure is repeated at least twice until the supernatant is clear. Erythrocytes from each species are combined by adding 0.1 ml of each type of packed blood cells to 100 ml of PBSA. The erythrocytes from different species may be kept separate or combined, as desired. To each flask, add 5 ml of the erythrocyte suspension, and incubate the flasks at 4°C for 30 minutes. Monolayers are washed twice with PBSA and examined for haemadsorption. If no haemadsorption is apparent, 5 ml of fresh erythrocyte suspension is added to each flask; the flasks are incubated at 20–25°C (room temperature) for 30 minutes, rinsed as before, and examined for haemadsorption. Separate flasks may be used for each incubation temperature if desired. Monolayers are examined for the presence of haemadsorption using an illuminated light box and microscopically. Non-inoculated monolayers are used as negative controls. The PBSA and fresh erythrocytes should prevent most nonspecific haemadsorption from occurring. If specific haemadsorption attributable to an extraneous agent is found, results are reported, and additional specific testing may be conducted.

~~5~~6.2. Example of specific agent exclusion testing of biologicals used in the production of veterinary vaccines

~~5~~6.2.1. Example of porcine epidemic diarrhoea virus (PEDV)

Trypsin presence is required at inoculation and in the culture medium for isolation of porcine epidemic diarrhoea virus (PEDV) in Vero cells (CCL81, ATCC) to ensure the virus can enter host

cells. Just confluent monolayers are required as under confluent monolayers (<90%) are more sensitive to the presence of trypsin. An over confluent or aging monolayer will not be sensitive for growth of PEDV. Maintenance media (MM) formulation consists of Earle's MEM (minimal essential medium) (with 5.6 M HEPES [N-2-hydroxyethylpiperazine, N-2-ethanesulphonic acid] and glutamine) + 0.3% Tryptose phosphate broth, 0.02% yeast extract and 4 µg/ml TPCK treated trypsin. The addition of the trypsin into MM should occur on the day the media is to be used.

Prior to inoculation, confluent 75 cm^2 monolayers are washed twice with MM to remove growth media containing FCS. Virus or cell seed (1 ml) is added with 1 ml of MM to each monolayer; incubate at 37°C for 2 hours, then add 30 ml/flask of MM. Negative control monolayers of the same size are set up prior to inoculation of test material. Positive and interference controls are set up last, and where possible, in a separate laboratory to avoid contamination. Assessment for sensitivity and interfering substances requires use of reference virus of known titre. A control for interference using co-inoculation of test sample and PEDV needs only to be set up on the first pass. Positive controls should be set up at every pass to ensure each monolayer used gives expected sensitivity. PEDV virus is titrated in log dilutions starting at 10^{-1} to 10^{-6} in MM (depending on the endpoint titre of reference virus) in duplicate rows of 6 wells of a 24-well tissue culture plate. For the interference test, PEDV is titrated in the same dilution series but using MM spiked with a 10% volume of test material. Decant off the growth media and discard. Wash plates to ensure no FCS is present. Two washes using approximately 400 µl/well MM (with trypsin added) are sufficient.

Add 100 µl of diluted virus on to each of two duplicate wells. Rock inoculated plates to distribute the inoculum evenly over the surface of the monolayer. Incubate at 37°C with 5% CO_2 for 2 hours then add a further 1 ml volumes/well of MM.

After 7 days, 75 cm^2 monolayers have cells disrupted using two freeze–thaw cycles at –80°C. Positive control plates are read for end-point titres, and these are compared with virus in the presence of test material to ensure titres are comparable and interference has not occurred. Freeze–thaw lysates are clarified at 2000 ***g*** for 5 minutes and re-passed on to newly formed monolayers as for the first passage. Passages are repeated until a total of four passages are completed at which point cell lysates are assessed by PCR for detection of PEDV and day 7 monolayers in 24-well plates are fixed and stained for FAT. If a seed virus is to be tested and requires neutralisation using antiserum, extra care in the isolation of PEDV needs to be considered. Trypsin is rendered inactive in the presence of serum proteins and without trypsin present, PEDV grows poorly, or not at all. Washing off the inoculum with two MM washes is required after an extended adsorption time of up to 4 hours to ensure acceptable sensitivity.

~~H~~. I. INFORMATION TO BE SUBMITTED WHEN APPLYING FOR AN IMPORT LICENCE

When undertaking risk analysis for biologicals, Veterinary Authorities should follow the *Terrestrial Code,* and the manufacturer should follow the requirements of the importing country. Requirements for each importing country should be accessible and published online. The manufacturer or the Veterinary Authority of the exporting country should make available detailed information, in confidence as necessary, on the source of the materials used in the manufacture of the product (e.g. substrates). They should make available details of the method of manufacture (and where appropriate inactivation) of the substrates and component materials, the quality assurance procedures for each step in the process, final product testing regimes, and the pharmacopoeia with which the product must conform in the country of origin. They should also make available challenge organisms, their biotypes and reference sera, and other means of appropriate product testing.

For detailed examples of a risk-based assessment of veterinary biologicals for import into a country refer to:

- European Commission (2015). The Rules Governing Medicinal Products in the European Union. Eudralex. Volume 6. Notice to applicants and regulatory guidelines for medicinal products for veterinary use.
- Department of Agriculture, Forest and Fisheries of Australia (2021b). Live veterinary vaccines Summary of information required for biosecurity risk assessment, Version 6 and Inactivated veterinary vaccines, Version 8.
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- Ministry of Agriculture and Rural Affairs, China (People's Rep. of), Regulations on the Administration of Veterinary drugs (revised in 2020).

When applying for an import licence other regulatory requirements may need to be addressed depending on the type of sample and if the sample needs to be shipped out of country to a testing laboratory. For example, cell seeds may come under certain requirements for permits such as the Convention for International Trade in Endangered Species of Wild Fauna and Flora (CITES), where a cell line is derived from an endangered species, e.g. the cell line and its derivatives. Applying for such a permit is time consuming and requires input from both the exporting and importing country.

Genetically modified organisms (GMOs) are becoming more frequent in use with changes in manufacturing technologies and specialised, time-consuming procedures need to be in place. A laboratory that accepts a GMO product for testing shall follow the procedures of the ~~Office of the Gene Regulator (OGTR)~~ national regulator to allow the GMO to be dealt with.

~~I.~~ J. RISK ANALYSIS PROCESS

Risk analysis should be as objective and transparent as possible and should be performed in accordance with Section 2 of the *Terrestrial Code*, and certification in line with Section 5 of the *Terrestrial Code*. Of necessity, assessment of the country and commodity factors and risk reduction measures will be based largely on manufacturers' data. These data depend on quality assurance at all stages of manufacture, rather than on testing of the final product alone.

Domestic exposure may be influenced by the approved usage of the product. Veterinary Authorities may place limits on usage of some products (e.g. restricting usage to institutions of appropriate biosecurity).

~~J.~~ K. BIOCONTAINMENT

Suitable biocontainment may be necessary for many forms of biologicals. In particular, the importation of exotic micro-organisms should be carried out in accordance with Chapter 1.1.4 *Biosafety and biosecurity: standard for managing biological risk in the veterinary laboratory and animal facilities*.

Laboratories using high risk agents should have well researched and documented risk assessments in place prior to working with such agents to ensure the safety of their staff and laboratory.

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NB: FIRST ADOPTED IN 1989. MOST RECENT UPDATES ADOPTED IN 2024.

Annexe 8. Section 2.1. Diagnostic de laboratoire (Chapitre 2.1.1. Méthodes de laboratoire utilisées pour les tests de sensibilité des bactéries aux antimicrobiens)

RÉUNION DE LA COMMISSION DES NORMES BIOLOGIQUES
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SECTION 2.1.

LABORATORY DIAGNOSTICS

CHAPTER 2.1.1.

LABORATORY METHODOLOGIES FOR BACTERIAL ANTIMICROBIAL SUSCEPTIBILITY TESTING

SUMMARY

With the increase in bacterial resistance to traditionally used antimicrobials, ~~it has become more difficult for clinicians to empirically~~ the use of antimicrobial susceptibility testing (AST) has become a key element in antimicrobial stewardship as clinicians select an appropriate antimicrobial agent. As a result, in-vitro *~~antimicrobial susceptibility testing (AST)~~ of the relevant bacterial pathogens, from properly collected specimens, should be performed using validated methods. Thus, AST is an important component of prudent antimicrobial use guidelines in animal husbandry worldwide and veterinarians in all countries should have ~~these~~ AST data available for informed decision-making.*

Although a variety of methods exist, the goals of ~~in vitro antimicrobial susceptibility testing~~ AST are either to provide a reliable predictor of how ~~an organism~~ pathogen is likely to respond to antimicrobial therapy in the infected host or to assess whether there has been a change in susceptibility for a given bacterial species over time for surveillance purposes ~~whether there has been development of resistance~~. This type of information aids the clinician in selecting the appropriate antimicrobial agent, aids in developing antimicrobial use policy, and provides data for epidemiological surveillance. ~~Such epidemiological surveillance data provide a base to choose the appropriate empirical treatment (first-line therapy) and to detect the emergence and/or the dissemination of resistant bacterial strains or resistance determinants in different bacterial species.~~ The selection of a particular AST method is based on many factors such as validation data, practicality, flexibility, automation, cost, reproducibility, accuracy, standardisation and harmonisation.

The use of genotypic approaches for detection of antimicrobial resistance genes or mutations associated with resistance has also been promoted as a way to increase the speed and ~~accuracy~~ resolution of ~~susceptibility testing~~ resistance surveillance. ~~Numerous DNA-based assays are being developed to detect bacterial antimicrobial resistance at the genetic level.~~ These methods, when ~~used in conjunction~~ validated with phenotypic analysis, offer the promise of increased sensitivity, specificity, scope, and speed in the detection of specific known resistance genes and mutations, and ~~can be used in tandem with~~ could in some circumstances replace traditional ~~laboratory~~ AST methods.

INTRODUCTION

The spread of multiple antimicrobial-resistant pathogenic bacteria has been recognised by the World Organisation for Animal Health (WOAH), the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) as a serious global human and animal health problem. The development of bacterial antimicrobial resistance is neither an unexpected nor a new phenomenon. It is, however, of increasing concern due to the frequency with which new emerging resistance phenotypes are occurring among many bacterial pathogens and commensal organisms, such as resistance to carbapenems, colistin, linezolid, macrolides, etc.

Historically, many infections could be treated successfully according to the clinician's past clinical experience or because susceptibility could be reliably predicted (i.e. empirical therapy)~~,~~; ~~however, this~~ which is becoming more ~~the exception than the rule~~ challenging ~~(Walker, 2007)~~. Resistance has been observed to essentially all of the antimicrobial agents currently approved for use in humans and ~~veterinary clinical medicine~~ animals. ~~This, combined with the variety of antimicrobial agents currently available, makes the selection of an appropriate agent an increasingly challenging task.~~ This situation has made clinicians more dependent on AST data~~,~~ ~~from *in-vitro* antimicrobial susceptibility testing, and~~ highlighting the importance of the diagnostic laboratory in clinical practice.

In this context, the use of AST allows clinicians to make more informed decisions about the most appropriate antimicrobial agent and provides valuable data to assess susceptibility trends over time for surveillance purposes.

~~A number of antimicrobial susceptibility testing (AST) methods are available to determine bacterial susceptibility to antimicrobials.~~ The selection of an AST method is based on many factors such as practicality, flexibility, automation, cost, reproducibility, accuracy, accessibility and individual preference. Standardisation and harmonisation of AST ~~methodologies,~~ methods and data interpretation used in epidemiological surveillance of antimicrobial drug resistance, are critical if data are to be compared among national or international surveillance/monitoring programmes of WOAH Members. ~~It is essential that AST methods provide reproducible results in day-to-day routine laboratory use and that the data be comparable with those results obtained by an acknowledged 'gold standard' reference method.~~ Currently the reference AST method endorsed by the Clinical and Laboratory Standards Institute (CLSI) is the broth micro-dilution method (Humphries *et al.,* 2023) [1] that determines minimum inhibitory concentration (MIC) as described by the ISO (International Organization for Standardization, ~~2006~~ 2019). In the absence of standardised methods or reference procedures, susceptibility results from different laboratories cannot be reliably compared. The method used to select samples for inclusion in ~~antimicrobial resistance~~ surveillance programmes, as well as the ~~methods~~ laboratory procedures used for ~~primary~~ bacterial isolation and identification, are also important factors that should be standardised or harmonised to allow direct comparison of data between different regions; consideration of these issues is addressed in ~~a~~ WOAH, Codex Alimentarius, WHO documents (Codex Alimentarius, 2021; ~~Dehaumont 2004~~; WHO, 2017; WOAH, 2024).

~~As the science of AST has progressed, a greater understanding of the multiple factors that could affect the overall outcome of susceptibility testing has become clearer (WHO, 2017).~~ This ~~document~~ chapter provides ~~guidelines and~~ guidance for using both standardised AST ~~standardisation for~~ methodologies, and interpretation of ~~antimicrobial susceptibility test~~ AST results.

1. Test requirements

The following requirements should be applied to ~~achieve standardisation of AST methods and~~ allow for comparability of AST results across national or international surveillance/monitoring programmes of WOAH Members:

i) the use of standardised AST methods is essential to generate accurate and reproducible data, and includes harmonised ~~including the harmonisation of~~ AST test parameters such as media, inoculum, incubation time, quality controls, choice of antimicrobial agents and subsequent interpretive criteria,

ii) standardised AST methods, including all critical specifications and interpretive criteria, should be clearly defined, documented in detail and used by all participating laboratories,

~~iii) all AST methods should generate accurate and reproducible data,~~

iii) quantitative susceptibility data (e.g. MIC frequencies, zone diameter frequencies) should be reported,

iv) establishment of national or regional reference laboratories, in cooperation with consensus standards-setting organisations (e.g. European Committee on Antimicrobial Susceptibility Testing [EUCAST], CLSI) is essential for the coordination of AST methodologies, interpretations and appropriate operational techniques used to ensure accuracy and reproducibility ~~(e.g. quality controls)~~,

~~[1] https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10281161/~~

v) microbiological laboratories should implement and maintain a formal quality management programme (see Chapter 1.1.5 *Quality management in veterinary testing laboratories*),

vi) laboratories should ~~have acquired~~ be accredited by a third party ~~accreditation~~ that ~~includes~~ covers the AST methodologies to be used within the scope of that accreditation. The accreditation body should meet accepted international Laboratory Accreditation Cooperation (ILAC) standards and guidelines regarding the standards used for the accreditation process. The accreditation standards used should include the requirement for participation in proficiency testing programmes,

vii) specific bacterial reference strains/quality control strains are essential for determining intra- and inter-laboratory quality control, quality assurance and proficiency testing.

2. Selection of antimicrobials for testing and reporting

Selecting the appropriate antimicrobials for ~~susceptibility testing~~ AST can be difficult given the vast numbers of agents available. The following ~~guidelines~~ best practices should be noted:

i) ~~the~~ FAO/WOAH/WHO expert workshop on non-human antimicrobial usage and antimicrobial resistance recommends ~~creating~~ laboratories or programmes create a list of veterinary and human critically important antimicrobials for ~~susceptibility testing~~ routine AST and reporting,

ii) selection of the most appropriate antimicrobials to test is a decision best made by each WOAH Member in consultation with the appropriate bodies and organisations,

iii) antimicrobials in the same class may have similar *in-vitro* activities against select bacterial pathogens. In these cases, a representative antimicrobial should be selected that predicts susceptibility to other members of the same class,

iv) certain ~~microorganisms~~ bacteria can be intrinsically resistant to particular antimicrobial classes; therefore it is unnecessary to test and misleading to ~~test~~ report results for certain agents ~~for activity *in vitro*. The type of intrinsic resistance has to be determined for these organisms from either the scientific literature or through testing.~~ WOAH Members may identify intrinsic resistance among tested bacteria from the literature,

v) the number of antimicrobials to be tested should comply with the guideline used (CLSI/EUCAST/ISO) and at least contain class representatives to ensure the relevance and practicality of AST (see also WHO, 2017),

vi) the panel of antimicrobials tested should depend on the target bacteria, the clinical or epidemiological relevance of these antimicrobials at national or regional level.

vii) the panel of antimicrobials tested should ideally be harmonised within national surveillance programme(s) to ensure continuity and comparability of data.

viii) the panel of antimicrobials to be tested may be prioritised based on their higher importance for human health, the national context, or their influence on the selection or co-selection of resistance.

Periodic review of ~~microorganisms~~ bacteria that are currently predictably susceptible to certain antimicrobial agents is recommended to ensure that emergent, unexpected resistance is detected and used to update local treatment guidelines. Timely AST can identify emerging resistances before clinical failure is widely observed ~~Emerging resistance may also be suspected following poor response or treatment failure to a standard antimicrobial treatment regime~~.

3. Antimicrobial susceptibility testing methodologies

The following requirements should be respected:

i) bacterial isolates subjected to AST must be ~~isolated in~~ derived from a pure culture from the submitted sample,

ii) standard ~~reference methods~~ laboratory procedures and culture methods should be used for identification so that the subject bacteria are consistently and correctly identified to the genus, ~~and/or~~ species, or serotype level, where relevant,

iii) bacterial isolates considered to be the most important, ~~and~~ other selected isolates, and reference/quality control (QC) strains should be stored for future analysis in conditions ensuring an absence in change in the characteristics and purity of the strain (either lyophilisation or cryogenic preservation at –70°C to –80°C).

The following factors influencing AST methods should be determined, optimised, and documented in a detailed standard operating procedure:

i) once ~~the~~ a bacterium has been isolated in pure culture, a standardised concentration of the inoculum must be prepared using a nephelometer or spectrophotometer to ensure a defined number of colony forming units to

obtain accurate and repeatable susceptibility results. Bacteria or other organisms used in AST ~~testing~~ should be from a fresh 24-hour culture,

ii) the composition and preparation of the agar and broth media used (e.g. pH, cations, thymidine or thymine, use of supplemented media) should comply with ~~guidelines~~ the validated test method (CLSI/EUCAST/ISO). Performance and sterility testing of media lots should also be determined and documented ~~as well as the procedures used~~,

iii) the content, range/interval and concentration of the antimicrobials used (microtitre plates, disk~~, strip, tablet~~) should follow guidelines (CLSI/EUCAST/ISO) and be relevant to the bacterial species tested,

iv) composition of solvents and diluents for preparation of antimicrobial stock solutions,

v) growth and incubation conditions (time, temperature, atmosphere e.g. CO_2),

vi) agar depth,

vii) the test controls to be used, including the reference organisms ~~used~~,

viii) ~~the subsequent~~ interpretive criteria (i.e. clinical breakpoints, epidemiological cut-off values ~~– ECOFFs~~).

For these reasons, special emphasis has to be placed on the use of documented procedures and validated, well documented methods, as sufficient reproducibility can be attained only through the use of such methodology.

4. Selection of antimicrobial susceptibility testing methodology

The selection of an AST methodology may be influenced by the following factors:

i) ease of performance,

ii) flexibility,

iii) adaptability to automated or semi-automated systems,

iv) cost,

v) reproducibility,

vi) reliability,

vii) accuracy,

viii) the organisms and the antimicrobials of interest ~~in~~to that particular WOAH Member~~,~~.

~~ix) availability of suitable validation data for the range of organisms to be susceptibility tested.~~

5. Antimicrobial susceptibility testing methods

These ~~following~~ three methods ~~have been shown to~~ consistently provide reproducible ~~and repeatable~~ results when following standardised procedures ~~followed correctly (Clinical and Laboratory Standards Institute [~~CLSI~~], 2008~~ 2024a~~; Walker, 2007)~~:

i) disk diffusion,

ii) broth dilution,

iii) agar dilution~~,~~.

5.1 Disk diffusion method

Disk diffusion refers to the diffusion of an antimicrobial agent from a disk ~~or tablet~~ containing a specified ~~concentration~~ amount of the agent ~~tablets~~ (i.e. disk content) into a solid culture medium (normally Mueller–Hinton agar) that has been inoculated on the surface with a pure culture (see Section 3). The disk diffusion test result is determined by measurement of the diameter of the inhibition zone around the disk, the diameter being proportional to the bacterial susceptibility to the antimicrobial present in the disk.

The diffusion of the antimicrobial agent into the culture media results in a gradient of the antimicrobial. When the concentration of the antimicrobial becomes so diluted that it can no longer inhibit the growth of the test bacterium, the zone of inhibition is demarcated. ~~The diameter of this zone of inhibition around the antimicrobial disk is related to MIC for that particular bacterium/antimicrobial combination; the zone of inhibition correlates inversely with the MIC of the test bacterium. Generally, the larger the zone of inhibition, the lower the concentration of antimicrobial required to inhibit the growth of the organisms.~~

~~However, this depends on the concentration of antimicrobial agent in the disk and its diffusibility.~~ Antimicrobial agents that are very large molecules (e.g. polymyxins) diffuse poorly in agar making disk diffusion methods unreliable for these compounds~~. For this reason disk diffusion methods are not recommended for example for the susceptibility testing of colistin/polymyxin~~ (Matuschek *et al.,* 2018).

Note: Disk diffusion tests based solely on the presence or absence of a zone of inhibition without regard to the measured size of the zone of inhibition are not acceptable AST methodology.

5.1.1. Considerations for the use of the disk diffusion methodology

~~Disk diffusion is easy to perform, reproducible if standardised, and does not require expensive equipment. Its main advantages are~~ The advantages of disk diffusion methodology include:

i) low cost,

ii) ease in performance,

iii) ease in modifying test by changing antimicrobial disks when required,

iv) can be used as a screening test against large numbers of isolates,

v) can identify a subset of isolates for further testing by other methods, such as ~~determination of MICs~~ broth dilution.

vi) the procedure is controlled by inclusion of appropriate quality control organisms for which a target zone size range is available (or has been derived) for each of the relevant antimicrobial agents being tested in the disk diffusion test procedure.

The disadvantages of disk diffusion methodology include:

i) Availability of antimicrobial agent disks of specific interest to veterinary medicine and at the concentrations needed.

ii) Limited direct relevance *in vivo* of zones of inhibition measurements as compared to MICs.

iii) Manual measurement of zones of inhibition may be time-consuming and can be subject to variability by technician.

iv) Not available for bacteria that require long incubation times (*Mycobacterium* spp., etc.).

Automated zone-reading devices are available that can be integrated with laboratory reporting and data-handling systems. The disks should be distributed evenly on the agar surface so that the zones of inhibition around antimicrobial disks in the disk diffusion test do not overlap to such a degree that the zone of inhibition cannot be determined. Generally, this can be accomplished if the disks are no closer than 24 mm from centre to centre, though this is dependent on disk concentration and the ability of the antimicrobial to diffuse in agar. Contamination of culture plates may be harder to detect using automated readers.

The diameter of the zone of inhibition obtained in disk diffusion tests is strongly influenced by the density of the bacterial inoculum applied, underlining the requirement to standardise the inoculum density in accordance with guidelines (CLSI, EUCAST, ISO). A denser inoculum than intended will result in reduced zones of inhibition and a sparse inoculum will result in increased zones of inhibition (BSAC [British Society for Antimicrobial Chemotherapy], 2015).

5.2. Broth and agar dilution methods

The aim of the broth and agar dilution methods is to determine the lowest concentration of the antimicrobial that inhibits the visible growth of the bacterium being tested ~~in either broth or on agar~~ (MIC, usually expressed in µg/ml or mg/~~litre~~). ~~The range of concentrations tested in broth and agar dilution methods generally includes the breakpoint (clinical or microbiological) with doubling dilutions either side of that value as considered appropriate. However, the MIC does not always represent exactly the concentration which was tested. The 'true' MIC is a point between the lowest test concentration that inhibits the growth of the bacterium and the next lower test concentration. Therefore, MIC determinations performed using a dilution series may be considered to have an inherent variation of ±1 dilution.~~

Tested antimicrobial concentration ranges should encompass both the ~~interpretive criteria~~ clinical breakpoint and epidemiological cut-off value, when available ~~(susceptible, intermediate and resistant)~~ for a specific bacterium/antimicrobial. The concentration range of each antimicrobial agent should also cover the full range of allowable results for the QC strain(s) used for each antimicrobial agent~~ biotic combination~~

~~and appropriate quality control reference organisms. Target MIC ranges should be available for each antimicrobial agent being tested~~.

~~Antimicrobial susceptibility dilution methods are more reproducible than agar disk diffusion which is why broth microdilution is the current reference test method. However, antibiotics are usually tested in doubling dilutions, which can produce inexact MIC data. The continuous range of zone diameter values obtained with disk diffusion can therefore be advantageous in certain circumstances, such as screening large numbers of susceptible isolates.~~

Any laboratory that intends to use a dilution method and set up its own reagents and ~~antibiotic~~ antimicrobial dilutions should have the ability to obtain, prepare and appropriately maintain stock solutions of reagent-grade antimicrobials, to account for the potency of the antimicrobial (supplied by the manufacturer) and to generate complex working dilutions on a regular basis. Published methods should be consulted. It is ~~then~~ essential that such laboratories use quality control organisms (see below) to assure accuracy and standardisation of their procedures.

5.2.1. Broth dilution

Broth dilution is a technique in which a suspension of a bacterium of a predetermined optimal ~~concentration~~ density is tested against varying concentrations of an antimicrobial agent (usually serial twofold dilutions) in a liquid test medium of predetermined, documented formulation. The broth dilution method can be performed either in tubes containing a minimum volume of 2 ml ~~(macrodilution)~~ or in smaller volumes using microtitration plates (microdilution). ~~Numerous~~ Commercially available microtitre plates contain~~ing~~ lyophilised or dried prediluted ~~antibiotic~~ antimicrobials within the wells~~, are commercially available. The use of the same batches of microdilution plates may assist in the minimisation of variation that may arise due to the preparation and dilution of the antimicrobials at different laboratories. The use of these plates, with a documented test protocol, including specification of appropriate reference organisms, will facilitate the comparability of results among laboratories.~~

The advantages of broth dilution methods include:

i) Numerous variations of commercially available plate formats (for microdilution).

ii) Reduced variability of results and concentration testing ranges using commercially available plate formats (for microdilution).

iii) Ease in increasing the capacity of testing with commercially available plates (for microdilution).

The disadvantages of broth dilution methods include:

i) Relatively high cost per commercially available plate.

ii) Specialised equipment is required for automatically reading MICs.

iii) Limited concentrations range and antimicrobials on commercially available plates.

~~Due to the fact that most broth microdilution antimicrobial test panels are prepared commercially, this method is less flexible than agar dilution or disk diffusion in adjusting to the changing needs of the surveillance/monitoring programme.~~

~~Because the purchase of antimicrobial plates and associated equipment may be costly, this methodology may not be feasible for some laboratories.~~

5.2.2. Agar dilution

Agar dilution involves the incorporation of varying concentrations of antimicrobial agent into an agar medium, usually using serial twofold dilutions, followed by the precise application of a defined bacterial inoculum to the agar surface of the plate. This method may be considered the most reliable for MIC determination for some antimicrobials (fosfomycin, mecillinam) and for certain bacteria where broth dilution methods are not ~~well established~~ fully validated.

The advantages of agar dilution methods include:

i) the ability to test multiple bacterial isolates (generally 32 to 60), except bacteria that swarm, on the same set of agar plates at the same time,

ii) the potential to improve the ~~identification of~~ measurement of more precise MICs ~~endpoints and extend the antibiotic concentration range~~ (i.e. test using concentrations that are less

than the traditional two-fold dilutions in the concentrations ranges tested, e.g. 3, 2, 1.5, 1, 0.75, 0.5, 0.25 µg/ml).

iii) the possibility to semi-automate the method using an inoculum-replicating apparatus. Commercially produced inoculum replicators are available and these can transfer with precision between 32 and 60 inocula prepared from different bacterial ~~inocula~~ isolates onto each agar plate.

Agar dilution methods also have certain disadvantages, for example:

i) if not automated, they are very laborious and require substantial economic and technical resources,

ii) once the plates have been prepared, they normally should be used within 1–3 weeks depending ~~in~~ on quality control results (or less, depending on the stability of the antimicrobials ~~tested~~ test),

iii) the endpoints are not always easy to read.

Agar dilution is often recommended as a standardised AST method for fastidious organisms (CLSI, ~~2015~~ 2017), such as anaerobes and *Helicobacter* ~~species~~ spp.

5.3. Other bacterial AST and specific antimicrobial resistance tests

Bacterial antimicrobial MICs can also be obtained using commercially available gradient strips that ~~diffuse~~ contain a predetermined ~~antibiotic~~ antimicrobial concentration gradient. However, the use of gradient strips can be expensive and MIC discrepancies can be found when testing certain bacteria and ~~/~~antimicrobials ~~combinations~~ compared with results of other methods (Ge *et al.*, 2002~~; Rathe *et al.*, 2009~~). Gradient strip methods are not recommended for testing the susceptibility of the antimicrobial agent colistin because of the large size of this molecule and its poor diffusion in agar (Matuschek *et al.,* 2018). An accurate, simple, and practical method for determining colistin MICs is the colistin broth disk elution test (Simner *et al.,* 2019).

Regardless of the AST method used, the procedures should be documented in detail to ensure accurate and reproducible results, and appropriate reference and quality control organisms should always be tested ~~every time AST is performed in order~~ to ensure accuracy and validity of the data.

The appropriate AST method choice can be dependent on the growth characteristics of the bacterium in question, as well as the objective of testing. In special circumstances, novel test methods and assays may be more appropriate for detection of ~~particular~~ specific resistance phenotypes. For example, chromogenic cephalosporin-based tests (CLSI, ~~2018~~ 2024a) (e.g. nitrocefin) may provide more reliable and rapid results for beta-lactamase ~~determination~~ production in certain bacteria, whereas inducible clindamycin resistance in Staphylococcus spp. may be detected using a disk diffusion method employing standard erythromycin and clindamycin disks in adjacent positions and measuring the resultant zones of inhibition (e.g. D-zone or D-test) (Zelazny *et al.,* 2005).

Similarly, extended-spectrum beta-lactamase (ESBL) (CLSI, ~~2018~~ 2024a) activity in certain bacteria can also be detected ~~by~~ using standard disk diffusion ~~susceptibility~~ test methods incorporating specific cephalosporins (cefotaxime and ceftazidime) separately and in combination with a beta-lactamase inhibitor (clavulanic acid) and measuring the resulting zones of inhibition. Penicillin-binding protein 2a (PBP 2a) can also be detected in methicillin resistant staphylococci with a latex agglutination test (Stepanovic *et al.,* 2006). It is essential that testing of known positive and negative control strains occurs alongside clinical isolates to ensure accurate results.

Susceptibility testing may also be performed using only concentrations at or near the clinical breakpoint values ~~specifically intended~~ to detect particular mechanisms of ~~bacterial~~ resistance of clinical or public health importance~~.~~. For example, resistance to the carbapenems, which are ~~used prudently~~ reserved to treat highly-resistant bacterial infections in humans (EUCAST, 2017).

~~Whenever possible, molecular testing may be useful in addressing or confirming inconclusive phenotypic results and may be used for the early detection or detection of resistant microorganisms of high public health importance.~~

5.4. ~~Future directions in antimicrobial susceptibility/resistance detection~~ Gene-based resistance detection

Whenever possible, molecular testing may be useful in addressing or confirming inconclusive phenotypic results and may be used for the early detection or detection of resistant microorganisms of high public health importance.

The use of genotypic approaches for detection of antimicrobial resistance genes has been promoted as a way to increase the rapidity and accuracy of susceptibility testing (Cai *et al.,* 2003; Chen *et al.,* 2005). Numerous DNA-based assays are being developed to detect bacterial resistance at the genetic level. The newest and perhaps most state-of-the-art approach is whole genome sequencing (WGS), which has been shown with a few bacterial pathogens to predict antimicrobial resistance phenotypes via identification and characterisation of known genes that encode resistance mechanisms.

5.4.1. Molecular tests

Methods that employ the use of comparative genomics, genetic probes, microarrays, nucleic acid amplification techniques (e.g. polymerase chain reaction [PCR]), and DNA sequencing offer the promise of increased sensitivity, specificity, and speed in the detection of specific known resistance genes (Cai *et al.*, 2003; Chen *et al.*, 2005~~; Perreten *et al.*, 2005~~). Genotypic methods have been successfully applied to supplement traditional AST phenotypic methods for ~~other organisms including~~ identifying methicillin-resistant staphylococci, vancomycin-resistant enterococci, and detecting fluoroquinolone resistance mutations (Cai *et al.*, 2003; Chen *et al.*, 2005~~; Perreten *et al.*, 2005~~). PCR methods have also been described for various antimicrobial resistance genes, including beta-lactamases, aminoglycoside inactivating enzymes, tetracycline efflux genes, and colistin resistance genes (Cai *et al.*, 2003; Chen *et al.*, 2005; Frye *et al.*, 2010; ~~Perreten *et al.*, 2005;~~ Rebelo *et al.,* 2018).

~~Technological innovations in DNA-based diagnostics should allow for the detection of multiple resistance genes and/or variants during the same test. The development of rapid diagnostic identification methods and genotypic resistance testing should help reduce the emergence of antimicrobial resistance, by enabling the use of the most appropriate antimicrobial when therapy is initiated. However, DNA techniques have to be demonstrated to be complementary to AST methods and results.~~

~~Additionally, new technological advances may facilitate the ability to probe bacterial species for large numbers of antimicrobial resistance genes quickly and cheaply, thereby providing additional relevant data for surveillance and monitoring programmes (Frye *et al.*, 2010). However, despite the new influx of genotypic tests, documented and agreed upon phenotypic AST methods will still be required in the near future to detect emerging resistance mechanisms among bacterial pathogens and to detect and characterise newly discovered mechanisms of resistance for the development and validation of genetic testing. A literature review (Ellington *et al.,* 2017) considered the role of whole genome sequencing (WGS) in antimicrobial susceptibility testing of bacteria and concluded there was insufficient published evidence to support the use of AST via WGS to replace phenotypic AST in clinical settings for all bacterial species, although certain bacteria (e.g. *Salmonella*, *Staphylococcus aureus*) had been well characterised for that purpose. Subsequently several publications have added support to the use of genetic AST (e.g. McDermott *et al.,* 2016, Zhao *et al.,* 2016). The future of genetic testing in the detection of antimicrobial resistance is promising, but phenotypic testing will remain an important mainstay.~~

5.4.2. Whole genome sequencing

In the past decade, it has become feasible to detect and monitor antimicrobial resistance on a very large scale using genomic approaches (EFSA, 2021; McDermott *et al.,* 2016). With the advent of high-capacity WGS technology, it is relatively inexpensive to quickly determine the genomic sequence of a microorganism (Allard *et al.,* 2019) and to identify the presence and location of antimicrobial resistance (and other) determinants comprehensively, even for compounds not routinely tested using traditional AST methods. A growing body of evidence shows that the presence of known resistance determinants correlates strongly with MICs at or above the 'resistant' clinical breakpoint for most agents in many pathogens and intermediate (decreased susceptibility) for some pathogens (Feldgarden *et al.,* 2019; Tyson *et al.,* 2015; 2017; Zhao *et al.,* 2016). At present, analysis of WGS data requires a high level of technical mastery and bioinformatics skills. Improvements to the technology and a reduction in start-up costs should make WGS more accessible to hospitals and clinics, as well as public health institutions conducting pathogen monitoring.

While complexities in WGS data management and interpretation require bioinformatics expertise, this can be supported to a certain extent by national service laboratories or by the international genomics database at the US National Institute of Health (NIH) and others, where bioinformatics is automated for antimicrobial resistance annotation and is freely available to all users.

An added benefit of WGS is that it can replace many traditional microbiology methods for characterising organisms that used to require dedicated instrumentation and specialised expertise, such as bacterial speciation, serotyping and pathotyping. It can also greatly improve tracking of antimicrobial resistant strains and determining the antimicrobial resistance source attribution. While many older genetic methods such as multiplex DNA amplification methods can also provide the resistance genotype, the advantages of WGS have obviated these *ad hoc* techniques for most purposes while also detecting new alleles not found by amplicon-based techniques. Furthermore, some studies show the promise of WGS to also predict actual MICs and not just susceptibility phenotype (Nguyen *et al.*, 2019; Tyson *et al.*, 2017), suggesting the possibility that WGS methods may eventually fully complement traditional AST methods across the range of susceptibility phenotypes.

Despite the rapidly expanding genetic and genomic data, documented and agreed upon phenotypic AST methods will still be required in the foreseeable future to detect emerging resistance mechanisms among bacterial pathogens and to detect and characterise newly discovered mechanisms of resistance for the development and validation of genetic testing. This process will be used to update canonical resistance gene databases, where artificial intelligence (AI) and machine learning tools can be applied to genome-based AST. The timeline for mature sequence-based AST will depend upon the number and diversity of genomes made available for training AI algorithms, and the possibility that stable signature sequences alone or in combination exist for reliably predicting MICs for major pathogens. Additionally, standardised laboratory methods and bioinformatic pipelines will be needed to harmonise WGS results across international entities.

6. Antimicrobial susceptibility ~~breakpoints and zone of inhibition~~ testing interpretive criteria

The primary objective of ~~in vitro~~ AST is to predict how a bacterial pathogen may respond to an antimicrobial agent *in vivo.* The results generated by ~~bacterial *in-vitro* antimicrobial susceptibility tests, regardless of whether disk diffusion or dilution methods are used,~~ AST are generally interpreted and reported as susceptible (S), intermediate (I), or resistant (R)~~, susceptible or intermediate to the action of a particular antimicrobial~~ by applying clinical breakpoints or as wild-type (WT) or non-wild-type (NWT) by applying epidemiological cut-off values. ~~No single formula for selection of optimal breakpoints has been established. The process involves a review of existing data and is influenced by the methods used to select appropriate breakpoints.~~

Generally, ~~antimicrobial susceptibility~~ clinical breakpoints and epidemiological cut-off values are established by ~~national~~ standard-setting organisations, professional societies or regulatory agencies. The relevant documents should be consulted. ~~However,~~ There can be notable differences in clinical breakpoints for the same antimicrobial agent within and among countries usually due to differences ~~between standards setting organisations and regulatory agencies and because of regional or national differences~~ in drug formulations and dosing regimens in the various jurisdictions (~~Brown & MacGowan, 2010;~~ de Jong *et al.*, 2009; Kahlmeter *et al.*, 2006).

~~As mentioned previously, antimicrobial susceptibility testing~~ AST results should be recorded quantitatively:

i) as distribution of MICs in mg/litre or µg/ml,

ii) or as inhibition zone diameters in millimetres.

The following ~~two~~ three primary factors enable a bacterial isolate's data to be interpreted as ~~susceptible or resistant to~~ S, I, R, WT, or NWT for an antimicrobial ~~agent~~:

i) The development and establishment of quality control ranges (CLSI, 2015), for disk diffusion or dilution testing, for quality control reference microorganisms.

Establishment of quality control ranges for control organisms is essential ~~for validating test~~ to validate results obtained using a specific AST method. ~~The allowable interpretive category ranges for reference control organisms should be established in addition to determining breakpoints for susceptibility or resistance. The use of reference organisms is a quality control and quality assurance activity.~~

ii) The ~~determination of the appropriate interpretive criteria regarding establishment~~ availability of clinical breakpoints (CLSI, ~~2015~~ 2024b).

Clinical breakpoints are established by standard setting organisations, professional societies, and regulatory agencies and ~~This~~ involve~~s~~ the ~~generation~~ integration of three distinct types of data:

a) MIC population distributions of the relevant microorganisms,

b) pharmacokinetic parameters and pharmacodynamic indices of the antimicrobial ~~agent~~, and when available

c) results of clinical trials and the outcome of treatment of clinical cases of disease correlated with AST results from pathogenic isolates.

iii) The availability of epidemiological cut-off values (CLSI, 2018; EUCAST 2021).

Epidemiological cut-off values are established by EUCAST (2021) (abbreviated ECOFFs) and by the CLSI (2018) (abbreviated ECVs). Both bodies evaluate AST data in very similar ways for the purpose of setting these values. The epidemiological cut-off value represents ~~The development of a concept known as 'microbiological breakpoints', or 'epidemiological cut-off values' (~~the highest MIC value for the bacterium and antimicrobial ~~agent under consideration~~, where the bacterium is devoid of any phenotypically expressed resistance to that antimicrobial ~~agent)~~. Application of these values is often ~~may be~~ more appropriate for ~~some~~ antimicrobial resistance surveillance programmes. ~~Epidemiological cut-off~~ These values are derived by statistically examining MIC population distributions for specific bacterial species and antimicrobials performed at several laboratories according to a standardised broth microdilution method (Turnidge *et al.,* 2006). Bacterial isolates that possess any acquired ~~phenotypic~~ resistance (that is, have an MIC above the epidemiological cut-off value) and therefore deviate from the normal ~~wild type~~ WT fully-susceptible population are designated as ~~non-wild type~~ NWT. ~~(also termed microbiologically resistant) and~~ This approach allows detection of more subtle shifts in susceptibility to the specific antimicrobial~~/bacterium combination can thus be monitored~~ (Kahlmeter, 2015; Kahlmeter *et al.*, 2006~~; Turnidge *et al.*, 2006~~). There is a great advantage in the recording of quantitative ~~susceptibility~~ AST data, as such ~~in that~~ data may be analysed according to clinical breakpoints ~~as well as by using~~ or epidemiological cut-off values, when available.

The development of clinical breakpoints ~~criteria~~ for disk diffusion tests usually involves comparing disk diffusion data against dilution data by creating a scattergram of the bacterial population distribution (representative bacterial isolates), by plotting the zone of inhibition against the logarithm to the base 2 of the MIC for each bacterial isolate for an individual bacterial species. The selection of breakpoints is then based on multiple factors, including regression line analysis that correlates MICs and zone diameters of inhibition, bacterial population distributions, error rate bounding, pharmacokinetics, and ultimately, clinical verification.

7. Antimicrobial susceptibility testing guidelines

~~A number of national standards and guidelines are currently available.~~ Internationally harmonised standards and guidelines for ~~antimicrobial susceptibility testing and subsequent interpretive criteria throughout the world~~ AST are available through the~~:~~ Clinical and Laboratory ~~and~~ Standards Institute (~~CLSI, 2018~~ CLSI, clsi.org) and the European Committee on Antimicrobial Susceptibility Testing (~~EUCAST, 2017~~ eucast.org).

~~At this time, only the CLSI has developed protocols for susceptibility testing of bacteria of animal origin and determination of interpretive criteria (CLSI, 2018). A veterinary sub-committee (VETCAST) has also been set up under the umbrella of EUCAST. However,~~ Protocols and guidelines are available from a number of standards organisations and professional societies, including those listed above for ~~susceptibility testing for~~ AST of similar bacterial species that cause infections in humans. It is possible that such ~~guidelines~~ methods can be adopted for susceptibility testing for bacteria of animal origin, but ~~each country must evaluate its own AST standards and guidelines~~ any extrapolation of AST data interpretations should be made with caution. ~~Additionally, efforts focusing on both standardisation and harmonisation of susceptibility/resistance breakpoints on an international scale are progressing. These efforts have primarily focused on the adoption of the standards and guidelines of CLSI and EUCAST, which provide laboratories with methods and quality control values enabling comparisons of AST methods and generated data (CLSI, 2018; Kahlmeter *et al.*, 2006). For those~~ WOAH Members ~~that do not have standardised AST methods in place, the~~ are encouraged to adopt~~ion of~~ either set of CLSI or EUCAST standards ~~would be~~ as an appropriate ~~initial~~ step towards ~~acceptable methods and~~ international harmonisation.

Many bacteria that cause disease in aquatic animals require growth conditions (e.g. lower temperatures, supplemented or semisolid media) that may vary considerably as compared to terrestrial bacterial pathogens. This necessitated the need for the development of ~~antimicrobial testing~~ AST methods for bacteria isolated from aquatic species. Further information with regards to methods for disk diffusion or broth dilution ~~antimicrobial susceptibility testing~~ AST and interpretive criteria for bacteria isolated from aquatic animals can be referenced in two CLSI documents (CLSI, ~~2006; 2014b~~ 2020a; 2020b). ~~Further information with regards to~~ Additionally, methods for disk diffusion ~~or~~ and broth dilution ~~antimicrobial susceptibility testing for~~ AST of infrequently isolated or certain fastidious bacteria (e.g. *Campylobacter*, *Pasteurella*) can also be ~~referenced~~ found in ~~the~~ CLSI's VET06 ~~M45-A document~~ supplement (CLSI, ~~2015~~ 2017). Specific media formulations for a few fastidious pathogens have also been published online by EUCAST (2022).

As a first step towards comparability of monitoring and surveillance data, Members should be encouraged to strive for harmonised and standardised ~~programme design~~ AST methods (~~Brown & MacGowan, 2010;~~ Kahlmeter *et al.*, 2006~~; White *et al.*, 2001~~). Data from countries using different methods ~~and programme design~~ may ~~otherwise~~ not be directly comparable ~~(Brown & MacGowan, 2010)~~. ~~Notwithstanding this, data collected over time in a given country may at least allow the detection of emergence of antimicrobial resistance or trends in prevalence of susceptibility/resistance in that particular country (Petersen *et al.*, 2003). However, if results achieved with different AST methods are to be compared, then comparability of results must be demonstrated and consensus on interpretation achieved. This will be best accomplished by the use of accurate and reliable documented AST methods used in conjunction with monitoring of AST performance while using well characterised reference microorganisms among participating laboratories.~~

Table 1. Phenotypic susceptibility testing AST methods available and their features

~~Susceptibility testing~~ AST method	International standard available?	Published methods available?	Used in national surveillance programmes?	~~Use in susceptibility testing for therapeutic purposes~~	~~Breakpoints that may be applied~~	Test output	~~Comparability of outputs~~	~~Features~~
Broth ~~(micro)~~ dilution ~~MIC determination~~	Yes (ISO 20776-1), CLSI, EUCAST	Yes (CLSI, EUCAST)	Yes, broth microdilution ~~MIC determination is preferred~~ widely used	~~Yes~~	~~Clinical breakpoints or epidemiological cut-off values (ECOFFs)~~	MIC	~~High~~	~~Current reference method. Recording MIC values allows interpretation of the test outputs using different breakpoints (e.g. clinical breakpoint or ECOFF), as well as re-evaluation of historical data if changes occur to breakpoints and evaluation of shifts in MIC. Numerous national surveillance programmes adopt this method. The MIC value can sometimes indicate the likely mechanism of resistance (e.g. high-level amikacin resistance and rRNA methylases) or provide an epidemiological marker.~~ Currently, this is the only method suitable for determining susceptibility to colistin.
Agar dilution ~~MIC determination~~	No	Yes (CLSI, EUCAST)	No, not ~~widely~~ generally used	~~Yes~~	~~Clinical breakpoints or ECOFFs~~	MIC	~~Dependent on congruity of methods used~~	~~Reference method. The breakpoints appropriate for broth dilution may not be directly applicable to agar dilution. Currently used in particular for testing certain fastidious organisms.~~
~~**Breakpoint method**~~	~~No~~	~~Yes (scientific literature)~~	~~Not widely used~~	~~Yes~~	~~The test is performed at a set breakpoint~~	~~Resistant or susceptible at selected breakpoint~~	~~Dependent on congruity of methods used~~	~~Changes to breakpoints in this method result in the inability to interpret historical data. Shifts in susceptibility within the S or R categories cannot be detected. The breakpoint method relies on the growth or absence of growth of bacteria in broth or on agar containing an antimicrobial at a single (breakpoint) dilution.~~
~~**Gradient strip method**~~**E-test**	No	Yes (manufacturer only)	No, not ~~widely~~ generally used	~~Yes~~	~~Clinical breakpoints or ECOFFs~~	MIC	~~High~~	~~Provide a convenient alternative method of determining MIC with minimal additional equipment required.~~
Disk diffusion test	No	Yes (CLSI, EUCAST) ~~A number of different methods are available. These are not in general equivalent.~~	Yes ~~May be used,~~ but broth ~~microdilution MIC determination is preferred~~	~~Yes~~	~~Clinical breakpoints (ECOFFs are also available for the EUCAST disc diffusion method).~~	Diameter of zone of inhibition~~, interpreted as resistant or susceptible according to test guidelines~~	~~Dependent on congruity of methods used~~	~~Frequently used to provide an indication of susceptibility for therapeutic purposes. Versatile in that different discs can be used, according to the antimicrobials authorised for treatment. Different methods are not usually equivalent (zone sizes obtained using one method cannot be interpreted using criteria from another, different method). The collection of zone size data can allow shifts in susceptibility to be detected. Disc diffusion methods may be harmonised to a degree with other methods, by using the same breakpoint.~~

The susceptibility testing method selected should provide details of the method, appropriate controls and quality control ranges and ~~breakpoints~~ interpretive criteria. The comparability of outputs obtained in surveillance programmes is not only dependent on the laboratory methodology used but is also dependent on the target population of livestock included in the study and method of sampling.

8. Comparability of results

To determine the comparability of results originating from different surveillance systems, results obtained from standardised AST should be reported quantitatively including information on the performance of the methods, the reference organisms and clinical breakpoints/epidemiological cut-off values used ~~and the antimicrobial~~.

Sample sources, analytical methods, AST methods, and interpretive criteria should be clearly described, and differences transparently explained to show where data may not be directly comparable.

AST data, consisting of cumulative and ongoing summaries of susceptibility patterns (antibiograms) ~~among clinically important and surveillance microorganisms~~ should be created, recorded and analysed periodically at regular intervals (CLSI, ~~2014a~~ 2022). Data must also be presented in a clear and consistent manner so that both new patterns of resistance can be identified and atypical findings confirmed or refuted. This data should be available on a central data bank and published yearly.

Cumulative AST data will be useful in monitoring susceptibility/resistance trends in a region over time and assessing the effects of interventions to reduce antimicrobial resistance.

9. Quality control (QC) and quality assurance (QA)

~~Quality control/quality assurance~~ QC and QA systems should be established in accordance with chapter 1.1.5 in laboratories performing AST:

i) ~~quality control~~ QC refers to the operational techniques that are used to ensure accuracy and reproducibility of AST results,

ii) ~~quality assurance~~ QA includes, but is not limited to, monitoring, record keeping, evaluating, taking potential corrective actions if necessary, calibration, and maintenance of equipment, proficiency testing, training and QC. A QA programme helps ensure that testing materials and processes provide consistent quality results.

The following components should be determined and monitored:

i) precision of the AST procedure,

ii) accuracy of the AST procedure,

iii) qualifications, competence, and proficiency of the laboratory personnel, as well as the personnel that interpret the results and those that are involved in monitoring of antimicrobial resistance,

iv) performance of the appropriate reagents.

The following requirements should be respected:

i) Strict adherence to specified and documented techniques in conjunction with ~~quality control~~ QC (i.e. assurance of performance and other critical criteria) of media and reagents.

ii) Record keeping of:

   a) lot numbers of all appropriate materials and reagents,

   b) expiration dates of all appropriate materials and reagents,

   c) equipment calibration and monitoring,

   d) critical specifications for AST performance (reference results, time, temperature etc.).

iii) The appropriate reference QC microorganism(s) based on availability of QC testing ranges for the antimicrobial(s) tested, should always be used ~~regardless of the AST method employed~~.

iv) Reference QC microorganisms are to be obtained from a reliable source ~~for example, from the~~ (e.g. American Type Culture Collection (ATCC®)~~, reliable commercial sources, or institutions with demonstrated reliability to store and use the organisms correctly~~.

~~v) Reference microorganisms should be catalogued and well characterised, including stable defined antimicrobial susceptibility phenotypes. Records regarding these reference organisms should include the established resistant and susceptible ranges of the antimicrobials to be assayed, and the reference to the method(s) by which these were determined.~~

~~vi) Laboratories involved in AST should use the appropriate reference microorganisms in all AST testing.~~

v) Reference QC strains should be kept as stock cultures from which working cultures are derived ~~and should be obtained from national or international culture collections~~. Reference ~~bacterial~~ QC strains should be stored

at designated centralised or regional laboratories. Working cultures should not be subcultured from day to day as this introduces contamination and the method of producing working cultures should ensure that stock cultures are rarely used. This may be accomplished with the production of an intermediate stock of cultures derived from the original cultures that are used to create day-to-day working cultures.

vi) The preferred method for analysing the overall performance of each laboratory should test the working stock of the appropriate reference QC microorganisms on each day that susceptibility tests are performed.

Because this may not always be practical or economical, the frequency of such QC tests may be reduced if the laboratory can demonstrate that the results of QC testing ~~reference microorganisms using the selected method~~ are reproducible. If a laboratory can document the reproducibility of the ~~susceptibility testing~~ AST methods used, testing may be performed on a weekly basis. If concerns regarding accuracy, reproducibility, or method validity emerge, the laboratory has a responsibility to determine the cause(s) and repeat the tests using the reference materials. Depending on the cause(s), daily reference material use and any other corrective action may be re-initiated.

vii) Reference ~~microorganisms~~ QC strains should be tested each time a new batch of media or microtitre plate lot or batch of disks is used, and on a regular basis in parallel with the ~~microorganisms~~ QC strains to be ~~assayed~~ tested.

viii) Appropriate biosecurity issues should be addressed in obtaining and dispersing ~~microorganisms~~ QC strains to participating laboratories.

10. External proficiency testing

Laboratories should participate in external ~~quality assurance~~ QA and/or proficiency testing programmes in accordance with chapter 1.1.5. Laboratories are also encouraged to participate in international inter-laboratory comparisons (e.g. WHO External Quality Assurance System) (Hendriksen *et al.*, 2009). All bacterial species subjected to AST should be included.

National reference laboratories should be designated with responsibility for:

i) monitoring the ~~quality assurance~~ QA programmes of laboratories participating in surveillance and monitoring of antimicrobial resistance,

ii) characterising and supplying to those laboratories a set of reference ~~microorganisms~~ QC strains,

iii) creating, managing, and distributing samples to be used in external proficiency testing,

iv) creating a central database available on the internet (e.g. European Antimicrobial Resistance Surveillance System [EARSS]) that contains the different susceptibility/resistance profiles for each bacterial species under surveillance.

11. Conclusion

Although a variety of AST methods exist, the goal of ~~*in-vitro* antimicrobial susceptibility testing~~ AST for clinical veterinary purposes, surveillance and monitoring is the same: to provide a reliable predictor of how a microorganism is likely to respond to antimicrobial therapy in the infected host. This type of information aids the clinician in selecting the appropriate antimicrobial agent, provides data for surveillance, and aids in developing antimicrobial judicious use policies (see Chapter 6.10 of the *Terrestrial Code* ~~*Manual*~~).

~~*In vitro* antimicrobial susceptibility testing~~ AST can be performed using a variety of formats, the most common being broth microdilution and disk diffusion methods~~, agar dilution, broth macrodilution, broth microdilution, and a concentration gradient test~~. ~~Each of~~ These procedures require~~s~~ ~~the~~ use of specific testing conditions and methods, including media, incubation conditions and times, and the identification of appropriate ~~quality control~~ QC organisms along with their specific QC concentration ranges. It is essential that AST methods provide reproducible results in day-to-day laboratory use and that the data be comparable ~~with those results obtained by an acknowledged 'gold standard' reference method~~ across geographical regions and time. In the absence of standardised methods or reference procedures, ~~antimicrobial susceptibility/resistance results~~ AST data from different laboratories cannot be reliably compared.

The ~~use~~ pursuit of using genotypic approaches for detection of antimicrobial resistance genes ~~has also been promoted as a way to increase the rapidity and accuracy of susceptibility testing. New technological advances in molecular techniques (e.g. microarray) may facilitate the ability to probe bacterial species for large numbers of antimicrobial resistance genes quickly and cheaply, thereby providing additional relevant data into surveillance and monitoring programs (Ojha & Kostrzynska, 2008; Poxton, 2005)~~ has made significant strides in the past few years, becoming more affordable, and offered with a large array of bioinformatics tools to interpret and report the data. While genomics can reliably identify known resistance genes, it has not yet been shown to predict exact MIC values across the range of microbial pathogens. Standardised phenotypic AST methods will still be required to detect novel

and emerging resistance mechanisms among bacterial pathogens and to validate their detection via genetic techniques (Ellington *et al.,* 2017).

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787 **NB:** There is a WOAH Reference Laboratory (https://www.woah.org/en/what-we-offer/expertise-
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790 Please contact the WOAH Reference Centres for any further information on antimicrobial resistance

791 **NB:** FIRST ADOPTED IN 2004. MOST RECENT UPDATES ADOPTED IN 2019.

Annexe 9. Chapitre 2.2.1. Mise au point et optimisation des méthodes de détection d'anticorps

RÉUNION DE LA COMMISSION DES NORMES BIOLOGIQUES
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CHAPTER 2.2.1.

DEVELOPMENT AND OPTIMISATION OF ANTIBODY DETECTION ASSAYS

INTRODUCTION

The WOAH Validation Recommendations ~~in Section 2.2~~ Validation of diagnostic tests *~~of this~~* Terrestrial Manual *provide detailed information and examples in support of ~~the WOAH Validation Standard that is published as~~ Chapter 1.1.6* Validation of diagnostic assays for infectious diseases of terrestrial animals. *~~The Term "WOAH Validation Standard" in this chapter should be taken as referring to that chapter.~~ An up-to-date compilation of the relevant validation standards (WOAH and non-WOAH) and guidance documents for all stages of diagnostic test validation and proficiency testing, including design, analysis as well as clear, complete and transparent reporting of validation studies with case studies is provided in Colling & Gardner (2021). Published standards for peer-reviewed reporting of accuracy studies (STARD) are available for infectious diseases of humans (Bossuyt* et al.*, 2015), and terrestrial animals (Gardner* et al.*, 2011; 2019; Kostoulas* et al.*, 2017; 2021). In their review of WOAH recommended diagnostic tests, Cullinane & Garvey (2021) concluded that enzyme-linked immunosorbent assay (ELISA) and molecular assays were the most widely used WOAH-recommended tests and further examples for these methods can be found in Mayo* et al. *(2021).*

Detection of antibodies that are elicited in response to infectious agents constitutes an indirect means of laboratory-based disease diagnosis. The most common antibody detection methods are enzyme-linked immunosorbent assay (ELISA), ~~classical~~ virus neutralisation test (VNT), ~~enzyme-linked immunosorbent assay (ELISA),~~ haemagglutination inhibition (HAI) and the complement fixation test (CFT). Other, less common, antibody detection tests are the agar gel immunodiffusion (AGID), the indirect fluorescent antibody test (IFAT), the serum agglutination test (SAT), the latex agglutination test (LAT), and the microscopic agglutination test (MAT). More recent novel methods include biosensors, bioluminometry, fluorescence polarisation, chemoluminescence and lateral flow devices also known as point of care or pen-side tests. Other immunological assays that use antibodies in antigen detection tests are described in Chapter 2.2.2.

When considering ~~a candidate~~ an assay type for disease diagnosis, one should include antibody detection assays because of their practicality, ease of sample collection and preparation, generally good diagnostic performance characteristics including the possibility to detect antibodies for long periods of time after infection, suitability for automation (high-throughput), low cost and fast turn-around time. They are particularly useful for processing large numbers of samples in epidemiological and population studies, or for mass diagnosis and surveillance programmes. Antibody assays are also widely used for export, import and trade of animals, and still represent the majority of WOAH recommended tests for international trade.

Comprehensive and well-designed experiments are required to develop and optimise assays with favourable analytical characteristics. The underlying principles are broadly applicable to all assay types and, when conducted with appropriate rigour, provide the foundations for high-quality diagnostic tests that are fit for their intended purpose(s) (Bowden et al.*, 2021). A characteristic of antibody assays is their capacity to indicate prior exposure to an infectious agent in the absence of detectable organisms or their analytes. They are also adaptable to a variety of matrices, such as serum, plasma, whole blood, milk, lacrimal secretions*

and saliva. Immunoglobulin isotype or subclass-specific test systems may selectively target early or late immune responses, e.g. IgM and IgG, respectively. Specifically designed detection systems allow differentiation between responses to vaccine and field strains and are available as commercial kits, e.g. the detection of antibodies to classical swine fever virus in pigs. Competitive or blocking formats allow use of the same basic assay for a variety of animal species while other formats are species specific. Many types of chemical or physical indicators are used to indicate the presence of specific antibody in a specimen (chromogens, fluorochromes, agglutinins, among many others). Because of the large number of antibody detection methods available, it is not possible to describe the best practices for validation of each of these assay types in this chapter. ~~The most widely used antibody detection system, the ELISA, will therefore be used as an example for application of best practices in antibody assays.~~ Most of the basic processes used to validate other types of assay systems will become evident by extension of those used to validate ELISAs.

A. ANTIBODY DETECTION ASSAY DEVELOPMENT PATHWAY

1. Intended purpose(s) of the antibody assay

The first consideration in assay development is to define clearly the specific purpose and application of the test to be developed. Many decisions in developing assays will be based on these first considerations. For antibody detection assays (hereafter in this chapter designated as "antibody assays") such as ELISA, such knowledge will guide the selection of the most appropriate type of antibody detection system to achieve the intended purpose. Many factors related to the assay's intended purpose, use, and suitability need to be taken into account ~~(see the WOAH Validation Standard for other possible purposes).~~ An example-based overview for the purpose-oriented use of diagnostic tests is provided in Gardner *et al.* (2021). Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals*, Table 1 highlights the relevance of assay parameters such as diagnostic sensitivity and specificity, predictive value and likelihood ratios for individual purposes.

The six basic intended purposes for diagnostic assays relevant in the WOAH context are stated in Chapter 1.1.6 and ~~the WOAH Validation Standard, and listed in the footnote to Table 1 below. Because antibody assays have such a broad range of applications, and can be configured for very specific purposes, it is useful to consider and evaluate several parameters when establishing the specific purpose(s) for the candidate assay. Table 1 summarises characteristics of antibody assays when applied for different purposes. Consideration of these characteristics will provide guidance in establishing the specific purposes for which the candidate assay will be fit.~~

~~Note – The reader is advised to read Section B.4. Programme implementation, as a primer for the following discussions. That section describes the inter-relationships between diagnostic sensitivity and specificity, false positive and negative test errors, and positive and negative predictive values. For a more in-depth discussion of predictive values as a function of prevalence, see Jacobson, 1998.~~

~~***Table 1.** Determinants of an antibody assay's fitness for its intended purpose*~~

~~***Assay characteristics***~~	~~***Determinants of fitness for purpose***~~						
	~~***1****~~		~~***2****~~	~~***3****~~	~~***4****~~	~~***5****~~	~~***6****~~
	~~**a**~~	~~**b**~~					
~~Diagnostic sensitivity (DSe)~~	~~+++~~	~~+++~~	~~+++~~	~~+++~~	~~+++~~	~~±~~	~~±~~
~~Diagnostic specificity (DSp)~~	~~±~~	~~±~~	~~±~~	~~±~~	~~+++~~	~~±~~	~~+++~~
~~Positive predictive value (PPV)~~	~~±~~	~~±~~	~~±~~	~~±~~	~~+++~~	~~±~~	~~+++~~
~~Negative predictive value (NPV)~~	~~+++~~	~~+++~~	~~+++~~	~~+++~~	~~+++~~	~~±~~	~~±~~
~~Throughput capacity~~	~~±~~	~~+++~~	~~++~~	~~±~~	~~–~~	~~++~~	~~++~~
~~Turn-around time of test~~	~~±~~	~~±~~	~~±~~	~~±~~	~~+++~~	~~–~~	~~±~~
~~QA capability~~	~~+++~~	~~+++~~	~~+++~~	~~+++~~	~~+++~~	~~+++~~	~~+++~~
~~Reproducibility~~	~~+++~~	~~+++~~	~~+++~~	~~+++~~	~~+++~~	~~+++~~	~~+++~~
~~Repeatability~~	~~+++~~	~~+++~~	~~+++~~	~~+++~~	~~+++~~	~~+++~~	~~+++~~

~~Other characteristics such as the technical sophistication of the assay, and the skill required for interpretation will be related to the disease or infection under investigation.~~

~~**Symbols:** +++ = essential; + = of less importance; – = not important.~~

~~*Basic purposes for which an assay may be deemed fit: 1.~~

include: 1. Contribute to the demonstration of freedom from infection in a defined population a) 'Free' with or without vaccination, b) Re-establishment of freedom after outbreaks. 2. Certify freedom from infection or presence of the agent in individual animals or products for trade/movement purposes; 3. Contribute to the eradication of disease or elimination of infection from defined populations; 4. Confirmatory diagnosis of clinical cases (includes confirmation of positive screening test); 5. Estimate prevalence of infection or exposure to facilitate risk analysis; 6. Determine immune status in individual animals or populations (post-vaccination). An example-based overview of diagnostic tests for each of the six purposes is provided in Gardner *et al.* (2021), and chapter 1.1.6 highlights the relevance of assay characteristics for each purpose.

Designing the method

- Has the design been shaped by the intended purpose of the assay?
- What is the specific application?
- What are the types and statistically relevant numbers of samples to be tested? (See Chapter 2.2.5)
- Will the test be field or laboratory based?

Note – The reader is advised to read Section B.4. *Stage 4 – Programme implementation*, as a primer for the following discussions. That Section describes the inter-relationships between diagnostic sensitivity and specificity, false positive and negative test errors, and positive and negative predictive values. For a more in-depth discussion of predictive values as a function of prevalence, see Jacobson (1998).

1.1. Purpose 1: Contribute to the demonstration of freedom from infection in a defined population

For disease freedom categories as given in purposes 1a and 1b (Table 1), antibody screening tests of tests or test algorithms favouring a high diagnostic sensitivity (DSe specificity (DSp) are the tests of choice generally considered as fit for purpose. As indicated in the purposes above, these tests would be applied to populations that have an apparent prevalence of zero. Tests of high DSe DSp demonstrate low false negative (FN positive (FP) rates and when applied to low prevalence populations, the negative predictive value (NPV) is at its highest level. However, DSe DSp and diagnostic specificity (DSp sensitivity (DSe) are usually inversely related and as such, a decrease in DSp DSe will result in an elevated false positive (FP negative (FN) rate. Other considerations, if this is to involve a continuous volume of surveillance samples, would include high throughput, low cost and technical simplicity. All screening test positive results should be subjected to some form of confirmatory testing to evaluate their true status. Confirmatory tests characteristically have high DSp and therefore a low FP rate. These tests are often more sophisticated, more costly and may require enhanced interpretive skills.

If demonstration of freedom from infection is to be achieved after an outbreak (1b), in which vaccination has been used for disease control, then screening of massive numbers of sera is often required. In addition to the considerations above, this also necessitates an antibody detection test which is able to distinguish between infected and vaccinated animals (i.e. a DIVA [differentiation of infected from vaccinated animals] test). At the same time an antigen or nucleic acid detection test may be warranted in some situations to prove that shedding and/or circulation of the infectious agent has ceased.

1.2. Purpose 2: Certify freedom from infection or presence of the agent in individual animals or products for trade/movement purposes

If the purpose is to qualify individual animals for international movement, antibody screening tests of high DSe are again the tests of choice. The same rationale as stated above applies with respect to the NPV. Again, all positive reactors will need to be subjected to some form of confirmatory testing to evaluate their true status or may be excluded from shipment without further testing. In cases where borderline positives are observed, it may be wise to request a repeat sampling of the animal(s) at a suitable time interval to ensure that herd/flock has not been very recently infected.

1.3. Purpose 3: Contribute to the eradication of disease or elimination of infection from defined populations

If the purpose of the test is the eradication of disease or elimination of infection from defined populations, antibody screening tests of moderate to high DSe are the tests of choice. However, the rationale is slightly different in that the testing will likely be done at herd or compartment level. At the beginning of the campaign, when the disease prevalence is high, moderate DSe and DSp are suitable as both FP and FN rates are less relevant at this juncture and a moderate level of test error is tolerable. Depending on the nature of the disease and rapidity of spread, high throughput and fast turn-around-times may become critical. Usually, decisions are made without confirmatory testing at this point.

In the latter stages of the campaign, a higher DSe is warranted as the FN rate becomes the more critical factor. Much like Purposes 1 and 2, positive reactors will need to be subjected to some form of confirmatory testing to

evaluate their true status. In these latter stages, antibody detection tests are often applied in conjunction with antigen and/or nucleic acid detection systems to detect subclinical cases and possibly, latent carriers.

1.4. Purpose 4: Confirmatory diagnosis of clinical cases (includes confirmation of positive screening test)

For the confirmatory diagnosis of clinical cases, antibody tests of high DSp are the tests of choice. In these cases, the idea is to minimise the FP rate and enhance the PPV of the test. As a general rule, infection is well established and the immune response is usually well underway. In some situations it may be preferable to carry out a screening test of high ~~DSn~~ DSe but a lower DSp, then following up positives with a high DSp confirmatory test. For some clinical cases, e.g. vesicular diseases in terrestrial animals, several tests may be required to rule out select pathogens that present similar clinical signs. In some cases, antigen and/or nucleic acid detection tests may be a better choice for confirmation of clinical cases provided that they offer a fast turn-around-time. A prime example would be highly pathogenic avian influenza infections where mortality may occur before an immune response is even detectable.

1.5. Purpose 5: Estimate prevalence of infection or exposure to facilitate risk analysis

For estimates of prevalence of infection or exposure to facilitate risk analysis, e.g. for health surveys, herd health status and to monitor disease control measures, antibody tests of moderate DSe & DSp are the tests of choice. In general, this would balance both FN & FP rates and result in a more accurate estimate of the true prevalence of infection in the target population. However, if accurate estimates of both DSe and DSp have been established, statistical approaches can be used to minimise bias attributable to FN & FP rates (see Chapter 2.2.5 *Statistical approaches to validation*).

1.6. Purpose 6: *Determine immune status in individual animals or populations (post-vaccination)*

For the determination of the immune status in individual animals or populations, e.g. post-vaccination, antibody tests of high DSp are required. Such tests have very low FP rates and as such provide a high degree of confidence in the PPV of the result. For use in individual animals, the use of virus neutralisation (VN) tests in cell culture for the detection of vaccine-induced neutralising antibodies against rabies virus in dogs and cats would be a prime example of a test with high DSp used for expression of titres in international units. However, these tests are technically sophisticated, expensive to maintain and run, and require strict biosafety procedures. For larger volume applications, such as monitoring regional vaccination programmes, ELISA-based tests would be more applicable, given their simplicity, cost effectiveness and high throughput. The same DSp considerations should be applied to these types of tests.

The experience of laboratory diagnosticians is not only essential in the choice of an appropriate test that will achieve the desired purpose, but is also required to determine reliably the scientific limitations of an assay and practical considerations such as cost, equipment and reagent availability, throughput capacity of the laboratory and test turn-around-times.

2. Assay development – experimentation

2.1. Reference materials, reagents and controls

2.1.1. Test samples

Samples to be tested in antibody assays should be handled as described in Chapter 1.1.2 *Collection, submission and storage of diagnostic specimens*. The sample matrix in which antibodies are usually detected is serum, but may also include plasma, whole blood, milk, meat juice, egg yoke, lacrimal secretions and saliva.

~~**Designing the method**~~

- ~~Has the design been shaped by the intended purpose of the assay?~~
- ~~What is the specific application?~~
- ~~What are the types and statistically relevant numbers of samples to be tested? (See Chapter 2.2.5)~~
- ~~Will the test be field or lab based?~~

2.1.2. Reference Standards

Antisera directed against the reference strain of a pathogen are known as reference sera or reference standards (Wright *et al.,* 1993; ~~WOAH Validation Standard~~ Chapter 1.1.6; Section 1.4 of Chapter 2.2.6 *Selection and use of reference samples and panels*). Such sera containing antibody of known concentration/activity are useful in the initial development of an assay. For a number of WOAH listed diseases, (e.g. avian influenza, foot and mouth disease, classical swine fever, etc.) international reference standards are available through WOAH Reference Laboratories and Collaborating Centres. When not available from other sources, it may be necessary to produce in-house reference standards against which working standards (process or quality controls) are calibrated.

Critical points to be addressed:

- ☐ Have you considered that concentrations of analyte in matrix significantly impact the lower limit of antibody detection and the operating range of the assay?
- ☐ Are the required antibody reagents (mono/polyclonal) available?
- ☐ Is available antigen sufficiently purified?
- ☐ Are reagents commercially available? If not, is it practical to produce them in-house?
- ☐ Are reference standard reagents available? If not, how are you going to resolve this deficiency? (See Chapter 1.1.6, Section A.2.6.)

2.1.3. Positive and negative reference panel

These sera, containing concentrations of antibody over the intended operating range, (also known as dynamic range) of the assay, should be used throughout the development and standardisation of an antibody assay. It is recommended that they be prepared in sufficient quantities so that they may be used in various aspects of validation. These samples should represent known infected and uninfected animals from the population that eventually will become the target of the validated assay. They should preferably be derived from individual animals, but they may represent pools of samples from several animals (Chapter 2.2.6). Sera samples have to be stored below –20°C for long-term storage and as pools/aliquots of manageable size and volume. The working stock must be in lower volumes so as to avoid repeated freeze–thaw cycles.

2.1.4. Monoclonal antibody reagents

The advent of monoclonal antibodies has greatly enhanced ~~enzyme~~ immunoassays. Whereas polyclonal anti-immunoglobulin conjugates are used in most indirect ELISAs, monoclonal antibody conjugates can be directed to specific immunoglobulin isotypes. Depending on the immunoglobulin epitope targeted, many of these monoclonals can be effectively used to detect antibodies in related species, e.g. ruminants. Using monoclonal conjugates to either light or heavy chain epitopes can effectively modulate the DSp and DSe of the indirect ELISA.

Monoclonal antibodies are best known for their application in competitive or blocking ELISAs. In this case, the monoclonal specificity is directed to epitopes on the pathogen in question. Depending on the epitope targeted, the analytical specificity of the assay can be modulated.

Monoclonal antibodies can also be used in sandwich ELISAs, either for trapping antigen to the plate or for subsequently detecting antigens that have been trapped. Depending on the size and complexity of the antigen in question, it is sometimes preferable to use a polyclonal antibody preparation for trapping as they generally contain antibodies of high binding affinity.

2.1.5. Antigens.

Antigens used in ELISAs are of critical importance to diagnostic performance given a particular application. Antigens expressing highly conserved epitopes, such as those found in some viral matrix or nucleoproteins, are generally useful in group-specific assays, such as ELISAs for the detection of responses to all Influenza A viruses. Other antigen epitopes can be used to restrict detection to certain serotypes. The choice of antigen must be carefully researched and considered.

Aspects affecting choice of test

- ☐ Is the assay to be used for screening or confirmatory purposes, or both?
- ☐ Will it be used for one or more species? Which ones?
- ☐ Is the test intended for detection of early or late infection?
- ☐ Will the test be used to measure serotype- or subtype-specific antibodies?
- ☐ Will the assay be used to confirm sero-conversion after vaccination?
- ☐ Will it be a DIVA assay (differentiation of infected from vaccinated animals)?
- ☐ Will the test be applied to trade?

Crude antigen preparations like cell lysates have had widespread use in the past, and are still deployed for some assays. However, antigens improved greatly as purification techniques advanced, e.g. affinity chromatography. Further improvements were achieved through the application of molecular cloning. Recombinant antigen technologies have greatly enhanced all aspects of ELISA performance, from analytical through diagnostic characteristics.

2.2. Design of test method

In designing a test, its intended application will influence the choice of assay format that is best suited for the task. For example, if its use is primarily for surveillance, then the type of ELISA needs to be conducive to achieving high DSe, as described in the 'Purposes' above. If, however, the screening assay's DSe is set so high that it generates many false positives, then a companion confirmatory test should also be considered at the same time. Many ELISA formats are available, each with their advantages and disadvantages that allow customisation of assays for very specific purposes ~~(Table 2)~~.

Practical matters in selecting an assay format

- Is high-throughput essential? Will it be automated?
- What is the anticipated turnaround time? Is that suitable?
- What level of sophistication is needed to run the assay?
- What skills are required to interpret the test?
- Will that assay be feasible for use in my laboratory?
- Will it be easily transferrable to other laboratories?

Important factors that influence the choice of an antibody assay format are availability of reagents and likely continuity of supply, not only for the design and optimisation stage but for operational scale application of the test. A limitation may be the unavailability of relevant antibody reagents for a particular format, e.g. competitive or blocking formats generally require antigen-specific monoclonal antibodies. Another example would be the need for an effective capturing antigen: a rather crude antigen may be acceptable for use in a sandwich-based ELISA screening assay, whereas a purified antigen would be necessary for a confirmatory assay. Other important considerations for choosing a particular ELISA format are which antibody isotypes, concentrations, avidities and antigenic specificities are diagnostically relevant; which antigen, and in particular which epitopes are relevant; and what is the desired operating range of the assay. All will play a large role in selecting a particular type of ELISA (Table ~~2~~ 1). If it is anticipated that the test will be used in different species, including wildlife, a competitive/blocking format may be useful (Berguido *et al.*, 2016; 2021; Soubrier *et al.*, 2022). Deciding on an assay format also requires that application of the assay be considered. Questions that should be addressed are detailed in the box above on "*Practical matters in selecting an assay format*" and practical questions in the boxes below Table ~~2~~ 1. It is essential to deal with such questions at this point in assay development as they are essential to a positive outcome and application.

Table ~~2~~ 1. *ELISA formats: advantages and disadvantages**

Type of ELISA	Advantage	Disadvantage
Indirect – bound Ab detected by anti-species conjugate or by Protein A/G conjugates.	Use and availability of high variety of antispecies-specific conjugates often targeting particular antibody subsets, such as anti IgM, IgG1, IgG2, etc. Protein A and Protein G conjugates have a wide species specificity and may give lower background signals than anti-Ig reagents. Wide use for screening large numbers of samples.	Variation in degree of nonspecific binding in individual sera. To compensate for this problem high starting dilutions are required. This can lead to a decrease in DSe in comparison to competitive/blocking formats. Can only be used for one or a few species at a time.
Sandwich – Ag presented on a solid-bound-phase capture antibody.	The capture antibody on the solid phase can help to orient the antigenic molecule, which improves the chance that the sample antibody will bind. Unpurified antigen preparations can be used because capture antibody selectively binds crude antigen. Pre-coating with capture antibody can reduce the potential for subsequent binding of nonspecific proteins during the test.	Antigens must have at least two antigenic sites or epitopes which limits this type to relatively large antigenic complexes or more complex proteins. Size and spatial relationship of epitopes can affect the assay.

Type of ELISA	Advantage	Disadvantage
Competition (indirect and sandwich types) – Test antibody in sample mixed with pre-titrated detection antibody, then added to wells coated with capture antigen, either in direct or inhibition/blocking format.	Can be used in different species for which no species-specific conjugated antibodies exist. Easy adaptation for use as antibody detection tests. When highly specific MAbs are used the antigen does not have to be highly purified. ~~Can be used in different species for which no conjugated antibodies exist~~ Advantage of competitive/blocking sandwich type relies on antigen capture. Sera can be tested in low dilutions without risk of interference due to non-specific antibodies binding. This may contribute to a higher sensitivity of this format. Different antibody concentrations can be used to favour either analytical sensitivity or specificity. This is particularly relevant for assays using polyclonal antibodies which are much more affected through the use of different dilutions of sera.	Generally more steps and more optimisation may be needed, e.g. pre-titration and optimisation for liquid and solid phase reagents. Higher level of technical sophistication required.

*Primary source is Crowther (2001).

2.3. Proof of concept experiments (feasibility studies)

Proof of concept

- Was the feasibility study conducted with at least 4 to 5 samples spanning the operating range of the assay?
- Did you include one or more reference standards if required for data normalisation?
- Was separation of results between negative, low positive and high positive samples adequate?

After choice of an ELISA format, initial experiments are designed to determine if the proposed assay is viable. A reference panel such as described in Section A.2.1.3 should be tested in the prototype assay. If a reference standard is to be used for normalisation of test data, it should be selected and incorporated at this point in assay development. To provide continuity in data assessment throughout, both the reference panel and any reference standards should be included in all remaining aspects of the validation studies. The reference panel used in the feasibility study should span the entire anticipated operating range of ~~the candidate assay~~ test under evaluation (TUE) and be run in replicates as a quick check for repeatability.

The assay should achieve good separation in OD values, spanning the operating range of antibody activity. Adequate separation is particularly important between the negative and low positive samples. The lower OD range should be 0.1 or less for the negative control in indirect ELISAs, or for the strong positive control in competitive/blocking ELISAs. OD values at the upper end of the operating range should not exceed 2.0, as above this value plate readers become rather inaccurate. If the assay appears promising, optimisation is the next step.

2.4. Samples and data expression

2.4.1. Preparation and storage serum panels for optimisation studies

A best practice for antibody assays to select several (a minimum of four to five) serum samples that range from negative to high levels of antibodies against the infectious agent in question. These samples are initially used in experiments designed to demonstrate proof of concept. A large volume (e.g. a minimum of 10 ml) of each serum sample is acquired and divided it into 0.1 ml aliquots for storage at or below –20°C. One aliquot of each sample is thawed, used for experiments, and ideally then discarded. If it is impractical to discard the aliquot, it may be held at 4°C between experiments for up to about 2 weeks; however, there is a possibility of sample deterioration under these circumstances. Then, another aliquot is thawed for further experimentation. This method provides the same source of serum with the same number of freeze–thaw cycles for all experiments (repeated freezing and thawing of serum can denature antibodies so should be avoided, Chapter 1.1.2). Also, variation is reduced when the experimenter uses the same source of serum for all experiments rather than switching among various sera between experiments. This approach has the added advantage of generating a data trail for the repeatedly run samples.

After the initial stages of assay validation are completed, one or more of the samples may be suitable as a reference standard for data expression and the entire panel may be used for repeatability assessments both within and between runs of the assay (Jacobson, 1998). They may also serve as in-house working standards, i.e. quality or process controls given that their reactivity has been well characterised; such controls provide assurance that runs of the assay are producing accurate data (Wright *et al.,* 1993).

2.4.2. Normalisation of results and their expression

An optical density (OD) reading in ELISA is a measurement of colour development that is a function the amount of antibody present in a sample. Because colour development is a function of a reaction of enzyme and substrate in the presence of a chromogen, results from day to day are subject to variation attributable to external factors such as temperature, reaction time, etc. Comparison of OD results for the same samples between runs of an assay in the same laboratory, or between laboratories, lacks precision because of variation in results of reference standards included in each run of the assay. Therefore, OD results of test samples need to be adjusted as a function of the OD(s) of one or more reference standards in a specific assay run. This process is known as "normalisation" of ELISA results (see ~~the WOAH Validation Standard~~ Chapter 1.1.6, Section A.2.7 for details). The method of normalisation and expression of data should be determined, preferably no later than at the end of the feasibility studies.

OD values may be expressed in several ways (Wright *et al.,* 1993). A simple method is to express all OD values as a percentage of a single high-positive serum control that is included on each plate. For such calculations, this control must yield results that are in the linear segment of the operating range of the assay. A more rigorous normalisation procedure is to calculate results from a standard curve generated by plotting observed OD values against concentration (or dilution) of antibody for several serum controls that span the range of antibody activity of the assay. It requires a more sophisticated algorithm, such as linear regression, log-logit, or 4 or 5 parameter logistic regression analysis, among others. This approach is more precise because it does not rely on only one high-positive control sample for data normalisation, but rather uses several serum controls, adjusted to expected values, to plot a standard curve from which the sample value is extrapolated. This method also allows for exclusion of a control value that may fall outside expected confidence limits.

2.5. Optimisation

Optimisation and standardisation

- Have all critical reagents been tested against each other in checkerboard titrations?
- Did you find optimal concentration/dilutions for each reagent?
- Did you incorporate quality or process control procedures and reagents?
- Did you incorporate methods for normalisation of test data?

For ELISAs, the most important variables that need to be optimised are concentration/dilution of antigen adsorbed to the solid phase, test serum working dilution, enzyme molarity, antibody-conjugate dilution, and substrate solution concentration. These are evaluated through checkerboard assessments (each variable compared against all other variables within one run of an assay that is repeated several times). Other variables that need consideration are pH and ionicity of reagents, molecular factors such as valency and epitope density of antigens, isotype of targeted antibody and antibody affinity (Bowden *et al.,* 2021). Precision of test results can be graphically depicted or expressed numerically by various statistical methods (Crowther, 2001). ELISA studies require that instrumentation (plate washers and readers, etc.) must be properly calibrated prior to use – part of the laboratory's quality control programme.

2.6. Inhibitory factors in sample matrix

Although ELISA antibody detection systems are rather resistant to inhibitory factors, ~~the WOAH Validation Standard~~ Chapter 1.1.6, Section A.2.4, and Greiner *et al.* (1997) provide descriptions of the type of inhibitors that could affect the assay. These references should be reviewed carefully to assure that all inhibitory factors are accounted for and controlled.

2.7. Calibration to reference standard sera

If international, national, or other-source reference sera are available, the assay should be calibrated to match the analytical sensitivity in terms of the metrological units ascribed to the calibration sera (Wright, 1998).

B. ASSAY VALIDATION PATHWAY

1. Stage 1 – Analytical performance characteristics

Factors that affect the analytical characteristics of diagnostic assays are numerous and may vary according to each assay type, for examples, the main factors affecting the analytical characteristics of serological tests are described in Bowden *et al.* (2021).

1.1. Repeatability

Analytical performance characteristics

- Has repeatability been established for a range of positive and negative samples within and between runs of the assay?
- Have upper and lower control limits of the assay been established?
- Have you defined ASe and ASp for this assay?
- Does the TUE compare favourably with a standard test method, based on objective quantitative and qualitative criteria?

Repeatability is the level of agreement between results of replicates of a sample, both within and between runs of the same method in one laboratory. The same or similar panel of samples used in the feasibility study is adequate. No less than three (preferably 5) samples covering the operating range of the assay, and of sufficient quantity for at least 20 runs of the assay over several days. Specifics of how the samples should be prepared and handled are provided in Chapters 2.2.6 and ~~in the WOAH Validation Standard~~ 1.1.6, Section B.1.1. It is valuable to include at least one reference sample in an indirect ELISA (a positive serum control) to which the test samples can be normalised by per cent of the positive control. The within run variation can be determined by the mean OD and coefficient of variation (CV) of the replicates of each sample. The CV should not exceed about ~~15%~~ 20% (Jacobson, 1998) (with the possible exception of negative and very low positive samples which may have higher (and meaningless CVs). If all of the samples have previously been calibrated to reference standards, and their expected ODs are thus known, the observed ODs for each sample in each run can normalised as a function of their expected ODs in linear regression analysis. This provides a correlation coefficient as evidence of closeness of fit to the expected value, and allows for normalised values to be plotted in control charts (Crowther, 2001).

1.2. Analytical specificity

Analytical specificity (ASp) is determined by testing sera from animals that are known to have been infected/exposed to all species/strains that the test should detect (Chapter 2.2.6, Section B.1). Cross reactivity with sera from animals infected with related species is used to evaluate the ASp. ELISAs are also subject to false positive results attributable to exogenous factors, such as nonspecific binding of serum or conjugate to the plastic surface that may require use of blocking agents (selectivity). Care must be taken to eliminate this source of error. Blocking and competitive ELISAs may also suffer specificity problems due to stearic hindrance preventing proteins binding to their target sites.

Analytical specificity in antibody detection assays, which can be further characterised according to selectivity, exclusivity and inclusivity, is assessed using well-characterised samples and should include (Bowden *et al.*, 2021):

i) Sera from animals of known exposure status, including:

   a) non-infected and non-vaccinated animals

   b) non-infected and vaccinated animals

   c) infected and non-vaccinated animals

   d) infected and vaccinated animals

ii) Details of the source of the material, including species, breed, age, sex, reproductive status, vaccination history, herd history, experimental or field origin, geographical region and the presence or absence of clinical signs

iii) Sera from animals known to have been infected with the species/strains that the test should detect

iv) Sera from animals infected with related organisms and pathogens that cause similar clinical signs and which may cause cross-reactions.

1.3. Analytical sensitivity

Analytical sensitivity (ASe) is synonymous with the lower limit of detection (LOD) of antibody concentration in a sample. The different types of antibody assays vary considerably in their inherent limit in antibody detection. For instance, LODs for eight different types of antibody assays range from 1000 ng/ml (radial immunodiffusion) to 0.01 ng/ml (chemiluminescence) (Nielsen *et al.,* 1996). LODs are usually determined by endpoint dilution in which replicates (preferably 10) of each dilution in a $\log_2$ dilution series are run in the assay.

1.4. Standard test method comparison with the ~~candidate~~ test ~~method~~ under evaluation (TUE)

The ~~candidate test method~~ TUE should be run in parallel with a~~n~~ WOAH or other accepted reference test method, using the same panel of samples on both, to determine whether the ~~candidate method~~ TUE exhibits the same quantitative and qualitative characteristics as the standard method. Favourable comparability lends strength to the belief that ~~candidate method~~ the TUE will be a successful substitute for the reference method (see also methods comparison studies, Chapter 2.2.~~5~~ 8).

2. Stage 2 – Diagnostic performance characteristics

DSe and DSp are the primary performance indicators of the validation process. Antibody assays are subject to the same general procedures to achieve estimates of DSe and DSp as required of all other assay types (see ~~the WOAH Validation Standard~~ Chapter 1.1.6, Section B.2 for essential details). The number of samples needed to establish these estimates for a particular antibody assay require a sampling design that considers many variables. This includes creation of a sample panel that is tailored particularly for the intended purpose of the assay (e.g. a screening versus confirmatory test). It also requires predetermined desired levels of DSe and DSp (indicating acceptable levels of false negative and false positive results), allowable error in the estimates of such DSe and DSp, and the confidence level required for these estimates.

The number of animals required to establish acceptable DSe and DSp estimates is a function of the level of confidence desired in DSe and DSp estimates and the accepted allowable error. For instance, for a pathogenic disease like FMD, it is necessary to reduce the likelihood that infected animals will be misclassified as uninfected, which reduces allowable error in the test result which, in turn, increases the number of samples needed to establish a high level of confidence in the DSe estimates. Alternatively, for a confirmatory assay it is desirable to reduce the likelihood that uninfected animals will be classified as infected. A high DSp is then desired with minimal allowable error, requiring a larger sample size of uninfected animals. All of these general issues related to sample size, confidence intervals and allowable error in the DSe and DSp estimates are described in ~~the WOAH Validation Standard~~ Chapter 1.1.6, Section B.2, Table 2 with additional detail and tables of sample numbers required available elsewhere (Jacobson, 1998).

It is often challenging to obtain a sufficient number of well characterised sera to achieve estimates of DSe and DSp that are sufficient for the intended purpose of the assay. Initially, it may be a compromise between what is statistically meaningful and practically feasible, resulting in an assay that is provisionally recognised (~~WOAH Validation Standard, Section B.2.6)~~ Chapter 1.1.6., Section B.2.6). Colling *et al.* (2018) describe a network approach for provisional recognition of an antibody ELISA for Hendra virus when only low sample numbers from infected animals are available. However, over time, with accumulation of more well characterised samples, the estimates of DSe and DSp may be strengthened (see Chapter 1.1.6, Section 5.4 below).

2.1. The challenge in establishing accurate estimates of DSe and DSp for antibody assays

Antibody assays undergoing validation pose unique problems when attempting to assemble known positive and known negative samples in sufficient quantity to establish assay performance characteristics. Antibody is an indirect indicator of the presence of, or prior exposure to, an infectious agent or its components. Inferences from detection of antibody (or the lack thereof) depend on the host's qualitative and quantitative responses to the organism. Factors that affect the concentration and composition of specific antibody in serum samples are inherent to the host (e.g. age, sex, breed, nutritional status, pregnancy, immunological responsiveness) or acquired (e.g. passively acquired antibody, or active immunity elicited by vaccination or infection). Theoretically, samples from animals that represent all of these variables should be included in the panels used for establishing DSe and DSp estimates. Clearly, this becomes a daunting, if not impossible task. To surmount this problem, the initial sample panels should be representative of the majority of animals in the target population to achieve initial estimates of DSe and DSp. In reality, it is necessary to enhance DSe and DSp estimates after the assay has been implemented as more well characterised samples become available (see Section 5.4, below).

Because it is often desirable to stretch the application of antibody detection assays to a huge number of animals spanning large geographical areas (e.g. as in screening assays for an entire continent), assembly of fully representative sample panels for such a large diagnostic window of variables may be nearly impossible. A useful alternative is to first establish DSe and DSp estimates for a rather homogeneous population of animals. If the assay is destined for use in disparate populations of animals, which may harbour a different infectious agent profile (with possibility of cross reactions not seen in the original targeted population), a reassessment of DSe and DSp may be necessary, drawing from data acquired using new sample panels that are representative of the population(s) targeted.

Diagnostic performance characteristics

- Are the criteria used to determine the positive and negative reference populations legitimate?
- Do the reference samples fully represent the population targeted by the assay?
- Were there difficulties in obtaining a sufficient number of samples? If so, how was the problem addressed?

2.2. Reference animal populations

2.2.1. Animals of "known infection status"

Reference animals of known infected and known uninfected status are the ideal source of samples for determining DSe and DSp. However, such samples are rare and difficult to establish. The most familiar term for reference animals or samples used in establishing DSe and DSp is the so called "gold standard", a misnomer commonly used to classify almost any reference animal as infected/exposed or uninfected, with samples from such animals classified as positive or negative (see ~~the WOAH Validation Standard,~~ Chapter 1.1.6. Section B.2.1–~~2.~~–3).

Assay developers should be aware of the advantages, and particularly the pitfalls, associated with various methods that are used to classify reference animals as infected or uninfected. The samples from such animals are deemed either positive or negative, and collectively become the *reference standard* upon which the ~~candidate assay's~~ TUE's DSe and DSp are based. It is, therefore, crucial to carefully consider the validity of various reference standards as exemplified in the following four examples:

i) An unequivocal reference standard: presence of the agent in the host or evidence of definitive (pathognomonic) histopathology

If an infectious agent or definitive histopathological criterion is detected in an animal, this generally constitutes an unequivocal reference standard for that animal. Serum samples derived from such animals usually are considered to be unequivocal serum reference standards for determining DSe and DSp of the ~~candidate assay~~ TUE. However, such samples may have their limitations. At the population level, a pathogen may be unequivocally present in some animals, but if the serum sample was taken from the animal early in the infection process, the immune response may not yet have produced detectable antibody. In this case, such serum samples used as reference standards would have been FN for the subset of animals in an early stage of infection. In contrast, for more chronic types of infection, using only reference animals that have confirmatory culture or histopathology may produce higher estimates of DSe than are realistic for the population targeted by the assay because the immune response will always be well established.

ii) A composite reference standard: verification of uninfected or unexposed animals

This standard is achieved by selecting reference animals from geographical areas where herd histories, clinical profiles, prior testing results and other parameters provide evidence suggesting the absence of the pathogen, and thus no specific host antibody response to the pathogen targeted by the ~~candidate assay~~ TUE. These types of reference materials, their strengths and limitations are described elsewhere (Jacobson, 1998), and must be considered carefully when using samples from such sources for establishing DSe and DSp for a ~~candidate assay~~ TUE.

iii) A relative reference standard: comparative serology

This standard is characterised by reference animals that have been classified for their infection status by comparison with the test results of another serological assay on the same samples. It often is the only practical source of reference material available for evaluation of a new serological test. If results of such a reference test are chosen as the standard for determining diagnostic performance characteristics of the ~~candidate assay~~ TUE, the resultant estimates of DSe and DSp are useful only insofar as the reference test has documentable, established and acceptable performance characteristics. A deficiency of relative reference standards is that they have their own established levels of FP and FN test results, which are sources of error that will be compounded in

estimates of DSe and DSp for the new assay. Generally, however, the use of other well described test methods is regarded as good practice to determine the status of reference animals, but only if the inherent bias introduced by the relative reference standard is accounted for.

iv) *An adjunct reference standard*: experimental infection or vaccination

(See ~~the WOAH Validation Standard,~~ Chapter 1.1.6, Section B.2.3 for significant limitations of this type of standard.) In some cases, the only way to obtain positive samples is by experimental infection. This approach is highly suitable to model the dynamics of the infection and to determine the 'diagnostic window' with the new assay. For example, it is possible to get estimates of the time interval between exposure to a pathogen and when antibody is first detectable, or when 25, 50, 75 and 100% of the infected animals return a positive test result. Nevertheless, there are pitfalls in use of time-series data that must be avoided. Data representing repeated observations from the same animals cannot be used in calculation of DSe and DSe because the statistical models used to establish DSe and DSp require independent observations (only one sample from each anima). For statistically legitimate time-course studies, or when single samples are used from each of many experimental animals, the strain of cultured organism, route and dose of exposure, infection with other related, cross-reactive and non-related, non-cross-reactive organisms are variables which may produce quantitatively and qualitatively atypical responses which are not found in natural infections in the target population. Experimental conditions typically lead to an overestimation of sensitivity and specificity for example by artificially high challenge doses and by using specific pathogen free animals as negative controls.

The time point of sample collection (days post-infection) must be indicated. Sources and history of experimental animals should be described. The validation should not be based solely on experimental animals as they do not represent natural populations of animals subject to pathogens by natural exposure.

v) Heuer & Stevenson (2021) describe procedures for diagnostic test validation studies when there is a perfect reference standard available for either positive or negative animals or both.

2.2.2. Latent-class models for estimation of DSe and DSp

For a discussion of this approach for estimation of diagnostic performance, see ~~the WOAH Validation Standard~~ Chapter 1.1.6, Section B.2.5 ~~and~~, Chapter 2.2.5, Cheung *et al.* (2021).

2.3. Threshold (cut-off) determination

The procedures for establishing the cut-off between negative and positive results of antibody assays are as described in ~~the WOAH Validation Standard~~ Chapter 1.1.6, Section B.2.4.

3. Stage 3 – Reproducibility and augmented repeatability estimates

Reproducibility is the measure of precision of an assay when used in several laboratories located in distinct regions or countries using the identical assay (protocol, reagents and controls) to test the same panel of samples. Factors affecting testing reproducibility among laboratories and practical examples of proficiency testing and interlaboratory comparison testing are provided by Johnson & Cabuang (2021) and Waugh & Clark (2021). A case study with FMD for selection and use of reference panels is presented in Ludi *et al.* (2021). The relevance of virtual biobanks for transparency purposes with respect to reagents and samples used during test development and validation is reported by Watson *et al.* (2021). Reproducibility assessments for antibody assays are not uniquely different from similar assessments for any other type of assay. Therefore, the reader is directed to ~~the WOAH Validation Standard~~ Chapter 1.1.6, Section 3, for details on reproducibility analysis and for reference samples and panels to Chapter 2.2.6.

4. Stage 4 – Programme implementation

4.1. Interpretation of results ~~and determination of predictive values~~

Best practices for programme implementation are general to all assay types ~~(WOAH Validation Standard, Section B.4)~~. However, as Ab-ELISA is often the assay of choice for surveillance programs to affirm absence of disease, or for eradication of disease or elimination of infection from defined populations, the issue of false positive results can become a significant problem when the disease prevalence drops, e.g. during proof-of-freedom testing after an outbreak even if the diagnostic specificity is very high. For the interpretation of test results, the predictive value (PV) and likelihood ratio (LR) are widely used. Depending solely on the combined

DSe and DSp the LR has the advantage of being prevalence independent (Chapter 1.1.6, Section B.4.2., Caraguel & Colling, 2021; Jacobson, 1998).

A common misperception is that a test with 99% DSp and DSe will only mis-classify animals as FP or FN 1% of the time. The FN and FP rates vary depending on the prevalence of infection in the targeted population. False positive reactions in a disease eradication campaign can vary significantly from the beginning of the campaign when prevalence is relatively high (for example, 10%) to near the end of the campaign when it has decreased to 0.1%. The predictive values of test results then become very important. Predictive values are probabilities that a test result is truly positive or truly negative. In our example using an assay with 99% DSe and DSp for testing a population of animals with a 10% prevalence of disease, the predictive value of a positive test result (PPV) is 91.7%, meaning that there is a 91.7% probability that the animal is truly infected. The predictive value of a negative test result (NPV) is 99.9. When the prevalence drops to 5%, the PPV and NPV are 83.9% and 99.9%, respectively. However, if the prevalence drops further to 0.1%, by successfully removing infected animals from the population, the same test will produce a PPV of 9% and a NPV of 99.9%, meaning that there is only a 9% chance that a positive test result is detecting a truly infected animal (of 1000 animals tested, only about 1 in 10 positive test results is indicative of an infected animal – the other 9 are false positive). So, if the test is intended for the purpose of eradication of a disease or elimination of infection from a population, the test developer is advised to consider moving the assay to a second cut-off that yields a higher DSp late in the campaign to reduce the probability of false positive reactions. It is instructive to examine a predictive value chart for assays of varying DSe and DSp, to visualise the effects of reduced prevalence on predictive values of an assay (WOAH Validation Standard, Table 2, and Jacobson, 1998).

5. Monitoring assay performance

5.1. Monitoring the assay

Once the assay is in routine use, internal quality control is accomplished by consistently monitoring the assay using quality control charts for assessment of repeatability and accuracy. Charts representing at least 30 runs will reveal trends or shifts in values of controls and standards. Lines representing the mean value of a control sample in at least 30 runs, plus/minus 3 standard deviations, are useful decision criteria for inclusion or exclusion of a run of the assay. The run is rejected if one control/standard exceeds ± 3 standard deviations (STD) or if 2 controls (or more) exceed ± 2 STD (Crowther, 2001). Decision criteria may need to be customised for a given assay because of inherent differences between assays attributable to the host pathogen system. Chapter 2.2.4 provides an example of how to apply measurement uncertainty for an antibody ELISA using a positive internal control sample.

Reproducibility of test results between laboratories should be assessed by External Quality Assurance at least once per year and is an essential requirement of ISO 17025 accredited laboratories. Membership in a consortium of laboratories that are interested in evaluating their output is valuable.

Monitoring assay performance

- Has the purpose of the assay changed?
- Has the epidemiology of the disease in question changed, e.g. prevalence, new serotypes or strains, etc.?
- Have critical reagents been changed, and if so, was comparability of the new reagents assessed?
- Are performance indicators included in day to day use of the assay (control charts, basic statistics)?
- Are upper and lower limits in control charts updated periodically as more experience with the control samples is achieved?
- Are procedures in place that determine what to do when controls are outside acceptance limits?
- Are test panels shared with other laboratories to assess reproducibility?
- Is proficiency testing included as part of continuing evaluation of the assay?

Crowther *et al.* (2006) provides a critical review of aspects of kit validation for tests used for the diagnosis and surveillance of livestock diseases: producer and end-user responsibilities.

5.2. Minor modifications of the assay – replacement of depleted reagents (Chapter 2.2.8)

When quality or process control samples are nearing depletion, it is essential to prepare and repeatedly test the replacement samples. The replacement samples should be included in at least 10 routine runs of the assay, with their results normalised against the existing reference standard. The activity of the replacement control should be comparable to the replaced control. If the reference standard requires replacement, care must be taken to select a replacement that matches all of the original serum characteristics as closely as possible, thus allowing use of the replacement to normalise test results with comparable outcomes (see also Chapter 2.2.8 *Comparability of assays after changes in a validated test method*). Kirkland & Newberry (2021) provide an

example-based review of verification studies of diagnostic tests and Reising *et al.* (2021) describe comparability studies of assays after minor changes in a validated test method.

When other reagents such as antigen for capture of antibody, must be replaced they should be produced or procured using the same protocols or criteria as used for the original reagents. They need to be assessed using sera from routine submissions in 5–10 parallel runs that include the current and the new reagent(s). A panel of representative samples, such as a proficiency panel, is also a useful tool for assessing the comparability of the reagents (Chapter 2.2.6).

5.3. Major modifications of the assay – changing to a new ELISA type or geographical region

If the assay is to be changed from, say, a sandwich ELISA to a competitive/blocking format, the assay will require revalidation because of the many variables that may affect the performance characteristics of the new assay. For an assay considered for implementation in another geographic region, e.g. from the northern to the southern hemisphere, it is essential to verify and potentially revalidate the assay by subjecting it to sera from populations of animals that reside under local conditions. Evaluation of reference sera that represent those populations ~~is done by using stages 3–5 in Figure 1 in the WOAH Validation Standard. It~~ is the only way to assure that the assay is valid for populations that are of different composition compared with the original population targeted by the assay.

5.4. Enhancing confidence in validation criteria

Due to the extensive set of variables that have an impact on the performance of serodiagnostic assays, it is useful to expand the number of reference sera wherever possible, recognising the principle that error is reduced with increasing sample size. An expanded reference serum bank should be accumulated with well characterised sera, and used periodically to update estimates for DSe and DSp for the population targeted by the assay.

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NB: FIRST ADOPTED IN 2014.

Annexe 10. Chapitre 2.2.2. Mise au point et optimisation des méthodes de détection des antigènes

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CHAPTER 2.2.2.

DEVELOPMENT AND OPTIMISATION OF ANTIGEN DETECTION ASSAYS

INTRODUCTION

The WOAH Validation Recommendations ~~in Section 2.2~~ Validation of diagnostic tests *~~of this~~* Terrestrial ~~Manual~~ *provide detailed information and examples in support of ~~the WOAH Validation Standard that is published as~~ Chapter 1.1.6* Validation of diagnostic assays for infectious diseases *of* terrestrial animals.~~ *The Term "WOAH Validation Standard" in this chapter should be taken as referring to that chapter.*~~ *An up-to-date compilation of the relevant validation standards (WOAH and non-WOAH) and guidance documents for all stages of diagnostic test validation and proficiency testing, including design, analysis, as well as clear, complete and transparent reporting of validation studies with case studies is provided in Colling & Gardner (2021). Published standards for peer-reviewed reporting of accuracy studies (STARD) are available for infectious diseases of humans (Bossuyt* et al., *2015*), and *terrestrial animals (Gardner* et al., *2011; 2019, Kostoulas* et al., *2017; 2021)*

Practical matters in selecting an assay format

- Is high-throughput essential? Will the test be automated?
- Will the test be used as a herd test or for testing individuals?
- What is the anticipated turnaround time? Is that suitable?
- What level of sophistication is needed to run the assay?
- What skills are required to interpret test results?
- Will the assay be feasible for use in my laboratory?
- Will it be easily transferrable to other laboratories?
- What are the costs to run the assay?
- What is the level of acceptance in the scientific & regulatory community?

The detection and identification of an agent is confirmatory evidence of either infection with or disease caused by a particular pathogen. There are many ~~different~~ diverse direct and indirect test methodologies available. Classical direct [1] agent detection assays include electron microscopy, light microscopy (e.g. observation of unique histopathological or pathognomonic features, identification of parasites in situ*, etc.), virus isolation, bacterial culture and parasitic digestion techniques. Many direct techniques require secondary procedures to assist in the characterisation and identification of these agents (e.g. haemagglutination inhibition or virus neutralisation tests, special bacteria or fungal stains, etc.). Representatives of indirect agent and in particular, antigen (Ag) detection tests include enzyme linked immunosorbent assays (ELISA), immunofluorescence or immunoperoxidase assays, western blotting techniques, micro-arrays, fluorescence activated cell sorting (FACS) and biosensors. Often*

[1] Definitions: In the context of direct and indirect detection of an analyte, the term "direct" is relevant to the organism or its antigens, e.g. direct detection by microscopy. The term "indirect" is relevant to the host response to the organism, (e.g., indirect detection inferred by detection of antibodies to the organism). In the context of diagnostic methods that are used to detect infection, microscopy is a means of direct observation of the organism, whereas using a reagent that infers the presence of the agent (such as an enzyme conjugated to a purified antibody that is specific for the agent) is an indirect method.

direct and indirect methods are used ~~in tandem~~ complementary, for example a direct technique may be used to isolate, enrich and/or extract the organism followed by indirect techniques to characterise and identify the agent.

The examples given above vary widely in terms of laboratory requirements. ~~Pen-side~~ Point-of-Care tests (PoCT) including lateral flow devices~~)~~ may also be antigen detection tests. They are easy to use and were developed for testing of animals in the field but may have applications in laboratories. ~~Cost~~ (Halpin et al.*, 2021; Hobbs, 2020). Specific validation requirements of PoCT are given in Fig. 1 in Halpin* et al. *(2021) and Chapter 1.1.6, Fig.1, laboratory infrastructure, biocontainment/safety, technical sophistication, interpretation skills, turn-around time, throughput capacity, diagnostic performance characteristics, repeatability and reproducibility are important parameters which need consideration when selecting the most appropriate assay. They also vary with respect to suitability for different diagnostic applications.*

In contrast to ~~serological or~~ antibody detection tests, Ag detection tests depend heavily on the time of clinical onset and pathogenesis of the disease and the concentration of the pathogen in certain tissues and/or fluids. Successful diagnosis depends on appropriate timing and selection of the sampling site (affected tissue/lesions, scrapings, swabs, blood, and other body fluids), storage conditions *and specimen integrity during transport (Chapter 1.1.2* Collection, submission and storage of diagnostic specimens *and Chapter 1.1.3* Transport of biological materials*). For certain applications, the testing of individual animals and/or samples may be appropriate (e.g. confirmatory testing); while for other purposes (e.g. screening), pooling of animals may be efficient and effective. Selection of the appropriate specimen requires good understanding of the disease and the effect of the sample matrix on the pathogen (e.g. cloacal or tracheal swabs for avian influenza).*

Nucleic acid detection (NAD) tests and more recently high throughput sequencing (HTS) are increasingly replacing classical antigen detection systems. For development and optimisation of NADs, please refer to Chapter 2.2.3 Development and optimisation of nucleic acid detection assays *and for HTS to Chapter 1.1.7* Standards for high throughput sequencing, bioinformatics and computational genomics*, and van Borm* et al. *(2016). Although ~~these~~ NAD tests may seem to be the tests of choice for many applications, they are not always the most practical or efficient. In most cases it is still necessary and prudent, at least for an index case, to culture the agent on selective media or in susceptible cell lines or eggs to facilitate further characterisation and identification. While genotyping is an important consideration, especially in molecular epidemiology, other means of agent characterisation such as serotyping, pathotyping or biotyping are also important and provide valuable information on the antigenic properties of the target agent. Cultured and preserved agents have tremendous historical value and are also an important source of reference materials, which can be used for future assay validation, specificity testing and pathogenesis trials for new and emerging agents (Watson* et al.*, 2021).*

In their review of WOAH recommended diagnostic tests, Cullinane & Garvey (2021) concluded that ELISA and molecular assays were the most widely used WOAH-recommended tests. Due to its worldwide application, the Ag ELISA is used as an example for application of best practices in antigen detection assays in this chapter. Most of the basic processes used to validate other types of antigen detection assays will become evident by extension of those used to validate ELISAs. Because of the many conceptual similarities between antigen and antibody detection assays this chapter frequently cross-refers to Chapter 2.2.1 Development and optimisation of antibody detection assays.

A. ANTIGEN DETECTION ASSAY DEVELOPMENT PATHWAY

1. Intended purpose(s) of the antigen assay

The intended purpose of ~~the~~ a test is a key factor which will guide decisions in the selection ~~and~~, early development and validation of a test. ~~the candidate assay. Given the WOAH-defined 'fitness for purpose' categories in Table 1, Ag detection systems may be appropriate for certain applications. Support for disease eradication or surveillance programmes generally require testing of high numbers of samples, with an emphasis on diagnostic sensitivity and throughput capacity. In contrast, confirmation of clinical cases does not entail high numbers to be tested, but diagnostic specificity and turn-around times become very important. At the outset, the questions posed in the text box, above, should be carefully considered.~~

~~***Table 1.** Determinants of an antigen assay's fitness for its purpose*~~

~~*Assay characteristics*~~	~~*Determinants of fitness for purpose*~~						
	~~*1**~~		~~*2**~~	~~*3**~~	~~*4**~~	~~*5**~~	~~*6**~~
	~~a~~	~~b~~					
~~Diagnostic sensitivity (DSe)~~		~~+++~~	~~+++~~	~~+++~~	~~+++~~		
~~Diagnostic specificity (DSp)~~		~~+~~	~~+~~	~~+~~	~~+++~~		
~~Positive predictive value (PPV)~~		~~+~~	~~+~~	~~+~~	~~+++~~		
~~Negative predictive value (NPV)~~		~~+++~~	~~+++~~	~~+++~~	~~+++~~		
~~Throughput capacity~~		~~+++~~	~~++~~	~~+~~	~~–~~		
~~Turn-around time of test~~		~~+~~	~~+~~	~~+~~	~~+++~~		
~~QA capability~~		~~+++~~	~~+++~~	~~+++~~	~~+++~~		
~~Reproducibility~~		~~+++~~	~~+++~~	~~+++~~	~~+++~~		
~~Repeatability~~		~~+++~~	~~+++~~	~~+++~~	~~+++~~		

~~Other characteristics such as the technical sophistication of the assay, and the skill required for interpretation will be related to the disease or infection under investigation.~~
~~NB NAD tests may also be used for agent detection, and are considered in Chapter 2.2.3.~~
~~**Symbols:** +++ = essential; + = of less importance; – = not important.~~

~~*~~Basic~~ The six basic intended~~ purposes for ~~which an assay may be deemed fit: 1.~~ diagnostic assays relevant in the WOAH context are stated in Chapter 1.1.6 and include: 1. Contribute to the demonstration of freedom from infection in a defined population: a) 'Free' with or without vaccination, b) Re-establishment of freedom after outbreaks. 2. Certify freedom from infection or presence of the agent in individual animals or products for trade/movement purposes; 3. Contribute to the eradication of disease or elimination of infection from defined populations; 4. Confirmatory diagnosis of clinical cases (includes confirmation of positive screening test); 5. Estimate prevalence of infection or exposure to facilitate risk analysis; 6. Determine immune status in individual animals or populations (post-vaccination). An example-based overview for the purpose-oriented use of diagnostic tests is provided in Gardner *et al.* (2021). Table 1 of Chapter 1.1.6 highlights the relevance of assay parameters such as diagnostic sensitivity and specificity, predictive value and likelihood ratios for individual purposes.

1.1. Purpose 1a: Contribute to the demonstration of freedom from infection in a defined population a) 'Free' with or without vaccination

In a population that is historically free of a particular disease/pathogen, the prevalence is zero (or close to zero). To be fit for purpose, the test or test algorithm aims to minimise the chances of false-positive results and ideally requires high DSp, high positive predictive value (PV+) and high positive likelihood ratios (LR+). This can be achieved by a single test with a high DSp or serial testing. In most countries, active surveillance at abattoirs and the screening of fallen stock identifies preclinical cases and cases in which there may have been unrecognised clinical signs. To detect the modified prion protein (PrP) in bovine spongiform encephalopathy in the brain, immunochemical methods including immunohistochemical (IHC) techniques, western immunoblot methods, and rapid test methods such as lateral flow assays and enzyme linked immunosorbent assays (ELISA) are used. Histopathology is no longer the diagnostic method of choice for investigation of animals or the screening of healthy populations. No diagnostic test is currently available for live animals.

Purpose 1b Contribute to the demonstration of freedom from infection in a defined population b) Re-establishment of freedom after outbreaks

For ~~disease freedom categories~~ re-establishing freedom after outbreaks as given in purpose ~~(Table 1), 1b,~~ antibody screening tests of high diagnostic ~~sensitivity (DSe~~ specificity (DSp) are usually the tests of choice provided that a detectable immune response is a significant indicator of infection. However, there may be certain disease situations where the humoral immune response can be misleading and pathogen detection may be

more appropriate (e.g. mycobacterial or trypanosomal infections). In these cases, Ag detection tests may be more effective. The antigen ~~screening test~~ testing algorithm should ~~demonstrate~~ favour a high ~~DSe. Tests~~ DSp. Algorithms of high ~~DSe~~ DSp and high LR+ demonstrate low false ~~negative (FN~~ positive (FP) rates, which is needed in populations where ~~and when applied to low~~ prevalence is low ~~populations, the NPV is at its highest level~~. As ~~DSe~~ DSp and diagnostic ~~specificity (DSp~~ sensitivity (DSe) are usually inversely related, selection of a more sensitive test may lead to a decrease in DSp, which will result in an elevated false positive (FP) rate. ~~Therefore, all screening test positive results should be subjected to some form of confirmatory testing to evaluate their true status. Confirmatory tests characteristically have high DSp and therefore a low FP rate. These tests are often more sophisticated and require enhanced interpretive skills.~~ A common approach to demonstration of freedom is to use highly sensitive screening tests and then subject positive results samples to some form of confirmatory testing to evaluate their true status. Confirmatory tests characteristically have high DSp and therefore a low FP rate. These tests are often more sophisticated and require enhanced interpretive skills, laboratory environment and highly skilled staff. Provided an appropriate confirmatory assay is selected, such serial testing approaches may present a more economical approach to surveillance testing, however maintaining high DSe in the testing algorithm is necessary to ensure adequate positive predictive values in low to zero prevalence populations.

1.2. Purpose 2: Certify freedom from infection or presence of the agent in individual animals or products for trade/movement purposes

For a number of diseases included in the *Terrestrial Manual*, agent identification is listed as the preferred test method for the purpose of determining "Individual animal freedom from infection prior to movement". Although in many cases this involves culture of the organism or detection of nucleic acid, there may be situations where an antigen detection test is appropriate for this purpose. ~~To avoid risk of disease spread through trade, a test offering high DSe is to be preferred.~~ For example, for ASF the antigen detection ELISA is described in Chapter 3.9.1. *African swine fever* (*infection with African swine fever virus*) as "recommended but with limitations". It may perform satisfactorily as an alternative method but, as its sensitivity is much lower than that of the molecular tests, it should not be used as the only method for virus detection and results should be confirmed by polymerase chain reaction (PCR). To avoid risk of disease spread through trade, a test or a combination of tests offering high DSe is to be preferred. This can be achieved through parallel testing where a sample is considered positive if any of the tests are positive. Therefore, parallel testing increases DSe but decreases DSp, when considering laboratory diagnosis within an epidemiological unit.

1.3. Purpose 3: Contribute to the eradication of disease or elimination of infection from defined populations

If the purpose of the test is the eradication of disease or elimination of infection from defined populations, antigen screening tests~~, like~~ and antibody screening tests, of moderate to high ~~DSe~~ DSp, are the tests of choice. However, the rationale is slightly different in that the testing will likely be done at the herd or compartment level. At the beginning of the eradication campaign, when the disease prevalence is high, moderate DSe and DSp are suitable as both FP and FN rates are less relevant at this juncture and a moderate level of error is tolerable. Depending on the nature of the disease and rapidity of spread, high throughput ~~and fast turn-around-times~~ may become critical. Usually decisions are made without confirmatory testing at this point.

In the latter stages of the campaign, a higher ~~DSe~~ DSp is warranted as the ~~FN~~ FP rate becomes the more critical factor. Much like Purpose 1, positive reactors will need to be subjected to some form of confirmatory testing to evaluate their true infection status. In these latter stages, antigen and/or nucleic acid detection systems are critical in the detection of sub-clinical cases, shedders and possibly, latent carriers.

1.4. Purpose 4: Confirmatory diagnosis of clinical cases (includes confirmation of positive screening test)

Although antibody tests of high DSp are often the tests of choice for ~~confirmatory diagnosis of clinical cases, antibody tests~~ confirmation of a positive result from a screening test, they may not ~~always~~ be ~~the tests of choice~~ ideal to confirm a clinical case, especially if clinical signs appear before an immune response is mounted~~. A~~, e.g. a prime example would be highly pathogenic avian influenza infections where mortality may occur before an immune response is even detectable. ~~Antigen~~ Under these circumstances antigen or nucleic acid detection tests are usually a better choice for confirmation of clinical cases provided that they offer a fast turn-around-time. In these cases, the idea is to maximise DSp and thereby minimise any potential FP reactions. For some clinical cases, e.g. vesicular diseases in terrestrial animals, several tests may be required to quickly rule out select pathogens that present similar clinical signs. In this category of test, fast turn-around-times are extremely critical in identifying potential outbreaks. An example of a confirmatory diagnosis would be a hydrolysis probe positive avian influenza (AI) virus swab and haemagglutinin (HA) and neuraminidase (NA) testing (including pathotyping/sequencing).

1.5. Purpose 5: Estimate prevalence of infection or exposure to facilitate risk analysis

For estimates of prevalence of infection and/or shedding to facilitate risk analysis, e.g. for health surveys, herd health status and to monitor disease control measures, antigen detection tests of moderate DSe and DSp are the tests of choice. In general, this would balance both FN and FP rates and result in a more accurate estimate of the true prevalence in the target population. However, if accurate estimates of both DSe and DSp have been established, statistical approaches can be used to minimise bias attributable to FN and FP rates (see Chapter 2.2.5 *Statistical approaches to validation*).

1.6. Purpose 6: *Determine immune status in individual animals or populations (post-vaccination)*

This purpose is not applicable to antigen detection assays.

2. Assay development – experimentation

2.1. Reference materials, reagents and controls

2.1.1. Test samples

Samples required for antigen detection assays should be handled as described in Chapter 1.1.2 *Collection, submission and storage of diagnostic specimens*. Sample matrixes for antigen detection assays can be very heterogeneous (e.g. blood, faeces, milk, skin, semen, saliva, blisters, vesicles or swabs from affected tissues such as oropharynx (Probang), trachea, genitals, cloaca, oesophagus, etc.). The ideal specimen is the one that is easy to obtain and with a high concentration of the analyte. In many cases blood or swabs are the specimens of choice but depending on the pathogen, other tissues or fluids are needed, e.g. skin, organs such as brain (rabies, transmissible spongiform encephalopathies [TSE]), lymphatic organs, such as spleen and lymph nodes (classical swine fever), kidney, liver, heart, parts of respiratory tract (avian influenza), digestive tract (parvovirus), milk, faeces, semen, saliva, tumour material (enzootic bovine leukosis), etc.

As stated in Chapter 1.1.2, the usual considerations apply to limit bacterial and fungal contamination of specimens. The use of preservatives and fixatives is not usually recommended and samples should be sent with minimal delay and under refrigeration to a diagnostic laboratory. During transport and storage it is important to be aware of the physical and chemical requirements of the pathogen (e.g. foot and mouth disease [FMD] virus is highly labile at low pH and requires equal amounts of glycerol and phosphate buffer to maintain a pH over 7). Transporting tissue samples in glycerol containing buffer may have an effect on the sensitivity of antigen detection assays, e.g. the rabies fluorescent antibody test. Rinsing tissue samples with phosphate-buffered saline before processing for testing is recommended as it helps to prevent inhibition of the fluorescence (Chapter 3.1.19 *Rabies*.).

If samples are to be tested as pools, experiments need to be undertaken to demonstrate that the assay is fit for that purpose (e.g. that the analytical sensitivity is sufficiently high to detect one infected animal e.g. in a pool of ~~5, 10, 50 or more~~ samples ~~from uninfected animals)~~ (Chapter 2.2.6 *Selection and use of reference samples and panels*).

2.1.2. Reference Standards

See Chapters 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals*, 2.2.1 *Development and optimisation of antibody detection assays* and 2.2.6 *Selection and use of reference samples and panels* and Watson *et al.* (2021).

2.1.3. Positive and negative reference panels

Samples, containing concentrations of antigen over the intended operating range of the assay, should be used as controls during the development and standardisation of an antigen detection assay. They may be obtained from field specimens ~~or produced in~~, the laboratory as a result of animal trials or as spiked samples (see Chapter 2.2.6). Negative samples should be obtained from known uninfected animals and this same matrix should be used when spiked samples are produced. Rahmadane *et al.* (2017) describes the development and validation of an immunoperoxidase antigen detection test for improved diagnosis of rabies in Indonesia, where 116 dog brains from animals exhibiting signs of rabies involved in human bite cases and 110 brains collected as part of government control measures thought to be rabies free were tested for the exclusion of rabies virus.

2.1.4. Purified and crude antigens for antibody production

~~In general, antigens to be used for the production of immunological reagents should be as natural as possible in terms of conformation to ensure that the presentation of epitopes mimics the orientation on the live organism. Therefore, isolation and/or purification methods used should preserve the antigenic integrity of the agent as much as possible.~~

~~For very large pathogens such as poxviruses, bacteria and protozoal parasites, protein microarrays may be useful to identify and select antigens which elicit strong immune responses.~~

~~2.1.5. Monoclonal and polyclonal antibodies for indirect antigen detection assays~~

~~Monoclonal antibodies demonstrate unique analytical specificities and are very useful in the detection of agent-specific epitopes at the group, strain or sub-strain levels. As such, they need to be considered carefully with respect to the application at hand and the desired specificity of the assay. Polyclonal antibodies, by nature, tend to demonstrate a broader range of specificities. Purified or semi-purified polyclonal antibodies are often the reagent of choice for trapping complex antigens because they usually demonstrate higher affinities than their monoclonal counterparts.~~

Antigens used to produce antibodies for use as immunological reagents will ideally match their naturally occurring form with regards to their structural conformation and any post-translational modifications. This ensures that the epitopes presented to the host's (the species chosen for antibody production) immune system mimic those presented by the live pathogen, which in turn provides the best chance of producing antibodies relevant to their diagnostic application. Therefore, regardless of whether a crude or purified antigen preparation is to be used, antigen isolation or purification methods should be designed to preserve the antigenic integrity of the target agent. To determine whether a crude or purified antigen preparation is required, it is important to understand the final diagnostic application of the antibodies and whether polyclonal or monoclonal antibodies (MAbs) will be used (see Section A.2.1.5 *Polyclonal and monoclonal antibodies for antigen detection assays* below). Failing to carefully consider these factors may detrimentally affect the specificity of the assay.

Preparations of antigen used to generate polyclonal immune sera or MAbs range from crude preparations of a complete pathogen (e.g. virions in tissue culture medium) to highly purified individual protein constituents derived from the pathogen (e.g. structural or non-structural proteins), and even purified subunits of such proteins (e.g. synthetic peptides). Crude preparations containing the pathogen of interest and other irrelevant antigenic elements will produce a multifaceted antibody response that may negatively affect the specificity of the final assay. Using the example of a crude preparation of virus in tissue culture medium containing fetal bovine serum (FBS), the immune response would be expected to produce antibodies specific to the virus and to the antigenic constituents of FBS, assuming the antiserum is raised in a non-bovine species, such as rabbits or mice, as is typically the case. Application of this antiserum to an immunohistochemical (IHC) examination of a clinical sample of bovine tissue for the presence of the virus of interest would produce confounding results due the nonspecific staining caused by anti-bovine antibodies present in the antiserum. It is worth noting here that this specificity limitation, caused by using a crude antigen preparation, is dependent on the application and may not be a problem where, for example, the IHC examination is of tissue from a non-bovine species. It is also only an issue where polyclonal antisera are used. Although hybridomas produced from mice immunised with crude antigen preparations will produce the same range of virus-specific and nonspecific MAbs, only hybridomas producing the virus-specific MAbs are selected for antibody production.

The use of purified antigen preparations in the generation of antibodies avoids the specificity problems associated with crude antigens by presenting only a single relevant immunogenic antigen or population of antigens to the host's immune system. Purified antigens may take the form of purified pathogen (e.g. purified virus or bacteria preparations), fractions of a pathogen (e.g. a cell wall preparation), individual protein components of a pathogen (e.g. a nucleoprotein preparation) or peptide fragments of such proteins. Techniques for purifying antigens range from differential and density gradient centrifugation to various chromatographic techniques.

Antigens must be immunogenic to generate antibodies. Typically, whole pathogens or full-length proteins derived of pathogens are inherently immunogenic; however, individual peptide fragments of proteins or synthetic peptides may not be. These non-immunogenic peptides are referred to as haptens and must be coupled to larger carrier molecules (e.g. keyhole limpet hemocyanin [KLH]) to make them immunogenic. The immunogenicity of an antigen may be increased using adjuvants, resulting in higher antibody titres. Repeated periodic exposure of a host to the immunogen will further increase the antibody titre. A typical immunisation schedule for the purpose of antibody generation might involve subcutaneous

immunisation of a mouse or rabbit with an immunogenic antigen in adjuvant followed by second and often third immunisations given at approximately 3-week intervals. High titre antiserum is collected approximately 2–3 weeks after the last immunisation (Rahmadane *et al.*, 2017; Shiell *et al.*, 2020.).

Other important considerations when choosing an appropriate antigen for use in the production of antibodies for detection of antigen are surface presentation and epitope immunodominance. Antibodies targeting antigens that appear on the surface of a pathogen will have the widest diagnostic applicability, being suitable for standard indirect antigen detection ELISAs, antigen capture/sandwich assays and immunohistological assays. Using antigens containing immunodominant epitopes will likely ensure robust seroconversion in the antibody-producing host, resulting in antisera of high titre and assay relevance, especially where the assay has a competition format. Where no information exists regarding an appropriate immunodominant antigen, protein or peptide microarrays probed with immune sera from animals exposed to the pathogen may be useful to identify antigens most likely to elicit strong immune responses.

2.1.5. Polyclonal and monoclonal antibodies for antigen detection assays

Polyclonal antibodies (PAbs) for antigen detection assays are typically used in the form of unpurified hyperimmune antisera, produced by harvesting serum from an animal that is successively immunised with an antigen of interest until sufficient specific titre is achieved. Polyclonal antisera are therefore constituted by the polyclonal population of antibodies targeting the antigen of interest, pre-existing host antibodies and all naturally occurring serum proteins. While it is possible to affinity isolate the target antibody away from the host's pre-existing antibodies (or vice versa), this requires complicated chromatographic techniques and should be unnecessary where careful assay design is employed.

Where complex antigenic preparations (e.g. inactivated virus or multimeric protein complexes) are used to raise the antisera, antibodies within the polyclonal population will target multiple proteins and multiple epitopes within each protein in the preparation. For these reasons, when compared with antigen detection assays developed using MAbs, PAbs demonstrate: i) a broader range of specificities, ii) generally higher avidities, iii) potential for greater sensitivity; iv) broader cross-reactivity with similar targets, and v) greater potential for background signal due to nonspecific binding.

Several of these properties of PAbs make them the preferred option for assays that trap complex antigens; namely, their broader range of specificity and high avidity, which contribute to higher sensitivities in these types of assays by trapping more antigen. Their ability to bind a broader range of targets on a pathogen of interest gives PAbs an advantage where genus-, species- or group-specific assays are desired, i.e. where specific strain or variant information is deemed unimportant for the diagnostic application. While consideration must be given to the greater propensity of polyclonal antisera to bind nonspecifically in assays, this issue is often able to be overcome using suitable assay parameters such as, for example, more stringent washes, suitable controls, or competition formats. It should be noted that MAbs can also be used in trapping assays and may present advantages where a sandwich-type assay format is chosen since two MAbs targeting different epitopes will not compete for binding sites.

MAbs for antigen detection assays are typically used as purified preparations due to their ease of purification using simple affinity chromatography methods. Being purified and mono-specific, MAbs tend to produce lower levels of assay background. However, they are also commonly used as crude tissue culture supernatant preparations that contain the secreted MAb and the constituents of the hybridoma culture medium, which may increase background. Current ethical guidelines have led to the elimination of MAbs produced in ascitic fluid as a detection reagent.

MAbs differ from PAbs in their ability to target one specific epitope. For this reason, they are favoured in assays where detection of a single specific antigen or antigenic epitope is desired. For example, where the serotype or subtype of a pathogenic species is determined by epitopic differences in one of its constituent proteins, MAbs may be developed to target each of the different epitopes, thereby providing typing capability. Of course, where an immunodominant epitope is represented more generally across a genus or species, an MAb may also be useful in higher order (e.g. pan-genotype) diagnostic assays.

Recombinant antibodies may be thought of as MAbs that can take many forms and offer a range of advantages, and while not as commonly used as traditional polyclonal and MAbs, they have been incorporated into several assays. Material required for production of recombinant antibodies can be stored virtually as a coding sequence data file (if gene synthesis capability is available) or physically as expression plasmids containing the antibody coding sequence. This eliminates the need for expensive storage of hybridomas in liquid nitrogen and the risk of loss where storage systems fail. Recombinant

antibodies are expressed in transformed/transfected cultured cells (bacterial, mammalian, etc.). They may be expressed in a range of forms from full-length IgG to various subunit forms such as single chain variable domain (scFv) or nanobodies. These smaller forms may be advantageous in diagnostic applications where permeation is a factor e.g. immunohistochemical examination of formalin-fixed paraffin-embedded tissue sections.

Recombinant antibodies may be modified using standard molecular biological cloning techniques to create antibody fusions with protein/peptide tags (e.g. HA or strep tags) or labels (e.g. green fluorescent protein [GFP] or alkaline phosphatase). Tags may be used to facilitate purification or as a target for secondary antibodies in indirect format assays, while the addition of labels allows for development of direct antigen detection, eliminating the need for a secondary antibody step. It should be noted that MAbs, once purified, are able to be chemically modified with tags or labels, however the nature of chemical coupling reactions means placement of the tag/label is random and may interfere with binding. Chemical coupling is also less efficient as it requires additional processing, expertise, time and cost.

Additional benefits of recombinant MAbs lie in the ability to customise the antibody's species (e.g. from mouse to rabbit or human IgG) or isotype (e.g. from IgG1 to IgG2a or IgE) by swapping determinant sequences within the antibody's constant heavy and light chains. Switching species or isotype provides greater flexibility in assay design and applicability by enabling the use of alternative secondary detection reagents. A practical application might be to switch the species of a recombinant MAb from mouse to rabbit to develop an immunohistochemical assay designed to detect antigen in mouse tissue. Using the rabbit version of the recombinant MAb would increase assay specificity by reducing background staining that would be evident where an anti-mouse secondary antibody conjugate would otherwise be required.

When designing assays using PAbs, MAbs or recombinant antibodies to detect antigens, careful consideration must first be given to the desired output of the assay in terms of diagnostic sensitivity and specificity. Within this context, informed decisions on assay format and the choice of antibody type will be pertinent to successful assay development.

2.2. Design of test method

2.2.1. Choice of test

The prologue to a proper test design requires careful consideration of many variables in the context of performance requirements. The choice of assay must be coupled with its intended application, which usually necessitates consensus between the assay developer, statisticians, and other stakeholders such as epidemiologists and regulatory bodies. The role and capacity of the laboratory may influence the test type chosen. If the purpose is to develop a screening test (e.g. during a post-outbreak surveillance period) the emphasis will be on high DSe, high throughput, low cost, technical simplicity, low interpretative skill, etc. If the purpose is to develop a confirmatory test (e.g. for the confirmation of clinical cases or confirmation of positive screening test reactors), a different set of priorities will come into play including high DSp, fast turn-around-times, technical sophistication and interpretative skills. Further purposes and related parameters are available in chapter 1.1.6, Table 1. There are a growing number of point-of-care or pen-side tests that have their own set of additional robustness and ruggedness requirements given the variable conditions of the environment in which they will be used and the skill level of the operator who will performing and interpreting the test.

Aspects affecting choice of test

- Laboratory resources
- Is the assay to be used for screening or confirmatory purposes, or both?
- Will the assay be used as a laboratory or field-based test?
- Will it be used for one or more species? Which ones?
- What are the preferred specimens?
- Will the test be used for typing organisms to group, serotype or strain-specific levels?
- Will the test be applied nationally or internationally?

The antigen ELISA is conceptually the same method employed for the antibody ELISA (Chapter 2.2.1), with the exception that antigen is the targeted analyte, and antibodies are the primary reagents used for capture and detection of antigens. Depending on whether the antigen is adsorbed directly on the microplate or captured by antibodies on a solid phase, along with subsequent detection steps, different formats are available.

It is not always apparent which assay format should be used to best fit the intended purpose. Availability of reagents and the limit of detection of the assay for a particular application may be significant factors

in limiting the choice. Since many of the systems now target highly specific antigens, the choice of antibody for both trapping and/or detection becomes critical.

Preparation of the test sample is also a critical consideration depending on the test format being used. The use of trapping or capture antibody in sandwich-type assays enhances selectivity and reduces potential matrix effects. For assays requiring direct application of the analyte to the solid phase, preparatory extraction, centrifugation or filtration methods may be necessary to remove extraneous material. For a more in-depth discussion of these different assay formats, please see Crowther (2001).

Of critical importance is the size and complexity of antigen and the availability of relevant reagents, such as capture antibodies (e.g. antigens to be detected in sandwich assays must have at least two unfettered epitopes) which limits this assay type to relatively large antigens or whole pathogens. The affinities of the immunological reagents come into play as the stability of the resulting antibody-antigen complexes in the microplate or on beads will affect the performance characteristics of the assay.

Practical concerns are availability and use of antigen standards for quality control and assurance purposes, repeatability, reproducibility, throughput capacity, turn-around-time of a test result, cost and technical sophistication and interpretation skills.

Working with exotic and/or zoonotic agents requires particular attention to biosafety and biosecurity regulations (see also Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*) and will inform the laboratory what personal protective equipment or biosafety level should be employed to ensure operators are kept safe during the testing.

2.3. Proof of concept experiments (feasibility studies)

The same types of experiments used in antibody detection tests are required for antigen detection tests (see Chapter 2.2.1).

2.4. Samples and data expression

2.4.1. Selection, storage and use of control samples for test development and validation studies

It is important to assess and monitor sensitivity and specificity of the test during development and validation. This is achieved by selecting several samples (4–5 is adequate) that range from negative to high levels of antigen of the analyte in question. These samples are used in experiments designed to optimise the assay. To achieve continuity of evidence requires care and forethought in preparation and storage of samples. A large volume (e.g. 10 ml) of each control sample is acquired and divided into 0.1 ml aliquots for storage at or below –80°C (Chapter 1.1.2.). One aliquot of each sample is thawed, used for experiments, and ideally then discarded. If it is impractical to discard the aliquot, it may be held at 4°C between experiments for up to about 2 weeks; however, there is a possibility of sample deterioration under these circumstances, which will vary with sample and antigen types. Then, another aliquot is thawed for further experimentation. This method provides the same source of sample with the same number of freeze–thaw cycles for all experiments (repeated freezing and thawing of samples can denature antigen and/or facilitates the growth of other unwanted microorganisms and should be avoided). Also, variation is reduced when the experimenter uses the same source of sample for all

Optimisation and standardisation

- Is the test able to distinguish between samples with and without the analyte (what is the limit of detection = analytical sensitivity?)
- Does the test cross-react with non-target antigen in the sample or sample matrix (analytical specificity)?
- What is the noise or background activity in a negative sample?
- Has the repeatability been assessed for a range of control samples over a number of days?
- Are sufficient positive and negative samples on hand to carry out the experiments for optimisation and validation?
- If yes, are reference and control samples dispensed and stored properly to avoid introduction of sample related bias (sample deterioration)?
- Have all critical reagents been run against each other in checkerboard titrations?
- ~~Did you find~~ Were optimal concentration/dilutions established for each reagent?
- ~~Did you include~~ Were reference reagents and working standards/controls included and did normalised ~~the~~ OD data ~~to~~ achieve the best possible comparative results?
- Are the controls in the same matrix as the test samples?

experiments rather than switching among various samples between experiments. This approach has the added advantage of generating a data trail for the repeatedly run samples. After the initial stages of assay validation are completed, one or more of the samples can become reference reagent(s) that are the basis for data expression and repeatability assessments both within and between runs of the assay (Jacobson, 1998). They may also serve as in-house working standards if their activity has been predetermined; such standards provide assurance that runs of the assay are producing accurate data (Wright, 1998).

2.4.2. Normalisation of results and their expression

The same normalisation procedures as used for antibody detection assays are applicable to antigen detection assays (see Chapter 2.2.1 for details).

2.5. Optimisation

The aim of this stage is to finalise test parameters related to reagents, consumables and equipment that will lead to a fixed protocol and will be used during the assay validation pathway Part B. (for details, see Chapter 2.2.1).

2.6. Inhibitory factors in sample matrix

Due to a higher variety and complexity of specimens, antigen detection assays are more likely to be influenced by matrix factors than antibody detection assays, which normally detect antibodies in serum. Inhibitory substances are frequently found in complex matrixes such as pus, semen, tracheal/nasal/cloacal swabs and may have an impact on the test result. ELISA antigen detection systems are rather resistant to inhibitory factors, please see ~~the WOAH Validation Standard,~~ Chapter 1.1.6, Section A.2.4~~,~~ and B1.2 and Greiner *et al.* (1997) for descriptions of the type of inhibitors that could affect the assay. These references should be reviewed carefully to assure that all inhibitory factors are accounted for and controlled.

2.7. Calibration to reference sample and comparison to standard test method

Chapter 2.2.1 contains information relevant to this procedure.

B. ASSAY VALIDATION PATHWAY

1. Stage 1 – Analytical performance characteristics

Factors that influence the analytical characteristics of diagnostic assays are numerous and may vary according to each assay type, e.g. the main factors affecting the analytical characteristics of serological (Chapter 2.2.1.) and molecular assays are described in Bowden *et al.* (2021).

1.1. Repeatability

~~Chapter 2.2.1.~~

Repeatability of an assay over time and when run by different operators is of vital importance to the validation pathway. For ELISAs, raw absorbance values are usually used at this early stage. To obtain preliminary estimates of repeatability it is recommended to perform three to four replicates of each control sample, run in at least five plates on five separate occasions. Coefficients of variation (CV) (SD of replicates divided by mean of replicates) of less than 20% are considered to be acceptable (Jacobson, 1998, Chapter 1.1.6. A2.5., B1.1. and Bowden *et al.*, 2021). Assessing the inter-operator variation is important if the test will be performed by different staff and provides information about robustness and fitness for purpose in a laboratory as a diagnostic assay.

Analytical performance characteristics

- Has repeatability been established for a range of positive and negative samples within and between runs of the assay?
- Have upper and lower control limits of the assay been established and is an alternative procedure in place if results fall outside limits?
- Has ASe and ASp for the assay been defined?
- Does the test under evaluation compare favourably with a standard test method, based on objective quantitative and qualitative criteria?

1.2. Analytical specificity

Analytical specificity (ASp) is defined as the degree to which the assay distinguishes between the target analyte and other components that may be detected in the sample matrix (see Chapter 2.2.6, Section B.1). The higher the ASp, the lower the number of false positive results. Selectivity, exclusivity and inclusivity are components of ASp (Chapter B1.2). **Selectivity** refers to the extent to which an antigen detection method can accurately quantify the targeted analyte in the presence of: 1) interferents such as matrix components (e.g. inhibitors of enzymes in the reaction mix; 2) degradants (e.g. toxic factors) and 3) nonspecific binding of reactants to a solid phase (e.g. conjugate of an ELISA adsorbed to well of microtiter plate). One way of establishing if there are inhibitory factors in a sample is to test a dilution series. For antigen ELISAs, the resulting optical density value will increase due to the dilution of the inhibitory substance. **Exclusivity** ~~ASp~~ should be determined by testing well characterised samples from similar or related pathogens, which produce similar lesions as the target pathogen or are frequently found in samples containing the target pathogen. For ~~example, to assess the ASp of~~ an FMD antigen ~~detection~~ ELISA ~~for one particular serotype (e.g.~~ that detects serotype O~~,~~ ~~), it is necessary to assess its reactivity of all sub-strains within this serotype (e.g. O Campos, O Manisa, etc.) to assess inclusivity. At the same time it is important to show that the test does not cross react with other serotypes such as A, Asia 1, C, SAT 1, 2 and 3. Finally, there is also a need to assess whether the test cross-reacts with agents from diseases which may produce similar signs, e.g.~~ a lack of cross-reaction with vesicular stomatitis, swine vesicular disease and swine vesicular exanthema would be an example of exclusivity testing. Another example is that the same test does not cross-react with other FMD serotypes such as A, Asia 1, C, SAT 1, 2 and 3. This would also define a confirmatory assay. **Inclusivity** can be assessed for one particular serotype e.g. serotype O by testing the assay's reactivity of sub-strains within this serotype, e.g. O Campos, O Manisa, etc. Another example for ASp is an ELISA designed to detect avian influenza virus: as a screening test the assay should detect the nucleoprotein or matrix antigen of all subtypes, e.g. H1-H16 and N1-N9 (inclusivity). However it should not cross-react with viruses which cause similar clinical signs such as Newcastle disease or infectious bursal disease (~~diagnostic specificity~~ Exclusivity) or with other non-specific components present in the matrix or on the solid phase~~. Some ELISAs may be subject to false positive results attributable to non-specific factors,~~ (Selectivity). Blocking agents such as bovine serum albumin and the use of detergents, for example Tween 20 in the wash buffer assist in the prevention of non-specific binding ~~of antibody conjugates to the plastic surface and may require use of blocking agents~~ and background reactivity. Care must be taken to eliminate these ~~types of errors~~ effects.

1.3. Analytical sensitivity

Analytical sensitivity (ASe) is synonymous with the lower limit of detection (LOD) of antigen concentration in a sample. LODs are usually determined by endpoint dilution in which replicates (preferably 10) of each dilution in a $\log_2$ dilution series are run in the assay. The larger the number of replicates, the more precise the determination of the dilution at which the antigen is no longer detectable. ~~Further information on LODs and ASe is given in the WOAH Validation Standard and in Chapter 2.2.5~~ or below the cut-off respectively (Chapter 1.1.6. B1.3.).

Screening assays or assays which are designed to detect sub-clinical infections or carriers should have a ~~very~~ high ASe. In these cases it may be difficult to obtain suitable samples ~~and~~ to determine the comparative ASe by running a panel of samples on the ~~candidate assay~~ test under evaluation (TUE) and on another independent assay. If available, serial samples from experimentally infected animals could provide temporal information about the assay's capacity to detect antigen over the course of infection. Further information about LOD and ASe experiments is given in Bowden *et al.* (2021), Chapter 1.1.6., Chapter 2.2.5. and Chapter 2.2.8 *Comparability of assays after changes in a validated test method.*

1.4. Standard test method comparison with the ~~candidate~~ test ~~method~~ under evaluation (TUE)

~~Chapter 2.2.1.~~

The TUE should be run in parallel with a WOAH or other accepted reference test method, using the same panel of samples on both, to determine whether the TUE exhibits the same quantitative and qualitative characteristics as the standard method. Favourable comparability lends strength to the belief that the TUE will be a successful substitute for the reference method (see also methods comparison studies, Chapter 2.2.8 and Reising *et al.*, 2021).

2. Stage 2 – Diagnostic performance characteristics

See also Chapter 2.2.1. and Chapter 1.1.6. B stage 2.

2.1. The challenge in establishing accurate estimates of DSe and DSp for antigen assays

Diagnostic performance characteristics

- Are the criteria used to determine the positive and negative reference populations legitimate?
- Do the reference samples with which the assay was validated fully represent the ~~population~~ target~~ed by~~ population where the assay will be applied?
- Were there difficulties in obtaining a sufficient number of samples? If so, how was the problem addressed?
- Were the samples collected and stored appropriately to minimise deterioration?

For all antigen detection assays including ELISAs, particular consideration must be given to the timing when the sample is taken as the probability of detecting antigen or the pathogen itself is very closely linked to the stage of infection. The diagnostic window will likely be much smaller in antigen/pathogen detection assays than in antibody detection tests as immune responses can normally be measured over an extended period of time. Dynamics of infection, e.g. acute, persistent, sub-acute, chronic or carrier-state are important determinants for sampling recommendations. For example, during an acute viral infection, the sample should be taken as early as possible after the onset of clinical signs. In persistent, sub-acute, chronic or carrier animals there is a balanced relation between the pathogen and the host and the agent may be present in minute concentrations which may be very difficult to detect. During the course of pathogenesis, other organ systems may become involved and different tissues or fluids may be more appropriate target tissues for sampling as the disease progresses. This information is provided in the disease-specific chapters of this WOAH *Terrestrial Manual*.

2.2. Reference animal populations

2.2.1. Animals of "known infection status"

Depending on the composition of positive and negative reference samples, the same test may have different estimates for DSe and DSp. Ideally the composition of reference samples and animals should match as closely as possible the samples which are expected from the target population for which the test was developed. It is important to have a clear case definition. This is a set of criteria used to decide whether an individual or group of animals is infected or not. The reference status must relate to the purpose of the testing. For example, if the purpose of the assay is to be used as a screening test to detect early infection of FMD (e.g. in free-ranging cattle with vesicles), then the majority of samples to determine DSe and DSp should be taken from this target population. Relevant information should be collected and summarised for all animals involved at this stage of validation (e.g. species, age, sex, breed), and information on other factors that are known to influence DSe and DSp (e.g. date and place of sampling, immunological status, vaccination and disease history, pathognomonic and surrogate tests used to define status of animals, prevalence within population and description how the reference status was derived).

A sample from a negative reference animal refers to lack of exposure to or infection with the agent in question. For example, a classical swine fever negative population could be defined as a region with pig herds without confirmed clinical cases of the disease in recent years, supported by negative serological tests and negative virological test results of suspected cases. Samples from these animals fulfil the status of negative reference samples. The negative reference population should be chosen with care so as to ensure that it is representative and matches the positive reference population (e.g. as to the breed and exposure to environmental challenge). Another source of negative reference animal samples depending on the sample type is sample collection pre-inoculation for animal trials; numbers for these sample types may be limited. If vaccination is carried out, it may interfere with antigen detection (e.g. vaccination with modified live viral vaccines). Samples from these animals should not qualify as negative reference samples.

The types and limitations of reference standards commonly used for evaluation of the performance characteristics of a new assay are listed here. An expanded description of each reference standard is provided in Chapter 2.2.1, Section B.2.2.1 and in Jacobson (1998). The strengths and limitations of these reference standards must be considered carefully when using samples, derived from animals that fall into any of the following four categories, as sources for establishing DSe and DSp for a ~~candidate assay~~ TUE (Jacobson, 1998).

i) An unequivocal reference standard

An unequivocal reference standard: presence of the agent in the host or evidence of definitive (pathognomonic) histopathology.

ii) A composite reference standard

A composite reference standard: verification of uninfected or unexposed animals.

iii) A relative reference standard

A relative reference standard: reference animals that have been classified for their infection status by comparison with the test results of another assay for antigen or nucleic acid detection on the same samples. As with antibody assays, the estimates of DSe and DSp are useful only insofar as the reference test has documented, established and acceptable performance characteristics. A deficiency of relative reference standards is that they have their own established levels of FP and FN test results, which are sources of error that will be compounded in estimates of DSe and DSp for the new assay. Generally, however, the use of other well described test methods is regarded as good practice to determine the status of reference animals, but only if the inherent bias introduced by the relative reference standard is accounted for.

iv) An adjunct reference standard: experimental infection or vaccination

See ~~the WOAH Validation Standard~~ Chapter 1.1.6, Section B.2.3 for significant limitations of this type of standard. Note: In the context of antigen detection, "comparative" tests should include pathogen detection by isolation, culture, NAD tests or histopathology or other *in-situ* techniques.

v) Heuer & Stevenson (2021) describes procedures for diagnostic test validation studies when there is a perfect reference standard available for either positive or negative animals or both.

2.2.2. Latent-class models ~~for sample selection~~ of DSe and DSp

For a discussion of this approach ~~to sample selection~~, see ~~the WOAH Validation Standard~~ Chapter 1.1.6, Section B.2.5 ~~and~~, Chapter 2.2.6 and Cheung *et al.* (2021).

2.3. Threshold (cut-off) determination

It is important to clearly describe the method and the samples used for selecting a cut-off. It is strongly recommended to conduct a receiver operating characteristic (ROC) analysis to show the potential performance of the test in other epidemiological settings (Chapter 1.1.6., section B.2.4.).

3. Stage 3 – Reproducibility and augmented repeatability estimates

Reproducibility assessments for antigen detection assays are not uniquely different from similar assessments for any other type of assay. Therefore, the reader is directed to ~~the WOAH Validation Standard,~~ Chapter 1.1.6, Section 3 and ~~for~~ details on reproducibility analysis and for reference samples and panels to Chapter 2.2.6 ~~WOAH provides guidelines~~. Further information about reproducibility is available from Johnson & Cabuang (2021) and Waugh & Clark (2021). Samples provided from Proficiency Test rounds can be used for ~~laboratory proficiency~~ comparability testing ~~(https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-5)~~ of a reference test and TUE (Johnson & Cabuang, 2021; Waugh & Clark, 2021).

4. Stage 4 – Programme implementation

4.1. Interpretation of results

See the Chapter 2.2.1, Section B.4.1.

5. Monitoring assay performance

5.1. Monitoring the assay

After performance characteristics of the ~~new test~~ TUE have been established, on-going monitoring, maintenance and enhancement are required. It is important to continue monitoring the repeatability and reproducibility of the assay over time, on a per run basis, quality control samples have to fall within pre-established limits. If not, the test result is not valid and ~~has~~ established alternative procedures have to be ~~repeated~~ followed. Monitoring the assay controls' performance over time is an important way to detect changes or trends in the assay. Simple analysis of results, e.g. statistical assessment of mean values, standard deviation and coefficient of variation are useful in this process and results can be plotted in a control chart. Participation in external quality control or proficiency testing programs is a requirement of ISO 17025 and useful to identify random and systematic errors and provide credibility in test results. Further information about this topic can be obtained from Crowther *et al.*

(2006) and in the Chapter 2.2.1, Section B.5.1. This also provides valuable information on the stability of assay reagents over a time period.

5.2. Minor modifications of the assay – replacement of depleted reagents

Over time changes in the test protocol may be necessary due to better or less costly reagents or because the target analyte has changed. Batch-to-batch variation of biological reagents is considered a major contributor to test variation. When reagents such as antibodies or antigen must be replaced they should be produced or procured using the same protocols or criteria as used for the original reagents. New biological reagents (e.g. control samples, antigen, capture or detection antibodies, conjugate, chemicals or consumables) need to be assessed for comparability. Chapter 2.2.8 *Comparability of assays after changes in a validated test method* gives an overview of acceptable comparability studies. A ground rule is never to change more than one reagent at a time in order to avoid the compound problem of evaluating more than one variable concurrently (see also Chapter 2.2.1, Section B.5.2, Chapter 2.2.8. and Reising *et al.*, 2021).

5.3. Major modifications of the assay – changing to a new ELISA type

It is a major challenge of laboratory diagnosis to keep up with the evolving nature of infectious pathogens. Over time pathogens may change their antigenic characteristics and new strains may emerge, e.g. the emergence of Bluetongue virus serotype 8 in northern Europe in 2009. This change necessitates a full test development and validation study. Another major change is the use of a test in a different species than that for which it was originally validated, e.g. there may be a requirement to use an FMD Ag detection ELISA, validated in cattle, for testing camelids or buffalo in different geographical and climatic regions (Kirkland & Newberry, 2021). Evaluation of reference samples that represent those populations of Stage 2 in Figure 1 of the ~~WOAH Validation Standard~~ Chapter 1.1.6 will accomplish this requirement (see also Chapter 2.2.1, Section B.5.3).

5.4. Enhancing confidence in validation criteria

Due to the extensive set of variables that have an impact on the performance of antigen detection assays, it is useful to expand the number of reference samples when possible, due to the principle that error is reduced with increasing sample size ~~(see also Chapter 2.2.1, Section B.5.4)~~.

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Annexe 11. Chapitre 2.2.3. Mise au point et optimisation des méthodes de détection de l'acide nucléique

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CHAPTER 2.2.3.

DEVELOPMENT AND OPTIMISATION OF NUCLEIC ACID DETECTION ASSAYS

INTRODUCTION

The WOAH Validation Recommendations ~~in Section 2.2~~ Validation of diagnostic tests *~~of this~~* Terrestrial Manual *~~provide~~ provide detailed information and examples in support of ~~the WOAH Validation Standard that is published as~~ Chapter 1.1.6* Validation of diagnostic assays for infectious diseases of terrestrial animals. *~~The Term "WOAH Validation Standard" in this chapter should be taken as referring to that chapter.~~ An up-to-date compilation of the relevant validation standards (WOAH and non-WOAH) and guidance documents for all stages of diagnostic test validation and proficiency testing, including design, analysis as well as clear, case studies are provided in the WOAH* Scientific and Technical Review *issue Diagnostic Test Validation Science (Colling & Gardner, 2021). Published standards for peer-reviewed reporting of accuracy studies (STARD) are available for infectious diseases of humans and terrestrial animals (paratuberculosis, Bayesian latent class model) (Bossuyt* et al., *2015; Gardner* et al., *2011; Kostoulas* et al., *2017).*

~~An ever increasing number of~~ Nucleic acid detection (NAD) tests are ~~now being used~~ a primary diagnostic tool for ~~diagnosis~~ the detection of infectious diseases in various species of animals and man. ~~The most common methods are the~~ Polymerase chain reaction (PCR)~~, of which there are a number of variations and~~ is the method of choice in the veterinary clinical laboratory with point-of-care (POC) devices and technologies, *such as isothermal amplification methods ~~(such as loop-mediated isothermal amplification)~~,* gaining traction in field studies*. In addition, solid-phase or liquid phase microarrays are ~~appearing as new~~ useful tools ~~of biotechnology based diagnosis~~ for screening changes in gene expression. The amplification techniques employed in these assays ~~provide them with~~ often result in high analytical sensitivity. The products of the amplification reaction can be detected ~~in a number of ways, for example~~, by visualisation in agarose gels~~,~~ or by using DNA-binding dyes, or labelled probes (e.g. ~~Taqman~~ hydrolysis probes~~), by the detection of the accumulation of the products in real time or by using arrays where specific probes are captured on a~~ for real-time (rt)PCR, and solid-surface matrix or ~~on~~ beads for microarrays). (Lauerman, 2004; Viljoen* et al., *2005*). *Different PCR assays can be multiplexed together in a single reaction well to detect several targets in one tube or to combine targeted analytes and controls that generate different amplification products~~, all in one reaction vessel~~. Whilst this has obvious advantages, ~~great care must be taken during~~ it also requires significant optimisation and validation to ensure that assay performance is not compromised. Similarly, a multiple outcome PCR can be created using one set of primers~~, but employing~~ and tagged probes, ~~which~~ that bind to different target sequences ~~in the~~ represented by different species or strains detected by the PCR* (*Wakeley* et al., *2005; 2006*).

NAD amplification techniques are usually based on the principle that there is an exponential amplification of the specific sequence targeted in the reaction. This provides ~~a~~ NAD with high analytical sensitivity and specificity~~. Due to this characteristic high level of sensitivity reached with NAD, special care,~~ but also requires uni-directional workflows and ~~indeed special~~ precautionary steps ~~have to be taken~~ to prevent NAD amplicon contamination ~~to~~ of the environment and subsequent sample analyses. ~~This~~ Contamination is more likely to be a problem where reaction tubes are opened in the laboratory for further processing, for example, to run

gels or to perform nested assays. To avoid such contamination, strict laboratory protocols should be employed involving separate rooms or cabinets for particular stages of the assays, changes of laboratory gowns and gloves, and stringent cleaning programmes (*Viljoen* et al., *2005; Subcommittee of Animal Health Laboratory Standards*). *For these reasons, tests based on closed tube systems are generally more suitable for diagnostic assays* (*Sawyer* et al., *2006*). *As with all assays, it is important to use appropriate controls to prove that the assay is performing as expected. All samples used in assay development should have well established provenance and ~~the~~ assay development and validation should be carried out within the framework of a quality system to ensure appropriate levels of training, equipment maintenance and monitoring, etc.* (*Burkhardt, 2000*).

A. ASSAY DEVELOPMENT PATHWAY

1. Definition of the intended purpose(s) for an assay

Purpose of the assay:

- Is it for a screening or confirmatory test, or both?
- Is it for detection of a group of pathogens?
- Is it for detecting a single disease agent?
- Is it for discriminating between vaccinated and infected animals?

The first consideration in assay development is to define ~~clearly~~ the specific purpose and application of the test to be developed. The purpose and ~~to understand how it~~ application will ~~be applied because this~~ inform~~s~~ many of the decisions of the development pathway. For example ~~one might choose~~, to develop a screening test to detect all avian influenza (AI) subtypes or variants in birds ~~for which~~, an inclusive and broadly reactive test is necessary~~, or~~. In contrast, to determine the haemagglutinin type, ~~in which case~~ a more specific test is needed. ~~For~~ Some tests ~~the requirement is~~ are designed to detect a group of viruses, e.g. the pan-Pestivirus PCR that detects bovine viral diarrhoea (BVD), border disease (BD) and classical swine fever (CSF) viruses. ~~For other tests, the requirement may be~~, whereas others are designed to detect a single agent, or ~~sometimes even to allow~~ a particular immune status using a DIVA (differentiating infected from vaccinated animals) approach. ~~An~~ Examples of such ~~a method is~~ methods include the ~~recently published~~ real-time ~~RT~~ PCR assays for CSF virus ~~which was developed for the genetic differentiation of naturally~~, and lumpy skin disease (LSD) virus, which are able to differentiate animals infected ~~from~~ with wild type viruses and vaccinated ~~wild boar~~ animals (Agianniotaki *et al.*, 2017; Liu *et al.*, 2009; Wolff *et al.*, 2021).

2. Assay development – experimentation

2.1. Quality assurance

It is important that assays are developed in laboratories where high standards of quality assurance and control are employed (see Chapter 1.1.5 *Quality management in veterinary testing laboratories*). The validation data for test performance and accuracy determined during the development and validation phase must be robust as it will form the basis for interpretation of disease status and consequent actions when the assay is routinely used. Laboratories should keep accurate records of samples, reagents and methods used in test validation and store summary data and reports in secure, communal databases for ready access and review. Where assays are to undergo accreditation with national agencies, comprehensive reports should be prepared describing samples used, replicates and statistical analyses for each validation stage.

2.2. Reference materials

- What kinds of samples will be used from the target population?
- What are the predilection sites for the agent in the host
- ~~Will samples be single or pooled?~~
- What sample collection, storage and transport methods are anticipated and what are the possible effects on results?
- Will samples in the validation panel be representative of the target population?

Sample selection (see Chapter 1.1.2 *Collection, submission and storage of diagnostic specimens*). For further information on biobanks and selection and use of reference samples please see Watson *et al.* (2021) and Chapter 2.2.6. *Selection and use of reference samples and panels*, respectively.

For most diseases, the samples required for NAD assays are likely to be similar to those used for ~~current~~ other detection methods ~~such as bacterial cultivation or virus isolation.~~, for example, detection of AI by real-time RT-PCR and virus isolation use the same samples. ~~Recently~~ Wherever possible, reference samples that will be used to develop and assess the sensitivity and

specificity of the assay should resemble the type of sample that will be used in the diagnostic setting. Therefore, it is important to understand the biology of the pathogen concerned and the tissues that will be infected to select an appropriate sample type. For assay validation, the choice of reference material must reflect the likely diagnostic setting and include consideration of: sample type, sample matrix, presence and effect of inhibitory substances, type of collection device, sample buffers and transport/storage conditions, target population/species and whether the assay will be performed in the laboratory or field/pen-side. Recent trends in sampling techniques to minimise or eliminate invasive methods must be considered when choosing reference materials. These changes in sampling have several benefits including improving ease of, and access to, sampling sites for the field practitioner, reduction in biohazard risks, such as tissue homogenisation and aerosol generation and minimising sample processing times in the laboratory and field. Examples include swabbing of fresh cuts in organ samples ~~proved to be a practical approach, replacing the laborious~~ rather than homogenisation of organ samples~~. Similarly, there is a trend to use samples, which can be obtained by non-invasive methods, such as~~ (Errington *et al.,* 2014), use of saliva for detection of porcine reproductive and respiratory syndrome (PRRS) in pigs (Prickett *et al.,* 2008), ~~or~~ collection of oral fluids using cotton ropes for detections of foot and mouth disease virus (FMDV) and African swine fever virus (ASFV) (Goonewardene *et al.,* 2021; Mouchantat *et al.,* 2014; Vosloo *et al.,* 2015) and bulk milk samples for determination of herd BVD status. Prior to the selection of pooled samples, such as bulk milk, test developers need to consider and assess the implications of dilution of the target analyte on diagnostic sensitivity and incorporate this into the validation plan (see below). Faecal samples are widely used for detection of different viruses associated with gastrointestinal diseases and hair samples can be used for detection of some viruses e.g. border disease virus (Kalaiyarasu *et al.,* 2019) and bovine viral diarrhoea virus (Singh *et al.,* 2011; Zoccola *et al.,* 2017).

~~It is important to understand the biology of the pathogen concerned and the nature of the sample collection devices. As in the case of AI, different subtypes or variants of the viruses have different predilection sites in host birds. For example cloacal samples are appropriate for some subtypes, whereas buccal samples are acceptable for others. Therefore, a test for use in a surveillance programme would need to incorporate both sampling sites and be validated using both types of sample. Another significant factor is the matrix in which the analyte resides in the host. Cloacal samples are more likely to contain PCR inhibitors than buccal samples. Another potential confounding factor is the type of swab used to collect the samples; some contain materials that inhibit PCR assays. Therefore, it is very important to specify precisely the preferred sample material and to describe fully the swabs and swabbing protocol, including the preferred buffers or transport media and storage conditions.~~

~~Consideration also should be given to whether samples should be tested singly or in pools and whether pools should be of different samples from a single or multiple animals. Any pooling strategy should be precisely defined and validated prior to use. Finally, if the target population is "birds", the validation study should cover a large representative population of different species to demonstrate that the assay is widely applicable, whilst concentrating on the most prevalent species or those used as sentinels of infection.~~

2.3. Design of test method (fitness for purposes)

2.3.1. ~~Choice~~ Purpose of test and target sequence

The purpose of the test under evaluation (TUE) will largely determine the choice of nucleic acid (NA) target sequence. For emerging animal disease surveillance usually ~~activities~~ large numbers of samples may be tested first by a broad screening assay~~. In the above example for AI, the screening assay described must detect all known subtypes or variants of Influenza A, either in a particular region or throughout the world. The test must be highly sensitive so it does not miss true positive samples, and analytically specific (inclusive) for detection of all viruses in the Influenza A group~~ followed by specific agent characterisation assays. Where broad screening strategies are necessary, consideration must be given to whether these will be conducted in a laboratory with automation to achieve high throughput, or without, or in the field using a pen-side assay.

- Is the test for use only in a particular region or world-wide?
- Is it sufficiently sensitive and is the analytical target inclusive enough to not miss positive samples?
- Consider the impact of a high proportion of false positive results that cannot be confirmed.
- Are rapid results possible and/or necessary?
- Will confirmatory tests (an analytical tool) be used to determine/confirm pathogen pathotype (strain)?
- Will determination of the strain have important bearing on the action taken?

Using AI as an example, the screening assay must detect all known subtypes or variants of influenza A, either in a particular region or throughout the world. The test must be highly sensitive and analytically specific (inclusive) for detection of all viruses in the Influenza A group. ~~Avian Influenza is a high profile disease. If a new test should~~ It should not, however, generate a high proportion of false positive results that cannot otherwise be confirmed, as this may make the infection status of the birds ~~would be~~ difficult

to resolve. Exclusion of closely related agents that are not of interest within the context of the intended purpose of the assay is therefore essential.

~~To continue with the AI screening example, it is also important for the test to produce rapid results so that disease control measures can be swiftly applied. So, for the detection of avian influenza, the logical choice of tests would be a real time PCR using TaqMan probes or a field based assay. For an AI screening assay, the primers and probes are likely to be based upon the Matrix (M) gene which is known to be present in all influenza type A isolates. Dependent on the assigned fitness for purpose, the scientists may choose to develop an additional assay for use in conjunction with the screening test to determine which particular strain of AI is present and whether the strain is highly pathogenic, because this has an important bearing on the notification requirements and control measures that may follow. Some test methods may qualify as secondary analytic tools that are applied to the analyte detected in the primary assays, and may be used to further characterise the product detected in the screening assay (see the WOAH Validation Standard, Section B.1.4). An example of such an analytic method would include sequence analysis of the matrix PCR amplicon detected during AI screening. Development of paired confirmatory assays such as real-time PCR assays that target other genes, such as the AI haemagglutinin or neuramindase to identify subtype or pathotype, would require that those assays also undergo all steps of development, optimisation and validation. Recently a novel, plexus primers cased PCR assay was developed at the WOAH Collaborating Centre for the Biotechnology based Diagnosis of Infectious Diseases in Uppsala, Sweden. This assay allows the simple and rapid determination of various RNA virus pathotypes. For example, avian influenza viruses and Newcastle disease viruses can be detected and evaluated with low-cost equipment (PCR machine) and in a very short period of time (less than three hours), in simply equipped laboratories or even on site, in the field, during outbreak situations (Leijon *et al.*, 2011; Yacoub *et al.*, 2012). This indicates a very rapid development and simplification in the field of NAD tests in diagnostics towards field/on-site applications.~~

~~2.3.2. Method design~~

~~The method should be carefully designed to fulfil the purpose that was defined at the outset. Important factors to consider will include application, types, and numbers of samples to be tested. This, in turn, leads to logistical and practical considerations for the candidate assay, such as whether it will be conducted in a laboratory, with or without automation to achieve high throughput, or in the field using a pen-side assay.~~

- ~~Was the assay first evaluated on a small sample panel (six to eight samples) to assess viability of the approach?~~
- ~~Was there good separation between test results of negative and positive samples?~~
- ~~Was a preliminary test made on a dilution series in matrix to assess preliminary relative analytical sensitivity?~~

All available information such as publications, sequences deposited in databases and in-house sequence data should be used~~. Sophisticated software is available to optimise primer and probe design; computer modelling of probable sequences for use in the assay is a first step.~~ for target sequence design. Software is available to model suitable target sequences and optimise primer and probe design. The critical design features are target identification; primer/probe characterisation; and assay optimisation. The first two steps are carried out by *in-silico* analysis, and the third by experimental investigation. For further information refer to Bustin & Huggett, 2017; Rodriguez *et al.*, 2015; Thornton & Basu, 2015; and Guide to PCR Qiagen[1]. The main features of the steps are:

i) Target identification – use the purpose of the TUE and *in-silico* alignments of representative isolates to determine the appropriate sequence target of the pathogen

ii) Primer and probe characterisation:

a) Melting temperature (Tm) – to obtain best performance for standard hydrolysis probe PCR assay, the Tm of the probe is normally 8–10°C higher than that of primers, and the Tms of the primers differs by less than 1°C;

b) Primer and probe length between 15 and 30 bp; PCR amplicon length is between 50 and 150 bp (optimum <80 bp).

c) Avoid formation of primer-dimer and hairpins within and between primers and probe.

d) Where degenerate nucleotides are used, avoid degeneracy in the three nucleotides at the 3' end; and try to design primers with less than four-fold degeneracy at any given position

[1] https://www.qiagen.com/us/knowledge-and-support/knowledge-hub/bench-guide/pcr/introduction/guidelines-for-pcr

iii) Assay optimisation via experimental methods of:

a) Primer and probe concentration, and combination

b) Annealing/extension temperature of PCR reaction

c) Concentration of Mg^{2+} and all enzymes (e.g. reverse transcriptase [if applicable] and DNA polymerase)

d) PCR additives and enhancers (e.g. dimethyl sulfoxide [DMSO], bovine serum album [BSA] and glycerol) for specific target sequences (e.g. GC rich).

2.3.2 Ancillary or supplementary tests

Ancillary or supplementary tests may be used in conjunction with the screening test to assist with agent characterisation, because this has an important bearing on the notification requirements and control measures that may follow (see Chapter 1.1.6, Section B.1.4). Examples include sequence analysis of the matrix PCR amplicon detected during AI screening and real-time PCR assays for haemagglutinin or neuraminidase. Ancillary assays that provide additional agent characterisation also require development, optimisation, and limited validation to determine fitness-for-purpose.

2.3.3 Point of care (POC) tests

During the last few years, POC (penside) testing has gained popularity for use in veterinary diagnostics (Halpin *et al.*, 2021; Hobbs *et al.*, 2020) because of its advantages such as (1) portability and ease of use, (2) rapid turnaround time for results; and (3) no need to ship samples to central laboratories (reviewed by Chander, 2023). The rapid rise in the development of POC tests reflects the desire of veterinarians, regulators, and producers to make timely decisions about animal health to instigate appropriate controls if needed.

- Optimal location? Use a clean room or cabinet system to minimise risk of contamination.
- Standardise sample extraction for manual and/or by automated preparation.
- ~~Optimal method of sample extraction to prepare samples? Done manually or by automation?~~

Isothermal amplification-based techniques, such as LAMP, helicase-dependent amplification (HAD) and recombinase polymerase amplification (RPA), have been applied as POC tests for a variety of agents (Bath *et al.*, 2020; Hassan *et al.*, 2022; Velayudhan & Naikare, 2022). Care must be exercised in the interpretation of negative results in suspect animals, to avoid false negatives being reported where viral loads are below the sensitivity threshold of the POC test. Where POC tests are supported by laboratory-based assays, comparative studies of both assays using, where possible, identical reference and diagnostic samples, should be conducted to fully appreciate any differences in sensitivity or specificity.

2.4. Feasibility study

Before embarking on the validation of a newly developed prototype assay, a feasibility study should be carried out using a small panel of ~~approximately~~ six or eight well-characterised samples, covering the operational range of the assay, to assess whether the system is viable. The panel should ~~consist of samples distributed across the operating range of the assay, i.e.~~ include at least two negatives, ~~at least~~ two unambiguous positives, and ideally samples falling mid-range. Ideally these samples should come from different animals. Samples from experimentally infected animals or viral isolates can add value to the feasibility study. The assay should achieve as wide-as-possible separation of test results for the ~~high~~ strong positive and negative samples in ~~this~~ the panel. It may be useful to test a dilution series (analyte diluted in matrix) at this point to assess relative analytical sensitivity, particularly if the test is a potential replacement for ~~another~~ an existing method where sensitivity is an important criterion (~~see~~ Chapter 2.2.8 *Comparability of assays after changes in a validated test method).* It is important to test different genetic strains/lineages of the target pathogen as well as genetically closely related pathogens to determine preliminary inclusivity and exclusivity, respectively. For example, a well-established PCR assay detected all known isolates for many years (Smith *et al.*, 2001) but failed on a recent novel variant (HeV-g2) (Annand *et al.*, 2022; Bowden *et al.*, 2021). Toohey-Kurth *et al.* (2020) describe guidelines for validation, verification and comparison of real-time PCR assays in veterinary diagnostic testing laboratories.

- Evaluate the assay on a small sample panel (six to eight samples) to assess viability of the approach.
- Is there good separation between test results of negative and positive samples?
- Use a dilution series in matrix to obtain preliminary relative analytical sensitivity.

2.5. Development and optimisation

The aim of this stage is to define and optimise the method that will be used to carry out the test in future routine testing. This includes description of appropriate facilities to carry out the assay. It is important to consider both the method of sample extraction required to prepare samples for the test and the assay procedure. For any PCR assay, ~~definition of~~ clean room protocols ~~is~~ are required to minimise contamination. If large numbers of samples are anticipated, selection of an automated extraction procedure may be essential (Jungkind, 2001).

Usually, it is necessary to assess a number of different test methodologies and vary concentrations of reagents, template additions and reaction times to optimise the extraction and the assay. It is important to ~~either~~ change only one variable at a time or use a multi-factorial design and analysis strategy (see the WOAH Validation Standard, Section A.2.5 on Robustness). ~~It is important to identify which~~ Factors ~~that~~ have ~~only~~ a narrow range of optimal activity must be identified, as these are critical points in the assay procedure and may affect an assay's robustness. Generally, the assay stages are relatively simple to optimise, but care needs to be taken to ensure a robust nucleic acid extraction, and that all assay reagents, and procedural steps are optimised and suitable for routine laboratory application (Burkhardt, 2000).

- Optimise reagent concentrations and PCR cycling conditions.
- ~~Have concentrations of reagents been tested for optimal reactivity?~~
- ~~Has extraction been optimised?~~
- ~~What are the template additions and reaction times to optimise the assay?~~
- Which factors have a narrow range in which they perform optimally?
- Has that range been defined?

Controls for PCR are many and varied. It is important to include appropriate controls to show that the assay is performing as expected. The various controls that should be considered include:

~~2.5.1. A host-species control~~

~~This control demonstrates that the target species has been appropriately sampled. In the AI screening assay example, the control verifies that the sample swab had been in contact with a target species and that the sample contained "bird" nucleic acid, which is available for extraction using the defined protocol. A possible target for this control is a housekeeping gene such as beta actin, which is present in the target host species. This is relatively simple if the host species is a single species e.g. chickens, but it will be more challenging to find a suitable target for all "bird" samples. A host-species control may not be required if a sample is added directly to the extraction process, such as a piece of tissue or blood. It is highly recommended for "indirect" samples such as those collected on swabs; where the host species control should be used for every sample tested.~~

~~Have you included all necessary controls to prove the assay is performing as expected?~~

- ~~Template control?~~
- ~~Inhibition control?~~
- ~~Positive sample control?~~
- ~~For RNA assay – reverse transcription control?~~

2.5.1. Analyte-positive control

This positive control, having a real-time PCR quantification cycle ~~threshold~~ (~~Ct~~ Cq) activity within the ~~defined operating~~ moderate to weak range of the assay, is used on each plate. ~~A suitable choice for this control may be a plasmid containing the target sequence, which~~ It can be ~~used to check for the expected level of amplification in the assay. However, this does not assess the efficacy of the~~ either an extraction ~~process, which requires~~ control prepared from a known field sample or its equivalent (such as a sample from an experimental infection) or a plasmid or *in-vitro* transcribed RNA containing the target sequence. The extraction control has the advantage of testing the entire sample processing pipeline, whereas a plasmid control tests only the DNA amplification step. Whichever is selected for use, positive controls should be prepared as a large batch, aliquoted into single-use vials and tested for homogeneity, to ensure suitable reactivity in the assay. The positive control batch then provides progressive monitoring of assay performance particularly during the optimisation steps.

- Has a large batch of quality-assured positive control material been prepared and aliquoted in single use vials to provide progressive monitoring of the TUE during optimisation and validation?
- Have both the advantages and disadvantages of including an internal control been considered?
- Is the purpose and application of the inhibition control clearly specified in the assay protocol?

2.5.2. No-template control (NTC)

The NTC consists of a well containing master-mix alone without addition of NA template. This control reveals whether contamination of the ~~sample~~ master-mix has occurred, resulting in amplified product when no amplified product should be present~~, as in a sample containing no target~~. Consideration should be given to the number and placement of ~~no template controls~~ NTCs in the assay set up template. Generally, a number (approximately 5% of wells) of ~~no template controls~~ NTCs are distributed randomly within the assay or over the plate when 96 and 386 well-plate formats are used.

2.5.3. Inhibition control

~~This control is needed to detect possible inhibitors of the PCR reaction. If an inhibition control yields a negative result, this infers that the sample contains inhibitory substances and that a negative test result for the test sample cannot be interpreted as "negative" because the assay did not perform correctly. Certain samples such as faeces and semen often contain inhibitors whereas this may be less of a problem when testing blood samples or cultured organisms (Ballagi-Pordány & Belák, 1996). Data collected during the validation process concerning assay performance using the sample matrices being targeted will allow for a risk based decision as to whether an inhibition control should be included for each sample or whether the test system is unlikely to be affected by inhibition. If inhibitory substances are a significant problem an inhibition control must be included for each test sample.~~

~~There is considerable debate about the most suitable and effective inhibition control. Examples include the following:~~

~~i) An artificial target, such as a length of DNA contained in a plasmid, which is added to the extracted sample and amplified with the same primers as the test target, but is of a different size or contains a different internal sequence so that it is identified as the internal control when the detection method is applied (Sawyer et al., 2006).The advantage of this approach is that it utilises the same primers employed in the test, and the control can be added at precise concentrations. Because the target for the assay and for the control is the WOAH Terrestrial Manual 2024 5 Chapter 2.2.3. Development and optimisation of nucleic acid detection assays same, competition for the primer and dNTPs may reduce the analytic performance of the assay. Care must be taken during assay optimisation, so that the analytical sensitivity of the assay is not detrimentally affected. Another disadvantage is that as an added component, this control only verifies the assay stage of the test and does not act as a control for the extraction stage.~~

~~ii) ii) An alternative strategy for an inhibition control is to amplify a housekeeping or structural gene such as β-actin which always is present in the target tissue, and thus the sample. If the housekeeping gene has been inhibited by substances in the sample, the inference is that amplification of the gene targeted by the test may also be inhibited. This conclusion is not always warranted. Housekeeping genes are often present in abundance, so sometimes can be detected even in the presence of inhibitory substances while the more limited amounts of the assay's targeted sequences may be inhibited from amplification. In this case, the amplified and detected housekeeping gene was not a sufficient control for inhibitors, resulting in significant risk of a false negative inference for the test result. However, with knowledge of the risk associated with housekeeping genes, which are naturally present in the sample housekeeping genes can be a useful control for inhibitors for the whole assay including sampling, storage and extraction.~~

Many common diagnostic samples, such as faeces, milk and blood, contain naturally occurring substances that inhibit NA amplification. Anticoagulants and transport media used for sample collection may also cause inhibition of NA amplification (Yan *et al.,* 2020). If an inhibition control yields a negative result, this indicates that the sample contains inhibitory substances, the assay is compromised and a negative test result for a diagnostic sample may not be reliable.

During validation studies, data assessing the effects of sample matrix on assay performance will guide a risk-based approach to the need for inhibition controls. If inhibitory substances are a significant problem, an inhibition control must be included for each test sample. In a recent review of inhibition monitoring in the veterinary setting, Yan *et al.* (2020) describe two categories of internal controls that can be used to detect inhibitory substances in the PCR reaction:

i) Endogenous internal controls

A common choice of inhibition control is an endogenous gene target, such as house-keeping or structural genes present in multiple species. This control also demonstrates that the target species

has been appropriately sampled and that NA extraction has been successful. Examples of endogenous controls include 18S ribosomal RNA (18S rRNA, all eukaryotes), 16S ribosomal RNA (16S rRNA, all prokaryotes), beta actin (ACTB, avian, bovine, porcine), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, equine, bovine, canine, apidae), etc.

Caution is required with interpreting results for endogenous genes as these are often present in high copy number, so may be detected even in the presence of inhibitory substances, while the more limited copies of the assay's targeted sequences may be inhibited from amplification. Endogenous internal controls may carry a significant risk of a false negative inference for the test result. However, with knowledge of the risk associated with endogenous genes, which are naturally present in the sample, endogenous genes can be a useful control for detection of inhibitors and for confirmation of successful sampling, storage and extraction (whole assay approach).

ii) Exogenous internal controls

An exogeneous internal control is an artificial target, such as a length of DNA contained in a plasmid or *in-vitro* transcribed RNA, which is usually spiked into lysis buffer in a defined concentration, prior to extraction. This provides a more stable, and standardised control to assess PCR amplification/inhibition. Exogeneous internal controls can be designed as homologous or heterologous templates.

a) Homologous exogeneous internal controls are synthetic templates containing identical primer binding sites as the test target, but when amplified produce an amplicon of a different size or use a specific probe to discriminate it from the pathogen target (Hoorfar *et al.*, 2004; Sawyer *et al.*, 2006; Schrader *et al.*, 2012). The advantages of this approach are that it utilises the same primers employed in the test, and the control can be added at precise concentrations. The disadvantages are that competition for the primer and dNTPs may reduce the analytic performance of the assay. Care must be taken during assay optimisation, so that the analytical sensitivity of the assay is not detrimentally affected.

b) Heterologous exogenous internal controls are designed using primers and probes unique and specific for the construct (Hoorfar *et al.*, 2004; Schrader *et al.*, 2012). Like homologous internal controls, optimisation is required to limit the competition for reagents used in PCR reaction. Heterologous exogenous internal controls are commonly used in the diagnostic laboratories providing efficient deployment for different animal species and matrices (Yan *et al.*, 2020).

~~2.6. Inhibitory factors in the sample matrix~~

~~Generally for NAD methods, pure cultures, blood and most tissues are the preferred samples because extraction and recovery of amplifiable NA is generally successful. Faecal samples, semen and autolysed tissue can be more challenging because they generally contain more inhibitors for NAD assays. It is vital to have a robust and repeatable sample extraction procedure, which is appropriate for the numbers of samples to be handled (automated if necessary) and to utilise inhibition controls as necessary (see section above).~~

2.6. Operating range of the assay

- For determining the operating range of the assay, were samples diluted in the matrix for which the test is intended?
- Does the operating range of the assay conform with the expected norms for such an assay?

The ~~operating~~ analytical range of the PCR assay ~~should~~ can be determined by ~~diluting a strong positive sample and plotting the range of results obtained versus known amounts of nucleic acid (concentration, dilution, number of genomic copies, etc.). This~~ titration of a reference sample ~~must be~~, such as a synthetic construct of known copy number or whole virus of known infectious dose (e.g. $TCID_{50}$). This reference sample must be diluted in the same matrix as the test sample, i.e. it is not appropriate to determine the operating range of a sample diluted ~~out~~ in buffer, if the usual matrix is blood. Each dilution in the titration series should be tested in replicate and resulting Cq values plotted against the reference sample concentration ($TCID_{50}$, dilution, number of genomic copies, etc.). Once the analytical range of the assay is known, titration of strong diagnostic positives can be used to provide a practical guide to the operating range and end-point Cq values. This data will define interpretation criteria for the positive, negative and suspect range of the assay.

2.7. Robustness

~~An~~ To provide reproducible results, when deployed in multiple laboratories, an assay should tolerate small changes in concentrations of reagents and/or slight variations in processing times and temperatures used for different stages of the assay ~~in order to be effectively deployed and provide reproducible results when used in multiple laboratories. This can be determined during assay optimisation when~~. Critical procedural steps, reagents, and equipment are identified~~. Such~~ during assay optimisation. The critical factors ~~that when not adhered to cause unacceptable variability~~ and their acceptable range of use should be well described in the ~~assay~~ protocol ~~so that particularly exacting processes are assured for carrying them out. This is a laborious process that ultimately is monitored for~~ to minimise assay variability. Ultimately, the precision and accuracy ~~by~~ of the assay are assessed through the preparation and use of quality assured internal and external ~~quality control samples run~~ controls prepared in bulk and progressively monitored every time the assay is performed.

2.8. Calibration verification of the assay to reference samples

In calibration verification, known quantities of a material are measured by the assay and results are compared to the known true value of the material (Burd, 2010). Calibration verification and quality control procedures are required to ensure that the expected performance of the assay is maintained throughout the life of the test (Burd, 2010). Ideally, international or national reference standards should be used to calibrate or verify the calibration of the assay. However, these are not always available so it may be necessary to create an in-house reference standard (see Chapter 2.2.6). A working standard(s), for inclusion in all runs of the assay, needs to be created, aliquoted, and stored in sufficient quantities for use in every run of the validation process and for routine use after the validation has been completed. The working standard(s) could be multiple aliquots of a particular sample, which can be used within each assay run. They could also consist of a plasmid containing the sequence of interest, spiked into sample matrix. Use of the latter allows the test developer to determine the number of genome copies that can be detected by the assay. In some instances, test sample results are "normalised" by comparison to the working standard sample(s) included in each run of the assay. This allows direct comparison of data between runs (Huggett *et al.,* 2005; and Chapter 1.1.6).

- Have you verified the calibration of the assay to external reference standards?
- If external reference standards not available, have you created an in-house reference standard?
- Have you made working reference standards in sufficient amounts for use in all development and validation experiments?

B. ASSAY VALIDATION PATHWAY

Once the protocol for the assay has been developed and optimised, it must be fixed and held constant while being evaluated through the stages of the validation pathway and during routine use. Some countries have validation templates specifically designed for molecular assays which assists in the standardisation, evaluation and transparency of the validation process (SCAHLS, 2008). Minor changes to a validated assay can be addressed using comparison studies to document that the assay continues to perform as originally defined (Chapter 2.2.8 *Comparability of assays after changes in a validated test method*).

1. Stage 1 – Analytical performance criteria

1.1. Repeatability (~~Ct~~ Cq or qualitative conventional PCR)

Repeatability of the assay is a measure of agreement between results (within and between runs) using the same samples and test method in one laboratory. ~~Usually~~ Ideally, ~~a small~~ panel of three (preferably five) ~~samples covering~~ batches of samples that are unequivocally positive and, cover the operating range of the assay ~~is selected~~, are prepared and tested using the entire assay procedure (including nucleic acid extraction). Each batch must be prepared to ensure homogeneity during preparation and aliquoting and contain enough single-use vials to permit repeated testing over several days/weeks by two or more operators. Within assay (intra-assay) variation is determined using multiple (at least five) replicates of each sample in ~~this~~ the panel in one assay run. Between run (inter-assay) variations are determined by testing these samples over several days, using several operators and at least 20 runs. The repeatability panel should be tested treating all samples and each of their replicates exactly like individual diagnostic samples, subjected to every step from sample preparation to data analysis. Accordingly, every replicate of every sample is subjected to an independent

- Has intra- and inter-assay repeatability been determined?
- Is repeatability within the accepted range of coefficient of variation (CV) limits?

extraction. This mimics the future use of the assay when implemented for diagnostic use and allows ~~for~~ robust determination of repeatability, both within and between runs~~ of the assay that mimic future runs of the assay when implemented for diagnostic use~~. Minimal variation in repeatability is important, particularly near the cut-off(s) that establish positive, inconclusive, and negative ranges, because high~~er~~ variability (especially at the cut-offs) can result in incorrect interpretations (~~see~~ Chapter 2.2.4 *Measurement uncertainty*). The assay should be designed so that the decision point (cycle quantification, Cq) lies on the steepest part of the amplification curve and is standardised for the assay (automated settings on the PCR cycler cannot be used). Standardisation of the assay threshold will ensure consistency of data analysis between runs and that repeatability will be optimal at the critical point of the assay.

The variation observed in repeated measurements (repeatability) can be expressed in several forms including standard deviation (SD), a percentage change or as a coefficient of variation (CV) defined as the ratio of the standard deviation to the mean or relative standard deviation (see Chapter 2.2.5 *Statistical approaches to validation*). Because the CV is independent of the measurement unit, it can be used to compare variability between data sets with different means. The SD, by contrast, is always dependent on the mean of the data. It is worth noting that when the analyte concentration is low (as in weak positives) the CV may be greater than that for strong positives because weak samples (near the negative threshold or cut-off point) often exhibit greater variability in Cq value and thus have a larger SD. Burd *et al.* (2010) suggest the use of precision profiles, plotting the SD or CV as a function of analyte concentration, to map changes in precision.

Currently, there are no specific recommendations for acceptable CV values when assessing real-time PCR repeatability in clinical, veterinary or food microbial detection. Accumulated data from the proficiency testing (PT) provider at the authors location indicates that CV<5% using PCR for repeated testing of PT batches, prepared in compliance with ISO 17043, is an indicator of homogeneous, stable samples and reliable repeated testing (unpublished data). However, if the quantitative PCR test is required, the CV should be based on template copy numbers (Bustin, 2009). In fields, such as for genetically modified organisms (GMO) and environmental microbial detection, CV values of up to 25–50% are considered acceptable, particularly when template copy numbers are fewer than 100 (Bowden *et al.*, 2021; Broeders *et al.*, 2014; European Network of GMO Laboratories [ENGL], 2015; Kralik & Ricchi, 2017).

Consideration should be given to whether samples are tested individually, in duplicate, or more replicates. Poor precision will affect the linearity of an assay therefore, it is important to assess repeatability (precision) as part of the linearity study (Chapter 1.1.6). In brief, to do this, batches of analyte are prepared covering the operating range of the assay (strong, moderate and weak positive), are aliquoted into single use tubes and are tested in replicate by two or more operators over multiple runs. Consultation with a statistician may be necessary to ensure suitable replicates and number of runs for the expected imprecision of the assay (CLSI, 2003) and use of appropriate result interpretation and statistical analysis.

~~Repeatability can be expressed as a coefficient of variation (see Chapter 2.2.5 Statistical approaches to validation). The assay should be designed so that the decision point (cut-off) lies on the steepest part of the real-time PCR Ct curve. If this is achieved, repeatability will be optimal at the critical point of the assay. (Larger CVs at the clearly negative and highly positive ends of the operating range of the assay do occur and have little impact on test result interpretation).~~

1.2. Analytical specificity (ASp)

Depending upon the intended purpose of an assay, its analytical specificity is determined by the selected genetic sequence(s) of organism(s) targeted by the assay. The assay can be designed to be highly selective, with analytical specificity for a single genetic sequence that is known not to be present in other organisms or strains of the targeted organism. Such an assay is said to exhibit **exclusivity** and connotes a confirmatory assay of high ASp.

Alternatively, the assay may be designed to target a conserved genetic sequence that is common to several strains of a given species, or several species of a genus. Such an assay has an ASp that exhibits **inclusivity**, making it useful as a screening test. For an inclusive screening assay, the analytical specificity should be determined by testing all lineages, strains, species, etc., that the assay is expected to detect. The assay should then be evaluated for its capacity to exclude related organisms such as non-pathogenic strains that are not of interest to the intended purpose of the ~~candidate assay~~ TUE.

In the example of an AI screening test, the assay should be evaluated, for inclusivity, against as many well-characterised isolates of AI virus as are available to assure ~~that all~~ detection of strains from a variety of geographical areas (Asia, Europe, North America) and hosts~~ are detected (i.e. to demonstrate inclusivity). This is generally done using laboratory~~ (chickens, migratory and non-migratory birds) Laboratory strains/cultures of AI or extracted nucleic acid~~. For AI, different lineages~~ may be used for this purpose. The purpose of the ~~virus exist such as Asian, European and North American. It is important to consider how~~ assay, including the ~~assay will be used and in which~~ geographical ~~regions. That~~ area in which it will be used, will ~~aid in determining~~ determine whether it is necessary to evaluate some or all of these lineages. This important requirement ~~is clearly~~ was demonstrated ~~with the recently developed~~ in a cooperative study by various WOAH Collaborating Centres to assess a rapid and simple RNA-virus pathotyping assays~~, which were~~ tested on a large collection of AI and Newcastle disease viruses, originating both from the Eastern and from the Western hemispheres (Leijon *et al.,* 2011; Yacoub *et al.,* 2012~~), in a collaboration between various WOAH Collaborating Centres. Another~~). For screening assays, it is important ~~factor is~~ to note that viruses can ~~change~~ evolve rapidly and the resultant mutations can render a diagnostic test sub-optimal. An example of this is the appearance of the 2009 pandemic strain of Influenza A H1N1 and the 2013 low pathogenic AI H7N9 outbreak in Southern China. The analytical sensitivity of the PCR for the traditional M gene was impaired for this strain ~~because~~ due to mutation in the target sequence~~ that rendered the assay specific had mutated~~. In most countries new primers have been introduced and used either as a new test or in combination with the traditional M gene primers as a combination test (Harmon *et al.,* 2010; Hatchette *et al.,* 2013; Shu *et al.,* 2011; Spackman *et al.,* 2002; WHO, 2013).

- Has a panel of as many well-characterised isolates of the target pathogen as possible, including isolates from a variety of geographical areas and hosts, been tested?
- Have related organisms and pathogens, which cause similar clinical syndromes, been tested?
- Have exclusivity, inclusivity and selectivity been assessed?

~~The~~ To demonstrate **exclusivity** the discriminatory power of the assay should be checked by testing organisms related to the AI virus. These would include pathogens which cause similar clinical syndromes such as Newcastle disease, infectious bursal disease, etc., and other organisms, which are likely to be found in the target sample ~~(i.e. to demonstrate exclusivity)~~.

Selectivity is the extent to which a method can detect and quantify the target analyte in the presence of specific reactants, such as interferents and degradants (e.g. matrix components, inhibitors of enzymes in the reaction mix). Selectivity is essentially the test for detection in the presence of inhibitors. Sample matrix variation is one of the most important factors contributing to interference, but may be among the least acknowledged sources of error in analytical measurements. Assessment of ASp should use matrices relevant to the intended purpose, such as solid tissue, whole blood and swabs, nasal versus cloacal. Interfering substances (inhibitors) can originate from the samples, e.g. haemoglobin, from the environment, or from sample collection and transport media (e.g. media and anticoagulants) (Yan *et al.,* 2020).

To assess multiplexed assays, samples containing each target organism should be used, not only to establish assay characteristics for that particular pathogen but also to rule out cross-reactivity or interference between the pathogen and the primers/probes designed to detect the other pathogens in the assay (Bowden *et al.,* 2021).

1.3. Analytical sensitivity (ASe)

The analytical sensitivity (or limit of detection, LOD) is the lowest concentration at which an analyte can be detected within statistical limits, i.e. 95%. There are two common approaches to assessing analytical sensitivity~~, also known as the limit of detection. The first is to~~; comparative sensitivity or quantitative sensitivity. Both approaches use a dilution series of the target pathogen (in this case AI virus) diluted in sample matrix and not buffer. ~~The~~ To perform a comparative sensitivity study the dilution series is ~~usually~~ tested using the ~~assay under validation~~ TUE and an existing standard method. For AI, the standard method could be virus isolation or another in-house standard method of detection. This approach yields a comparative measure of the two methods (~~see~~ Chapter 2.2.5). ~~The second~~ In the quantitative approach~~ is to use~~, a target of known concentration, such as a plasmid construct or *in-vitro* transcribed RNA~~, containing the target sequence and test this as a dilution series~~ of known copy number or virus of known $TCID_{50}$, is serially diluted in sample matrix~~. In this manner,~~ and assessed in the TUE to yield the smallest concentration quantifiable e.g. the number of genome copies ~~detectable by the test method can be estimated~~ (or number of virulent particles).

Genetic variants (e.g. different lineages, genotypes/subtypes/strains of pathogen) may not have the same ASe in an assay, due to differences in the avidity of primer/probe binding and amplification efficiency. For example, when assessing an ASFV real-time PCR assay using several genotypes, variations in ASe (measured as viral genomic copies) were found between different genotypes (Bowden *et al.,* 2021). Ideally, evaluation of ASe

should include all pathogen variants or, at a minimum, the genotypes/lineages prevalent in the region where the test is intended to be applied.

1.4. Standard test method for comparison with the ~~candidate test method~~ TUE

- Has the new test performed in a satisfactory manner compared to a standard method of comparison?
- Is preliminary reproducibility data acceptable
- Does the assay merit proceeding to studies on full validation (Stages 2–4)?

On some occasions it is not possible to complete a full validation exercise either because samples of known analyte status are scarce (e.g. exotic diseases) or, an emergency situation arises, and the assay is required for use before it can be fully validated. Provisional recognition can be achieved ~~provided that~~ where results through Stage 1 of the validation process compare favourably with results of a standard test method or a known established, and preferably published, method. ~~Another choice for a standard method is the one used routinely in the laboratory.~~ It is important to recognise that different methods identify different morphological or functional entities of the organism. It is therefore possible that comparison between a standard culture-based method and a new NAD technique will give rise to discrepant results (see Section B.2, Stage 2, below, for discussion on resolution of discrepancies). The choice of assessment panel is very important and it should be as extensive as possible (~~see~~ Chapter 2.2.6). If only a small panel of samples is available for evaluation, it is useful to determine if the assay passes reproducibility expectation, i.e. withstands the rigors of use in other laboratories. This requires that both laboratories use the same protocol, and same reagents, same panel of samples and similar (if not identical) equipment.

~~If lack of samples prevents continuing through the next stage of the validation pathway (Diagnostic Performance of the assay),~~ It is acceptable to use an NAD assay that has been provisionally accepted by ~~having been thoroughly validated through~~ satisfactory completion of Stage 1 of validation ~~(see the WOAH~~ if lack of samples prevents continuing through the next stage of the validation pathway (Chapter 1.1.6. Validation ~~Standard~~ of diagnostic assays for infectious diseases of terrestrial animals, Section B.2.6). Acceptance of provisional validation is fully dependent upon approval by local authorities, or through bilateral agreements between countries.

1.5. Analytical accuracy of supplementary tests or procedures

~~Analytical tools, designed to provide information~~ Supplementary tests, used to characterise samples that are detected using a screening assay, only require validation of their analytical performance characteristics~~. So, there~~ (Chapter 1.1.6., B.1.4. Analytical accuracy of adjunct tests or procedures). It is ~~no requirement~~ not necessary to determine diagnostic sensitivity or diagnostic specificity ~~in~~ of such ~~cases~~ tests. Examples ~~of such approaches~~ include ~~PCR~~ H-typing PCR assays ~~to determine whether~~ of a matrix-positive influenza A strain ~~is H5 or H7, or methods to determine~~ and determination of antibiotic resistance ~~which are only applied to~~ of cultured bacteria. ~~A~~ nucleic acid based ~~technology employed for~~ technologies such ~~tests includes~~ as nano-array ~~based~~ methods, which introduce their own challenges due to the large amount of data generated for each sample tested. Before implementing such assays, consideration should be given to how this analysis can be accomplished. A simpler approach is to compare the results of a new analytical tool to a standard tool and permit its use as long as the results of the new and existing techniques compare favourably (Anjum *et al.,* 2007; Batchelor *et al.,* 2008).

2. Stage 2 – Diagnostic performance criteria

2.1. Diagnostic sensitivity and diagnostic specificity

Diagnostic sensitivity (DSe) and specificity (DSp) provide the principal performance indicators for use of a diagnostic assay~~. When determining these estimates it is vital to select sufficient~~ and require adequate numbers of samples ~~which represent~~ representing the target population for the test under assessment. It can be difficult to obtain large numbers of samples (particularly positive samples) for some exotic diseases, and negative samples for endemic diseases. In such cases, with few samples available, the amount of error allowed in estimates of DSe and DSp, of necessity, may be rather large (see the WOAH Validation Standard, Section B.2, Table 2). Employing an appropriate sampling design and different, independent test methods to test the samples, it is possible to obtain estimates of DSe and DSp by using Bayesian methods (latent class models) (Chapter 2.2.5 and Cheung *et al.,* 2021). Negative reference samples are often selected from animals living in regions where the disease is not present, while positive samples are usually obtained from animals with clinical signs which have been confirmed in the laboratory. This can lead to overly optimistic estimates of DSe and DSp because the samples do not represent the whole spectrum of the disease process, ranging from non-clinical

animals which may have pathogen loads that are much different from animals experiencing fulminant or chronic disease.

Samples are often categorised as positive or negative using ~~current test~~ classical methods such as virus isolation (VI) or bacterial culture. However, this can be problematic when validating new molecular tests, because the basis of the two test systems is different. For example, a positive bacterial culture is dependent upon the presence of a viable organism whereas ~~nucleic acid-based~~ molecular methods detect genomic sequences of both live and dead organisms ~~as long as the nucleic acid is still present in the sample~~. VI methods can be particularly susceptible to inhibitors and contaminants present in the sample matrix, leading to an underestimate of "true positives". This can result in apparent discrepancies where samples are positive using the new molecular test and negative by traditional methods. Various strategies for resolving such anomalies include, but are not limited to, sequencing (which can demonstrate that the pathogen of interest was present in a particular sample), or testing using another molecular approach.

- **Cut-off** is the test (Ct) value selected for distinguishing between negative and positive results on a continuous scale of test values.
- **Indeterminate, intermediate, suspicious, borderline, grey zone or equivocal** are terms used synonymously for a zone of test values falling between the positive and negative cut-offs.

To calculate DSe and DSp estimates of the ~~candidate assay~~ TUE, the test results first must be reduced to categorical (positive, negative, or indeterminate) status (Chapter 2.2.5). This is accomplished by insertion of one or two cut-off points (decision limits) in the continuous scale of test results. The cut-off points are determined by several factors in the assay pipeline and may differ with target pathogen and/or test purpose. For example, in some circumstances it is appropriate to use a cut-off for a real time PCR assay in the region of 35 ~~Ct~~ Cq, which means that some samples that produce a higher ~~Ct~~ Cq value are categorised as negative or inconclusive. For a different PCR assay, however, any sample which merely registers a ~~Ct~~ Cq and a typical amplification curve, may be categorised as positive. The performance of a particular real-time PCR, comparative validation data, the ultimate application of the results generated, and any relevant veterinary information should be taken into account when considering the use of a cut-off.

3. Stage 3 – Reproducibility and augmented repeatability estimates

Reproducibility is a measure of the agreement between results obtained in different laboratories using the same protocol, similar (preferably the same) equipment and the same panel of samples. Ideally the panel would consist of 20–30 samples, including a few ~~which are~~ present as quadruplicates~~. The panel should consist of samples~~, that cover the dynamic range of the test ~~with~~and several ~~that have activity~~with reactivity close to, and on either side of, the test-cut-off(s). The same panel used for determination of repeatability could be used for this evaluation, but with enhanced numbers of replicates. Measurements of precision can be estimated for both the repeatability and reproducibility data (see Chapter 2.2.4 for further explanation of this topic and its application). Chapter 2.2.6 provides further information about the selection and use of reference panels. See also Johnson & Cabuang (2021) about proficiency testing and ring trials and Waugh & Clark (2021) about factors affecting test reproducibility among laboratories.

4. Stage 4 – Programme implementation

4.1. Interpretation of test results

Best practices for programme implementation are general to all assay types (~~see WOAH Standard~~ Chapter 1.1.6). For NADs, an inherent advantage is the possibility of follow-up genomic sequencing to characterise pathogens and resolve apparent false positive results. ~~Assays including PCRs are often validated~~ The advantage of network validation studies is that they can provide a preliminary evaluation of molecular assays when used in different laboratories using ~~similar numbers of positive and negative~~ a panel of well characterised samples. However, in surveillance programs, assay results are often applied to affirm the absence of the disease in question in locales where disease prevalence is very low and often approaching zero. In such circumstances, false positive results can be a significant problem even if the diagnostic specificity of a particular assay is high. If the DSp of an assay is 99.5% this means that one in 200 test-positive results will be false if the prevalence is close to zero. If a large number of samples are tested from a population of zero or very low prevalence, such false positive results can significantly out-number true positive results (~~see the WOAH Validation Standard~~ Chapter 1.1.6, Section B.4.2 for further explanation of predictive values of test results as a function of disease prevalence). Likelihood ratios offer a convenient advantage because they depend solely on the combined diagnostic sensitivity of the test and therefore do not vary with prevalence (Caraguel & Colling 2021). For NAD assays used in such circumstances, it would be good practise to confirm PCR-positive results by sequencing. This approach is problematic for weak positive samples with high Cq values because DNA sequencing is less sensitive than real-time PCR and may not yield sequence data. In this case additional sampling, and further

testing using other independent tests may assist with a confirmatory result (e.g. real-time targeting different genomic region of the same pathogen).

Pooling of samples may be appropriate to increase the efficiency of testing large numbers, for example for prevalence studies. Where pooled samples yield positive results, it may be useful to test each sample from the pool individually to identify diseased animals. Whether pools are different samples from a single or multiple animals, any pooling strategy should be precisely defined and validated prior to use to minimise loss of sensitivity.

5. Monitoring of assay performance after initial validation

5.1. Monitoring the assay and continued fitness for purpose

Monitoring of repeatability by charting the ~~Ct~~ Cq values obtained for working standard control samples provides re-assurance that the assay is performing as expected. Similarly, participation in proficiency testing schemes issued by external providers of quality assurance samples provides evidence of on-going reproducibility ~~and also allows comparison of test accuracy if a reference standard(s) are included in each run for "normalisation" of data~~, assay performance and overall laboratory competence. Re-testing of a proportion (usually in the range of 1–5% depending on throughput) of retained samples is also employed by some laboratories to demonstrate that the assay is performing consistently between runs.

In time it may be necessary to modify the assay because the target analyte has changed, e.g. if the assay for avian influenza is to be applied in another part of the world or if new strains or lineages of a virus have emerged (see Section B.1.2, above, on the evolution of the new pandemic lineage of H1N1). RNA viruses evolve rapidly and point mutations can occur, so it is advisable to regularly confirm the nucleotide sequences of the primer and probe sites to ensure that they remain appropriate. Verification is required when an assay is used in a new environment (Kirkland & Newberry, 2021).

5.2. Minor modification of an existing validated assay

5.2.1. Technical modifications

Modifications of an assay are likely to be required, over time. For example, use of different equipment, use of a different extraction protocol or automation of particular stages will minimally require comparison of the original validated assay with the modified version (~~see~~ Chapter 2.2.8). If results of the modified version fall outside of the operating range or performance expectation of the original assay, a revalidation may be necessary.

5.2.2. Replacement of depleted reagents

It is important to assign unique identification numbers to all batches of reagents and to record the components used for particular assays. The most critical components in PCR based assays are the probes, the primers and the enzymes. Current and new batches of critical reagents should be tested in parallel prior to their introduction. However, for other reagents such as buffers and nucleotides it is usually sufficient to monitor batches to inform troubleshooting, should that become a necessity.

5.3. Major change in assay requiring re-validation

Upon occasion, application of the assay may need to be extended beyond the scope of the original intended purpose of the assay. Examples are inclusion of another host species or a population of animals from a different geographical area. In such cases it is important to revalidate the assay because of new biological considerations with their many associated variables. The precise details will depend on the extent of the change. Moving the assay into a new geographical area might mean that the analytical characteristics of the assay are still valid but that the diagnostic criteria need to be re-defined. Similarly modifications may be made to PCR primer or probe sequences to allow detection of new strains. It will then be necessary to demonstrate how the new reagents behave in terms of analytical and diagnostic accuracy compared with the previous version of the assay.

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NB: FIRST ADOPTED IN 2014.

Annexe 12. Chapitre 2.2.5. Méthodes statistiques de validation

(Ce chapitre a été largement révisé et mis à jour par rapport à la version précédente adoptée en 2014. Bien que certaines parties du texte existant aient été incorporées, dans un souci de clarté, le texte nouveau et le texte supprimé n'ont pas été marqués.)

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CHAPTER 2.2.5.

STATISTICAL APPROACHES TO VALIDATION

INTRODUCTION

The statistical methods used to analyse data collected from diagnostic tests are determined based on the design of the study and the nature of the data (binary, ordinal, or continuous). Design considerations include whether the data are paired or independent and whether there is an infallible reference method (gold standard) available for comparison (Wilks, 2001). While analysis occurs after data collection, it is imperative that studies are properly designed so that analysis can be conducted (Gardner et al.*, 2019). Consultation with a statistician should be done during the design phase prior to commencement of the validation studies.*

Definitions of scales of measurement:

Binary (dichotomous): Either positive or negative because that is how the test result is presented, or positive/negative at a selected threshold (cut-off) value when results are measured on an ordinal or continuous scale.

Ordinal: Measured on a scale with discrete values where higher values typically indicate more analyte, e.g. serum virus neutralisation titres.

Continuous: An infinite number of measured values are theoretically possible, depending on the measurement system, e.g. enzyme-linked immunosorbent assay optical density values, cycle-to-threshold values in real-time polymerase chain reaction assays.

This chapter presents some commonly used statistical approaches but does not consider all methods that may be used in practice. Design considerations, analysis methods, and examples will be presented for assessing repeatability/precision, analytical sensitivity, analytical specificity, and for diagnostic sensitivity and diagnostic specificity. Analysis methods for direct comparison after a modification is made to a validated assay are discussed in detail in Chapter 2.2.8 Comparability of assays after changes in a validated test method*. Such methods are also discussed by Reising* et al. (*2021*).

When reporting the results of experiments conducted to assess the diagnostic performance of a test, it is imperative to report both the design of the studies and the results in an accurate and complete manner. The Standards for Report of Diagnostic Accuracy (STARD) statement was published in 2003 (Bossuyt et al., *2003a; 2003b) and updated in 2015 (Bossuyt* et al., *2015). The update includes a checklist of items to include when presenting diagnostic accuracy studies. An updated version, STARD-BLCM for reporting diagnostic accuracy studies that use latent class models and Bayesian methods was published by Kostoulas* et al. *in 2017*.

A. ANALYTICAL SENSITIVITY

Analytical sensitivity is synonymous with limit of detection (LOD). Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals* defines the LOD as an amount of analyte in a specified matrix that would produce a positive result at least a specified percent of the time. Prior to designing the study, the “specified percent” should be determined (e.g. 50% or 95%). In most instances, a conservative estimate of the LOD is acceptable (Chapter 1.1.6, Section B.1.3. *Analytical sensitivity*). When the LOD is defined as the concentration corresponding to a 95% probability of detection, a dilution-to-extinction (DTE) experiment can be used and the lowest concentration in which all replicates are positive is reported as a conservative estimate of the LOD (Bowden *et al.,* 2021). The DTE experiment may present unique challenges

for tests intended to detect antibodies (rather than antigen or nucleic acid) as it may not be possible to obtain an objective measure of the concentration of antibodies (Bowden *et al.,* 2021). There are times where the reciprocal of the dilution in which all replicates exceed background is reported as the relative LOD.

Alternatively, statistical models can be used to determine LODs. Logistic regression or probit regression can be used. Both are generalised linear regression models, but the difference is in the link function. Logistic regression uses a logit link (i.e. $\log(\pi/(1-\pi))$ where π is the probability of detection) and probit regression uses a probit link function (i.e. $\varphi^{-1}(\pi)$ where φ is the cumulative distribution function (CDF) of the standard normal distribution). Both models produce symmetric S-shaped curves. The complimentary log-log (cloglog) with link function, $\log(-\log(\pi))$, is another type of curve but is not symmetric. It was originally described in a paper by Fisher (1922) and the development in the context of a dilution assay can be found in McCullagh & Nelder (1989).

When designing an LOD study there may be a desire to have a more exact estimate than a DTE can offer. Furthermore, a DTE will not allow for a measure of uncertainty (e.g. confidence interval). In such instances, a two-stage experiment would be used. The first stage would be the DTE study to provide information on the span of dilutions to test in the second stage. In the second stage a smaller dilution factor would be used along with an increased number of samples tested at each dilution. Figure 1 provides an example of the results of a second stage study for the validation of a real-time polymerase chain reaction (PCR) along with generalised linear model fits using different link functions (logit, probit, and cloglog) to estimate the LOD.

The model fits for the logit and probit are very close (Figure 1) and visually difficult to distinguish which might be preferred. The estimates of the LOD using the probit link is 1.1 times higher than the LOD estimate using the logit link. McCullagh & Nelder (1989) indicate that it is usually difficult to distinguish between the two based on goodness-of fit. Hardin & Hilbe (2007) discuss various tools such as deviance, Akaike information criterion (AIC), and Bayesian information criterion (BIC), that can be used to help select a link function. In this example, visually there appears to be more lack-of-fit using the model with the cloglog link and this is confirmed by a dispersion estimate of 3.7. There may be scientific reasons that help one select a particular link function. An example of a dilution assay is presented in McCullagh & Nelder (1989) and provides the theoretical motivation for using a cloglog link.

Table 1 provides the estimate of the LOD along with 95% confidence intervals for each link function. The confidence interval for the cloglog link function used an adjusted variance (adjusting for the dispersion). One link function should be used when presenting results of such an experiment. All were included here only for illustration. In this example, the LODs estimates using the clog-log link functions. If this difference is meaningful in terms of acceptability of the assay, further investigation into the selection of link function may be warranted. However, there may not be a real practical difference in drawing conclusions of the acceptability of the assay in terms of the differences in the LOD estimates based on the link function chosen. In this particular example, a large dispersion estimate for the cloglog function suggests some lack of fit and can be used to rule out the cloglog link. Ultimately, if the differences in the LOD estimates between using the probit or logit link is crucial, the remaining performance characteristics will aid in determining the fitness for purpose of the assay. In this example, the LOD was estimated to be 79 copies/100 µl using the logit link and 126 copies/100 µl using the probit link which is approximately 1.5 times higher than the LOD using the logit link.

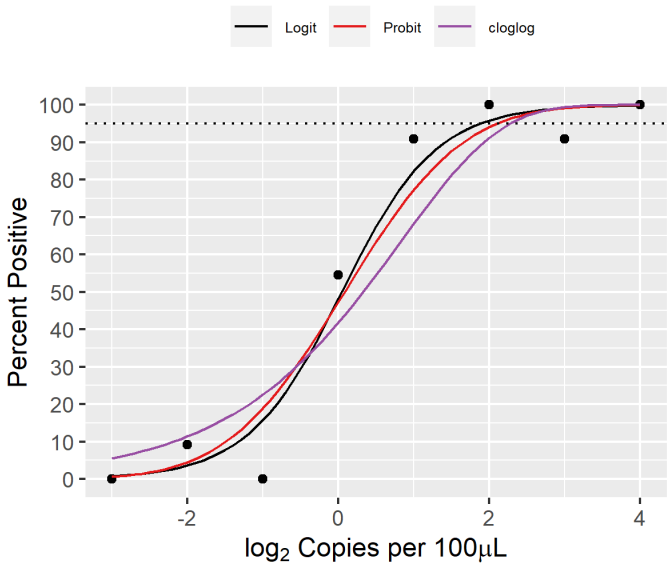


Fig. 1. *LOD data plotted with different models using various link functions.*

Table 1. *LOD estimates for various link functions*

Link	LOD (95% confidence interval)
Logit	1.9 (95% *CI*: 1.0, 2.8)
Probit	2.1 (95% *CI*: 1.2, 3.0)
Complementary log-log	2.3 (95% *CI*: 1.1, 3.5)

For a real-time PCR, another design consideration is whether to dilute before or after extraction which changes the interpretation. More sources of variability are incorporated into the design if samples are extracted at each dilution. Obviously, the extraction efficiency of the nucleic acid extraction method and amplification efficacy of a real-time PCR method can separately impact the estimate of LOD.

It is important to keep in mind that it is not sufficient to report the LOD as number of copies without identifying the concentration, including the per unit basis. The probabilities will depend on units (per ml, per aliquot, etc.). Specifically, a test method will have a higher observed probability of detecting one copy per aliquot than detecting one copy per mL if the size of aliquot for testing is smaller than 1 ml. It is also beneficial to report the method used for determining the concentration of a sample.

B. ANALYTICAL SPECIFICITY

Analytical specificity includes selectivity, exclusivity, and inclusivity. The latter two measures can both be assessed through a table where isolates tested are reported on a per lineage, isolate, species or genus basis, as appropriate for the target analyte and the intended purpose of the test. This table would include samples that the assay should detect (inclusivity) and those genetically similar that the assay was designed not to detect (exclusivity). Selectivity refers to the extent to which a method can accurately quantify the targeted analyte in the presence of inhibitors or degradants. Selectivity also includes assessment of nonspecific binding. A diagnostic assay that is intended to distinguish infected from vaccinated animals (DIVA) should demonstrate that a positive result is only obtained from infected and not vaccinated animals. Kirkland & Newberry (2021) provide a useful discussion regarding considerations of selection of negative animals when assessing analytical specificity.

C. REPEATABILITY

Stage I of the Validation Pathway includes repeatability and preliminary reproducibility. Repeatability is defined in Chapter 1.1.6 as the level of agreement between results of replicates of a sample both within and between runs of the same test method given a laboratory.

Bowden *et al.* (2021) provides a guide for assessing repeatability of antibody detection assays and nucleic acid detection assays. In both instances, the recommendation is to test a panel of three to five samples covering the operating range of the assay. For antibody detection assays, it is recommended that three to four replicates are tested of each sample in each run as well as five replicates for the nucleic acid methods. For all assay types discussed, the panel is tested (with sample replicates) on a minimum of 20 assays where two or more operators perform testing over several days. Bowden *et al.* states “the within- and between-run variations are estimated determining the coefficient of variation (CV)”. A CV is the ratio of the standard deviation to the mean.

Another approach commonly used to determine intra- and inter-assay variability involves testing at one or more analyte concentrations repeatedly in each of several runs of the assay system (Reising *et al.,* 2021; Toohey-Kurth *et al.* 2020). These variability estimates are expressed as standard deviations, rather than CVs. Such statistical analysis methods are described in Vardeman & Jobe (1999) while details regarding study design are presented in Reising *et al*. (2014). Bowden *et al.* (2021) also acknowledges the importance of assessing repeatability using the entire test procedure. Specifically, there is mention of including nucleic acid extraction for real-time PCR as extraction can be a significant source of variability.

A common approach to assess repeatability for a method that does not produce quantitative results is Cochran’s Q (Cochran, 1950). This is a hypothesis test that assumes, under the null hypothesis, that all percentages are the same when the same samples are tested on different days, for example. While this is a common method, it is a hypothesis test designed to detect differences, rather than conclude similarities.

D. DIAGNOSTIC SENSITIVITIY AND DIAGNOSTIC SPECIFICITY

Diagnostic performance of an assay is commonly assessed by estimating diagnostic sensitivity (DSe) and diagnostic specificity (DSp) or a combined measure of DSe and DSp such as the likelihood ratio of a positive or negative results. DSe and DSp can be estimated when the reference or comparison method is infallible (perfect sensitivity and perfect specificity) or when the reference method is imperfect. The methods used for estimation will depend on the type of reference method. In general, most reference test methods are not infallible, especially for test methods intended to test ante-mortem samples. Figure 2 provides a flow chart describing study design, nature of the data, data analysis and reporting of results for common scenarios encountered when attempting to determine the diagnostic performance characteristics of a test under evaluation (TUE).

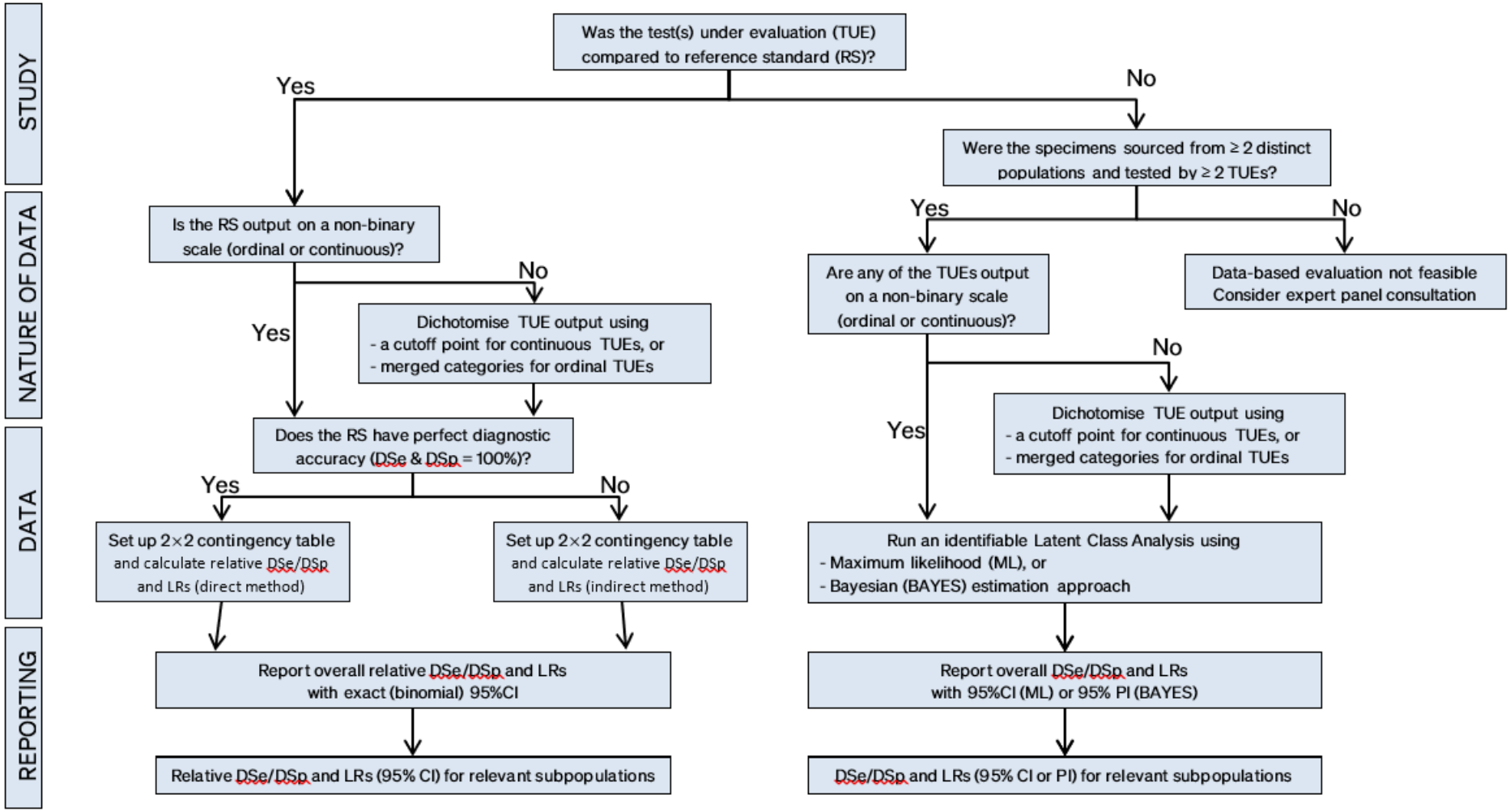
Sample selection is an important consideration in terms of estimating DSe and DSp. Animal samples typically come from one of the following: 1) reference bank with samples of known infection status, 2) outbreak or surveillance samples where animal status is unknown, but population status (infected or not infected) is known, 3) population where neither animal nor population status is known, or 4) experimental challenge studies. Chapter 1.1.6 provides a discussion of each source of samples along with advantages and disadvantages of each approach.

Mathematically, DSe and DSp are conditional probabilities, namely, P(T+|D+) and P(T-|D-), respectively. These are read as the probability of test positive given disease positive and the probability of test negative given disease negative, respectively, and commonly described as the chance of a test being positive for a positive animal and the chance of a test being negative for a negative animal. Within a herd of animals, the probability of disease positive, P(D+), is described as the herd prevalence. Something not discussed much in the literature but that perhaps warrants some thought is how to define disease positive in the context of estimating sensitivity and specificity. If one is validating a test method intended to detect antibodies, does 'disease positive' refer to a clinically diseased animal, an animal with active infection, or one that simply has antibodies present even if the disease pathogen is no longer present or the animal has recovered from the clinical presentation of the disease. A clear case definition helps to keep focus on the specific purpose of the test.

Questions are often presented in terms of the number of samples, but careful considerations should also be given to the types of samples as well. If samples are obtained from repositories, are all samples considered strong positives (with large amounts of target analyte) or do their reactivities span the operating range of the TUE? Have the samples undergone some type of treatment (such as freezing for long term storage) that would not be incorporated into routine testing and how might that impact the outcome? How do the samples represent the intended target population? While it is imperative to have a sufficient number of samples for evaluation, it is important to understand the strengths and limitations of the sample set chosen to assess the performance of the TUE.

When selecting samples with known status, estimating DSe and DSp is generally a simple calculation. If the TUE produces ordinal or continuous responses, dichotomise the test results into positive/negative using the established cut-off prior to determining DSe and DSp. Confidence intervals can be obtained using the Clopper-Pearson method (Clopper & Pearson, 1934) or the Wilson score method (Wilson, 1927). Newcombe (1998) compares seven different methods that could be used in this scenario to obtain confidence intervals. The same statistical methods can be used when the reference method is infallible.

Below is an example of estimating DSe and DSp using samples with known disease status or an infallible reference method (direct method). The same procedure can be used to present relative DSe and DSp to a test method that is not infallible (indirect method) but it should be noted that the resulting estimates would be relative to a reference test, rather than to true disease status. The Clopper-Pearson method was used in obtaining the exact confidence intervals.



Abbreviations: DSe = diagnostic sensitivity; DSp = diagnostic specificity; LR = likelihood ratio
CI = confidence interval; PI = probability interval.

Fig. 2. Flow chart for suggested methods of statistical analysis when a single test under evaluation is evaluated with and without a reference standard.

Table 2. *Contingency table*

	Disease status	
Test Result	Positive	Negative
Positive	195	3
Negative	5	203

True positive (TP): test positive and disease positive

True negative (TN): test negative and disease negative

False positive (FP): test positive and disease negative

False negative (FN): test negative and disease positive

Disease positive: TP + FN

Disease negative: TN + FP

DSe: TP/(TP+FN)

DSp: TN/(TN+FP)

DSe estimate and exact confidence interval: 195/(195 + 5) = 0.98 (95% CI: 0.94, 0.99)

DSp estimate and exact confidence interval: 203/(203+3) = 0.99 (95% CI: 0.96, 1.00)

LR+: 0.98/(1-0.99) = 98

LR–: (1-0.98)/0.99 = 0.02

The likelihood ratio (LR) is an inherent characteristic of the test; it depends solely on the combined diagnostic sensitivity and diagnostic specificity and therefore does not vary with prevalence (Caraguel & Colling, 2021). Likelihood ratios are extremely powerful, as they can be used to calculate the "post-test" probability of a disease. See equations above for LR+ and LR–. Below are the likelihood ratio estimates from the previous example.

LR+: Likelihood ratio of a positive test result = DSe/(1 – DSp)

LR–: Likelihood ratio of a negative test result = (1 – DSe) /DSp

LR+: 0.98/(1-0.99) = 98

LR–: (1-0.98)/0.99 = 0.02

The LR ranges from zero to infinity. If the LR of a given test result is greater than one, this test result supports the presence of the infection, i.e., this test result is more likely to occur in infected animals than in non-infected animals. Conversely, if the LR is lower than one, the test result support the absence of the infection, i.e., this test result is less likely to occur in infected animals than in non-infected animals. An LR equal to '1' means that the test result has no diagnostic power (i.e. the test result is as likely to occur an infected animal as it is in a non-infected animal) (Caraguel & Colling, 2021; Zhou *et al.*, 2011). The further the LR is away from one, towards either zero or infinity, the stronger the evidence provided by the test result. In the clinical context, test outputs with LR > 10 or LR < 0.1 are considered good diagnostic evidence of the infection being either present or absent, respectively.

Two additional metrics that are useful in test result interpretation are the positive and negative predictive values. The positive predictive value is the probability that an animal is indeed positive given it has tested positive. Likewise, the negative predictive value is the probability that an animal is negative given it has a negative test result. Both values are dependent upon the prevalence of the target population. Further details, including calculations, can be found in Section 4.2 of Chapter 1.1.6.

Statistical programs such as MedCalc or Epitools (Sergeant, 2018) can be used. They are specialised to compute most of the estimates used to assess diagnostic accuracy which are required for validation of diagnostic tests.

1. Bayesian latent class modelling for diagnostic test validation

A Bayesian latent class analysis (BLCA) is the WOAH-recommended approach (Cheung *et al.,* 2021), where insufficient reference samples are available for such an analysis, not assuming the true disease status of the animals from which the samples are derived. Sample size considerations for BLCA are similar to those for studies that estimate population proportions (e.g., prevalence) and include the expected proportion(s) (e.g., the estimated true population prevalence); the desired degree of precision of the parameters to be estimated (e.g., ± 2%, or 5%); the level of confidence (typically 95% or 99%); and any complexities of the study design, such as clustering. Three principles are considered in designing a BLCA model (BLCM): the disease prevalence is not the same among the populations studied, the diagnostic sensitivity and specificity must be constant across different populations, and diagnostic tests under evaluation are independent and conditional on an individual's true disease status.

Considering the above principles, a BLCM can be fit (Branscum *et al.,* 2005). The conditional dependencies between the diagnostic tests can be modelled (Bronsvoort *et al.,* 2006; Symons *et al.,* 2021) when neither test is perfect (Van Dreumel *et al.,* 2015), and the true status of the samples is unknown. To design a model structure, when k tests are evaluated for p populations, the data required are 2k tables per population (e.g., if k = 3 tests are evaluated, a 2×2×2 table is required). Considerations of model identifiability inform the choice and number of populations, the availability of appropriate prior information and the feasibility of data collection (Cheung *et al.,* 2021).

The BLCM can be run on the BUGS statistical packages, such as WinBUGS or OpenBUGS (freeware; can be downloaded via https://www.mrc-bsu.cam.ac.uk/software/bugs/portal). The code can be run in R Project (R core team, 2019) or JAGS using suitable libraries like R2Bugs.The analysis code includes the following specifications:

1. The model form for the data is based on:
    i) Hui & Walter (1980) equations for the relationship between the latent class and the observed data (apparent prevalence k × k tables for each population under consideration)
    ii) Prior probability distributions, which may incorporate further complexity, such as mixture distributions in the prior specification of true prevalence for certain populations to allow for disease freedom (i.e. zero prevalence) (Branscum *et al.,* 2004) or a logit function for the true prevalence, which allows for the inclusion of fixed and random effects (Mathevon *et al.,* 2017; Wood *et al.,* 2021)
    iii) Any outputs calculated based on inferred distributions, such as Youden's index, likelihood and odds ratios for any risk factors considered as fixed effects and predicted probabilities, such as the prevalence within a randomly selected herd or the probability of disease freedom at a specified prevalence (see sample code in Wood *et al.,* 2021)
2. Data is in the structure readable to the BUGS program.
3. Initials: a call to execute the BUGS program with control parameters, such as the number of chains, the number of Markov chain Monte Carlo (MCMC) iterations per chain, parameters to monitor, and initialising values for each unobserved parameter for each MCMC chain.
4. Model diagnostics to check that the model specification, model fit, chain length, convergence, and posterior probability distributions are appropriate to draw inferences from the model.

Illustrative examples of different BLCMs considering conditionality between diagnostic tests, population prevalences, and prior estimates are available in the public domain. These models have been derived from various publications and were reviewed by Cheung *et al.* (2021). Some model improvisations were constructed at the WOAH Centre for Diagnostic Test Validation Science in the Asia Pacific Region, Australian Centre for Disease Preparedness, Geelong in Australia.

E. REPRODUCIBILITY

Chapter 1.1.6 defines reproducibility as "the ability of a test method to provide consistent results, as determined by estimates of precision, when applied to aliquots of the same samples tested in different laboratories, preferably located in distinct or different regions or countries using the identical assay (protocol, reagents and controls)."

Waugh & Clark (2021) discuss factors that will affect the reproducibility of a test among different laboratories. They indicate that the ideal assessment would involve testing of identical samples in multiple laboratories where all the laboratories use the same reagent(s) and controls, but obviously the equipment would be unique to the laboratory, and material not provided in the test kit would be an additional source of variability. They also provide a discussion regarding the importance of technical training and ongoing professional development.

A common method of assessing reproducibility is by using a panel of samples sent to multiple laboratories from a central reference laboratory (Waugh & Clark, 2021). Chapter 1.1.6 recommends three or more laboratories to perform the test on the same samples. Statistically, including six or more laboratories, if possible, in the assessment of reproducibility is ideal if variance components are to be estimated (Burdick *et al.,* 2005).

Figure 3 provides plot of Ct (cycle threshold) values of 15 positive samples from a panel of 20 samples, which included five negatives, tested at six laboratories. To isolate the laboratory effect due to amplification only, all laboratories were provided extracted RNA and all used the same thermocycler with the same version of software. A model was fit to the data from the positive samples that included a fixed effect for sample and a random effect for laboratory. All negative samples were negative (i.e. no Ct value observed at the completion of cycling, data not shown). Reproducibility of the amplification process is reported as the standard deviation associated with laboratory and was estimated to be 1.2. In this example one laboratory, Lab A, was consistently lower than the remaining laboratories. Lower Ct results are associated with more nucleic acid present in the sample. In this example, the noted difference would not result in any misclassifications and therefore was not considered problematic. In general, Labs B and D produced the highest Cts, but it was deemed to be within an acceptable range of the remaining labs.

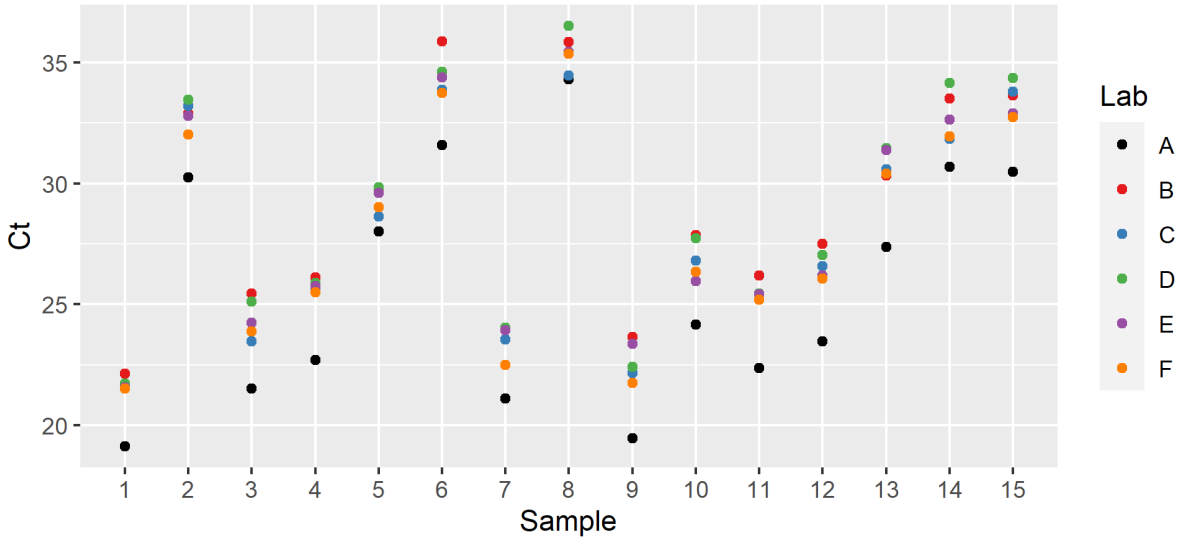


Fig. 3. *Ct values plotted for 15 positive samples from a panel tested by six laboratories to assess and compare differences in amplification of nucleic acid.*

Johnson & Cabuang (2021) discuss proficiency testing and ring trials. Both are used to assess reproducibility. “Ring trials” is common nomenclature for inter-laboratory comparison for a newly developed test. Proficiency tests are used for ongoing or periodic assessment of inter-laboratory test performance. Waugh & Clark (2021) describe that the choice of nucleic acid extraction method and other factors such as choice of enzyme and master mix, amount of template used in PCR, introduction of redundant nucleotides into primer/probe sequences and, for real-time PCR, choice of DNA dyes and probe chemistries have an impact on the assay’s reproducibility. Therefore, there is a need to include these variables in general assessments unless specific components need to be analysed in isolation as in Figure 3.

F. DISCUSSION

The design of a study and the nature of the data are key elements in selecting a statistical method for data analysis. Studies should be well planned with a clear objective. When designing a study, the nature of the data will be known, and this knowledge will allow for selection of a statistical method prior to data collection. Planning well-designed studies minimises the potential for problems during the analysis stage.

Formal statistical methods aid in determining whether an assay is fit for its intended purpose. Data visualisation and data summaries are valuable tools in the data assessment as well.

Latent class analysis continues to grow in popularity and offers a means of estimating diagnostic sensitivity, diagnostic specificity, and prevalence of disease within populations when there is no infallible gold standard method. Data analysis tools are readily available to aid in such analyses.

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NB: FIRST ADOPTED IN 2014.

Annexe 13. Chapitre 2.2.8. Comparabilité des épreuves suite à des changements introduits dans une méthode de test validée

(Ce chapitre a été largement révisé et mis à jour par rapport à la version précédente adoptée en 2016. Bien que certaines parties du texte existant aient été incorporées, dans un souci de clarté, le texte nouveau et le texte supprimé n'ont pas été marqués.)

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CHAPTER 2.2.8.

COMPARABILITY OF ASSAYS AFTER CHANGES IN A VALIDATED TEST METHOD

INTRODUCTION

Assay validation is a process that determines the fitness of an assay for an intended purpose. There will be instances over time when a validated assay may need to be modified or changed: see Figure 1, Chapter 1.1.6 Validation of diagnostic assays for infectious diseases of terrestrial animals, *which illustrates stages 1–4 of the validation pathways and validation status retention. Modifications may be driven by such things as the availability of less expensive or better reagents, the need for standardisation, access to reagents at scale in the event of an outbreak, or the availability increased throughput of a test using robotic methods. Minor changes need to be evaluated through a comparability process to demonstrate the test method remains fit for its intended purpose. In 2021, the WOAH* Scientific and Technical Review *published a special edition entitled* Diagnostic Test Validation Science*. In that special edition, Reising* et al. (*2021*) *discuss guidelines for evaluating a minor modification to a validated assay, including methods for data analysis. Chapter 2.2.5* Statistical approaches to validation *gives an overview of statistical methods that are relevant for validation of diagnostic tests and Chapter 2.2.6* Selection and use of reference samples and panels *describes the use of reference samples and panels for different purposes. This chapter will include several examples of experiments and data analyses previously discussed* (*Reising* et al., *2021*) *that may be used when evaluating the impact of a minor modification and to determine if the assay remains fit for its intended use. The examples only apply if the assay has been validated and if the modification can be classified as minor. Determining if a modification is minor may not always be straightforward and controversy can exist about what constitutes a "minor" and a "major" change for a diagnostic assay. There are some changes that are regarded as major because the biological basis of the assay is fundamentally altered, for example, evolutionary changes or mutations in the nucleic acid make-up of a pathogen will require adjustments to be primers and probes. Similarly, a change from an indirect to a competitive enzyme-linked immunosorbent assay* (*ELISA*) *format using a highly specific monoclonal antibody is considered a major change that would warrant complete re-validation of the assay. Rigorous and well-designed comparability studies provide an objective assessment of whether the assay, when used with a minor change, is as comparable to, and fit for the intended use, as the validated assay. The outcome of the experimental study will determine whether or not the candidate assay requires full re-validation and consequently whether or not it can be used with confidence. Approving use from additional species* (*cattle versus buffalo*) *or different specimen types* (*blood versus semen*) *is different from a minor modification to a validated assay and is discussed throughout chapter 1.1.6 and in Kirkland & Newberry* (*2021*). *This topic will not be discussed further in this chapter.*

This chapter provides examples of experiments for different comparisons (*e.g. limit of detection* [*LOD*] *or performance on diagnostic samples*) *including methods for data visualisation and metrics for quantifying the agreement. One should always visually inspect the data in a manner that is appropriate for the nature of the data. This is an easy method to reveal any glaring problems such as data transcription errors. Data visualisation tools are also useful to reveal patterns within the data,*

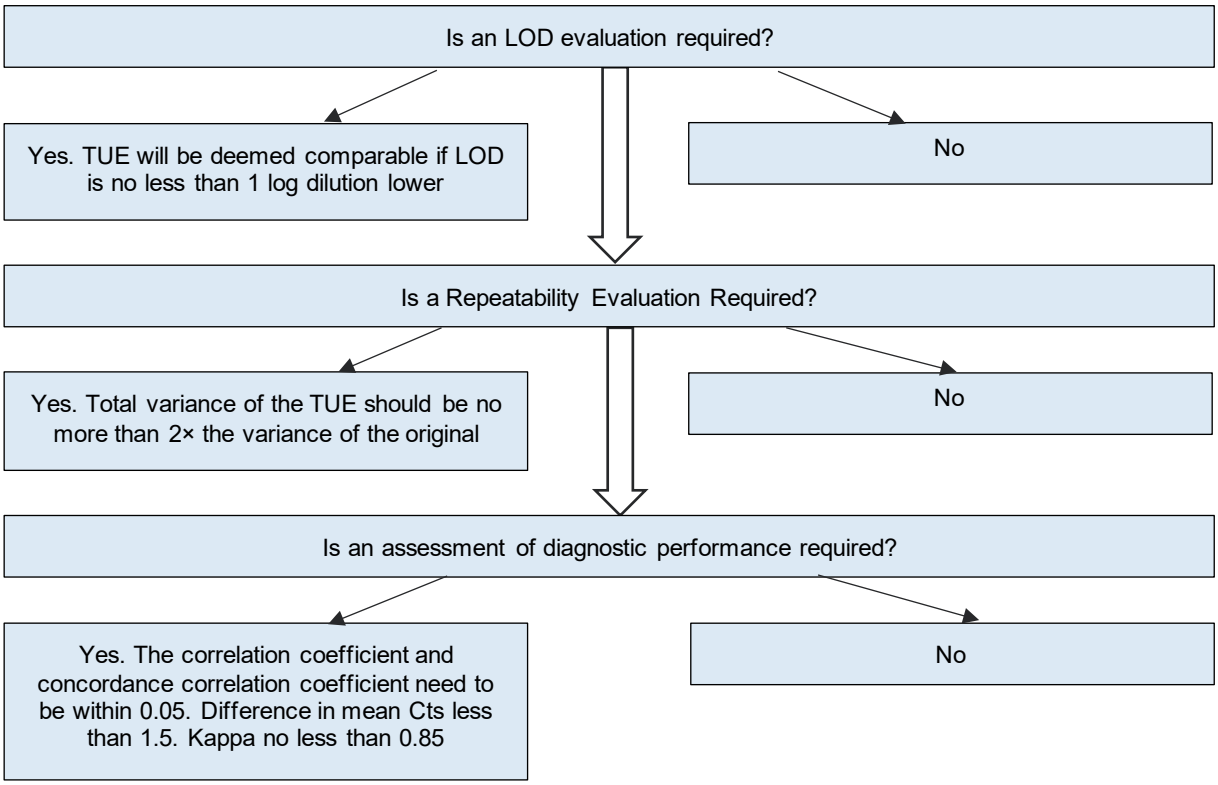
expected or unexpected. Metrics for quantifying agreement offer quantitative evidence for decision making.

The examples presented are from real-time PCR tests, but it can be assumed that the concepts are generally applicable to other test methods. The examples also discuss experimental set-up as care should be taken not to include unwanted sources of variation. Masking of samples and reagents should be incorporated into all comparability study designs. When determining the experiments to evaluate the minor modification, the purpose of the assay should be used as a guide. Typically, the evaluation is limited to Stage 1 (analytical sensitivity and specificity, repeatability) and Stage 2 (diagnostic sensitivity and specificity) characteristics as described in Chapter 1.1.6.

A. DETERMINING EVALUATION AND CONCLUSION CRITERIA

The following flowchart is intended to illustrate a thought process in determining the experiments necessary for demonstrating that the test under evaluation (TUE) is comparable to the original assay. There is no prescription that would be appropriate for all comparability evaluations. The type of change being made and the nature of the diagnostic test will help guide decisions regarding necessary studies. Care should be taken when determining whether a particular study is necessary and to provide justification for including or not including each evaluation into the comparability assessment. Decision criteria need to be included prior to study initiation when possible. The decision criteria presented here are for illustrative purposes only and not intended to be prescriptive. The way the limit of detection (LOD) is typically assessed is to make comparisons at consecutive dilutions on the log10 scale. If it important to get a more precise comparison, then a second stage comparison can be done. If the answer for any required evaluation is no, the investigator can move forward with the next study. If the answer is yes, the study should be conducted and determine if the objective was met. If the objective is met, the investigator can continue to the next study. If the objective is not met, the modified method may be deemed unacceptable, and no further investigation is needed. There are times when all studies are conducted, and the final decision is based on the collection of results. An example of this may happen when a reagent is discontinued and needs replacement immediately. All studies were conducted and the only noted difference between the method was one log dilution in LODs. On closer examination the difference was due to a single negative replicate resulting in the inferior LOD. Therefore, the TUE was deemed acceptable.

Fig. 1. *Illustration of one possible pathway for a comparability assessment for two molecular tests including decision criteria.*



B. LIMIT OF DETECTION

The LOD is a measure of analytical sensitivity. The LOD is the estimated amount of analyte in a specified matrix that would produce a positive result at least a specified percent of the time. For initial validation, the study may use spiked samples. In this example, the objective was to compare the current real-time PCR chemistry/mastermix to one made by a different commercial vendor. The mastermix made by the new vendor would be the TUE in this example. The motivation was to increase the availability of reagents in the event of an unexpected surge in testing. Therefore, the interest lies in the ability to directly compare the LOD between the two mastermixes. To set-up the experiment, nucleic acid extractions were performed for each of the three isolates. The extracted material was pooled for each isolate (not across all isolates) and serially diluted. Each dilution contained sufficient volume to test six aliquots (three per mastermix, current and TUE). On each of the three testing occasions, one operator tested an aliquot from each tenfold dilution using each mastermix on the same PCR plate. The experiment was designed so the variation associated with extraction and operator was not included and to avoid confounding the operator with mastermix. The data are displayed in Figure 2. The concentration of the last dilution showing 100% response was used as the conservative estimate of the LOD. As this was a direct comparison, quantifying the concentration of the analyte was not essential, as the endpoint dilution could be used as the basis for comparison. These data were also used to estimate amplification efficiency (AE), calculated as (10^[1/slope])–1, where the slope is obtained from the regression model. The LODs expressed as $\log_{10}$ dilutions are provided in Table 1 and AEs in Table 2. A two-stage approach could be taken to obtaining a more accurate estimate of the LOD which would require testing samples created using a smaller dilution factor and increasing replicates at each dilution. This two-stage approach may rarely be necessary for a comparability study.

Prior to study initiation, a decision should be made regarding conditions in which the modification would be deemed acceptable. Several external considerations often affect the decision of determining acceptability of a minor modification such as cost, availability, ease of testing, etc. In this instance it was decided that the comparability study would continue if the LODs of the TUE were no less than one $\log_{10}$ dilution worse than the current with similar efficiency estimates. Both conditions were met.

Fig. 2. *Cycle threshold (Ct) plotted against $\log_{10}$ dilution for three viral isolates to compare a new (TUE) mastermix with the current mastermix.*

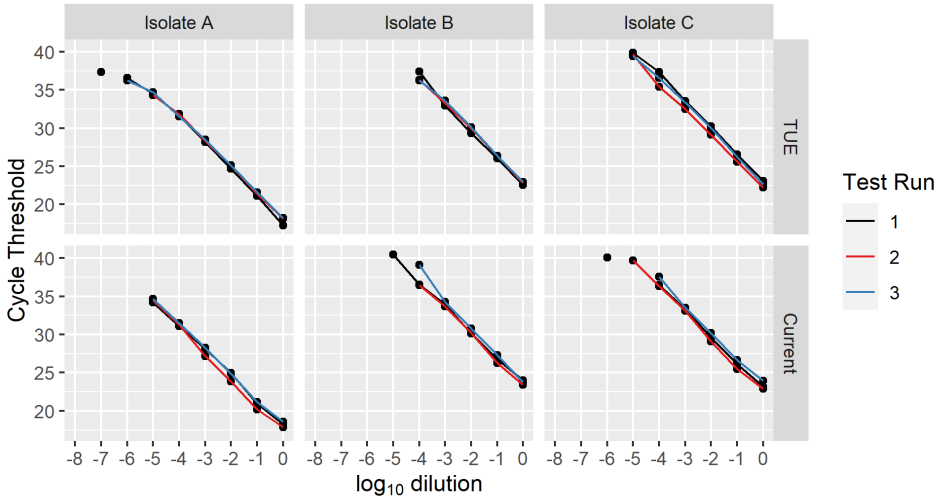


Table 1. *LOD estimates expressed as $\log_{10}$ dilution*

Mastermix	Isolate A	Isolate B	Isolate C
TUE	–5	–4	–5
Current	–5	–4	–4

Table 2. *Amplification efficiency estimates*

Mastermix	**Isolate A**	**Isolate B**	**Isolate C**
TUE	99	94	96

Mastermix	Isolate A	Isolate B	Isolate C
Current	101	95	96

C. REPEATABILITY

Repeatability is defined as the level of agreement between results of replicates of a sample both within and between runs of the same test method in a laboratory. Section 1.1 of Chapter 1.1.6 states that each replicate is run through all the steps of the assay for validation. In a comparability study, running all replicates through each step of the assay may not be necessary when attempting to isolate the impact of the minor modification. In this example where the modification is to the mastermix, the starting material was a pool of extracted nucleic acid diluted into low, medium, and high concentrations. Testing was conducted across multiple days by the same operator. The data are displayed in Figure 3. Table 3 provides estimates of within run and between run variability (expressed as standard deviations). A linear mixed effects model was used to estimate the inter- and intra-assay variability (reported as standard deviations). It is clear from Figure 3 that there is increased variance for the low concentration, which is not uncommon for a real-time PCR. Therefore, the intra-assay variance was estimated separately for each concentration. These measures are sensitive to any extreme values. There was nothing in the data to suggest that the TUE would perform worse than the current mastermix.

The study design here used three concentrations. Alternatively, a study could be designed to test at a single concentration near the assay's limit of detection.

Fig. 3. *Cycle threshold plotted against test run. The colours correspond to concentration level and the plots correspond to which mastermix was used.*

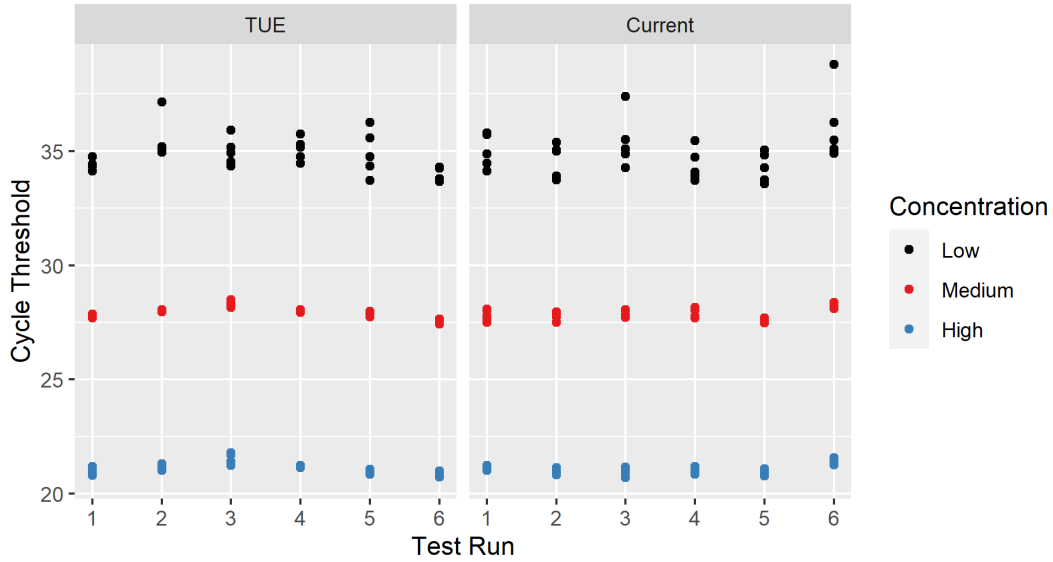


Table 3. Standard deviation estimates for inter- and intra-assay variability

Mastermix	Inter	Intra (high)	Intra (medium)	Intra (low)
TUE	0.19	0.13	0.17	1.04
Proposed	0.25	0.14	0.09	0.71

Another example in which repeatability is assessed would be when a control sample is being replaced. In this situation, testing can occur using a matched-pairs design. For instance, 30 routine PCR plates tested with both the current positive amplification control and the proposed replacement (TUE), resulting in 30 sets of paired data. These data can be visualised using a correlation scatterplot or a Bland–Altman plot (examples provided below).

D. DIAGNOSTIC CHARACTERISTICS

It is likely that estimating diagnostic sensitivity and diagnostic specificity for a minor modification to an assay is not necessary. Instead testing positive and negative diagnostic samples using the original assay and the assay after modification (i.e. TUE) would be sufficient to assess agreement for a comparability study.

Sample selection is a key factor in designing a study to evaluate the diagnostic performance or creating a panel of samples to be tested (Kirkland & Newberry, 2021). While there is never a one-size-fits-all answer to the question of how many samples should be tested, often 30 positive and 30 negatives is sufficient, for comparability, assuming they are a diverse set (Reising *et al.,* 2021). The selection of samples to span the range of reactivity of the assay is perhaps more important than the actual number. The question of sample size is perhaps one of the most common. When the goal is to demonstrate that a treatment has some desired effect using a statistical hypothesis test, a power analysis is used to determine an appropriate sample size. As discussed below, hypothesis testing is not appropriate for a comparability study as the objective is not to demonstrate a difference, but rather to conclude similarity.

A study was conducted to determine if a laborious and time-consuming sample processing step could be removed from processing swab samples prior to extraction and testing on a real-time PCR used for screening. Sample throughput would be substantially increased if the sample processing step could be removed without compromising the test results. A limit of detection and repeatability study were conducted prior to testing diagnostic samples. For the comparability study, 195 samples submitted for routine testing were tested (prospectively) side-by-side including and excluding the processing step in question. More samples were tested than what would typically be necessary for a comparability study when known positive and negative samples were selected randomly from a repository. Table 4 provides a 2×2 cross-classification table of the results indicating that out of 124 samples that tested negative in the original test, 15 samples tested positive in the TUE and out of 71 samples that tested positive in the original test, 13 tested negative in the TUE. Table 5 provides estimates of Cohen's Kappa, the Pearson correlation coefficient, the concordance correlation coefficient, and the mean difference, along with 95% confidence intervals as metrics to assess agreement. Test results are displayed using a scatterplot in Figure 4 (scatterplot) and a Bland–Altman/Tukey median difference plot (Bland & Altman, 1999; 2007) in Figure 5.

Table 4. *Cross classification of results with* (*current*) *and without* (*TUE*) *sample processing step*

	TUE	
Current	Negative	Positive
Negative	109	15
Positive	13	58

Table 5. *Summary metrics to assess agreement*

Metric	**Estimate (95% confidence interval)**
Cohens Kappa	0.69 (95% *CI*: 0.59, 0.80)
Correlation	0.93 (95% *CI*: 0.88, 0.96)
Concordance Correlation	0.92 (95% *CI*: 0.87, 0.95)
Mean Difference	–0.37 (95% *CI*: -0.80, 0.07)

The number of samples that disagreed with the positive/negative classification was about the same in both directions (i.e. positive using the current method and negative using the TUE versus negative using the current method and positive using the TUE). The closeness in values of the correlation and concordance correlation suggests good agreement and this is visually confirmed in the scatterplot as well as the Bland-Altman plot. The average difference (TUE – current) is a small negative value with a confidence interval that includes zero. The individual data in Figure 4 appear to be scattered symmetrically around the average difference. The range of observed values on the left side of the plot are smaller than on the right side of the plot indicate increasing variance from left to right. However, this visual assessment may be a result of fewer samples with lower Ct values (higher analyte concentrations). Most importantly, the Bland–Altman 95% limits of agreement of –3.58 and 2.85 suggest that for most samples we can expect the discrepancy between including and excluding the laborious sample processing step, should be no greater than about 3.5 cycle thresholds in either direction, which is just over one $\log_{10}$ (assuming perfect amplification). This statistic may be compared with a (preferably predefined) criterion for practical or diagnostic relevance.

Whereas the estimate of Kappa seems low, it is important to remember there is no universal interpretation for Kappa (Agresti, 2002). The estimate of Kappa is dependent upon the prevalence in the sample set tested.

The collection of test results allowed for the removal of the laborious and time-consuming processing step without the fear of loss of test sensitivity.

Fig. 4. *Scatterplot of Ct values from the TUE (without the sample processing step) and the current method (with the sample processing step). The diagonal line represents the 45° line of agreement. The number in the top right corner is the number of samples that were not detected by either method.*

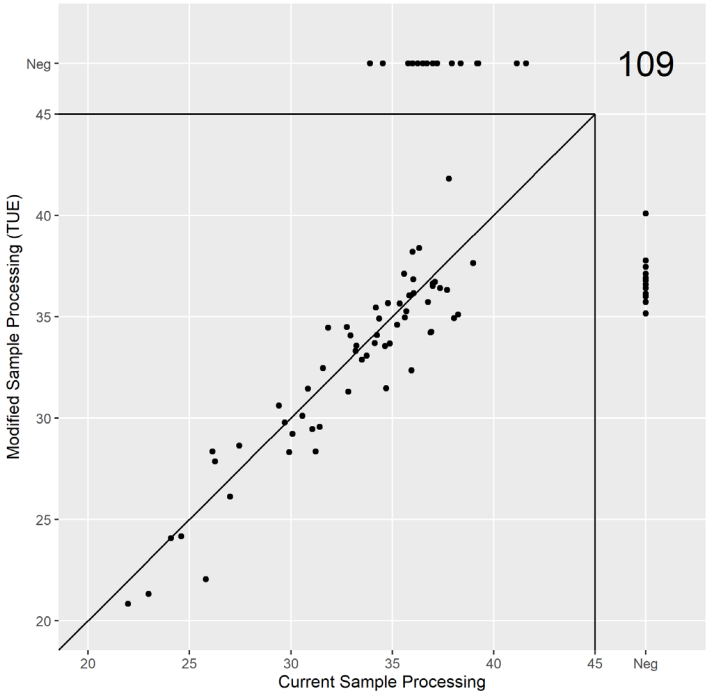
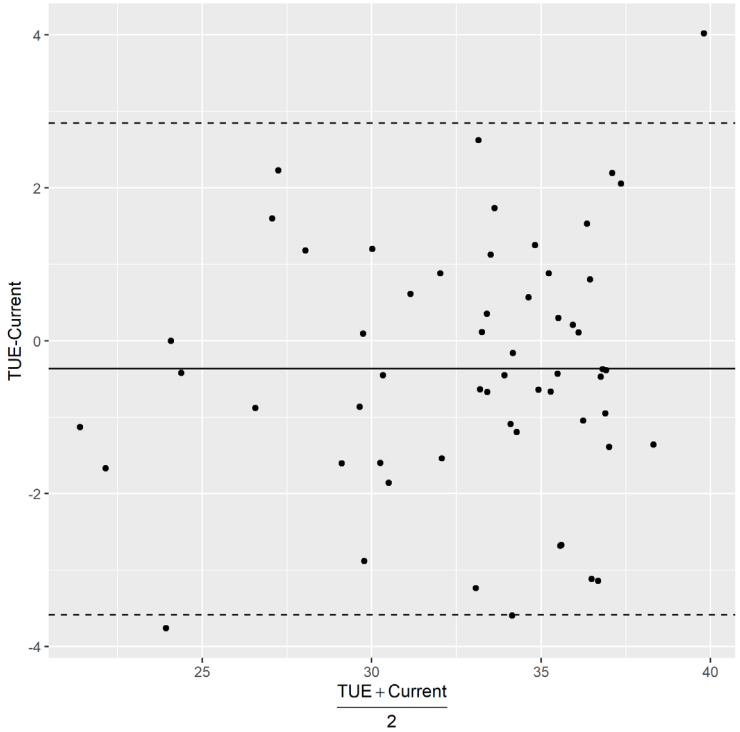


Fig. 5. *Bland–Altman Plot. The solid horizontal line is drawn at the average of the paired differences (TUE – current), and the dashed lines are the 95% limits of agreement.*

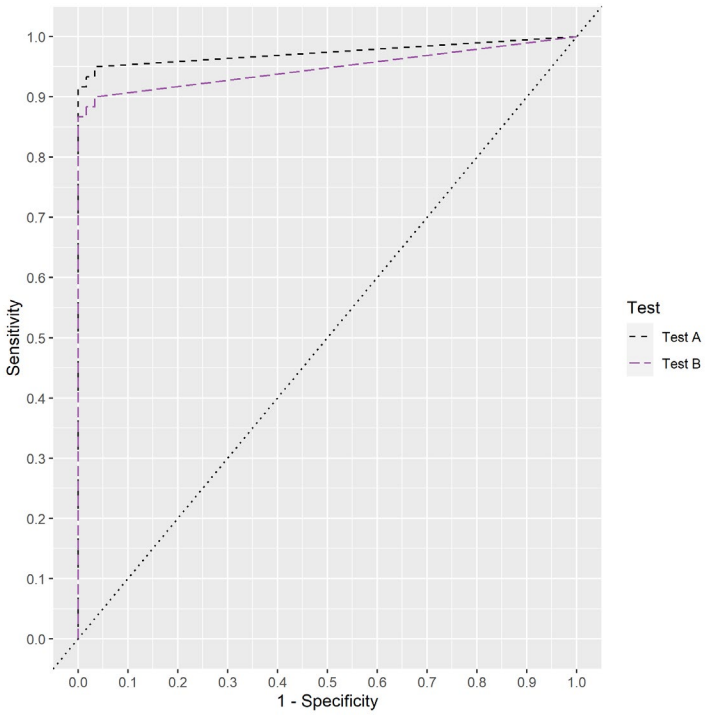


E. COMPARISON OF ROC CURVES

Receiver operating characteristic (ROC) analysis is used to compare the accuracy of diagnostic test methods at different cut-offs of one or more different tests when the same samples have been tested using different test methods. Zhou *et al.* (2011) indicate that the AUC (area under the ROC curve) has several different interpretations: 1) the average sensitivity for all possible values of specificity; 2) the average value of specificity for all possible values of sensitivity; and 3) the probability that a randomly selected diseased/infected individual would have a test result that is more indicative of disease/infection that a randomly selected individual that is not diseased/infected. They also provide examples of incorrect interpretations of AUC.

In this example, a methods comparison study was conducted to evaluate a new thermocycler (TUE). A panel of 120 samples, 60 positive and 60 negatives, were tested where the extracted material from each sample was amplified on each thermocycler. ROC curves and the AUC were used to evaluate the data. The ROC curves are displayed in Figure 6. The AUC for the current method (current thermocycler, Test A) was estimated to be 0.98 and the AUC for the new thermocycler (TUE, Test B) was estimated to be 0.95. The difference in AUC was estimated to be 0.03 (95% *CI*: –0.003, 0.05). It was determined that the thermocycler would be an appropriate alternative to the current one because the difference in AUCs was very close to zero and the confidence interval included zero, and was very narrow.

Fig. 6. *ROC curves comparing two thermocyclers* (*current = test A, proposed = Test B*). *The diagonal line would represent a ROC curve to a test with an AUC of 0.5.*



F. DISCUSSION

There are many reasons why comparability studies are necessary including depleted reagents, more cost-effective reagents, changes in instrumentation, etc. Analytical and diagnostic characteristics can be evaluated to determine whether the TUE is comparable to the original format of the test. While statistical analysis and objective assessments aid in the final decision, often practical considerations are used including, but not limited to, cost, ease of use, throughput capacity and increasing surge capabilities in determining acceptable conclusion criteria.

A comparability study is intended to evaluate a minor modification to an already validated assay. The intention is to thoroughly assess the impact the modification has to the assay without requiring revalidation. Scientific and clinical considerations guide decisions and reliance should not be solely on the data. While there may be changes, such as time or temperature during PCR cycling, that may require some optimisation work, a comparability assessment is a direct comparison of the TUE to the current, validated method. It is not meant to be an evaluation of all possible alternatives but as a direct comparison to a proposed alternative.

Validation, comparability, and verification studies are all intended to demonstrate the fitness for purpose of an assay. WOAH's formal validation standard for diagnostic assays for infectious diseases of terrestrial animals is chapter 1.1.6. This document has provided examples and considerations for a comparability study. Verification is discussed

in Kirkland & Newberry (2021). There are times when a verification or a comparability study may be used such as changes in batches of test reagents or a new lot of a test control. Verification would be used when introducing a test method into a different laboratory, in a different geographical region, or a new animal population and is discussed in Kirkland & Newberry (2021). Verification studies would not require the same direct comparison that a comparability study would. For both the diagnostic sample evaluation of a comparability study and the construction of the panel of test samples for a verification, a good representation of the population of interest should be used. Both recommend testing 30 positive and 30 negative samples. Both are tools used when a change is not made to the biological basis of the assay. The goal of both types of evaluation is to ensure the assay remains fit for the intended purpose.

Various data visualisation and statistical analysis tools commonly used in comparability studies including pros and cons are included in Table 6 (reproduced from Reising *et al.* [2021]). More extensive discussion of such tools and others may be found in a recent, comprehensive text by Choudhary & Nagaraja (2017).

Table 6. *Commonly used statistical methods and data visualisation tools to assess agreement with pros and cons (from Reising* et al. [*2021*])

Method	**Pros**	**Cons**
Data visualisation - scatterplot	Using a square plot with a line of agreement, agreement or specific bias can be evaluated visually	Does not provide a metric for agreement or bias. Subtle patterns may be less evident than in the mean-difference plot.
Correlation coefficient	Used with visualisation and concordance correlation, can help quantify degree of association	Often misused and misinterpreted. High correlation does not always correspond to agreement.
Linear regression	Allows quantitative assessment of systematic (intercept) or proportional (slope) bias	Measurement error in the independent variable (usually the original method) is not accounted for and estimates of the slope and intercept will be biased. (More advanced regression procedures can address these flaws)
Mean-difference plot	Easy to visualise bias when compared to a horizontal line. The expected extent of disagreement may be quantified using Bland–Altman 95% limits of agreement, which must be evaluated for practical significance	May need to be modified to quantify proportional bias or proportional error. Method is not resistant to outliers
Null hypothesis test for differences		Never appropriate when attempting to demonstrate a change has no practical impact. Do not use for method comparison
Equivalence test for differences	Careful consideration is necessary to set equivalence margins defining practically irrelevant differences	May require large sample sizes
Kappa	Popular measurement of agreement	Does not have absolute interpretation and is often misrepresented by comparing against predetermined scales of agreement. Sensitive to prevalence of positives in the sample, which is unrelated to method comparability
Area under the receiver operating-characteristic curve	Represents a trade-off between sensitivity and specificity. Can be used to compare diagnostic performance of the modified and original methods	Requires a sufficient number of diagnostic samples to be tested in a side-by-side fashion

G. DATA ANALYSIS

All data were stored in Microsoft EXCEL. Analysis (Pinheiro *et al.,* 2022; Wickham, 2011) and plotting (Wickham, 2016) were performed using R: A Language and Environment for Statistical Computing, Version 3.6.3 (R Core Team 2020).

All data were stored in Microsoft EXCEL. Analysis and plotting were performed using R: A Language and Environment for Statistical Computing, Version 3.6.3.

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NB: FIRST ADOPTED IN 2016.

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CHAPTER 3.1.2.

AUJESZKY'S DISEASE (INFECTION WITH AUJESZKY'S DISEASE VIRUS)

SUMMARY

Description and importance of the disease*: Aujeszky's disease, also known as pseudorabies, is caused by ~~an alphaherpesvirus~~ Varicellovirus suidalpha 1, a DNA virus belonging to the Order Herpesvirales that infects the central nervous system and other organs, such as the respiratory tract, in a variety of mammals~~.~~ ~~except~~ infection of humans and the tailless apes is rare. It is associated primarily with suidae (pigs or wild boar), the natural host, which remain latently infected following clinical recovery (except piglets under 2 weeks of age, which die from encephalitis). The disease is controlled by containment of infected herds ~~and by~~ the use of vaccines ~~or~~ and removal of latently infected animals.*

~~A diagnosis of Aujeszky's disease is established~~ Assays that detect the virus (by virus isolation or polymerase chain reaction [PCR]), as well as the serological response are useful in the diagnosis of Aujeszky's disease ~~by detecting the agent (by virus isolation or polymerase chain reaction [PCR]), as well as by detecting a serological response in the live animal~~.

Identification of the agent: *Isolation of Aujeszky's disease virus can be made by inoculating a tissue homogenate, for example of brain and tonsil or material collected from the nose/throat, into a susceptible cell line such as porcine kidney (PK-15 or SK6), or primary or secondary kidney cells. The specificity of the cytopathic effect ~~is~~ can be verified by immunofluorescence, immunoperoxidase or neutralisation with specific antiserum. The viral DNA can also be identified using PCR; this can be accomplished using real-time PCR techniques.*

Serological tests: *Aujeszky's disease antibodies are demonstrated by virus neutralisation, latex agglutination or enzyme-linked immunosorbent assay (ELISA). A number of ELISA kits are commercially available world-wide. A WOAH International Standard Reference Serum defines the lower limit of sensitivity for routine testing by laboratories that undertake the serological diagnosis of Aujeszky's disease.*

~~It is possible to distinguish between antibodies resulting from natural infection and those from vaccination with gene-deleted vaccines.~~

Requirements for vaccines: *Vaccines should prevent or at least limit the excretion of virus from infected pigs. Recombinant DNA-derived gene-deleted or naturally deleted live Aujeszky's disease virus vaccines~~,~~ lack a specific glycoprotein (gG, gE, or gC), which enables the use of companion diagnostic tests to differentiate vaccinal antibodies from those resulting from natural infection.*

A. INTRODUCTION

Aujeszky's disease, also known as pseudorabies, is caused by ~~*Suid herpesvirus 1* (SHV-1)~~ *Varicellovirus suidalpha 1* (SuAHV1, also called PRV [pseudorabies virus]), a member of the subfamily *Alphaherpesvirinae* and the family *Ortho*herpesviridae. The virus should be handled with appropriate biosafety and containment procedures as determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*). The virus infects the central nervous system and other organs,

such as the respiratory tract, of a variety of mammals (such as dogs, cats, cattle, sheep, rabbits, foxes, minks, etc.) ~~with the exception of humans and the tailless apes~~. Some recent reports from China (People's Rep. of) described human encephalitis associated with new variant SuAHV1 strains (Bo & Li, 2022; Liu *et al.*, 2021) ~~It~~ SuAHV1 is associated primarily with pigs, the natural host, which remain latently infected following clinical recovery (except piglets under 2 weeks of age, which die from encephalitis). The pig is able to survive the productive infection and ~~In consequence, the pig is the only species able to survive a productive infection and therefore,~~ serves as the reservoir host. In pigs, the severity of clinical signs depends on the age of the pig, the route of infection, the virulence of the infecting strain and the immunological status of the animal. Young piglets are highly susceptible with mortality rates reaching 100% during the first 2 weeks of life. These animals show signs of hyperthermia and severe neurological disorders: trembling, incoordination, ataxia, nystagmus to opisthotonos and severe epileptiform-like seizures. When pigs are older than 2 months (grower-finisher pigs), the respiratory forms become predominant with hyperthermia, anorexia, and mild to severe respiratory signs: rhinitis with sneezing and nasal discharge that may progress to pneumonia. The frequency of secondary bacterial infections is high, depending on the health status of the infected herd. In this group of pigs, the morbidity can reach 100%, but in cases of the absence of complicated secondary infections, mortality ranges from 1% to 2% (~~Pejsak & Truszczynski, 2006~~ Mettenleiter *et al.*, 2019). Sows and boars primarily develop respiratory signs, but in pregnant sows, the virus can cross the placenta, infect and kill the fetuses, inducing abortion, return to oestrus, or stillborn fetuses. Virus may be found in the semen of infected boars (Mettenleiter *et al.*, 2019 ~~van Rijn *et al.*, 2004~~).

In the other susceptible species, ~~the disease is fatal,~~ the predominant sign being intense pruritus causing the animal to gnaw or scratch part of the body, usually the head or hind quarters, until great tissue destruction is caused. For that reason, the disease was named "mad-itch" in the past, replaced nowadays by "pseudorabies".

Focal necrotic and encephalomyelitis lesions occur in the cerebrum, cerebellum, adrenals and other viscera such as lungs, liver or spleen. In fetuses or very young piglets, white spots on the liver are highly suggestive of their infection by the virus. Intranuclear ~~lesions~~ inclusions are frequently found in several tissues (Mettenleiter *et al.*, 2019).

Aujeszky's disease is endemic in many parts of the world, but several countries have successfully completed eradication programmes, e.g. the United States of America, Canada, New Zealand and many Member States of the European Union.

The disease is controlled by containment of infected herds ~~and~~ by the use of vaccines ~~or~~ and removal of latently infected animals (~~Pejsak & Truszczynski, 2006~~ Mettenleiter *et al.*, 2019). Stamping out has been or is used in several countries usually when the infected farms are small or when the threat to neighbouring farms is very high in free countries.

Whereas isolation of the Aujeszky's disease virus or detection of the viral genome by the polymerase chain reaction (PCR) are used for diagnosis in the case of lethal forms of Aujeszky's disease or clinical disease in pigs, serological tests are required for diagnosis ~~of latent infections and~~ after the disappearance of the clinical signs. Affected animals except suids~~,~~ usually do not live long enough to produce any marked serological response. Serological tests are recommended to confirm freedom from previous infection ~~are the tests to be used to detect subclinically or latently infected pigs, especially in the case of qualification of the health status of the animals for international trade or other purposes~~.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of Aujeszky's disease and their purpose

Method	Purpose					
	Population freedom from infection[a]	**Individual animal freedom from infection prior to movement[b]**	**Contribute to eradication policies[c]**	**Confirmation of clinical cases[d]**	**Prevalence of infection – surveillance[e]**	**Immune status in individual animals or populations post-vaccination[f]**
Detection and identification of the agent[a]						
Virus isolation	–	–	–	+++	–	–
Real-time PCR	–	+	+	+++	+	–
Detection of immune response						
Latex agglutination	+++	+++	+++	+	+++	++ ~~+~~

Method	Purpose					
	Population freedom from infection[a]	Individual animal freedom from infection prior to movement[b]	Contribute to eradication policies[c]	Confirmation of clinical cases[d]	Prevalence of infection – surveillance[e]	Immune status in individual animals or populations post-vaccination[f]
ELISA gB	+++	+++	+++	+	+++	++~~+~~
ELISA gE	++	++	+++	+	++	++
VN	+	+	+	+	+	+++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay; VN = virus neutralisation.
[a]A combination of agent identification methods applied on the same clinical sample is recommended.
[a]See Appendix 1 of this chapter for justification table for the scores given to the tests for this purpose.
[b]See Appendix 2 of this chapter for justification table for the scores given to the tests for this purpose.
[c]See Appendix 3 of this chapter for justification table for the scores given to the tests for this purpose.
[d]See Appendix 4 of this chapter for justification table for the scores given to the tests for this purpose.
[e]See Appendix 5 of this chapter for justification table for the scores given to the tests for this purpose.
[f]See Appendix 6 of this chapter for justification table for the scores given to the tests for this purpose.

1. Detection and identification of the agent

1.1. Virus isolation

The diagnosis of Aujeszky's disease can be confirmed by isolating the virus from the oro-pharyngeal fluid, nasal fluid (swabs) or tonsil swabs from living pigs, or from samples from dead pigs or following the presentation of clinical signs such as encephalitis in herbivores or carnivores. For post-mortem isolation of SuAHV1, samples of brain, tonsil, and lung are the preferred specimens. In cattle, infection is usually characterised by a pruritus, in which case a sample of the corresponding section of the spinal cord may be required in order to isolate the virus. ~~In latently infected pigs, the trigeminal ganglia is the most consistent site for virus isolation, although latent virus is usually non infective unless reactivated, making it difficult to recover in culture.~~

Tissue samples are homogenised in normal saline or cell culture medium with antibiotics. The method used should be suitable for the subsequent diagnostic test. The amount of tissue homogenised should take into account a possible non-homogeneous distribution of the virus (e.g. in the brain). A tissue homogenate of approximately 10% is recommended. The resulting suspension is clarified by low-speed centrifugation, e.g. at 900 ***g*** for 10 minutes. The supernatant fluid is used to inoculate any sensitive cell culture system. Numerous types of cell line or primary cell cultures are sensitive to SuAHV1, but a porcine kidney cell line (PK-15 or SK6) is generally employed. The overlay medium for the cultures should contain antibiotics (such as: 200 IU/ml penicillin; 100 µg/ml streptomycin; 100 µg/ml polymyxin; and 3 µg/ml fungizone).

SuAHV1 induces a cytopathic effect (CPE) that usually appears within 24–72 hours, but cell cultures may be incubated for 5–7 days. The monolayer develops accumulations of birefringent cells, followed by complete detachment of the cell sheet. Syncytia also develop, the appearance and size of which are variable. In the absence of any obvious CPE, it is advisable to make one blind passage into further cultures after freeze–thawing the cell culture vessel. Additional evidence may be obtained by staining infected cover-slip cultures with haematoxylin and eosin to demonstrate the characteristic herpesviral acidophilic intranuclear inclusions with margination of the chromatin. Virus identity should be confirmed by immunofluorescence, immunoperoxidase, neutralisation using specific antiserum following the method described in Section B.2.1. or by PCR.

~~The isolation of SHV-1 makes it possible to confirm Aujeszky's disease, but failure to isolate does not guarantee freedom from infection.~~

1.2. Identification of virus by the polymerase chain reaction

The PCR can be used to identify SuAHV1 genomes in secretions or organ samples. Many individual laboratories have established effective protocols, but there is as yet no internationally agreed standardised approach.

~~The PCR is based on the selective amplification of a specific part of the genome using two primers located at each end of the selected sequence. In a first step, the complete DNA may be isolated using standard procedures (e.g. proteinase K digestion and phenol–chloroform extraction) or commercially available DNA extraction kits. Using cycles of DNA denaturation to give single-stranded DNA templates, hybridisation of the primers, and synthesis of complementary sequences using a thermostable DNA polymerase, the target sequence can be amplified up to 10^6 fold.~~ The primers must be designed to amplify a sequence conserved among SuAHV1 strains, for example parts of the gB, ~~,~~ gC or gD genes that code for essential glycoproteins have been used (~~Mengeling *et al*., 1992; Van Rijn *et al*., 2004; Yoon *et al*., 2006~~). Real-time PCRs have been developed that can differentiate gE-deleted vaccine viruses from wild-type virus based on the specific detection of gB and gE genes (Ma *et al*., 2008; Wernike *et al*., 2014).

Real-time PCR

Pathogen/ target gene	Primer/probe (5'–3')	Concentration	Cycling parameters[(a)]
Method 1: Ma *et al.* (2008)			
gB	Fwd primer: ACA-AGT-TCA-AGG-CCC-ACA-TCT-AC Rev primer: GTC-YGT-GAA-GCG-GTT-CGT-GAT Probe: ACG-TCA-TCG-TCA-CGA-CC	10 pmol/reaction 5 pmol/reaction	40 cycles of 94°C/15 sec and 62°C/60 sec Cutoff: 35Ct
gE	Fwd primer: CTT-CCA-CTC-GCA-GCT-CTT-CTC Rev primer: GTR-AAG-TTC-TCG-CGC-GAG-T Probe: TTC-GAC-CTG-ATG-CCG-C	10 pmol/reaction 5 pmol/reaction	40 cycles of 94°C/15 sec and 62°C/60 sec. Cutoff: 39Ct
Method 2: Wernike *et al.* (2014)			
gB	Fwd primer: ACC-AAC-GAC-ACC-TAC-ACC-AAG Rev primer: CCT-CCT-CGA-CGA-TGC-AGT-TG Probe: CGG-GCT-TCT-ACC-ACA-CGG-GCA-CCT	20 pmol/reaction 2.5 pmol/reaction	42 cycles of 95°C/1 min, 58°C/30 sec, and 72°C/30 sec
gE	Fwd primer: CTG-TAC-GTG-CTC-GTG-ATG-AC Rev primer: CTC-CTT-GRT-GAC-CGT-GAC-G Probe: TCG-CCA-CCT-GGG-ACT-ACA-CGC-TCG	20 pmol/reaction 2.5 pmol/reaction	42 cycles of 95°C/1 min, 58°C/30 sec, and 72°C/30 sec

[(a)] A denaturation step prior to cycling has not been included.

Conventional PCR

Pathogen/ target gene	Primer (5'–3')	Cycling parameters[(a)]
Method 1: Ma *et al.* (2008); amplicon size :195pb		
SuAHV1	Fwd: ACG-GCA-CGG-GCG-TGA-TC- Rev: GGT-TCA-GGG-TCA-CCC-GC	40 cycles of 94°C/20 sec, 55°C/30 sec, and 72°C/60 sec
Method 2: Müller *et al.* (2010); amplicon size :217pb		
gD	Fwd: CAC-GGA-GGA-CGA-GCT-GGG-GCT Rev: GTC-CAC-GCC-CCG-CTT-GAA-GCT	40 cycles of 95°C/15 sec, 60°C/10 sec, and 72°C/10 sec

[(a)] A denaturation step prior to cycling has not been included.

~~The amplified product may be identified from its molecular weight as determined by migration in agarose gel, with further confirmation where possible by sequencing the amplified product. More recent techniques include the use of fluorescent probes linked to an exonuclease action and real-time monitoring of the evolution of product, enabling simultaneous amplification and confirmation of the template DNA thus increasing the rapidity and specificity of the PCR assays.~~

~~In all cases, the main advantage of PCR, when compared with conventional virus isolation techniques, is its rapidity; with the most modern equipment, the entire process of identification and confirmation can be completed within one day. However, because of the nature of the test, many precautions need to be taken to avoid contamination of samples with extraneous DNA from previous tests or from general~~

~~environmental contamination in the laboratory (see Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*). This may limit the value of the test for many laboratories unless care is taken to avoid DNA carry-over contamination.~~ The use of an internal control is recommended so as to avoid false-negative results by ensuring adequate efficiency of DNA extraction and confirming the absence of PCR inhibitors in each sample. In practice, different systems can be used for detection of endogenous or exogenous genes (Hoffman *et al.,* 2009). Kits for the test are commercially available (Pol *et al.,* 2013).

2. Serological tests

Virus neutralisation (VN) has been recognised as the reference method for serology ~~(Moennig *et al.*, 1982)~~, but for general diagnostic purposes it has been widely replaced by the enzyme-linked immunosorbent assay (ELISA) because of its suitability for large-scale testing (Moennig *et al.*, 1982). The tests can be performed on a variety of matrices (e.g. serum, whole blood, milk, muscular exudates, and filter paper), but the preferred matrix is serum.

A latex agglutination test has also been developed and can be used for screening for antibodies. Kits for the test are commercially available (Schoenbaum *et al.*, 1990).

Serological tests are <u>usually</u> carried out only for suids, as <u>most often</u> other animals (herbivores and carnivores) die too quickly to produce antibodies. In free areas where pigs are not vaccinated, an active epidemiological survey can be carried out using ELISA gB or gE or latex agglutination kits. As antibodies can be detected between 7 and 10 days post-infection, these serological tools can also be used to confirm infection in pigs in the case of a suspected outbreak. In area<u>s</u> where pigs are vaccinated with gE deleted vaccines, the ELISA gE kits permit the differentiation between infected and vaccinated pigs (DIVA), but to assess the level of immunity induced by vaccination, gB ELISA, latex agglutination kits or viral neutralisation should be used. <u>Moreover, in Aujeszky's disease free areas, the ELISA gE can be used to confirm or deny a positive or doubtful result obtained with ELISA gB on serum collected from unvaccinated pigs.</u>

Any serological technique used should be sufficiently sensitive to give a positive result with the WOAH International Standard Reference Serum or a calibrated secondary serum. Reference serum can be obtained from the WOAH Reference Laboratory for Aujeszky's disease in France<u>[1]</u> ~~(see Table given in Part 4 of this *Terrestrial Manual*)~~. For international trade purposes, the test should be sensitive enough to detect the standard serum diluted 1/2. To authorise pig movement from an area where deleted gE vaccines are used to a free area, serological assays should be able to detect at least the dilution of 1/8 for ELISA gE of the WOAH International Standard Reference Serum as prescribed by the European Commission (~~2008~~ <u>2020</u>).

2.1. Virus neutralisation

VN in cell culture can be performed in several ways, which vary according to the length of incubation of the virus/serum mixtures (e.g. 1 hour at 37°C or 24 hours at 4°C) and the presence or absence of complement. Most laboratories use a reaction period of 1 hour at 37°C in the absence of complement, because this is easy and rapid. However, the sensitivity can be improved by increasing the incubation period to 24 hours at 4°C, which facilitates the detection of antibody levels 10–15 times lower than in the 1-hour method. For international trade purposes, the test method should be validated as being sensitive enough to detect the WOAH Standard Reference Serum diluted 1/2.

VN cannot be used to differentiate antibodies of vaccinal origin from those caused by natural infection. It is one of the two tests available that complies with the requirement in the WOAH *Terrestrial Animal Health Code* chapter when it refers to "a diagnostic test to the whole virus".

i) Cells

Cells susceptible to infection with S<u>uA</u>HV1 are used; they may be cell lines (e.g. PK-15, SK6, MDBK), or primary or secondary cell cultures (e.g. porcine kidney).

ii) Cell culture medium

The medium depends on the type of cells. For example, the medium for PK-15 cells is Eagle's minimal essential medium (MEM) + 10% fetal bovine serum <u>(FBS)</u> and antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin or, alternatively, 50 µg/ml gentamycin).

iii) Maintenance of the cells

The cells are cultured in cell culture vessels of, for example, 75 cm^2. They ~~are~~ <u>can be</u> trypsinised once or twice per week. ~~For weekly trypsinisation, the cells are usually cultured in 50 ml of medium,~~

[1] <u>Please consult the online list: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/</u>

~~with a multiplication rate of 5. For two trypsinisations a week, the cells are cultured in 30 ml of medium, with a multiplication rate of 3.~~ For trypsinisation, the growth medium is removed once the cell sheet is complete. The cell sheet is washed with about 5 ml of recently thawed trypsin/ethylene diamine tetra-acetic acid (EDTA) (0.25%) in an isotonic buffer. The washing fluid is discarded and the preparation is washed again, retaining only a few drops of trypsin. The container is placed in an incubator at 37°C for 5–10 minutes until the cells have become detached. Once the sheet is detached and the cells are well separated, for twice weekly passage they ~~are~~ can be suspended in 90 ml of growth medium, and this suspension is distributed into three 75 cm^2 cell culture vessels. For weekly trypsinisation the cells ~~are~~ can be suspended in 150 ml of growth medium and the suspension distributed into five 75 cm^2 cell culture ~~bottles~~ flasks. These dilution rates may be adapted to the cell line used.

iv) Virus

A suitable strain of SuAHV1, such as the Kojnok strain or the NIA-3 strain, is stored at a temperature of –65°C or below, or in freeze-dried form at 4°C.

v) Preparation of stock virus suspension

The culture fluid is removed from a cell culture vessel containing a complete cell sheet. About 1 = 3 ml of stock virus suspension of known titre (about 10^7 $TCID_{50}$/ml [50% tissue culture infective dose]) is added, depending on the surface of the cell culture flask (25 or 75 cm^2), ~~and~~ The vessel is then incubated at 37°C±2°C for 1 hour with a gentle shake. Remove the inoculum and add 10–50 ~~30~~ ml of culture medium ~~is added and~~ containing 2–5% of FBS. Then the vessel is again incubated at 37°C±2°C. The vessel is examined frequently until there is about 75% cell destruction (after about 18–48 hours). It is then frozen at a temperature of –65°C or lower to disrupt the cells.

The vessel is then thawed and shaken vigorously. Medium is collected and centrifuged at 1500 ***g*** for 15 minutes. The supernatant fluid is divided into portions (of about 0.5 ml) in small tubes that are labelled (date and virus reference) before being stored at a temperature of –65°C or lower until required.

vi) Titration of the stock virus suspension

Titration of the stock suspension is performed by the method of Reed & Muench or that of Kärber, and the titre is expressed per 50 µl and per ml.

The VN test requires an internal quality control serum with a known titre of neutralising antibody to SuAHV1 (it can be calibrated against an international standard serum or a secondary standard prepared from that serum), and a negative control serum (from a specific antibody free pig, e.g. from an official Aujeszky's disease free herd). The test sera themselves should be of good quality, clearly labelled, of known provenance with clinical history, stored in refrigeration at all times, free from fungal or bacterial contamination, non-haemolysed and of sufficient quantity. Serum should be separated from the coagulum without delay, thereby preventing toxicity.

There are qualitative and quantitative procedures for VN, both of which are described below.

2.1.1. Qualitative virus neutralisation technique

i) Complement in the serum samples is destroyed by heating in a water bath at 56–59°C for 30 minutes.

ii) Each undiluted serum sample is placed in two to three wells, at 50 µl per well, of a 96-well cell-culture grade microtitre plate. Each serum can also be diluted 1/2 in the MEM, before being placed in two other wells.

iii) 50 µl of virus suspension containing 100 $TCID_{50}$ (or 2 × 10^3 $TCID_{50}$/ml), obtained by diluting stock virus suspension of known titre with MEM, is added to each well.

iv) The plate is gently shaken and placed in an incubator for 1 hour at 37°C (±2°C) (5% CO_2 optional).

v) 150 µl of cell suspension containing about 150,000 cells/ml is added to each well.

vi) The plate is covered (for incubation in CO_2), or a plastic sheet is sealed carefully around the edges of the plate (for incubation in air). The plate is shaken gently to obtain an even distribution of cells at the bottom of the wells, and placed in the incubator at 37°C (±2°C) (CO_2 optional) for 3–5 days.

vii) *Controls:* Each set of plates must include the following controls:

a) Virus control

This is to verify the amount of virus actually used for the test. The virus dose used for VN (target titre 100 $TCID_{50}$/50 µl) is diluted with MEM at 1/10, 1/100 and 1/1000. Of each dilution, 50 µl is placed in at least four wells, to which 50 µl of medium is added before the wells are incubated for 1 hour at 37°C (±2°C). The cell suspension is added in the same way as for the sera under test.

b) Cell control

150 µl cell suspension and 100 µl MEM are placed in each of at least four wells.

c) Positive serum control

A serum of known SuAHV1 neutralising antibody titre is used. Five dilutions are prepared in the same way as for the sera under test: a dilution corresponding to the serum titre, two-fold and four-fold dilutions, and 1/2 and 1/4 dilutions (equivalent to 4T, 2T, T, T/2 and T/4, where T is the serum titre, i.e. undiluted serum for the qualitative test). Add 50 µl of virus suspension containing 100 $TCID_{50}$/50 µl to 50 µl of positive control sample dilutions. The cells are incubated and the cell suspension is added in the same way as for the sera under test.

d) Serum control

This is to verify the absence of a toxic effect of the sera on the cells. Wells containing 50 µl of each serum are incubated for 1 hour at 37°C in the presence of 50 µl of medium. Then, 150 µl of cell suspension is added in the same way as for the sera under test.

e) Negative serum control

This is ~~done~~ to verify the specificity of the test. A negative control serum, collected from a known negative pig, is analysed in the same way as for sera under test.

viii) *Reading the results:* An inverted-image microscope (×100) is used to examine the wells for toxic effects and CPEs after 3 to 5 days. The controls must give the following results if the tests are to be considered valid:

a) Virus control

The titre of the viral suspension should be between $10^{1.5}$ and $10^{2.5}$ ~~30 and 300~~ $TCID_{50}$/50 µl.

b) Cell control

The cell sheet must be intact.

c) Positive serum control

The titre obtained must be equal to the predicted titre, within one dilution.

d) Serum control

Examination for a CPE should take into account a possible toxic effect on cells.

e) Negative serum control

A CPE should be present.

ix) For the sera under test if distributed in three wells, the following results may be seen:

a) presence of a CPE in three wells = negative result;

b) absence of a CPE in three wells on day 3 = positive result;

c) presence of a CPE in one well but not in the others = inconclusive result, test must be repeated;

d) small plaques indicating a CPE on day 3 = inconclusive result, test must be repeated;

e) toxicity in serum control and test wells = unreadable result, test must be repeated. (NB replacement of medium with fresh medium after 16 hours' incubation will reduce the toxicity without affecting the titre of specific antibody.) Plates can be read until day 5 of incubation.

f) If the serum was initially diluted 1/2 and distributed in two wells, it is considered positive if CPE is absent in one of the two wells, and it is highly recommended to retest using the quantitative technique. Diluting the serum to 1/2 can prevent the toxicity effect of the tested sera.

x) *Interpretation of the results:* This test is capable of detecting the presence or absence of neutralising antibody to SuAHV1. It is incapable of distinguishing vaccinated animals from infected animals.

The technique described (VN for 1 hour at 37°C) can give false-negative and false-positive results. The sensitivity can be increased (leading to fewer false negatives) by adopting a method based on neutralisation involving 24 hours of contact between virus and serum at 4°C, before the addition of cells.

A qualitative technique such as this one, which employs undiluted serum samples (1/2 final dilution), can give a false-positive result in certain cases due to nonspecific neutralisation of the virus. This problem can be addressed by carrying out a confirmatory test using the quantitative technique (see Section B.2.1.2 below).

Samples giving inconclusive results may be tested by an alternative technique with better sensitivity such as an ELISA or the animal should be re-bled to confirm status.

2.1.2. Quantitative virus neutralisation technique

The quantitative VN technique is similar to the qualitative procedure, but each serum is used both undiluted and in a series of dilutions. Depending on the desired precision, the purpose of testing and the expected titre, two wells are used for each dilution of serum, and a range of dilutions appropriate for the purpose. The procedure below describes the test for an initial maximum dilution of 1/16. It is possible to reach higher titres using more wells (e.g. A1 to A12 for 1/256 dilution).

i) Complement in the serum samples is destroyed by heating in a water bath at 56–59°C for 30 minutes.

ii) 75 µl of MEM is added to well A2 and 50 µl of MEM is added to wells A1, and A3 to A6 of a 96-well cell-culture grade microtitre plate and continued for comparable wells in rows B, C, etc., for additional serum samples.

iii) 75 µl of undiluted serum sample is added to well A2, and continued for wells in rows B, C, etc., with other serum samples.

iv) Using a multichannel pipette, the contents of wells in column 2 are mixed, then 50 µl is transferred to column 1 and 3, and so on to column 6 or further to a predetermined row, using the same nozzles. The 50 µl portions remaining after the last row are discarded.

v) 50 µl of virus suspension containing 100 $TCID_{50}$ (or 2×10^3 $TCID_{50}$/ml), obtained by diluting stock virus suspension of known titre with MEM, is added to each well in columns 2 to 6. No virus is added to wells in column 1, this is a control column of serum samples.

vi) The plate is shaken and placed in an incubator for 1 hour at 37°C (±2°C) (5% CO_2 optional).

vii) 150 µl of cell suspension containing about 150,000 cells/ml is added to each well.

viii) The plate is covered (for incubation in CO_2), or a plastic sheet is sealed carefully around the edges of the plate (for incubation in air). The plate is shaken lightly to obtain an even distribution of cells at the bottom of the wells, and placed in the incubator at 37°C (±2°C) (CO_2 optional) for 3–5 days.

ix) Controls are set up as described for the qualitative technique.

x) *Reading the results:* The neutralising titre of a serum is expressed by the denominator of the highest initial dilution that brings about complete neutralisation of the CPE of the virus in 50% of the wells. Neutralisation at any dilution (even undiluted, equivalent to a final dilution of 1/2) is considered to be positive. If the serum shows neutralisation only when undiluted (with growth of virus and CPE at the 1/2 and subsequent dilutions), it would be advisable to apply alternative tests (ELISA or latex agglutination) to provide confirmation of the result, or to request another sampling of the animal, at least 8 days after the first.

2.2. Enzyme-linked immunosorbent assay

The sensitivity of the ELISA is generally superior to that of the VN test using 1-hour neutralisation without complement. Some weak positive sera are more readily detected by VN tests using 24-hour neutralisation, while others are more readily detectable by ELISA.

ELISA kits, which are available commercially, use indirect or competitive techniques for detecting antibodies. They differ in their mode of preparation of antigen, conjugate, or substrate, in the period of incubation and in the interpretation of the results. Their general advantage is that they enable the rapid processing of large numbers of samples. This can also be automated and the results analysed by computer. Some of these kits make it possible to differentiate between vaccinated and naturally infected animals when used with a 'matching' vaccine (~~Eloit *et al.*, 1989; Van Oirschot *et al.*, 1986~~ Freuling *et al.*, 2017). Alternatively, non-commercial ELISA protocols may be adopted (Toma & Eloit, 1986; Cano-Terriza *et al.*, 2019) provided they are shown to detect the WOAH International Standard Reference Serum as positive at a dilution of 1/2 (the minimum sensitivity for international trade purposes). It is recommended to use a kit or in-house assay that has been validated to this standard or a secondary standard prepared against the International Reference Standard by external quality control tests by an independent laboratory. A suitable test protocol for whole virus antibodies is presented below (Toma & Eloit, 1986).

2.2.1. Preparation of antigen

i) A cell line sensitive to SuAHV1, such as PK-15 or fetal pig testis, is used. It must be free from extraneous viruses, such as bovine viral diarrhoea virus. The cells should be split and seeded into fresh 75 cm^2 flasks the day before inoculation. A suitable medium such as MEM, without serum, is used to overlay the cultures.

ii) Virus inoculated, and control uninoculated flasks are processed in parallel throughout. A suitable well characterised strain of SuAHV1 is used, e.g. Kojnock strain. When a confluent cell monolayer has developed (approximately 24 hours after seeding), it is inoculated with 10^8 $TCID_{50}$ SuAHV1 in 5 ml medium; and 5 ml medium (without virus) is placed in control flasks. The cultures are left for adsorption for 30 minutes at 37°C, and then overlaid with 20 ml medium.

iii) When CPE is just beginning, the supernatant medium is discarded and 4 ml KCl (4 mM solution) and glass beads are added. The flasks are shaken gently to detach cells.

iv) Cells are washed by centrifuging three times at 770 ***g*** in 4 mM KCl. The pellet is resuspended in 4 mM KCl with 0.2% Triton X-100 (1 ml per flask) by applying 60 strokes with a glass homogeniser

v) The cell homogenate is layered on to 0.25 mM sucrose in 4 mM KCl and centrifuged for 10 minutes at 770 ***g***.

vi) The pellet is resuspended in antigen-diluting buffer, pH 9.6 (0.1 M Tris, 2 mM EDTA, 0.15 mM NaCl) at 1/50 the volume of the original culture medium. It may then be stored in small aliquots at –70°C. Antigen is stable in this form for 2 years.

2.2.2. Coating microtitre plates

i) Virus antigen and control (no virus) antigen are diluted in diluting buffer, pH 9.6 (see above) to a dilution predetermined in chequerboard titrations.

ii) 200 µl of antigen is dispensed into each well of 96-well ELISA-grade plates, coating alternate rows with SuAHV1 positive and control antigen. Incubation is for 18 hours at 4°C.

iii) The plates are washed three times with washing solution (Tween 20, 0.5 ml/litre).

iv) Coated plates are stored at –20°C or –70°C. They are stable for several months.

2.2.3. Test procedure

i) Test serum samples are diluted 1/30 in PBS/Tween buffer, pH 7.2 (137 mM NaCl, 9.5 mM phosphate buffer, 0.5 ml/litre Tween 20).

ii) Diluted samples are added to virus and control antigen coated wells, and incubated at 37°C for 30 minutes.

iii) The plates are washed three times with washing solution (0.5 ml/litre Tween 20).

iv) Protein A/peroxidase conjugate is added to all wells at a predetermined dilution in PBS/Tween buffer, pH 7.2 (see above), with added bovine serum albumen fraction V (10 g/litre), and the plates are incubated at 37°C for 30 minutes.

v) The plates are washed three times with washing solution (0.5 ml/litre Tween 20).

vi) A suitable chromogen/substrate mixture, such as tetra methyl benzidine (TMB)/hydrogen peroxide, is added to each plate.

vii) The reaction is stopped with 2 M sulphuric acid. The absorbance is read at 492 nm.

The test must be fully validated using known positive and negative sera, and calibrated against the WOAH International Standard Reference Serum. It is highly recommended to carry out a batch control for each batch of the test, to determine sensitivity and specificity in relation to the original validation criteria (criteria to accept or refuse the batch have to be set). For routine analysis, all tests must include positive and negative internal controls, including at least one weak positive sample that, when diluted at the appropriate dilution for the test, has equivalent activity to a 1/2 dilution of the WOAH International Standard Reference Serum. Internal controls are also used to monitor the sensitivity, specificity and reproducibility of the test over time. For further details see Toma & Eloit (1986) and Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals*. Commercial ELISA kits also have to be validated in the setting in which they are going to be used.

As well as testing sera, the ELISA can be adapted to test pools of sera, filter paper disks that have been moistened with a small quantity of blood obtained by puncturing a superficial vein (Banks, 1985; Toma *et al.,* 1986), or muscle exudates (Carella *et al.,* 2023; Le Potier *et al.,* 1998). These techniques make it convenient to collect blood samples from large numbers of pigs (Vannier *et al.,* 2007). The disks are air-dried before shipment to the laboratory. The (analytical) sensitivity may be lower than for a standard ELISA due to the type of sample or unavoidable dilution of the sample. Use of an adapted ELISA is therefore more appropriate for testing at the population level rather than for individual testing (e.g. prior to animal movement), unless a validation study has shown a comparable (analytical) sensitivity to the standard ELISA.

Requirements for the detection of gE antibodies by ELISA in pigs destined for slaughter that are to be introduced into zones free from Aujeszky's disease have been defined by several control authorities. For example, in the European Union, ELISA gE kits must be able to detect activity at least equivalent to a 1/8 dilution of the WOAH International Standard Reference Serum (European Commission, ~~2008~~ 2020). The WOAH *Terrestrial Animal Health Code* specifies circumstances in which gE-specific tests may be used. The gE ELISA can also be adapted to test blood on filter paper disks depending on its sensitivity.

C. REQUIREMENTS FOR VACCINES

1. Background

1.1. Rationale and intended use of the product

Aujeszky's disease may be controlled by the use of vaccines containing either modified live or inactivated virus antigens. However, these conventional vaccines have been supplemented by recombinant DNA-derived gene-deleted or naturally deleted live SuAHV1 vaccines. These vaccines, referred to as marker or DIVA-vaccines, are made with a virus that lacks a specific glycoprotein (most commonly gE-, although gG- or gC-deleted vaccines have also been described, as have vaccines with multiple deletions[2]). These gene-deleted DIVA-vaccines have the advantage over conventional whole virus vaccines that it is possible to distinguish infected animals from non-infected vaccinated animals. This is done by testing for the antibodies directed against the protein coded for by the deleted gene, which will be absent in non-infected DIVA-vaccinated pigs but present in field-infected pigs. Therefore, in countries with infected pigs, where the eradication of Aujeszky's disease is planned, these DIVA-vaccines are the vaccines of choice (~~Pensaert *et al.*, 2004~~ Vannier *et al.,* 2007). Standards applicable to the manufacture of live and inactivated virus vaccines are described. For DIVA-vaccines, the tests should include demonstrable absence of a serological response in vaccinated pigs to the protein coded for by the deleted gene, and in addition a demonstrable response to the same protein in vaccinated pigs that become infected by field virus.

[2] The nomenclature for the genes changed several years ago, but the old designation is still in the literature. The old and the new nomenclature is: gII = gB; gIII = gC; gp50 = gD; gI = gE; gX = gG; gp63 = gI. Note that some commercial serological kits may still be named by the old nomenclature.

Other vaccines are inactivated and constituted of adjuvanted, viral subunit of purified and concentrated immunogenic glycoproteins (except the gE) allowing differentiation of vaccinated from infected pigs.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

2. Outline of production and minimum requirements for conventional vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

Vaccines are made using a seed-lot system in which a master seed virus (MSV) is prepared from a suitable strain of Aujeszky's disease virus. A number of strains are used for vaccine manufacture. The antigen in an inactivated vaccine can be one of a number of wild-type strains, or the naturally deleted Bucharest virus. Modified live conventional vaccines use numerous strains, such as Bartha or are derived from Aujeszky's original isolate or from other field isolates, such as the NIA-3 strain (~~Marchioli *et al*., 1987; McFerran & Dow, 1975;~~ Van Oirschot *et al*., ~~1990~~ 1996~~; Visser & Lutticken, 1988~~).

It is recommended that for differentiating between infected and vaccinated animals, gene-deleted strains should be used.

A virus identity test (using either a fluorescent antibody test, neutralisation test, [constant serum/decreasing virus method], or any other suitable identity test as full genome sequencing) must be conducted on the MSV.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

Guidelines are given in Chapter 2.3.4. *Minimum requirements for the production and quality control of vaccines.*

Most of the cell lines used to propagate ~~SHV-1~~ SuAHV1 are continuous lines, such as the PK-15 line. ~~A master cell stock (MCS) is established at a specified passage level. The MCS and the highest passage level (MCS × n) intended for use in the preparation of a biological product is specified in an Outline of Production. Both MCS and MCS × n are monitored by a variety of procedures to characterise the cell line and to ensure freedom from adventitious agents. The extraneous agents to be detected are generally defined in monographs or guidelines (e.g. European Pharmacopoeia, US Code of Federal Regulations, EU guidelines, etc.). In general, the type of agents to be looked for is founded on a risk analysis depending on the history of the viral strain and cells on which the vaccinal strain was isolated and on which it is cultivated. The MCS must be monitored for species of origin. A minimum of 50 mitotic cells should be examined at both the MCS and MCS × n passage levels. The modal number in the MCS × n must not exceed 15% of the modal number of the MCS. Any marker chromosomes in the MCS must also be present in the highest cell passage.~~

~~If there is evidence that the cell line may induce malignancies in the species for which the product is intended, the cell line is tested for tumorgenicity and oncogenicity.~~

Both the MSV and the master cell stock (MCS) must be shown to be free from mycoplasma, bacteria, fungi, cytopathogenic or haemadsorbing viruses, porcine parvovirus, cytopathic and noncytopathic ovine and bovine pestiviruses and other extraneous agents, such as circovirus or coronaviruses, as determined by culturing and by fluorescent antibody procedures or others, such as PCR, or deep-sequencing.

2.2. Method of manufacture

2.2.1. Procedure

Only MSV that has been established as pure, safe and immunogenic may be used as seed for a vaccine product. Cells from the MCS are propagated in a variety of growth media. All batches of vaccine must be from the first to the twentieth passage of MCS.

2.2.2. In-process controls

It is necessary to carry out tests at each critical step of the manufacturing process. The control tests are also carried out on intermediate products with a view to verifying the consistency of the production process and the final product.

2.2.3. Final product batch tests

It is essential to differentiate the tests that are carried out on a routine basis to release batches of final product from those that are performed to define the biological properties of a vaccine. The trials carried out for batch release are not the same as the ones carried out once only to determine the safety and efficacy of a vaccine. The batch release controls are always short-term trials, as inexpensive as possible, and not always carried out in pigs. Their purpose is mainly to attest the reproducibility of the quality of the finished product, which has to be in compliance with the quality initially defined in the application for marketing ~~authorisation~~ approval.

i) Sterility and purity

Tests must be carried out for sterility and freedom from contamination (see chapter 1.1.9 and Section C.2.1.2 of this chapter).

Each batch of ~~SHV-1~~ SUAHV1 vaccines must be tested for freedom from extraneous viruses. Using a minimum amount of a monospecific antiserum, the live vaccinal strain is neutralised and inoculated into cell cultures known to be sensitive to viruses pathogenic for pigs. No CPE and no haemadsorbing agents should be detected. The vaccines have to be free from pestiviruses.

ii) Inactivation

For inactivated vaccines, inactivation must be checked using two passages in the same type of cell culture as used in the production of the vaccine. Tests can be carried out by vaccinating susceptible animals such as rabbits.

iii) Identity

Where necessary, a specific test for virus identification should be carried out.

iv) Safety

Safety tests in target animals are not required by many regulatory authorities for the release of each batch. Where required, standard procedures should be statistically relevant and target the smallest number of animals required for the relevant regulatory approval.

v) Batch potency

The potency of the vaccine must be demonstrated using a suitable method, the results of which have to be correlated with the efficacy tests described previously.

In this kind of test, the most difficult point is to determine an acceptability threshold for using or rejecting the batch according to the results that are obtained.

Virus content tests should be carried out using each of at least three containers. The virus titre of the vaccine must be determined and must normally not be higher than 1/10 of the dose at which the vaccine has been shown to be safe, and not lower than the minimum release titre.

vi) Preservatives

If no preservative is included in the final product, the manufacturer must demonstrate that the product remains acceptable for its recommended period of use after opening the vial.

The efficacy of preservatives in multidose containers must be demonstrated. The concentration of the preservative in the final filled vaccine and its persistence throughout shelf life must be checked.

vii) Precautions (hazards)

All information about possible adverse reactions induced by the vaccine must be indicated. Any putative risk for human health if the user is accidentally given a small quantity of the

product has to be indicated. The manufacturer should indicate all the conditions of use of the vaccine: mixing, reconstitution, storage, asepsis, length of needle, route of administration and health status of the vaccinated animals.

2.2.4. Stability tests

Tests have to be carried out to verify the shelf life proposed by the manufacturer. These tests must always be real-time studies; they must be carried out on a sufficient number of batches (at least three) produced according to the described production process and on products stored in the final container, and normally include biological and physicochemical stability tests. The manufacturer has to provide the results of analyses that support the proposed shelf life under all proposed storage conditions. Usually, the proposed shelf life corresponds to the period for which the product is considered to be stable minus 3 months.

2.3. Requirements for ~~authorisation~~ regulatory approval

2.3.1. Safety requirements

Local and general reactions must be examined. When a live vaccine is used, it is necessary to differentiate the exact safety properties of the vaccinal strain from those of the finished product if this includes an adjuvant.

Objective and quantifiable criteria to detect and measure adverse reactions should be used; these would include temperature changes, weight gain, litter size, reproductive performance, etc., of vaccinated and control groups. The tests must be performed by administering the vaccine to the pigs in the recommended dose and by each recommended route of administration.

In general, safety is tested initially under experimental conditions, following the requirements of the WOAH *Terrestrial Animal Health Code,* Chapter 7.8 *Use of animals in research and education*. When the results of these preliminary tests are known, it is necessary to increase the number of animals vaccinated in order to evaluate the safety of the vaccine under practical conditions.

i) Laboratory testing

All tests must be carried out on pigs that do not have antibodies against Aujeszky's disease virus or against a subunit of the virus.

a) General effects

1. Live vaccines

Intranasal tests and vaccination of 3- to 5-day-old piglets are very useful for ascertaining the degree of safety of a strain. At least five piglets should be used.

It is also essential to assess the vaccine's properties in the target animals under normal conditions of use and at the youngest age intended for vaccination, e.g. fattening pigs, which are generally vaccinated between 9 and 12 weeks of age. When the manufacturer allows the use of the vaccine in pregnant sows and when such vaccination is authorised, the vaccine's properties should also be assessed. No clinical signs, including significant thermal reactions should be observed after vaccination: data must be recorded before vaccination and on a schedule such as 6 hours, 24 hours and 48 hours post-vaccination and then on a daily basis during the observation period. These assessments have to be performed on at least ten vaccinated pigs, with five unvaccinated pigs as controls.

The test carried out should be consistent with VICH GL41 (Examination of live veterinary vaccines in target animals for absence of reversion to virulence, 2008[3]). Reversion to virulence following serial passage must be examined. Primary vaccination is done by the intranasal route. Series of at least four passages in piglets are made. No fewer than two fully susceptible animals must be used for each passage. The object of these assays is to test the genetic stability of live vaccine strains. The tests appear to be less necessary when a genetically modified live strain is concerned, especially if it is produced by gene deletion.

[3] https://www.ema.europa.eu/en/documents/scientific-guideline/vich-gl41-target-animal-safety-examination-live-veterinary-vaccines-target-animals-absence-reversion_en.pdf.

It is recommended to test for possible excretion of the vaccine strain. For this purpose, no fewer than 14 piglets, 3–4 weeks old each receive one dose of vaccine by the recommended route and at the recommended site (except for vaccines administered by the intranasal route). Four unvaccinated piglets are kept as controls. Suitably sensitive tests for the virus are carried out individually on the nasal and/or oral secretions of vaccinated and in-contact pigs as follows: nasal and oral swabs are collected daily from 1 day before vaccination to 10 days after vaccination. Vaccine strains that are isolated from the nasal/oral secretion collected from pigs in which the vaccine was administered by the parenteral route are not recommended for eradication purposes.

The ability of the Aujeszky's disease vaccine strain to spread from vaccinated pigs to unvaccinated ones (lateral spread) must be tested by using the recommended route of administration that presents the greatest risk of spread (except for vaccines administered by the intranasal route). A repetition of the assays (four times) is necessary as this phenomenon is difficult to detect. Four piglets should be used each time for vaccination and placed in contact, 1 day later, with two unvaccinated piglets. It may also be necessary to examine the spread of the strain to nontarget species that may be susceptible to the vaccine strain.

Live attenuated vaccine strains are tested with regard to their general effects by administering to 5- to 10-day-old piglets ten times the field dose. This administration of an overdose makes it possible to detect reactions not produced under normal conditions of use. Such reactions may be produced inadvertently when large numbers of animals are vaccinated. If vaccines are administered by the intranasal route, the manufacturer has to indicate clearly that the vaccine will spread from vaccinated pigs to unvaccinated ones.

2. Inactivated vaccines

It is essential to test inactivated vaccines in the target animals under normal conditions of use for fattening pigs and for sows when this use is claimed by the manufacturer and ~~authorised~~ received regulatory approval (European Pharmacopoeia, ~~2008~~ 2022; Vannier *et al.*, 2007). As described previously, it is fundamental to use objective and quantifiable criteria to detect and to measure adverse reactions, such as temperature changes, weight performance, litter size, reproductive performance, etc., on vaccinated and control groups. The tests must be performed by administering the vaccine in the recommended dose and by each recommended route of administration to the pigs for which it is intended.

Pigs or sows are usually observed until there is no further evidence of vaccine reaction. The period of observation must not be fewer than 14 days from the day of administration. This period has to be extended when, for example, the vaccine is used in pregnant sows and it is necessary to assess the possible effects of the vaccine on reproductive performance. In this case, the period of observation lasts the full duration of the pregnancy.

Control authorities generally request vaccination with a double dose so that adverse reactions, which may be at the limit of detection when a single dose is administered, are more likely to be detected.

b) Local reactions

Local reactions are often associated with the use of inactivated vaccines, as these side-effects can be induced by adjuvants, particularly oil adjuvants. However, some Aujeszky's disease live vaccines are mixed with different adjuvants, which modify what has been observed in the past.

Local reactions are mainly inflammatory and can be more or less complicated (necrotic or suppurative), depending on the nature of the adjuvants used and the aseptic conditions of the vaccination. Oil adjuvants can induce a variety of effects including muscular degeneration, granuloma, fibrosis and abscessation. In addition to the nature of the oil used (the intensity of the reaction is reduced when metabolisable oils are used in the vaccine), the type of emulsion used (water/oil, oil/water, water/oil/water) induces these reactions to a greater or lesser extent. In consequence, it is necessary to observe the site of injection not only from the outside, but also by dissection after slaughter, especially for growing and finishing pigs.

ii) Field testing

Field trials are necessary to assess the safety of an Aujeszky's disease vaccine in a large number of pigs or sows. In Europe (European Pharmacopoeia, ~~2008~~ 2022), tests must be carried out in each category of animals for which the vaccine is intended (sows, fattening pigs). At least three groups of no fewer than 20 animals each are used with corresponding groups of no fewer than 10 controls. The rectal temperature of each animal is measured at the time of vaccination, 6, 24 and 48 hours later. At slaughter, the injection site must be examined for local reactions. If the vaccine is intended to be used in sows, reproductive performances have to be recorded. Field trials are supplemented by laboratory studies of efficacy correlated to vaccine potency.

2.3.2. Efficacy requirements

i) Laboratory trials

All tests must be carried out on pigs that do not have antibodies against Aujeszky's disease virus or against a subunit of the virus, except that some tests may be done using maternally immune animals.

a) Assessment of passive immunity

To test the efficacy of vaccines, it is important to mimic the natural infection conditions (European Pharmacopoeia, ~~2008~~ 2022). SuAHV1 infection gives rise to important losses of young piglets from nonimmune sows. Thus, when vaccinating sows, the main goal is to protect the young piglets through passive immunity conferred by the colostrum ingested immediately after birth, with the secondary objective of preventing abortion.

To measure this passive immunity and the protection induced by vaccinating the sows, experimental models have been established. The sows are vaccinated according to the vaccinal protocol during pregnancy. When the piglets are, for example, 6–10 days old they are given an intranasal challenge exposure with a virulent SuAHV1 strain. It is preferable to use a strain titrated in median lethal doses (LD_{50}). Pigs should be inoculated by the nasal route, 10^2 LD_{50} per pig in 1 ml. The efficacy of the vaccine is assessed by comparing clinical signs, but also and more importantly, mortality, or humane euthanasia, in piglets from unvaccinated dams with that observed in piglets from vaccinated sows.

Piglets from vaccinated sows can be found to have 80% protection against mortality compared with those from the control sows. In order for the results to be significant, it is recommended that eight vaccinated sows and four control sows be used (subject to satisfactory numbers of piglets from each sow).

b) Assessment of active immunity

1. Clinical protection

Several criteria can be considered when measuring active immunity induced by vaccinating pigs. Generally, pigs are vaccinated at the beginning of the growing period, i.e. when they are between 9 and 12 weeks old. Laboratory trials are performed by challenging pigs at the end of the finishing period, when they weigh between 80 and 90 kg.

In general, at least three criteria, such as rectal temperature, weight loss and clinical signs, along with mortality, are used to measure the clinical protection of pigs after vaccination and challenge (De Leeuw & Van Oirschot, 1985). The antibody titres have little predictive value for the efficacy of the vaccines. Weight loss compared between the vaccinated and control groups is the most reproducible and reliable parameter when the challenge conditions are well standardised. The measure of the difference in weight gain or loss between the two groups of pigs and, in the interval of time between challenge (day 0 and day 7), has a very good predictive value for the efficacy of the vaccines (Stellmann *et al.*, 1989). Significant results can be obtained when weight performances are compared between one group of at least eight vaccinated pigs and another group of eight unvaccinated control pigs

For challenge, it is usually preferable to use a high titre of a virulent strain, as this makes it possible to obtain a more marked difference between vaccinated and control pigs. On the basis of previous work, a challenge dose with at least 10^6 $TCID_{50}$/ml virulent strain having undergone not more than three passages on primary cells can be sufficient, but a higher titre ($10^{7.5}$ $TCID_{50}$/ml) is recommended. The oro-nasal route

should be used to challenge the pigs by introducing the virulent strain in an appropriately high volume (≥4 ml).

This method of evaluating the efficacy of SuAHV1 vaccines is now well tested and has made it possible to establish an objective index for determining the efficacy of a vaccine. This index, which compares the relative weight losses between vaccinated and control pigs, can also be used for potency testing batches before release and for batch efficacy testing. However, the value of the cut-off index will be different as the conditions of the assay will not be identical. The influence of passively acquired, maternally derived antibodies on the efficacy of a vaccine must be evaluated adequately.

2. Virulent virus excretion

Additionally, it is desirable that vaccines should prevent or at least limit viral excretion from infected pigs ~~(Vannier *et al.*, 1991)~~. When a control programme against Aujeszky's disease is based on large-scale vaccination, it is essential to choose the vaccines or the vaccinal scheme that best limits the replication of virulent virus in infected pigs. Several assays have been performed to compare vaccines on that basis.

Generally, the pigs are vaccinated and challenged at different periods. It is better, but more time-consuming, to infect pigs at the end of the finishing period. To measure the virus excretion, nasal swabs (taken at 10 cm depth in the nostrils) are taken daily from each pig from the day before challenge to at least 12 days after challenge. The swabs can be weighed before the sampling and immediately after to calculate the exact weight of collected mucus. Medium is then added to each tube containing a swab. The virus is titrated from the frozen and thawed medium.

Different indexes can be used to express the quantity of virulent virus excreted by pigs, taking into consideration the duration and the level of viral excretion, and the number of pigs excreting virulent virus.

3. Duration of immunity

It is recommended that any claims regarding the onset and duration of immunity should be supported by data from trials. Assessment of duration of immunity can be based on challenge trials or, as far as it is possible, on immunological and serological tests.

ii) Field trials

In general terms, it is extremely difficult to assess vaccine efficacy in animal populations. In order to do this, it would be necessary to vaccinate the animals in the absence of the pathogen that the vaccine protects against, then to await the moment of infection and to compare the effects of infection in vaccinated animals (or the offspring of vaccinated dams) with the effects in the unvaccinated animals of the same age, in the same building and in the same batch as the vaccinated animals (or those protected passively). As all these conditions are difficult to achieve in the field, field trials are certainly more appropriate to safety testing than to efficacy testing, except for the development of DIVA-vaccines that offer the opportunity to evaluate the effectiveness of vaccines under field conditions (Bouma, 2005).

2.3.3. Stability

Tests have to be carried out to verify the shelf life proposed by the manufacturer. These tests must always be real-time studies; they must be carried out on a sufficient number of batches (at least three) produced according to the described production process and on products stored in the final container, and normally include biological and physicochemical stability tests. The manufacturer has to provide the results of analyses that support the proposed shelf life under all proposed storage conditions. Usually, the proposed shelf life corresponds to the period for which the product is considered to be stable minus 3 months.

3. Vaccines based on biotechnology

3.1. Vaccines available and their advantages

Biotechnology combined with a better knowledge of the functions and characteristics of the SuAHV1 glycoproteins helped to develop new vaccines. For example, Quint *et al.* (1987) deleted glycoprotein E-

coding sequence from the NIA3 strain. This resulted in an efficient DIVA-vaccine against Aujeszky's disease, allowing differentiation of vaccinated from infected animals (DIVA vaccines). Most of the vaccines used at the moment are obtained from recombinant DNA-derived gene-deleted virus. The deletion of the genes coding for the glycoprotein E is the most commonly used, allowing an attenuated live virus vaccine to be obtained but still protecting against the clinical signs and reducing significantly the level of the viral excretion by the pigs vaccinated and infected. Because of the ability of some glycoproteins of SuAHV1 to induce strong immune responses, efficiencies of DNA vaccines, consisting of plasmids encoding these glycoproteins, were tested. Indeed, DNA vaccination has a number of advantages: ease of construction and standardised production of plasmids, no handling of infectious particles, induction of humoral and cellular immune responses, bypass of the maternal derived immunity. The pioneering study on DNA vaccination against Aujeszky's disease infection was published in 1997 (Gerdts *et al.,* 1997). The use of a novel generation of plasmid amplifying the level of gene transcription of the proteins of interest (Dory *et al.,* 2005) have been shown to be efficient strategies. These vaccines are not yet commercialised.

3.2. Special requirements for biotechnological vaccines, if any

Criteria to assess quality, safety and efficacy of the vaccines derived from the biotechnology are the same as the ones defined for conventional vaccines (see section C.2). Nevertheless, special attention has to be paid to the stability of the recombinant DNA construction.

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NB: There are WOAH Reference Laboratories for Aujeszky's disease (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for Aujeszky's disease

NB: FIRST ADOPTED IN 1991. MOST RECENT UPDATES ADOPTED IN 2018.

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Appendix 1: Aujeszky's disease
Intended purpose of test: population freedom from infection

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
Virus neutralisation (VN) Score: + Species: *Suidae*	Serum	VN was the reference method for serology Must detect at least the WOAH Standard Reference Serum diluted 1/2	Historical study conducted in the 1980s Comparison of three serological tests for the detection of antibodies to SUAHV1 in a population of 1285 pig 5.9% of sera positive by VNT NVT less sensitive than ELISA at the individual level	See references	1. High specificity	1. Low sensitivity 2. Time consuming (3–5 days) and labour intensive so not suitable for high throughput. 3. Requires incubators and microscope 4. Requires highly trained technicians 5. No DIVA properties	Goyal *et al.* (1987) Schoenbaum *et al.* (1990)
Latex agglutination Manual or semi-automatic Score: +++ Species: *Suidae*	Serum	Semi-automatic latex agglutination (LA) test was compared with manual LA, ELISA and VN	Study conducted in the 1990s on 1191 swine sera Overall, the sensitivities of the four tests were as follows: semiautomated LAT = manual LAT > ELISA > SNT. For 74 samples of known pseudorabies antibody status, the overall specificities were as follows: semi-automated LAT = manual LAT = SNT > ELISA	See references WOAH Expert opinion: the recommended test to be used at the herd level to assess the freedom of infection in absence of vaccination	1. High sensitivity	1. No DIVA properties 2. Few kits commercially available	Rodgers *et al.* (1996) Schoenbaum *et al.* (1990)
ELISA gB Score: +++ ELISA gE Score: ++ Species: *Suidae*	Pig or wild boar: Serum Clotted blood on filter paper Muscle exudates	Reference test: VN	Comparative studies of VN, LA and ELISA were conducted in the 1990s ELISA kits currently commercialised are more robust, sensitive and specific They are able to detect: for ELISA gB at least the WOAH Standard Reference Serum diluted 1/2 For ELISA gE at least the WOAH Standard Reference Serum diluted 1/8	See references + WOAH expert opinion based on ELISA kits initial and batch-to-batch controls conducted by the French national reference laboratory And on the inter-laboratory comparative test for serology organised every 2-years by the WOAH Reference Laboratory The recommended test to be used at the individual and herd level to assess the absence of infection in presence or absence of vaccination (gE deleted vaccine)	1. High sensitivity, detects persistently infected animals 2. Commercially available 3. Easy to handle 4. Rapid (<24 hours) 5. Possibility of automation for high throughput 6. ELISA gB can be used for individual serum or pool of five sera, and for individual filter paper or pool of five filter papers 7. DIVA: combination of ELISA gB and ELISA gE when using gE deleted vaccine to check the absence of infection in a vaccinated group of pigs at the individual or herd level	1. ELISA gB: Low per cent of false positive results (≤2%), mainly due to insufficient quality of sera 2. ELISA gE to be used only on individual serum 3. ELISA gE: Risk of false negative gE at the individual level because of delay of seronconversion to gE in vaccinated pigs 4. Requires a microplate absorbance reader	Rodgers *et al.* (1996) Schoenbaum *et al.* (1990)

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Appendix 2: Aujeszky's disease
Intended purpose of test: individual animal freedom from infection prior to movement

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
Virus neutralisation (VN) Score: + Species: *Suidae*	Serum	VN was the reference method for serology Must detect at least the WOAH Standard Reference Serum diluted 1/2	Historical study conducted in the 1980s Comparison of three serological tests for the detection of antibodies to SUAHV1 in a population of 1285 pig 5.9% of sera positive by VNT NVT less sensitive than ELISA at the individual level	See references	1. High specificity	1. Low sensitivity 2. Time consuming (3–5 days) and labour intensive so not suitable for high throughput. 3. Requires incubators and microscope 4. Requires highly trained technicians 5. No DIVA properties	Goyal *et al.*, 1987 Schoenbaum *et al.*, 1990
Latex agglutination Manual or semi-automatic Score: +++ Species: *Suidae*	Serum	Semi-automatic latex agglutination (LA) test was compared with manual LA, ELISA and VN	Study conducted in the 1990s on 1191 swine sera Overall, the sensitivities of the four tests were as follows: semiautomated LAT = manual LAT > ELISA > SNT. For 74 samples of known pseudorabies antibody status, the overall specificities were as follows: semi-automated LAT = manual LAT = SNT > ELISA	See references WOAH Expert opinion: the recommended test to be used at the herd level to assess the freedom of infection in absence of vaccination	1. High sensitivity	1. No DIVA properties 2. Few kits commercially available	Rodgers *et al.* (1996) Schoenbaum *et al.* (1990)
ELISA gB Score: +++ ELISA gE Score: ++ Species: *Suidae*	Serum Clotted blood on filter paper Muscle exudates	Reference test: VN	Comparative studies of VN, LA and ELISA were conducted in the 1990s ELISA kits currently commercialised are more robust, sensitive and specific They are able to detect: for ELISA gB at least the WOAH Standard Reference Serum diluted 1/2 For ELISA gE at least the WOAH Standard Reference Serum diluted 1/8	See references + WOAH expert opinion based on ELISA kits initial and batch-to-batch controls conducted by the French national reference laboratory And on the inter-laboratory comparative test for serology organised every 2-years by the WOAH Reference Laboratory The recommended test to be used at individual level to assess the absence of infection in presence or	1. High sensitivity, detects persistently infected animals 2. Commercially available 3. Easy to handle 4. Rapid (<24 hours) 5. Possibility of automation for high throughput 6. ELISA gB can be used for individual serum or individual filter paper 7. DIVA: ELISA gB/gE when using gE-deleted vaccine	1. ELISA gB: Low per cent of false positive results (<2%), mainly due to insufficient quality of sera 2. ELISA gE to be used only on individual serum 3. ELISA gE: Risk of false negative gE at the individual level because of delay of seronconversion to gE in vaccinated pigs 4. Requires a microplate absorbance reader	Rodgers *et al.* (1996) Schoenbaum *et al.* (1990)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
				absence of vaccination (gE deleted vaccine)			
PCR Score: + Species: *Suidae*	On live suid: nasal or ejaculate swabs	Reference test: VI	Müller *et al.* (2001): Experimental infection of 18 European wild boars and 16 domestic pigs: three groups of four wild boars were inoculated with SuAHV1 Bartha, Kaplan, and a wild-boar isolate (BFW1) and housed with uninfected pigs. Two groups of domestic pigs (four and eight pigs/group, respectively) were inoculated with various doses of BFW1. Animals were observed daily for clinical signs, and samples were tested for SuAHV1 excretion and homologous antibodies. After reactivation of latent infection by induced immunosuppression, SuAHV1 was detected in tissues of necropsied animals, using cell culture and a PCR	See references + WOAH expert opinion based on PCR kits initial and batch-to-batch controls conducted by the French national reference laboratory And on the inter-laboratory comparative test for virology (PCR) organised every 2-years by the WOAH Reference Laboratory Suitable in limited circumstances: e.g.: in Insemination centres or selection herds, before movement; always in combination with serological testing	1. High sensitivity 2. High specificity 3. Commercially available	1. Nasal swabs: to be used only in acute disease with respiratory clinical signs Risk of false negatives for two main reasons: a) nasal excretion duration is short (2–15 days post-infection). b)cannot detect latent infection 2. Requires extraction and thermal cycler equipment	Müller *et al.* (2001) Pol *et al.* (2013)

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Appendix 3: Aujeszky's disease
Intended purpose of test: contribute to eradication policies

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
Virus neutralisation (VN) Score: + Species: *Suidae*	Serum	VN was the reference method for serology Must detect at least the WOAH Standard Reference Serum diluted 1/2	Historical study conducted in the 1980s Comparison of three serological tests for the detection of antibodies to SUAHV1 in a population of 1285 pig 5.9% of sera positive by VNT NVT less sensitive than ELISA at the herd level	See references WOAH Expert opinion: VN is not useful at individual level for clinical case confirmation because of a delay of around 15 days post-infection for the detection of neutralising antibodies. Low level of interest at herd level	1. High specificity	1. Low sensitivity 2. Time consuming (3–5 days) and labour intensive so not suitable for high throughput. 3. Requires incubators and microscope 4. Requires highly trained technicians 5. No DIVA properties	Goyal *et al.* (1987)
Latex agglutination Manual or semi-automatic Score: +++ Species: *Suidae*	Serum	Semi-automatic latex agglutination (LA) test was compared with manual LA, ELISA and VN	Study conducted in the 1990s on 1191 swine sera Overall, the sensitivities of the four tests were as follows: semiautomated LAT = manual LAT > ELISA > SNT. For 74 samples of known pseudorabies antibody status, the overall specificities were as follows: semi-automated LAT = manual LAT = SNT > ELISA	See references WOAH Expert opinion: can be used at the individual or herd level to confirm the presence of infection	1. High sensitivity 2. High specificity	1. No DIVA properties 2. Few kits commercially available	Rodgers *et al.* (1996) Schoenbaum *et al.* (1990)
ELISA gB Score: +++ ELISA gE Score: +++ Species: *Suidae*	Serum Clotted blood on filter paper Muscle exudates	Reference test: VN	Comparative studies of VN, LA and ELISA were conducted in the 1990s ELISA kits currently commercialised are more robust, sensitive and specific They are able to detect: for ELISA gB at least the WOAH Standard Reference Serum diluted 1/2 For ELISA gE at least the WOAH Standard Reference Serum diluted 1/8	See references + WOAH expert opinion based on ELISA kits initial and batch-to-batch controls conducted by the French national reference laboratory And on the inter-laboratory comparative test for serology organised every 2-years by the WOAH Reference Laboratory The recommended test to be used at the herd level to assess the efficacy of	1. High sensitivity, detects persistently infected animals 2. Commercially available 3. Easy to handle 4. Rapid (<24 hours) 5. Possibility of automation for high throughput 6. ELISA gB can be used for individual serum or pool of five sera, and for individual filter paper or pool of five filter papers 7. DIVA: ELISA gB/gE when using gE-deleted vaccine	1. ELISA gB: Low per cent of false positive results (<2%), mainly due to insufficient quality of sera 2. ELISA gE to be used only on individual serum 3. ELISA gE: Risk of false negative gE at the individual level because of delay of seronconversion to gE in vaccinated pigs 4. Requires a microplate absorbance reader	Rodgers *et al.* (1996) Schoenbaum *et al.* (1990)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
				eradication policies of infection			
PCR Score: + Species: *Suidae*	On live suid: nasal or ejaculate swabs On dead suid: tonsil; lung, trigeminal lymph node; sacral lymph node	Reference test: VI	Müller *et al.* (2001): Experimental infection of 18 European wild boars and 16 domestic pigs: three groups of four wild boars were inoculated with SuAHV1 Bartha, Kaplan, and a wild-boar isolate (BFW1) and housed with uninfected pigs. Two groups of domestic pigs (four and eight pigs/group, respectively) were inoculated with various doses of BFW1. Animals were observed daily for clinical signs, and samples were tested for SuAHV1 excretion and homologous antibodies. After reactivation of latent infection by induced immunosuppression, SuAHV1 was detected in tissues of necropsied animals, using cell culture and a PCR	See references + WOAH expert opinion based on PCR kits initial and batch-to-batch controls conducted by the French national reference laboratory And on the inter-laboratory comparative test for virology (PCR) organised every 2-years by the WOAH Reference Laboratory	1. High sensitivity 2. High specificity 3. Commercially available	1. Nasal swabs: to be used only in acute disease with respiratory clinical signs Risk of false negatives for two main reasons: a) nasal excretion duration is short (2–15 days post-infection). b)cannot detect latent infection 2. Requires extraction and thermal cycler equipment	Müller *et al.* (2001) Pol *et al.* (2013)

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Appendix 4: Aujeszky's disease
Intended purpose of test: confirmation clinical cases

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
Virus neutralisation (VN) Score: + Species: *Suidae*	Serum	VN was the reference method for serology Must detect at least the WOAH Standard Reference Serum diluted 1/2	Historical study conducted in the 1980s Comparison of three serological tests for the detection of antibodies to SUAHV1 in a population of 1285 pig 5.9% of sera positive by VNT NVT less sensitive than ELISA at the herd level	See references WOAH Expert opinion: VN is not useful at the individual level for clinical case confirmation because of a delay of around 15 days post-infection for the detection of neutralising antibodies. Low level of interest at herd level	1. High specificity	1. Low sensitivity 2. Time consuming (3–5 days) and labour intensive so not suitable for high throughput. 3. Requires incubators and microscope 4. Requires highly trained technicians 5. No DIVA properties	Goyal *et al.* (1987)
Latex agglutination Manual or semi-automatic Score: + Species: *Suidae*	Serum	Semi-automatic latex agglutination (LA) test was compared with manual LA, ELISA and VN	Study conducted in the 1990s on 1191 swine sera Overall, the sensitivities of the four tests were as follows: semiautomated LAT = manual LAT > ELISA > SNT. For 74 samples of known pseudorabies antibody status, the overall specificities were as follows: semi-automated LAT = manual LAT = SNT > ELISA	See references WOAH Expert opinion: can be used at the individual or herd level to confirm the presence of infection	1. High sensitivity 2. High specificity	1. No DIVA properties 2. Few kits commercially available	Rodgers *et al.* (1996)
ELISA gB Score: + ELISA gE Score: + Species: *Suidae*	Serum Clotted blood on filter paper Muscle exudates	Reference test: VN	Comparative studies of VN, LA and ELISA were conducted in the 1990s ELISA kits currently commercialised are more robust, sensitive and specific They are able to detect: for ELISA gB at least the WOAH Standard Reference Serum diluted 1/2 For ELISA gE at least the WOAH Standard Reference Serum diluted 1/8	See references + WOAH expert opinion based on ELISA kits initial and batch-to-batch controls conducted by the French national reference laboratory And on the inter-laboratory comparative test for serology organised every 2-years by the WOAH Reference Laboratory Can be used at the herd level to confirm the detection of infection	1. High sensitivity, detects persistently infected animals 2. Commercially available 3. Easy to handle 4. Rapid (<24 hours) 5. Possibility of automation for high throughput 6. ELISA gB can be used for individual serum or pool of five sera, and for individual filter paper or pool of five filter papers 7. DIVA: ELISA gB/gE when using gE-deleted vaccine	1. ELISA gB: Low per cent of false positive results (<2%), mainly due to insufficient quality of sera 2. ELISA gE to be used only on individual serum 3. ELISA gE: Risk of false negative gE at the individual level because of delay of seronconversion to gE in vaccinated pigs 4. Requires a microplate absorbance reader	Rodgers *et al.* (1996)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
PCR Score: +++ Species: Suidae	On live suid: nasal swabs On dead suid: tonsil; lung, trigeminal lymph node; sacral lymph node	Reference test: VI	Müller *et al.* (2001): experimental infection of 18 European wild boars and 16 domestic pigs: three groups of four wild boars were inoculated with PrV Bartha, Kaplan, and a wild-boar isolate (BFW1) and housed with uninfected pigs. Two groups of domestic pigs (four and eight pigs/group, respectively) were inoculated with various doses of BFW1. Animals were observed daily for clinical signs, and samples were tested for PrV excretion and homologous antibodies. After reactivation of latent infection by induced immunosuppression, PrV was detected in tissues of necropsied animals, using cell culture and a polymerase chain reaction (PCR)	See references + WOAH expert opinion based on PCR kits initial and batch to batch controls conducted by the French national reference laboratory And on the ILCT for virology (PCR) organised every 2-years by the WOAH Reference Laboratory	1. High sensitivity 2. High specificity 3. Commercially available 4. Nasal swabs, tonsils , lung: To be used in acute disease with respiratory clinical signs, as nasal excretion duration is short (2–15 days post-infection) 5. Trigeminal and sacral lymph nodes and tonsils: useful to detect latently infected pig 6. Most recommended at individual level for clinical cases confirmation	1. Requires extraction and thermal cycler equipment	Müller *et al.* (2001) PoI *et al.* (2013)
VI Score: +++ Species : Suidae	On live suid: nasal swabs On dead suid: tonsil; lung, trigeminal lymph node; sacral lymph node	Reference test: VI	Müller *et al.* (2001): experimental infection of 18 European wild boars and 16 domestic pigs: three groups of four wild boars were inoculated with PrV Bartha, Kaplan, and a wild-boar isolate (BFW1) and housed with uninfected pigs. Two groups of domestic pigs (four and eight pigs/group, respectively) were inoculated with various doses of BFW1. Animals were observed daily for clinical signs, and samples were tested for PrV excretion and homologous antibodies. After reactivation of latent infection by induced immunosuppression, PrV was detected in tissues of necropsied animals, using cell culture and a polymerase chain reaction (PCR)	See reference Most recommended at individual level for clinical cases confirmation, permits virus isolation and identification of the virus by sequencing	1. Medium sensitivity 2. High specificity 3. Nasal swabs, tonsils , lung: To be used in acute disease with respiratory clinical signs, as nasal excretion duration is short (2–15 days post-infection) 4. Trigeminal and sacral lymph nodes : useful to detect latently infected pig	1. Medium sensitivity 2. Time consuming (up to three passages of 5 days) and labour intensive so not suitable for high throughput. 3. Requires incubators and microscope 4. Requires high trained technicians	Müller *et al.* (2001)

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Appendix 5: Aujeszky's disease
Intended purpose of test: prevalence of infection – surveillance

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
Virus neutralisation (VN) Score: + Species: *Suidae*	Serum	VN was the reference method for serology Must detect at least the WOAH Standard Reference Serum diluted 1/2	Historical study conducted in the 1980s Comparison of three serological tests for the detection of antibodies to SUAHV1 in a population of 1285 pig 5.9% of sera positive by VNT NVT less sensitive than ELISA at the herd level	See references	1. High specificity	1. Low sensitivity 2. Time consuming (3–5 days) and labour intensive so not suitable for high throughput. 3. Requires incubators and microscope 4. Requires highly trained technicians 5. No DIVA properties	Goyal *et al.* (1987)
Latex agglutination Manual or semi-automatic Score: +++ Species: *Suidae*	Serum	Semi-automatic latex agglutination (LA) test was compared with manual LA, ELISA and VN	Study conducted in the 1990s on 1191 swine sera Overall, the sensitivities of the four tests were as follows: semiautomated LAT = manual LAT > ELISA > SNT. For 74 samples of known pseudorabies antibody status, the overall specificities were as follows: semi-automated LAT = manual LAT = SNT > ELISA	See references WOAH Expert opinion: the recommended test to be used at the herd level to assess the prevalence of infection	1. High sensitivity 2. High specificity	1. No DIVA properties 2. Few kits commercially available	Rodgers *et al.* (1996) Schoenbaum *et al.* (1990)
ELISA gB Score: +++ ELISA gE Score: ++ Species: *Suidae*	Serum Clotted blood on filter paper Muscle exudates	Reference test: VN	Comparative studies of VN, LA and ELISA were conducted in the 1990s ELISA kits currently commercialised are more robust, sensitive and specific They are able to detect: for ELISA gB at least the WOAH Standard Reference Serum diluted 1/2 For ELISA gE at least the WOAH Standard Reference Serum diluted 1/8	See references + WOAH expert opinion based on ELISA kits initial and batch-to-batch controls conducted by the French national reference laboratory And on the inter-laboratory comparative test for serology organised every 2-years by the WOAH Reference Laboratory The recommended test to be used at the herd level to assess the prevalence of infection	1. High sensitivity, detects persistently infected animals 2. Commercially available 3. Easy to handle 4. Rapid (<24 hours) 5. Possibility of automation for high throughput 6. ELISA gB can be used for individual serum or pool of five sera, and for individual filter paper or pool of five filter papers 7. DIVA: ELISA gB/gE when using gE-deleted vaccine	1. ELISA gB: Low per cent of false positive results (<2%), mainly due to insufficient quality of sera 2. ELISA gE to be used only on individual serum 3. ELISA gE: Risk of false negative gE at the individual level because of delay of seronconversion to gE in vaccinated pigs 4. Requires a microplate absorbance reader	Rodgers *et al.* (1996) Schoenbaum *et al.* (1990)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
PCR Score: + Species: *Suidae*	On live suid: nasal or ejaculate swabs On dead suid: tonsil; lung, trigeminal lymph node; sacral lymph node	Reference test: VI	Müller *et al.* (2001): Experimental infection of 18 European wild boars and 16 domestic pigs: three groups of four wild boars were inoculated with SuAHV1 Bartha, Kaplan, and a wild-boar isolate (BFW1) and housed with uninfected pigs. Two groups of domestic pigs (four and eight pigs/group, respectively) were inoculated with various doses of BFW1. Animals were observed daily for clinical signs, and samples were tested for SuAHV1 excretion and homologous antibodies. After reactivation of latent infection by induced immunosuppression, SuAHV1 was detected in tissues of necropsied animals, using cell culture and a PCR	See references + WOAH expert opinion based on PCR kits initial and batch-to-batch controls conducted by the French national reference laboratory And on the inter-laboratory comparative test for virology (PCR) organised every 2-years by the WOAH Reference Laboratory Suitable in limited circumstances: e.g.: in insemination centres or selection herds, before movement; always in combination with serological testing	1. High sensitivity 2. High specificity 3. Commercially available	1. Nasal swabs: to be used only in acute disease with respiratory clinical signs Risk of false negatives for two main reasons: a) nasal excretion duration is short (2–15 days post-infection), b)cannot detect latent infection 2. Requires extraction and thermal cycler equipment	Müller *et al.* (2001) Pol *et al.* (2013)

Appendix 6: Aujeszky's disease

Intended purpose of test: immune status in individual animals or populations post-vaccination

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
Virus neutralisation (VN) Score: +++ Species: *Suidae*	Serum	VN was the reference method for serology Must detect at least the WOAH Standard Reference Serum diluted 1/2	Historical study conducted in the 1980s on 56 pigs A study of pseudorabies virus (PRV)-vaccinated pigs comparing the immune responses detected by the latex agglutination test (LAT) with responses detected by other routine tests for pseudorabies antibodies indicated that LAT was more sensitive than either the ELISA or the serum virus neutralisation test (SVNT). The LAT detected antibodies sooner than ELISA and SVNT in unvaccinated pigs after challenge with virulent SUAHV1. The specificities of the 3 tests were found to be near 100%.	See references	1. Quantification of neutralising antibodies: useful for evaluation of vaccination at individual level, can be used to assess a boost effect 2. High specificity	1. Low sensitivity 2. Time consuming (3–5 days) and labour intensive so not suitable for high throughput. 3. Requires incubators and microscope 4. Requires highly trained technicians 5. No DIVA properties	Schoenbaum *et al.* (1990)
Latex agglutination Manual or semi-automatic Score: ++ Species: *Suidae*	Serum	Semi-automatic latex agglutination (LA) test was compared with manual LA, ELISA and VN	See above (VN)	See references	1. High sensitivity, detects persistently infected animals 2. Commercially available 3. Easy to handle 4. Rapid (<24 hours) 5. Possibility of automation for high throughput 6. At the herd level, permits the percentage of vaccinated pigs to be assessed	1. No DIVA properties 2. Few kits commercially available 3. At the individual level does not permit quantification of antibodies, can be used to assess a boost effect at the herd level	Schoenbaum *et al.* (1990)
ELISA gB Score: ++ ELISA gE Score: ++ Species: *Suidae*	Serum Clotted blood on filter paper Muscle exudates	Reference test: VN	Comparative studies of VN, LA and ELISA were conducted in the 1980s ELISA kits currently commercialised are more robust, sensitive and specific They are able to detect: for ELISA gB at least the WOAH Standard Reference Serum diluted	See references + WOAH expert opinion based on ELISA kits initial and batch-to-batch controls conducted by the French national reference laboratory And on the inter-laboratory comparative	1. High sensitivity, detects persistently infected animals 2. Commercially available 3. Easy to handle 4. Rapid (<24 hours) 5. Possibility of automation for high throughput 6. ELISA gB can be used for individual serum or pool of	1. ELISA gB: Low per cent of false positive results (<2%), mainly due to insufficient quality of sera 2. ELISA gE to be used only on individual serum 3. ELISA gE: Risk of false negative gE at the individual level because of delay of	Schoenbaum *et al.* (1990)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
			1/2 For ELISA gE at least the WOAH Standard Reference Serum diluted 1/8	test for serology organised every 2-years by the WOAH Reference Laboratory	five sera, and for individual filter paper or pool of five filter papers 7. DIVA: ELISA gB/gE when using gE-deleted vaccine 8. ELISA gB: at the herd level, permits the percentage of vaccinated pigs to be assessed	seronconversion to gE in vaccinated pigs 4. Requires a microplate absorbance reader 5. ELISA gB: a) at the individual level does not permit quantification of antibodies. b) can be used to assess a boost effect at the herd level	

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CHAPTER 3.1.8.

FOOT AND MOUTH DISEASE (INFECTION WITH FOOT AND MOUTH DISEASE VIRUS)

SUMMARY

Foot and mouth disease (FMD) is a ~~highly~~ very contagious disease of mammals and has a great potential for causing severe economic loss in susceptible cloven-hoofed animals. There are seven serotypes of FMD virus (FMDV), namely, O, A, C, SAT 1, SAT 2, SAT 3 and Asia 1. Infection with one serotype does not confer immunity against another. FMD cannot be differentiated clinically from other vesicular diseases, such as swine vesicular disease, vesicular stomatitis, disease caused by Senecavirus A (also known as Seneca Valley virus) and vesicular exanthema~~ ~~and Seneca Valley virus infection~~. Laboratory diagnosis of any suspected FMD case is therefore a matter of urgency.

Typical cases of FMD are characterised by ~~a vesicular condition of~~ the appearance of vesicles on the feet, on the buccal mucosa and, in females, on the mammary glands. Clinical signs can vary from mild to severe, and fatalities may occur, especially in young animals. In some species the infection may be subclinical, e.g. African buffalo (Syncerus caffer). *The preferred tissue for diagnosis is epithelium from unruptured or freshly ruptured vesicles or vesicular fluid. Where collecting this is not possible, blood ~~and/~~or oesophageal–pharyngeal fluid samples taken by probang cup in ruminants or throat swabs from pigs provide an alternative source of virus. Myocardial tissue or blood can be submitted from fatal cases, but vesicles are again preferable if present.*

It is vital that samples from suspected cases be transported under secure conditions and according to international regulations. They should only be dispatched to authorised laboratories.

Laboratory diagnosis of FMD is conducted by ~~virus isolation or by the demonstration~~ detection of FMD viral antigen or nucleic acid in appropriate samples ~~of tissue or fluid~~. Detection of virus-specific antibody can also be used for diagnosis, and antibodies to viral nonstructural proteins (NSPs) can be used as indicators of infection, irrespective of vaccination status. Confirmation of a case of FMD should take account of all relevant clinical, epidemiological and laboratory findings.

Detection and identification of the agent: *The presence of FMD virus is confirmed by ~~demonstration~~ detection of nucleic acid or specific antigen ~~or nucleic acid~~, with or without prior amplification of the virus in cell culture through ~~(~~virus isolation in susceptible cells~~)~~. Due to the ~~highly~~ very contagious nature and economic importance of FMD, the laboratory diagnosis and serotype identification of the virus should be done in a laboratory with an appropriate level of bio-containment, determined by risk analysis in accordance with Chapter 1.1.4* Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities.

Sample materials can be tested by reverse transcription polymerase chain reaction (RT-PCR) or virus isolation using susceptible cells to amplify any nucleic acid or live virus that may be present. The cultures should preferably be of primary bovine (calf) thyroid, but pig, lamb or calf kidney cells, or cell lines of comparable sensitivity may be used. Once cytopathic effect (CPE) is complete in the cultures, harvested fluids can be tested for FMDV using enzyme-linked immunosorbent assays (ELISA) or RT-PCR. ~~Enzyme-linked immunosorbent assays (~~ELISA~~)~~ can also be directly used to detect FMD viral

antigens and for serotyping. Lateral flow devices (LFD) ~~are also becoming more readily available and~~ can also be used to detect FMD viral antigens even on-site. The ELISA has replaced complement fixation (CF) ~~in most laboratories~~ as it is more specific and sensitive and it is not affected by pro- or anti-complement factors. ~~If the sample is inadequate or the diagnosis remains uncertain, sample materials can be tested by reverse transcription polymerase chain reaction (RT-PCR) and/or virus isolation using susceptible cell to amplify any nucleic acid or live virus that may be present. The cultures should preferably be of primary bovine (calf) thyroid, but pig, lamb or calf kidney cells, or cell lines of comparable sensitivity may be used. Once a cytopathic effect (CPE) is complete in the cultures, harvested fluids can be tested for FMDV using ELISA, CF or RT-PCR.~~

***Serological tests:** The demonstration of specific antibodies to nonstructural proteins (NSPs) or structural proteins in nonvaccinated animals is indicative of prior infection with FMDV. This is particularly useful in mild cases or where epithelial tissue cannot be collected. Tests for antibodies to some NSPs of FMDV are useful in providing evidence of previous or current viral replication in the host, irrespective of vaccination status. NSPs, unlike structural proteins, are highly conserved and therefore are not serotype specific and as a consequence, the detection of these antibodies is not serotype restricted.*

Virus neutralisation tests (VNTs) and ELISAs for antibodies to structural proteins are used as serotype-specific serological tests. VNTs depend on ~~tissue~~ cell cultures and are therefore more prone to variability than ELISAs; they are also slower and subject to contamination. ELISAs for detection of antibodies have the advantage of being faster, and are not dependent on cell cultures. The ELISA can be performed with inactivated or recombinant antigens, thus requiring less restrictive biocontainment facilities.

***Requirements for vaccines:** Inactivated virus vaccines of varying composition are available commercially. Typically, virus is used to infect a suspension or monolayer cell culture and the resulting preparation is clarified, inactivated with ethyleneimine and concentrated. The antigen is usually blended with oil or aqueous adjuvant for vaccine formulation. Many FMD vaccines are multivalent to provide protection against the different serotypes, or to accommodate antigenic diversity likely to be encountered in a given field situation.*

The finished vaccine must be shown to be free from residual live virus. This is most effectively done by evaluating the inactivation kinetics and by using in-vitro *tests on concentrated inactivated virus preparations prior to formulation of the vaccine and freedom from live virus is subsequently confirmed during* in-vivo ~~*and/or*~~ in-vitro *tests on the finished product. Challenge tests are also conducted in vaccinated cattle to establish a PD_{50} (50% protective dose) value or protection against generalised foot infection (PGP), although a serological test, which can be performed to reduce the number of animals being used, is considered to be satisfactory where a valid correlation between protection, and specific antibody response has been established.*

FMD vaccine production facilities should also have an appropriate level of bio-containment, determined by risk analysis in accordance with Chapter 1.1.4.

Diagnostic and reference reagents are available from the WOAH Reference Laboratories for FMD or the FAO (Food and Agriculture Organization of the United Nations) World Reference Laboratory for FMD (The Pirbright Institute, UK).

A. INTRODUCTION

Foot and mouth disease (FMD) is caused by a virus of the genus *Aphthovirus*, family *Picornaviridae*. There are seven serotypes of FMD virus (FMDV), namely O, A, C, SAT 1, SAT 2, SAT 3, and Asia 1, that infect cloven-hoofed animals. Infection with any one serotype does not confer immunity against another. Within serotypes, many strains can be identified by ~~biochemical~~ molecular and immunological tests. Serotype C has not been detected since 2004 and is now considered extinct (Paton *et al.*, 2021).

Of the domesticated species, cattle, pigs, sheep, goats and water buffalo (*Bubalus bubalis*) are susceptible to FMD (Food and Agricultural Organization of the United Nations [FAO]; 1984). Many species of cloven-hoofed wildlife may become infected, and the virus has occasionally been recovered from other species as well. Amongst the camelidae, Bactrian camels and new world camelids have been shown to be susceptible (Larska *et al.*, 2009). In Africa, SAT serotypes of FMD viruses are often maintained by African buffalo (*Syncerus caffer*). There is periodic spillover of infection into livestock or sympatric cloven-hoofed wildlife. Elsewhere in the world, cattle are usually the

main reservoir for FMD viruses, although in some instances the viruses involved appear to be specifically adapted to pigs (such as the pig-adapted Cathay strain of type O FMDV) and that requires cells of porcine origin for primary isolation. Small ruminants can play an important role in the spread of FMDV, but it is not clear whether the virus can be maintained in these species for long periods in the absence of infection of cattle. Strains of FMDV that infect cattle have been isolated from wild pigs, antelope and deer. The evidence indicates that, in the past, infection of deer was derived from contact, direct or indirect, with infected domestic animals, and that apart from African buffalo, wildlife has not, so far, been shown to be able to maintain FMD viruses independently for more than a few months.

Infection of susceptible animals with FMDV can lead to the appearance of vesicles on the feet, in and around the oral cavity, and on the mammary glands of females. The vesicles rupture and then heal whilst coronary band lesions may give rise to growth arrest lines that grow down the side of the hoof. The age of lesions can be estimated from these changes as they provide an indicator of the time since infection occurred (Ministry of Agriculture, Fisheries and Food [of the UK], 1986). Mastitis is a common sequel of FMD in dairy cattle. Vesicles can also occur at other sites, such as inside the nostrils and at pressure points on the limbs – especially in pigs. The severity of clinical signs varies with the strain of virus, the exposure dose, the age and breed of animal, the host species and the immunity of the animal. The signs can range from a mild or inapparent infection to one that is severe. Death may result in some cases. Mortality from a multifocal myocarditis is most commonly seen in young animals: myositis may also occur in other sites.

On premises with a history of sudden death in young cloven-hoofed livestock, close examination of adult animals may often reveal the presence of vesicular lesions if FMD is involved. The presence of vesicles in fatal cases is variable.

~~In animals with a history of vesicular disease,~~ The detection of FMDV in samples of vesicular fluid, epithelial tissue, oesophageal–pharyngeal (OP) sample, milk, or blood is sufficient to establish a diagnosis. Diagnosis may also be established by the detection of FMDV in the blood, heart or other organs of fatal cases. A myocarditis may be seen macroscopically (the so-called “tiger heart”) in a proportion of fatal cases.

FMD viruses may occur in all the secretions and excretions of acutely infected animals, including expired air. Transmission ~~is~~ generally ~~effected~~ occurs by direct contact between infected and susceptible animals or, more rarely, indirect exposure of susceptible animals to the excretions and secretions of acutely infected animals or by consumption of uncooked (meat) products from infected animals. Following recovery from the acute stage of infection, infectious virus disappears, ~~with the exception of~~ however low levels ~~that~~ may persist in the oropharynx of some ruminants. Live virus or viral RNA may continue to be recovered from oropharyngeal fluids and cells collected with a probang cup. FMD virus has also been shown to persist in a non-replicative form in lymph nodes (Juleff *et al*., 2008). Animals in which the virus persists in the oropharynx for more than 28 days after infection are referred to as carriers. Pigs do not become carriers. Circumstantial evidence indicates, particularly in the African buffalo, that carriers are able, on rare occasions, to transmit the infection to susceptible domestic animals with which they come in close contact~~:~~ and the mechanism involved is unknown. The carrier state in cattle usually does not persist for more than 6 months, although in a small proportion, it may last up to 3 years. In African buffalo, individual animals have been shown to harbour the virus for at least 5 years, but it is probably not a lifelong phenomenon. Within a herd of buffalo, the virus may be maintained for 24 years or longer. Sheep and goats do not usually carry FMD viruses for more than a few months, whilst there is little information on the duration of the carrier state in Asian buffalo species and subspecies.

FMD is considered a negligible zoonotic risk. However, because of its ~~highly~~ very contagious nature for animals and the economic importance of FMD, all laboratory manipulations with live viral cultures or potentially infected/contaminated material such as tissue and blood samples must be performed at an appropriate containment level determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*). Countries lacking access to appropriate containment facilities should send specimens to a WOAH FMD Reference Laboratory. Vaccine production facilities should also meet these containment requirements.

Diagnostic and standard reagents are available in kit form or as individual items from WOAH Reference Laboratories for FMD. The use of inactivated antigens in the enzyme-linked immunosorbent assay (ELISA), as controls in the antigen-detection test or to react with test sera in the liquid-phase blocking or solid-phase competitive ELISA, reduces the disease security risk involved compared with the use of live virus. Reagents are supplied freeze-dried or in glycerol or non-glycerinated but frozen and can remain stable at temperatures between +1°C and +8°C, –30°C and –5°C and –90°C and –50°C, respectively, for many years. There are a number of commercially available diagnostic test kits, for the detection of virus antigens or antibodies.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of FMD and their purpose

Method	Purpose					
	Population freedom from infection	**Individual animal freedom from infection prior to movement**	**Contribute to eradication policies**	**Confirmation of clinical cases**	**Prevalence of infection – surveillance**	**Immune status in individual animals or populations post-vaccination**
Detection and identification of the agent[a]						
Virus isolation	–	+	+++	+++	–	–
Antigen detection ELISA	–	–	+++	+++	–	–
~~CFT~~	–	–	~~+~~	~~+~~	–	–
LFD	–	–	+++	+++	–	–
Real-time RT-PCR	+	+	+++	+++	+	–
Conventional RT-PCR	+	+	+++	+++	+	–
Detection of immune response						
NSP Ab ELISA	+++	++	+++	+++	+++	–
SP Ab ELISA[b]	++	++	+++	+++	++	+++
VNT[b]	++	++	+++	+++	++	+++
AGID[b]	+	+	+	+	+	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
ELISA = enzyme linked immunosorbent assay; ~~CFT = complement fixation test;~~ LFD: lateral flow device; RT-PCR = reverse-transcriptase polymerase chain reaction; AGID = Agar gel immunodiffusion; NSP Ab ELISA = ELISA for antibodies against nonstructural proteins; SP Ab ELISA = ELISA for antibodies against structural proteins; VNT = Virus neutralisation test.
[a]It is essential to confirm the presence of FMDV following virus isolation by an antigen or nucleic acid detection test.
[b]The tests do not distinguish infected from vaccinated animals.

For laboratory diagnosis, the tissue of choice is epithelium or vesicular fluid. Ideally, at least 1 g of epithelial tissue should be collected from an unruptured or recently ruptured vesicle, usually from the tongue, buccal mucosa or feet. To avoid injury to personnel collecting the samples, as well as for animal welfare reasons, it is recommended that animals be sedated before any samples are obtained.

Epithelial samples should be placed in a transport medium composed of equal amounts of glycerol and ~~0.04 M phosphate buffer, pH 7.2–7.6, preferably with added antibiotics (penicillin [1000 International Units (IU)], neomycin sulphate [100 IU], polymyxin B sulphate [50 IU], mycostatin [100 IU])~~ transport fluid (composed of 0.08 M phosphate buffer containing 0.01% bovine serum albumin, 0.002% phenol red, antibiotics [1000 units/ml penicillin, 100 units/ml mycostatin, 100 units/ml neomycin, and 50 units/ml polymyxin], and adjusted to pH 7.2). If ~~0.04 M phosphate buffer~~ transport medium is not available, tissue culture medium or phosphate-buffered saline (PBS) can be used instead, but it is important that the final pH of the glycerol/buffer mixture be in the range pH 7.2–7.6. FMDV is extremely labile in low pH and buffering of the transport media is critical for successful sample collection. Samples should be kept refrigerated or on ice until received by the laboratory. In case of a short transportation time and in circumstances where such medium is not present, transporting the samples to the laboratory refrigerated or on ice without transport medium can be considered.

Where epithelial tissue is not available from ruminant animals, for example in advanced or convalescent cases, or where infection is suspected in the absence of clinical signs, samples of OP fluid can be collected by means of a probang (sputum) cup (or in pigs by swabbing the throat) for submission to a laboratory for virus isolation or reverse-transcription polymerase chain reaction (RT-PCR). Viraemia may also be detected by examining serum samples by means of RT-PCR or virus isolation. For the collection of throat swabs from pigs up to 25 kg weight, the animal should be held on its back in a wooden cradle with the neck extended. Holding a swab in a suitable instrument, such as an artery forceps, the swab is pushed to the back of the mouth and into the pharynx. For larger pigs, it is recommended that standing animals be restrained by a snare attached to the upper snout and that swab sampling focus on peripheral surfaces in the mouth. Alternative sample matrices for diagnosis may consist of milk (Armson *et al.*, 2018; 2019), swine oral fluids collected with cotton ropes (Hole & Nfon, 2019; Senthilkumaran *et al.*, 2017; Vosloo *et al.*, 2015), and swine meat exudates (Yeo *et al.*, 2020). Samples collected on lateral flow devices (Fowler *et al.*, 2014; Romey *et al.*, 2018) or FTA cards (Abosrer *et al.*, 2022; Biswal *et al.*, 2016) can also tested by RT-PCR.

Before the collection of OP samples from cattle or large ruminants (e.g. buffalo), 2 ml transport fluid (composed of 0.08 M phosphate buffer containing 0.01% bovine serum albumin, 0.002% phenol red, antibiotics [1000 units/ml penicillin, 100 units/ml mycostatin, 100 units/ml neomycin, and 50 units/ml polymyxin], and adjusted to pH 7.2) should be added to a container of around 5 ml capacity capable of withstanding freezing above dry ice (solid carbon dioxide) or liquid nitrogen (Kitching & Donaldson, 1987).

An OP sample is collected by inserting a probang over the tongue into the oro-pharyngeal area and then passing it vigorously backwards and forwards 5–10 times between the first portion of the oesophagus and the back of the pharynx. The purpose is to collect oro-pharyngeal fluid and especially superficial epithelial cells from these areas, including the proximal part of the oesophagus, the walls of the pharynx, the tonsillar crypts and the surfaces of the soft palate. If the sample does not contain adequate cellular debris the actions may be repeated.

After collection of OP fluid by probang, the contents of the cup should be poured into a wide-necked transparent bottle of around 20 ml capacity. The fluid is examined and should contain some visible cellular material. Where mucus and cells are mainly attached to the surface of the probang cup, for example after sample collection from small ruminants, a small volume of buffered medium (pH 7.0) may be placed in a wide-necked sample container, into which the head of the probang may be immersed and agitated to detach and collect the sample. Care should be taken not to overdilute the sample with a large volume of medium as this may reduce the probability of virus detection. This fluid is then added to an approximately equal volume of transport fluid, ensuring that cellular material is transferred; the mixture is shaken gently and should have a final pH of around 7.6. Samples contaminated with ruminal contents may be unsuitable for culture. Samples seen to contain blood are not entirely satisfactory. Repeat sampling can be done after the mouth and throat of the animal have been rinsed with water or PBS. Where several animals are to be sampled, the probang must be cleaned and disinfected between each animal. This is done by washing the probang in tap water, immersing it in a suitable disinfectant (e.g. 0.5% [w/v] citric acid in tap water) and then rinsing off all the disinfectant with water before sampling the next animal.

OP samples from small ruminants are collected by putting 2 ml of transport fluid into a wide-necked bottle of about 20 ml capacity and, after collection, rinsing the probang cup in this transport fluid to discharge the OP sample. This is then transferred to a container of about 5 ml capacity for transport. The small container should be capable of withstanding freezing ~~above~~ in dry ice or liquid nitrogen (e.g. polypropylene, not polystyrene) and be leak-proof (Kitching & Donaldson, 1987).

Samples of OP fluid should be refrigerated or frozen immediately after collection. If they are to remain in transit for more than a few hours, they should preferably be frozen by being placed ~~either above~~ in dry ice or liquid nitrogen. Before freezing, the containers should be carefully sealed using airtight screw caps or with silicone film. This is particularly important when using dry ice, as introduction of CO_2 into the OP sample will lower its pH, inactivating any FMDV that may be in the samples. Glass containers and polystyrene vials should not be used because there is a risk that they will explode or crack on defrosting in the event of liquid nitrogen leaking into them. Samples should reach the laboratory in a frozen state or, if this is not feasible, maintained under reliable cold chain conditions during transit.

Special precautions are required when sending perishable suspect FMD material both within and between countries. The International Air Transport Association (IATA), Dangerous Goods Regulations (DGR) have explicit requirements for packaging and shipment of diagnostic specimens by ~~all commercial means of transport~~ air. These are summarised in Chapter 1.1.2 *Collection, submission and storage off diagnostic specimens* and Chapter 1.1.3 *Transport of biological materials*. Forms and guidance on sample submission, packing dangerous goods and specifications for manufacture of probang cups can be found on the website of the Pirbright WOAH Reference Laboratory at https://www.wrlfmd.org/laboratory-protocols. Procedures (in Spanish) for collection and shipment of field samples for the diagnosis of vesicular diseases and its differential diagnosis can be found at the Pan-American FMD WOAH Reference Laboratory in the technical information that is available at https://www.paho.org/es/panaftosa/laboratorio-referencia ~~http://www.panaftosa.org.br~~

1. Detection and identification of the agent

A range of sample types, including vesicular fluids, epithelium, OP samples, milk, serum and heart muscle from cases with myocarditis, may be examined by RT-PCR or virus isolation ~~or RT-PCR~~. By contrast, ELISA, ~~CF~~ and the lateral flow device (LFD) are suited to the examination of epithelial suspensions, vesicular fluids or infected cell culture supernatants, but are insufficiently sensitive for the direct examination of OP samples or serum. It is essential to confirm the presence of FMDV following virus isolation by an FMDV-specific antigen or nucleic acid detection test. A virus isolate or an RT-PCR product can be further characterised by sequencing, but a viral isolate is needed for some *in-vitro* serological tests and for *in-vivo* studies of transmission, virulence and vaccine induced protection.

1.1. Virus isolation

The epithelium sample should be ~~taken~~ removed from the PBS/glycerol transport media, may be washed at least twice in cell culture medium containing 2% antibiotics to reduce bacterial contamination, blotted dry on absorbent paper to reduce the glycerol content, which is toxic for cell cultures, and weighed. A suspension should be prepared by grinding the sample in sterile sand in a sterile pestle and mortar with a small volume of tissue culture medium (or PBS) and 2% antibiotics. Grinding can also be carried out by a tissue-lyser apparatus. Further medium should be added until a final volume of nine times that of the epithelial sample has been added, giving a 10% suspension. This is clarified on a bench centrifuge at 2000 ***g*** for 10 minutes. Once clarified, ~~such~~ suspensions of field samples suspected to contain FMDV are inoculated onto cell cultures. Sensitive cell culture systems include primary bovine (calf) thyroid cells and primary pig, calf or lamb kidney cells, but there are challenges associated with sourcing these cells from FMDV-negative animals (particularly in FMD endemic countries), and the costs and time required to regularly prepare batches of cells. Established cell lines, such as BHK-21 (baby hamster kidney), ZZ-R 127 (fetal goat tongue cell line), LFBK-αvβ6 (fetal porcine kidney cell line expressing the bovine integrin receptor) and IB-RS-2 (porcine kidney cell line) cells, may also be used but their sensitivity to field FMD viruses can be variable (Gray *et al.*, 2020) ~~are generally less sensitive than primary cells for detecting low amounts of infectivity~~. The sensitivity of any cells used should be tested routinely with standard preparations of FMDV to confirm that cells with different passage histories maintain sensitivity to FMDV. It is recommended to determine the maximum number of passages for which a batch of cells can be used. The use of IB-RS-2 cells and LFBK-αvβ6 aids the differentiation of swine vesicular disease virus (SVDV) and Senecavirus A (SVA) from FMDV (as SVDV will usually only grow in cells of porcine origin) and is often essential for the isolation of porcinophilic strains, such as FMDV O Cathay. The cell cultures should be examined for cytopathic effect (CPE) for 48 hours. If no CPE is detected, the cells should be frozen and thawed, used to inoculate fresh cultures and examined for CPE for another 48 hours. In the case of OP fluids, pretreatment with an equal volume of chloro-fluoro-carbons may improve the rate of virus detection by releasing virus from immune complexes.

Alternatively, FMDV may be rescued by chemical transfection or electroporation of FMD viral RNA into cultured cells (Dill & Eschbaumer, 2019; Romey *et al.*, 2018).

1.2. Immunological methods

1.2.1. Enzyme-linked immunosorbent assay

The ~~preferred~~ main procedure for the detection of FMD viral antigen and identification of viral serotype is the ELISA (Ferris & Donaldson, 1992; Roeder & Le Blanc Smith, 1987). This is an indirect sandwich test in which different rows in multiwell plates are coated with 'capture' rabbit antisera to each of the seven serotypes of FMDV. ~~These are the 'capture' sera.~~ Test sample suspensions are added to each of the rows, and appropriate controls are also included. Guinea-pig antisera to each of the serotypes of FMDV are added next, followed by rabbit anti-guinea-pig serum conjugated to an enzyme. Extensive washing is carried out between each stage to remove unbound reagents. A colour reaction on the addition of enzyme substrate and chromogen indicates a positive reaction. With strong positive reactions, this will be evident to the naked eye, but results can also be read spectrophotometrically at an appropriate wavelength. In this case, an absorbance reading greater than 0.1 above background indicates a positive reaction; the serotype of FMDV can also be identified. In case of cross-reactions between serotypes, (strong) positive samples can be diluted 1/5 and retested. Values close to 0.1 should be confirmed by retesting or by amplification of the antigen by tissue culture passage and testing the supernatant once a CPE has developed. Alternatively, cut-off OD values of 0.1 above background (doubtful, requires retesting or prior amplification) and 0.2 above background (positive) may be used. A suitable protocol is given below. Other protocols are available with slightly different formats and interpretation criteria (Alonso *et al.*, 1993). As an alternative to guinea-pig or rabbit antisera, suitable monoclonal antibodies (MAbs) can be used coated to the ELISA plates as capture antibody or peroxidase-conjugated as detecting antibody (Grazioli *et al.*, 2020).

Depending on the species affected and the geographical origin of samples, it may be appropriate to simultaneously test for SVDV, Senecavirus A (SVA) or vesicular stomatitis virus (VSV). Ideally a complete differential diagnosis should be undertaken in all vesicular ~~conditions~~ cases to obtain a final diagnosis.

Rabbit antiserum to the 146S antigen of each of the seven serotypes of FMDV (plus SVDV or VSV if required) is used as a trapping antibody at a predetermined optimal concentration in carbonate/bicarbonate buffer, pH 9.6.

Control antigens are prepared from selected strains of each of the seven types of FMDV (plus SVDV or VSV if appropriate) grown on monolayer cultures of BHK-21 cells (IB-RS-2 cells for SVDV or VSV). The unpurified supernatants are used and pretitrated on ELISA plates. The final dilution chosen is that which gives an absorbance at the top of the linear region of the titration curve (optical density approximately 2.0), so that the five-fold dilutions of the control antigens used in the test give two additional lower optical density readings from which the titration curve can be derived. PBS containing 0.05% Tween 20 and phenol red indicator is used as a diluent (PBST).

Guinea-pig antisera prepared by inoculating guinea-pigs with 146S antigen of one of the seven serotypes of FMDV (plus SVDV or VSV if required) and preblocked with normal bovine serum (NBS) is used as the detecting antibody. Predetermined optimal concentrations are prepared in PBS containing 0.05% Tween 20, and 5% dried, nonfat skimmed milk (PBSTM). Rabbit (or sheep) anti-guinea-pig immunoglobulin conjugated to horseradish peroxidase and preblocked with NBS is used at a predetermined optimum concentration in PBSTM. ~~As an alternative to guinea-pig or rabbit antisera, suitable monoclonal antibodies (MAbs) can be used coated to the ELISA plates as capture antibody or peroxidase-conjugated as detecting antibody. A validated ready-to-use kit is available, based on pre-selected monoclonal antibodies, for detection and serotyping of six of the seven FMDV serotypes.~~

Validation of diagnostic assays, whether these are (commercial) (MAb-based) kits or in-house methods, should be performed according to Chapter 1.1.6. *Validation of diagnostic assays for infectious diseases of terrestrial animals* and the supporting chapters in Section 2.2. *Validation of diagnostic tests* of this *Terrestrial Manual*.

1.2.2. Test procedure

i) ELISA plates are coated with 50 µl/well rabbit antiviral sera in 0.05 M carbonate/bicarbonate buffer, pH 9.6. Rows A to H receive, respectively, antisera to serotypes O, A, C, SAT 1, SAT 2, SAT 3, Asia 1 and SVDV or VSV (optional). In countries from regions where SAT and Asia 1 serotypes have never circulated those serotypes are not routinely included.

ii) Leave overnight at 4°C in a stationary position or place on an orbital shaker set at 100–120 revolutions per minute in a 37°C incubator for 1 hour.

iii) Prepare test sample suspension (10% original sample suspension or undiluted clarified cell culture supernatant fluid).

iv) The ELISA plates are washed five times in PBS.

v) On each plate, load wells of columns 4, 8 and 12 with 50 µl PBST. Additionally, add 50 µl of PBST to wells 1, 2 and 3 of rows A to H on plate 1. To well 1 of row A of plate 1 add 12.5 µl of control antigen type O, to well 1 of row B add 12.5 µl of control antigen type A; continue in this manner for control antigen of types C, SAT 1, SAT 2, SAT 3, Asia 1 and SVDV or VSV (if appropriate) in order to well 1, rows C to H. Mix diluent in well 1 of rows A to H and transfer 12.5 µl from well 1 to 2 (rows A to H), mix and transfer 12.5 µl from well 2 to 3, mix and discard 12.5 µl from well 3 (rows A to H) (this gives a five-fold dilution series of each control antigen). It is only necessary to change pipette tips on the micropipette between antigens. The remainder of the plate can be loaded with the test sample(s). Add 50 µl of sample one to wells 5, 6 and 7 of rows A to H, the second sample is placed similarly in columns 9, 10 and 11, rows A to H.

If more than two samples are to be tested at the same time, the other ELISA plates should be used as follows:

Dispense 50 µl of the PBST to the wells (rows A to H) of columns 4, 8 and 12 (buffer control columns). Note that the control antigens are not required on these plates. These test samples may be added in 50 µl volumes in rows A to H to columns 1, 2, 3; 5, 6, 7; 9, 10, 11, respectively.

vi) Cover with lids and place on an orbital shaker at 37°C for 1 hour.

vii) Wash the plates by flooding with PBS – wash three times as before and empty residual wash fluid. Blot the plates dry.

viii) Transfer 50 µl volumes of each guinea-pig serum dilution to each plate well in the appropriate order, e.g. rows A to H receive, respectively, antisera to serotypes O, A, C, SAT 1, SAT 2, SAT 3, Asia 1 and SVDV or VSV (optional).

ix) Cover the plates with lids and replace on the orbital shaker. Incubate at 37°C for 1 hour.

x) The plates are washed again three times, and 50 µl of rabbit anti-guinea-pig immunoglobulin conjugated to horseradish peroxidase is added to each well. The plates are incubated at 37°C for 1 hour on a rotary shaker.

xi) The plates are washed again three times, and 50 µl of substrate solution, containing 0.05% % H_2O_2 plus orthophenylene diamine (OPD) or a suitable ~~alternative~~ safer chromogen such as 3,3',5,5' tetramethylbenzidine (TMB), is added to each well.

xii) The reaction is stopped after 15 minutes by the addition of 50 µl of 1.25 M sulphuric acid. The plates are read at an appropriate wavelength on a spectrophotometer linked to a computer. For example, 492 nm ~~on a spectrophotometer linked to a computer~~ for OPD and 450 nm for TMB.

1.2.3. Lateral flow device test

There are commercially available pan-serotype or serotype-specific lateral flow devices (LFD) for the detection of FMDV antigens in the field (Ferris *et al.*, 2009)~~, but the OIE has not yet received a validation dossier for these tests. As soon as a dossier is received, the manufacturer could apply for inclusion on the OIE test register~~. Currently one ready-to-use LFD kit[1], validated for the detection of FMDV from East Asia of serotypes O, A and Asia-1 in epithelium samples or fluid from blisters or ruptured lesions of suspected swine or cattle, has been certified by WOAH as fit for purpose for a period of 5 years. Validation of test kits should be performed according to Chapter 1.1.6 and the supporting chapters in Section 2.2. *Validation of diagnostic tests* of this *Terrestrial Manual*. The results obtained with non-certified LFDs should thus be confirmed by validated laboratory assays. LFDs can be shipped to the laboratory and used for molecular detection and characterisation of FMDV (Fowler *et al.*, 2014; Romey *et al.*, 2018).

~~1.2.4. Complement fixation test~~

~~In general, the ELISA is preferable to the complement fixation test (CFT) because it is more sensitive and it is not affected by pro- or anti-complementary factors (Ferris & Dawson, 1988). If ELISA reagents are not available, or if subtyping is pursued, the CFT may be performed as follows:~~

~~The CF50% protocol in tubes used widely in South America for typing, subtyping and for establishing serological relationships (r values) is performed as follows: 0.2 ml antiserum to each FMDV serotype diluted at a predetermined optimal dilution in veronal buffer diluent (VBD) or borate-saline solution (BSS) is placed in separate tubes. To these, 0.2 ml of test sample suspension is added, followed by 0.2 ml of a complement dilution containing 4 units of complement. The test system is incubated at 37°C for 30 minutes prior to the addition of 0.4 ml 2% standardised sheep red blood cells (SRBC) in VBD or BSS sensitised with rabbit anti SRBC. The reagents are incubated at 37°C for further 30 minutes and the tubes are subsequently centrifuged and read. Samples with less than 50% haemolysis are considered positive.~~

~~Other protocols performed in microplates are available and are performed as follows: antisera to each of the seven types of FMDV are diluted in VBD in 1.5 fold dilution steps from an initial 1/16 dilution to leave 25 µl of successive antiserum dilutions in U-shaped wells across a microtitre plate. To these are added 50 µl of 3 units of complement, followed by 25 µl of test sample suspension(s). The test system is incubated at 37°C for 1 hour prior to the addition of 25 µl of 1.4% SRBC in VBD sensitised with 5 units of rabbit anti-SRBC. The reagents are incubated at 37°C for a further 30 minutes and the plates are subsequently centrifuged and read. Appropriate controls for the test suspension(s), antisera, cells and complement are included. CF titres are expressed as the reciprocal of the serum dilution producing 50% haemolysis. A CF titre ≥36 is considered to be a positive reaction. Titre values of 24 should be confirmed by retesting an antigen that has been amplified through tissue culture passage.~~

[1] vdrg-fmdv-3diff-pan-ag-rapid-kit-1.pdf (woah.org)

1.3. Nucleic acid ~~recognition~~ detection methods

RT-PCR can be used to amplify genome fragments of FMDV in a wide range of diagnostic materials including vesicular fluids, epithelium, milk, serum, whole blood and OP samples. Real-time RT-PCR ~~has a sensitivity comparable to that of virus isolation~~ is very sensitive and automated procedures enhance sample throughput (Reid *et al.,* 2003). ~~Serotyping primers have also been developed (Vangrysperre & De Clercq, 1996).~~ Simplified RT-PCR systems for potential field-use ~~are under development~~ have been developed (~~Callahan *et al.,* 2002~~ Goller *et al.,* 2018; Hole & Nfon, 2019; Howson *et al.,* 2018).

1.3.1. ~~Agarose gel-based~~ Conventional RT-PCR assay

A gel-based RT-PCR procedure is described (Reid *et al.,* 2000). The RT-PCR assay consists of the three successive procedures of (i) extraction of template RNA from the test or control sample followed by (ii) RT of the extracted RNA, (iii) PCR amplification of the RT product and (iv) detection of the PCR products by agarose gel electrophoresis.

1.3.2. Test procedure

i) Add 200 µl of test sample to 1 ml of RNA extraction reagent in a sterile tube. Store at –70°C until required for RNA extraction.

ii) Transfer 1 ml of the solution from i) into a fresh, sterile tube containing 200 µl of chloroform. Vortex mix for about 10–15 seconds and leave at room temperature for 3 minutes.

iii) Centrifuge for 15 minutes at 20,000 ***g***.

iv) Transfer 500 µl of the aqueous phase into a fresh, sterile tube containing 1 µl of glycogen (20 mg/ml) and add 500 µl of iso-propyl-alcohol (propan-2-ol). Vortex mix for a few seconds.

v) Leave at room temperature for 10 minutes then centrifuge for 10 minutes at 20,000 ***g***.

vi) Carefully discard the supernatant fluid from each tube taking care not to dislodge or lose any pellet at the bottom of the tube. Add 1 ml of 70% ethanol and vortex mix for a few seconds.

vii) Centrifuge for 10 minutes at 20,000 ***g***.

viii) Carefully remove the supernatant fluid from each tube taking care not to dislodge or lose any pellet at the bottom of the tube.

ix) Air dry each tube at room temperature for 2–3 minutes.

x) Resuspend each pellet by adding 20 µl of nuclease-free water to the tube.

xi) Keep the extracted RNA samples on ice if the RT step is about to be performed. Otherwise store at –70°C.

NOTE: As an alternative to phenol/chloroform, RNA extraction can be performed using commercially available kits based on chaotropic salt lysis and silica RNA affinity. The RNA can be extracted through both manual and (semi-)automated techniques.

xii) For each sample to be assayed, add 2 µl of random hexamers (20 µg/ml) and 5 µl of nuclease-free water into a sterile 0.5 ml microcentrifuge tube. It is recommended to prepare the dilution in bulk for the total number of samples to be assayed but allowing for one extra sample.

xiii) Add 5 µl of RNA from the extraction procedure described above to give a volume of 12 µl in each tube. Mix by gently pipetting up and down.

xiv) Incubate at 70°C for 5 minutes.

xv) Cool at room temperature for 10 minutes.

xvi) During the 10-minute incubation period, prepare the RT reaction mixture described below for each sample. Prepare the reaction mixture in bulk in a sterile 1.5 ml microcentrifuge tube for the number of samples to be assayed plus one extra sample.

First strand buffer, 5× conc. (4 µl); bovine serum albumin (acetylated), 1 mg/ml (2 µl); dNTPs, 10 mM mixture each of dATP, dCTP, dGTP, dTTP (1 µl); DTT, 1 M (0.2 µl); Moloney Murine Reverse Transcriptase, 200 U/µl (1 µl).

xvii) Add 8 µl reaction mix to the 12 µl of random primer/RNA mix. Mix by gently pipetting.

xviii) Incubate at 37°C for 45 minutes.

xix) Keep the RT products on ice if the PCR amplification step is about to be performed, otherwise store at –20°C.

xx) Prepare the PCR mix described below for each sample. It is recommended to prepare the mix in bulk for the number of samples to be tested plus one extra sample.

Nuclease-free water (35 µl); PCR reaction buffer, 10× conc (5 µl); $MgCl_2$, 50 mM (1.5 µl); dNTPs, 10 mM mixture each of dATP, dCTP, dGTP, dTTP (1 µl); forward primer ~~1~~, 10 pmol/µl (1 µl); reverse primer ~~2~~, 10 pmol/µl (1 µl); *Taq* Polymerase, 5 units/µl (0.5 µl).

xxi) Add 45 µl of PCR reaction mix to a well of a PCR plate or to a microcentifuge tube for each sample to be assayed followed by 5 µl of the RT product to give a final reaction volume of 50 µl.

xxii) Spin the plate or tubes for 1 minute in a suitable centrifuge to mix the contents of each well.

xxiii) Place the plate in a thermal cycler for PCR amplification and run the following programme:

94°C for 5 minutes: 1 cycle;
94°C for 1 minute, 55°C for 1 minute, 72°C for 2 minutes: 30 cycles;
72°C for 7 minutes: 1 cycle.

Times and temperatures may need to be optimised to the particular enzymes, reagents and PCR equipment used in individual laboratories.

xxiv) Mix a 20 µl aliquot of each PCR reaction product with 4 µl of staining solution and load onto a 1.5% agarose gel. After electrophoresis a positive result is indicated by the presence of a 328 bp band corresponding to FMDV sequence in the 5' untranslated region of the genome.

1.3.3. Stock solutions

i) Nuclease-free water, RNA extraction reagent, chloroform, glycogen, iso-propyl-alcohol (propan-2-ol), ethanol, random hexanucleotide primers, First, strand buffer, BSA (acetylated), dNTPs, DTT, Moloney Murine Reverse Transcriptase, PCR reaction buffer (10×), $MgCl_2$ and *Taq* Polymerase are commercially available.

ii) Primers at a concentration of 10 pmol/µl: Forward primer ~~1~~ sequence 5'-GCCTG-GTCTT-TCCAG-GTCT-3' (positive strand); Reverse primer ~~2~~ sequence 5'-CCAGT-CCCCT-TCTCA-GATC-3' (negative strand).

1.3.4. Real-time RT-PCR assay

Real-time RT-PCR assays employ total RNA extracted from the test or control samples followed by RT and subsequent PCR amplification of the extracted RNA. ~~The real-time RT-PCR assay can use the same procedures of extraction of total RNA from the test or control sample followed by RT of the extracted RNA as for the conventional agarose gel-based procedure. Automated extraction of total nucleic acid from samples followed by automated pipetting programmes for the RT and PCR steps (Reid *et al.,* 2003) can be used as an alternative to the manual procedures described above. PCR amplification of the RT product is performed by a different procedure. A~~ These simple~~r~~ one-step method~~s~~ ~~for combining~~ that combine the RT and PCR steps ~~has also been described~~ (Shaw *et al.,* 2007) ~~and is~~ are widely used by laboratories. Detection of the PCR products in agarose gels is not required following real-time amplification.

i) ~~Take the RT products from step xix (see above).~~ Extract RNA from the sample according to a locally validated method or a protocol provided by a WOAH Reference Laboratory for FMD. NB: Different methods for RNA extraction (manual, commercial conventional column or magnetic bead-based rapid RNA extraction methods) are available and selection of an appropriate method needs to consider the assay sensitivity.

ii) Prepare the RT-PCR reaction mix ~~described below~~ for each sample containing the one-tube RT-PCR reaction master mix, real-time PCR forward primer, 10 pmol/µl; real-time PCR reverse primer, 10 pmol/µl; labelled probe, 5 pmol/µl, with the remaining volume accommodated by nuclease-free water. ~~Again~~ It is recommended to prepare the mix in bulk for the number of samples to be tested plus one extra sample~~: nuclease-free water (6 µl); PCR reaction master mix, 2× conc. (12.5 µl); real-time PCR forward primer, 10 pmol/µl (2.25 µl); real-time PCR reverse primer, 10 pmol/µl (2.25 µl); labelled probe, 5 pmol/µl (1 µl)~~.

iii) Add ~~24~~ 20 µl RT-PCR reaction mix to a well of a real-time PCR plate or to a PCR tube for each sample to be assayed followed by ~~1~~ 5 µl of the ~~RT product~~ extracted RNA to give a final reaction volume of 25 µl. NB: Final reaction volumes used in the test can vary according to the source of the commercial one-step RT-PCR master mix.

iv) Spin the plate/tube for 1 minute in a suitable centrifuge to ~~mix the contents of each well~~ ensure that the fluids are at the bottom of the wells/tubes.

v) Place the plate/tube in a real-time PCR machine for RT and PCR amplification and run the following programme:

50°C for ~~2~~ 15 minutes: 1 cycle;
95°C for ~~10~~ 2 minutes: 1 cycle;
95°C for 15 seconds, 60°C for 1 minute: 50 cycles.

Times and temperatures may need to be optimised to the particular enzymes, reagents and PCR equipment used in individual laboratories.

vi) *Reading the results:* Assign a threshold cycle (C_T) value to each PCR reaction from the amplification plots after verification of the sigmoidal shape of the plot and establishment of the baseline (a plot of the fluorescence signal versus cycle number; different cut-off values may be appropriate for different sample types; Parida *et al.,* 2007). The C_T values used to assign samples as either FMDV positive, ~~or~~ negative, "borderline" or doubtful should be defined by individual laboratories using appropriate reference material. ~~For example at the OIE Reference Laboratory at Pirbright, negative test samples and negative controls should have a CT value at >50.0. Positive test samples and positive control samples should have a CT value <40.~~ Samples ~~with CT values falling within the range 40–50 are~~ designated "borderline" ~~and can~~ or doubtful should be retested. Strong positive FMD samples have a C_T value below 20.0 (Reid *et al.,* 2001).

Simultaneous detection in a duplex format of an internal quality control, such as ß-actin, can be performed to verify PCR performance, sample quality and performance of the RNA extraction, and is thus a preventative measure against false-negative results (Gorna *et al.*, 2016; Moniwa *et al.*, 2007). A positive amplification control can be synthesised and used to detect potential false-positive results due to contamination if a wild-type virus is used as positive control (Moniwa *et al.*, 2007).

1.3.5. Stock solutions for real-time PCR assay

i) Nuclease-free water and real-time PCR reaction master mixes are available from commercial suppliers.

ii) ~~Either~~ Any of the ~~two~~ following primers and probe sets can be used for real-time PCR of FMDV:

5'UTR (Reid *et al.,* 2001) Forward primer: CACYT-YAAGR-TGACA-YTGRT-ACTGG-TAC; Reverse primer: CAGAT-YCCRA-GTGWC-ICITG-TTA and labelled probe: CCTCG-GGGTA-CCTGA-AGGGC-ATCC.

3D (Callahan *et al.,* 2002) Forward primer: ACTGG-GTTTT-ACAAA-CCTGT-GA; Reverse primer: GCGAG-TCCTG-CCACG-GA and labelled probe: TCCTT-TGCAC-GCCGT-GGGAC.

3D (Moniwa *et al.*, 2007) Forward primer: ACTGG-GTTTT-AYAAA-CCTGT-GATG; Reverse primer: TCAAC-TTCTC-CTGKA-TGGTC-CCA and labelled probe: ATCCT-CTCCT-TTGCA-CGC.

Addition of nonspecific tail sequences to the 5'-terminus of these primers improves the robustness and overall performance of the real-time PCR assays, in particular of the 5'UTR assay (Vandenbussche *et al.*, 2016; 2017).

RT-PCR assays should meet the WOAH Standards for validation (Chapter 1.1.6 and the supporting chapters in Section 2.2. *Validation of diagnostic tests* of this *Terrestrial Manual*) and should be able to detect a broad spectrum of globally circulating FMDV strains.

1.3.6. Molecular epidemiology

The molecular epidemiology of FMD is based on the comparison of genetic differences between viruses. Dendrograms showing the genomic relationship between vaccine and field strains for all seven serotypes based on sequences derived from the 1D gene (encoding the VP1 viral protein) have been published (Knowles & Samuel, 2003; see also https://www.wrlfmd.org/). A list of FMDV prototype strains that can form the basic reference points of a phylogenetic tree is available at: https://foot-and-mouth.org/FMDV-nomenclature-working-group/prototype-strains. VP1 sequencing can also be used as an alternative method for serotyping by immunological methods.

In areas where the circulating lineages of FMDV are well known and where sequencing capability is difficult to access, lineage-specific real-time RT-PCR assays can allow for rapid characterisation at a relatively low cost (Bachanek-Bankowska *et al.*, 2016; Chestley *et al.*, 2022; Saduakassova *et al.*, 2023). Comparison of whole genome sequences can provide further discrimination between closely related viruses and help to recreate the transmission pathways between farms within outbreaks (Cottam *et al.,* 2008). RT-PCR amplification of FMDV RNA, followed by nucleotide sequencing, is the current preferred option for generating the sequence data to perform these comparisons. Many laboratories have developed molecular techniques for performing these studies, and WOAH Reference Laboratories hold databases containing ~~over 6000 partial~~ several thousands of FMDV sequences.

The recommended method for VP1 ~~analysis~~ Sanger sequencing is to:

i) ~~Extract~~ Purify FMDV RNA directly from epithelial suspensions or from ~~a~~ low passage cell culture ~~passage~~ material.

ii) Perform an RT-PCR of the complete 1D gene (or if only part of the 1D gene, then the 3' end of the gene is more useful).

iii) Determine the nucleotide sequence of the PCR product (or at least 170 nucleotides [preferably 420 for the SAT types] at the 3' end of the gene).

A protocol complete with primer sequences, is available (Knowles *et al*., 2016) ~~or can be downloaded from the following World Wide Web URLs~~ at: https://www.wrlfmd.org/laboratory-protocols/vp1-sequencing ~~http://bvs.panaftosa.org.br/~~

Other methods to obtain full or partial FMDV VP1 sequences by Sanger sequencing are described by Dill *et al.* (2017) and Amaral-Doel *et al.* (1993), respectively.

Full genome FMDV sequences can be obtained by either Sanger sequencing (Carrillo *et al.*, 2005; Cottam *et al.*, 2008; Dill *et al.*, 2017), or by high-throughput sequencing (Logan *et al.*, 2014).

2. Serological tests

Serological tests for FMD are performed in support of four main purposes namely: 1) to certify individual animals prior to import or export (i.e. for trade); 2) to confirm suspected cases of FMD; 3) to substantiate absence of infection; 4) to demonstrate the efficacy of vaccination. For substantiating freedom from infection, different approaches are required according to whether the population has been vaccinated or not and if vaccination has been used, whether this has been applied as an emergency application or as part of an ongoing programme of vaccination. Different tests and different interpretations of test results will be appropriate according to the above-mentioned purposes and the validation of the selected procedure must take account of the purpose. For example, test cut-offs may be set at a different threshold for herd-based serosurveillance than is appropriate for certifying freedom from infection for individual animals for the purposes of international trade.

Serological tests for FMD are of two types; those that detect antibodies to viral structural proteins (SP) and those that detect antibodies to viral nonstructural proteins (NSPs).

The SP tests are relatively serotype-specific and detect antibodies elicited by vaccination and infection; examples are the virus neutralisation test (VNT) (Golding *et al*., 1976), the solid-phase competition ELISA (SPCE; Brocchi *et al*., 1990; Chenard *et al*., 2003; Mackay *et al*., 2001; Paiba *et al*., 2004) and the liquid-phase blocking ELISA (LPBE; Hamblin *et al*., 1986; 1987). These tests are highly sensitive, ~~providing that~~ provided the virus or antigen used in the test is closely matched to the strain circulating in the field. ~~They~~ These are the tests used to certify animals prior to movement, including for international trade purposes, ~~and are appropriate for~~ confirming previous or ongoing infection in nonvaccinated animals ~~as well as for~~ and monitoring the immunity conferred by vaccination in the field. The VNT requires cell culture facilities, the use of live virus and takes 2–3 days to provide results. The ELISAs are blocking- or competition-based assays that use serotype-specific polyclonal antibodies (PAbs) or MAbs, are quicker to perform and are not dependent on tissue culture systems and the use of live viruses. The SP ELISAs can be cross-reactive between serotypes and caution must be taken when they are used for surveillance where multiple FMDV serotypes may be circulating (Ludi *et al.*, 2022). Furthermore, low titre false-positive reactions can be expected in a small proportion of the sera in either ELISA formats. An approach combining screening by ELISA and confirming the positives by the VNT minimises the occurrence of false-positive results. Reference sera to standardise FMD SP serological tests for some serotypes and subtypes are available from the WOAH Reference Laboratory at Pirbright.

The detection of antibody to the NSPs of FMDV can be used to identify past or present infection with any of the seven serotypes of the virus, whether or not the animal has also been vaccinated. Therefore, the tests can be used

to confirm suspected cases of FMD and to evaluate prevalence of infection or to substantiate freedom from infection on a population basis. For certifying animals for trade, the tests have the advantage over SP methods that the serotype of virus does not have to be known. However, there is experimental evidence that some cattle, vaccinated and subsequently challenged with live virus and confirmed persistently infected, may ~~not be detected in some anti-NSP tests, causing~~ give false-negative results (Brocchi *et al.*, 2006). These assays measure antibody to NSPs using antigens produced by recombinant techniques in a variety of *in-vitro* expression systems. Antibody to the polyproteins 3AB or 3ABC are generally considered to be the most reliable indicators of infection (Mackay *et al.*, 1997). In animals seropositive for antibody to 3AB or 3ABC, antibody to one or more of the other NSPs can aid in the final interpretation of the test (Bergmann *et al.*, 2000; Mackay *et al.*, 1997). However, lack of vaccine purity ~~may affect diagnostic specificity as the presence of NSPs~~ in some vaccine preparations may result in the presence of antibodies to NSPs in ~~misclassification in~~ animals that have been repeatedly vaccinated, potentially leading to misclassification of the animals as FMDV infected. Procedures for evaluating vaccine purity are covered in Section D of this chapter.

International standard sera for testing of cattle have been developed and are among others available from the WOAH Reference Laboratories in Brazil and UK (Campos *et al.*, 2008). ~~In the future,~~ Standard sera ~~will~~ are also ~~be made~~ available for sheep and pigs. Bovine serum panels have also been established to compare the sensitivity of NSP tests (Parida *et al.*, 2007).

2.1. Virus neutralisation test

The quantitative VNT ~~microtest~~ for antibodies to FMDV ~~antibody~~ is performed with IB-RS-2, BHK-21, lamb or pig kidney cells in flat-bottomed tissue-culture grade microtitre plates.

Stock virus is grown in cell monolayers and stored at –20°C after the addition of 50% glycerol~~.~~ (virus has been found to be stable under these conditions for at least 1 year~~.~~) or stored at –70°C or colder. To decrease complement activity, the sera are ~~inactivated~~ placed in a water bath at 56°C for 30 minutes before testing. The control standard serum is ~~21 day~~ well-characterised convalescent or post-vaccination serum. A suitable medium ~~is~~ can be Eagle's complete medium/LYH (Hank's balanced salt solution with yeast lactalbumin hydrolysate) with HEPES buffer and antibiotics.

The test is an equal volume test in 50 µl amounts.

2.1.1. Test procedure

i) Starting from a 1/4 dilution, sera are diluted in a twofold, dilution series down or across the plate, using at least two ~~rows of~~ wells per serum~~, preferably four rows,~~ dilution and a volume of 50 µl.

ii) ~~Previously titrated~~ Virus with a known titre is added; each 50 µl ~~unit~~ volume of virus suspension should ideally contain about 100 $TCID_{50}$ (50% tissue culture infective dose) ~~within an accepted range (e.g. 32–320 $TCID_{50}$)~~. A back titration must be carried out at the time of testing to ensure that the virus dose used to neutralise the sera falls within the accepted range of 32–320 ($10^{1.5}$–$10^{2.5}$) $TCID_{50}$.

iii) Controls should include a standard antiserum of known titre, a cell control and a medium control~~, and a virus titration used to calculate the actual virus titre used in the test~~.

iv) Incubate at 37°C for 1 hour with the plates covered.

v) A cell suspension ~~at~~ of approximately 10^6 cells/ml is made up in medium containing 10% bovine serum (specific antibody negative) for cell growth. A volume of 50 µl of cell suspension is added to each well.

vi) Plates are sealed ~~with pressure sensitive tape~~ and incubated at 37°C for 2–3 days. Alternatively, the plates may be covered with loosely fitting lids and incubated in an atmosphere of 3–5% carbon dioxide at 37°C for 2–3 days.

vii) Microscope readings may be feasible after 48 hours. The plates ~~are finally~~ can be fixed and stained ~~routinely~~ on the third day. Fixation is effected with 10% formol/saline for 30 minutes. For staining, the plates are immersed in 0.05% methylene blue in 10% formalin for 30 minutes. An alternative fixative/stain solution is naphthalene blue black solution (0.4% [w/v] naphthalene blue black, 8% [w/v] citric acid in saline). The plates are rinsed in tap water if deemed non-infectious by local risk assessments.

viii) Positive wells (where the virus has been neutralised and the cells remain intact) are seen to contain blue-stained cells sheets; the negative wells (where virus has not been neutralised) are completely or partially empty. Titres are expressed as the final dilution of serum present in the serum/virus mixture where 50% of wells are protected (Kärber, 1931). The test is

considered to be valid when the back-titration of the virus confirms that the amount of virus used per well is in the range ~~$\log_{10}$ 1.5–2.5 $TCID_{50}$,~~ of 32–320 ($10^{1.5}$–$0^{2.5}$) $TCID_{50}$ and the positive standard serum is within twofold of its expected titre.

ix) Interpretation of tests can vary between laboratories in regard to the negative/positive cut-off threshold. Laboratories should establish their own criteria by reference to standard reagents that can be obtained from the WOAH Reference Laboratory at Pirbright. In general, a titre of 1/45 or more of the final serum dilution in the serum/virus mixture is regarded as positive. A titre of less than 1/16 is considered to be negative. For certification of individual animals for the purposes of international trade, titres of 1/16 to 1/32 are considered to be doubtful, and further serum samples may be requested for testing; results are considered to be positive if the second sample has a titre of 1/16 or greater. For the purposes of herd-based serosurveillance as part of a statistically valid serological survey, a cut-off of 1/45 may be appropriate. Cut-off titres for evaluating immunological protection afforded by vaccination have to be established from experience of potency test results with the relevant vaccine and target species.

2.2. Solid-phase competition enzyme-linked immunosorbent assay

The method described (Paiba *et al.,* 2004) can be used for the detection of antibodies against each of the seven serotypes of FMDV. As an alternative to guinea-pig or rabbit antisera, suitable monoclonal antibodies (MAbs) can be ~~used~~ coated to the ELISA plates as capture antibody or peroxidase-conjugated as detecting antibody (Brocchi *et al.,* 1990). Commercial kits based on ~~monoclonal antibodies~~ MAbs are available from different manufacturers for serotype O (Chenard *et al.,* 2003), serotype A, serotype Asia 1, serotype SAT-1 and serotype SAT-2 ~~with a~~ in different formats but with similar performance characteristics (Ludi *et al.,* 2022). Validation of test kits should be performed according to Chapter 1.1.6 and the supporting chapters in Section 2.2. *Validation of diagnostic tests* of this *Terrestrial Manual*.

Serotype-specific rabbit antiserum to the 146S antigen of ~~one of the seven types of~~ FMDV is used as the trapping antibody at a predetermined optimal concentration in carbonate/bicarbonate buffer, pH 9.6.

Antigens are prepared by inactivating viruses propagated in cell culture with ethyleneimine using the procedures described for vaccine manufacture. The final dilution chosen is that which, after addition of an equal volume of diluent, gives an absorbance on the upper part of the linear region of the titration curve (optical density approximately 1.5). PBS containing 0.05% Tween 20, 10% NBS and 5% normal rabbit serum and phenol red indicator is used as a diluent (blocking buffer).

Serotype-specific guinea-pig antisera, prepared by inoculating guinea-pigs with 146S antigen of ~~one of the seven serotypes~~ FMDV and preblocking with normal bovine serum (NBS), is used as the detecting antibody. Predetermined optimal concentrations are prepared in blocking buffer (PBS containing 0.05% Tween 20, and 5% dried, nonfat skimmed milk [PBSTM]).

Rabbit (or sheep) anti-guinea-pig immunoglobulin conjugated to horseradish peroxidase and preblocked with NBS is used as conjugate at a predetermined optimum concentration in PBSTM blocking buffer.

Test sera are diluted in PBST blocking buffer.

The solid-phase competitive ELISA is more specific but as sensitive as the liquid-phase blocking ELISA (Mackay *et al.,* 2001; Paiba *et al.,* 2004). Methods have been described for the development of secondary and working standard sera (Goris & De Clercq, 2005a) and for charting assay performance (Goris & De Clercq, 2005b).

2.2.1. Test procedure

i) ELISA plates are coated with 50 µl/well rabbit antiserum homologous to the antigen being used, diluted in carbonate/bicarbonate buffer, pH 9.6, and left overnight in a humid chamber at 4°C.

ii) The ELISA plates are washed three times with PBS.

iii) Then 50 µl of the FMDV antigen diluted in blocking buffer is added to each well of the ELISA plates (blocking buffer: 0.05% [w/v] Tween 20, 10% [v/v] NBS, 5% [v/v] normal rabbit serum). The plates are covered and placed on an orbital shaker at 37°C for 1 hour, with continuous shaking.

iv) After washing three times with PBS, 40 µl of blocking buffer is added to each well, followed by 10 µl of test sera (or control sera), giving an initial serum dilution of 1/5.

v) Immediately 50 µl of guinea-pig anti-FMDV antiserum diluted in blocking buffer is added, giving a final serum dilution of 1/10.

vi) The plates are covered and incubated on an orbital shaker at 37°C for 1 hour.

vii) After washing three times with PBS, 50 µl of anti-guinea-pig Immunoglobulin conjugate (preblocked by incubation for 1 hour at room temperature with an equal volume of NBS) diluted in blocking buffer is added. The plates are covered and incubated for 1 hour at 37°C on an orbital shaker.

viii) After washing three times with PBS, 50 µl of substrate solution, containing 0.05% H_2O_2 plus orthophenylene diamine or a suitable alternative chromogen, is added to each well.

ix) The reaction is stopped after 10 minutes by the addition of 50 µl of 1 M sulphuric acid. The plates are read at 492 nm on a spectrophotometer linked to a computer.

x) *Controls:* On each plate two wells are used for conjugate control (no guinea-pig serum), four wells each for strong and weak positive sera, two wells for negative sera, and four wells for 0% competition (no test sera).

xi) *Interpretation of the results:* A percentage of inhibition is calculated for each well, either manually or using a suitable computer programme (100 – [optical density of each test or control ~~value~~ sera/mean optical density of the 0% competition] × 100%), representing the competition between the test sera and the guinea-pig anti-FMDV ~~anti~~sera for the FMDV antigen on the ELISA plate. Laboratories should validate the assay in terms of the cut-off value above which sera should be considered positive in relation to (i) the particular serotypes and strains of virus under investigation (ii) the purpose of testing (iii) the population under test, using the methods described in Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals*. This cut-off value for a positive result can either be a fixed or dynamic value. In the latter case this cut-off value is calculated for each individual test based on the per cent competition obtained for strong positive, weak positive, cut-off and negative working standards (Goris & De Clercq, 2005a). Working standards are also used for monitoring and charting cross-session assay performance (Goris & De Clercq, 2005b). ~~At the WOAH Reference Laboratory at Pirbright, for serotype O, for all species, for the purposes of demonstrating freedom from infection in a naïve population, greater than 60% inhibition is considered positive (Paiba *et al.*, 2004). For maximum sensitivity, for example when certifying individual animals for international trade, an inconclusive range may be set between 40 and 60%.~~

2.3. Liquid-phase blocking enzyme-linked immunosorbent assay

Antigens are prepared from selected strains of FMDV grown on ~~monolayers of BHK 21~~ cell cultures. The unpurified supernatants are used and pre-titrated in a twofold dilution series but without serum. The final dilution chosen is that which, after addition of an equal volume of diluent (see below), gives an absorbance on the upper part of the linear region of the titration curve (optical density approximately 1.5). PBS containing 0.05% Tween 20 and phenol red indicator is used as a diluent (PBST). The other reagents used in the test are the same as those in the solid-phase ~~blocking~~ competition ELISA. An example of the test procedure is described below. Temperature and incubation times can vary depending on the protocol.

2.3.1. Test procedure

i) ELISA plates are coated with 50 µl/well rabbit antiserum homologous to the antigen being used and left overnight in a humid chamber at room temperature.

ii) The ELISA plates are washed three times with PBS.

iii) In U-bottomed multiwell plates (carrier plates) 50 µl of a duplicate, twofold series of each test serum is prepared, starting at 1/8. To each well, 50 µl of a constant dose of viral antigen that is homologous to the rabbit antisera used to coat the plates is added and the mixtures are left overnight at 4°C, or incubated at 37°C for 1 hour. The addition of the antigen increases the final serum dilution to 1/16.

iv) Then 50 µl of serum/antigen mixtures is transferred from the carrier plates to the rabbit-serum coated ELISA plates and the plates are incubated at 37°C for 1 hour on a rotary shaker.

v) After washing, 50 µl of guinea-pig antiserum homologous to the viral antigen used in the previous step (iv) (preblocked with NBS and diluted in PBST containing 5% skimmed milk powder) is added to each well. The plates are then incubated at 37°C for 1 hour on a rotary shaker.

vi) The plates are washed and 50 µl of rabbit anti-guinea-pig immunoglobulin conjugated to horseradish peroxidase (preblocked with NBS and diluted in PBST containing 5% skimmed milk powder) is added to each well. The plates are incubated at 37°C for 1 hour on a rotary shaker.

vii) The plates are washed again three times and 50 µl of substrate solution, containing 0.05% H_2O_2 plus orthophenylene diamine or a suitable alternative chromogen, is added to each well.

viii) The reaction is stopped after 15 minutes by the addition of 50 µl of 1 M sulphuric acid. The plates are read at 492 nm on a spectrophotometer linked to a computer.

ix) Controls: A minimum of four wells each of strong positive, weak positive and negative bovine reference sera at a final dilution of 1/32 should be included on each plate together with an equivalent number of reaction (antigen) control wells containing antigen in diluent alone without serum. For end-point titration tests, duplicate twofold dilution series of positive and negative homologous bovine reference sera should be included on at least one plate of every run.

x) *Interpretation of the results:* Antibody titres are expressed as the 50% end-point titre, i.e. the dilution at which the reaction of the test sera results in an optical density equal to 50% inhibition of the median optical density of the reaction (antigen) control wells (Kärber, 1931). The median is calculated as the mean of two mid-values of the reaction control wells, eliminating from the calculation the highest and lowest values (alternatively, the mean value can be used after setting suitable tolerance limits to control for inter-well variation). In general sera with titres greater than or equal to 1/90 are considered to be positive. A titre of less than 1/40 is considered to be negative. For certification of individual animals for the purposes of international trade, titres of greater than 1/40, but less than 1/90 are considered to be doubtful, and further serum samples may be requested for testing; results are considered to be positive if the second sample has a titre of 1/40 or greater. For the purposes of herd-based serosurveillance as part of a statistically valid serological survey, a cut-off of 1/90 may be appropriate. Cut-off titres for evaluating immunological protection afforded by vaccination have to be established from experience of potency test results with the relevant vaccine and target species.

2.4. Nonstructural protein (NSP) antibody tests

Antibody to expressed recombinant FMDV NSPs (e.g. 3A, 3B, 2B, 2C, 3ABC) can be measured by different ELISA formats or immunoblotting. These ELISAs either use purified antigens absorbed directly to microplates or use PAbs or MAbs to trap specific antigens from semi-purified preparations (Bergmann *et al*., 2000; De Diego *et al*., 1997; Mackay *et al*., 1997; Sorensen *et al*., 1998). The screening method used in Panaftosa is described in detail below. Other indirect and competitive ELISAs detecting bovine antibodies to 3ABC have been shown to have equivalent diagnostic performance characteristics (Brocchi *et al*., 2006). This same study corroborates ~~preliminary~~ data from Panaftosa that suggests that the diagnostic performance characteristics of these tests are similar in cattle, sheep and pigs. Commercial kits validated for identification of antibodies against FMDV NSPs in cattle and other species are also available. Validation should be performed accordingly Chapter 1.1.6 and the supporting chapters in Section 2.2. *Validation of diagnostic tests* of this *Terrestrial Manual.*

2.4.1. Indirect enzyme-linked immunosorbent assay

a) Preparation of recombinant antigens

See Section B.2.4.2. *Enzyme-linked immunoelectrotransfer blot assay.*

b) Test procedure

i) Microplates are coated overnight at 4°C with 1 µg/ml of the fusion antigen 3ABC in carbonate/ bicarbonate buffer, pH 9.6 (100 µl per well). Antigen 3ABC was expressed and purified as indicated for the EITB (enzyme-linked immunoelectrotransfer blot) tests (Neizert *et al.,* 1991).

ii) The plates are washed six times with PBS, pH 7.2, supplemented with 0.05% Tween 20 (PBST).

iii) Test sera (100 µl per well) are added in a 1/20 dilution in blocking buffer consisting of PBS, 0.05% Tween 20, 5% nonfat dry milk, 10% equine sera and 0.1% *Escherichia coli* lysate. Each plate includes a set of strong and weak positive and negative controls calibrated against the International Standard Sera described below.

iv) The plates are incubated for 30 minutes at 37°C and washed six times in PBST.

v) Horseradish-peroxidase-conjugated rabbit anti-species IgG is diluted optimally in the blocking buffer, added at 100 µl per well and the plates are incubated for 30 minutes at 37°C.

vi) After six washings, each well is filled with 100 µl of 3'3', 5'5'-tetramethylbenzidine plus 0.004% (w/v) H_2O_2 in phosphate/citrate buffer, pH 5.5.

vii) The reaction is stopped after 15 minutes of incubation at room temperature by adding 100 µl of 0.5 M H_2SO_4. Absorbance is read at 450 nm and at 620 nm for background correction.

viii) *Interpreting the results:* Test results are expressed as per cent positivity relative to the strong positive control [(optical density of test or control wells/optical density of strong positive control) × 100] or alternatively as a test to control (T/C) index relative to a cut-off (i.e. threshold positive) control. Profiling the NSP antibody reactivity levels in herds along with age/vaccination stratification aids interpretation of herd infection status in vaccinated populations (Bergmann *et al.,* 2003). Test cut-off values, with or without suspicious zones, need to be determined with consideration to the purpose of testing and the intended target population. Inconclusive results may be followed up using confirmatory tests, retesting with EITB or a second NSP ELISA (taking account of the conditional dependence of the two tests). The overall test system sensitivity and specificity must be taken into account when designing the serosurveillance programme. Although not a suitable test for certifying animals prior to movement, NSP ELISAs may be a valuable adjunct in circumstances where the serotype or subtype of virus in the originating country is not known.

2.4.2. Enzyme-linked immunoelectrotransfer blot assay (EITB)

The EITB assay has been widely applied in South America as a confirmatory test for the above-described screening method. Further information is available from the WOAH Reference Laboratory, Panaftosa, PAHO/WHO.

a) Preparation of test strips containing the recombinant antigens

i) The five bioengineered FMDV NSPs 3A, 3B, 2C, 3D and 3ABC are expressed in *E. coli* C600 by thermo-induction. The 3D polypeptide is expressed in its complete form (McCullough *et al.,* 1992) whereas the rest of the proteins are obtained as fusions to the N-terminal part of the MS-2 polymerase gene (Strebel *et al.,* 1986).

ii) The expressed polymerase is purified over phosphocellulose, followed by poly(U) Sepharose columns. The fused proteins 3A, 3B, 2C and 3ABC are purified by sequential extraction of the bacterial extracts with increasing concentrations of urea. The 7M fraction containing the fusion proteins is further purified on a preparative 10% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis). The fusion protein band is excised from the gel and electroeluted (McCullough *et al.,* 1992).

iii) A mixture containing 20 ng/ml of each one of the purified recombinant polypeptides is separated on 12.5% SDS-PAGE and electrophoretically transferred to nitrocellulose (McCullough *et al.,* 1992).

b) Test procedure

i) The required amount of test strips should be assessed, taking into account that for each nitrocellulose sheet, which defines one transferred gel, a positive, a weakly positive, a cut-off and a negative control serum should be assayed. In general, 24 nitrocellulose strips, each 3 mm wide, should result from a gel.

ii) A volume of 0.8 ml of saturation buffer (50 mM Tris/HCl, pH 7.5; 150 mM NaCl; 0.2% Tween 20; 5% nonfat dry milk; and 0.05% bacterial *E. coli* lysate) is added to each well. The antigen-coated strips are blocked by placing the trays on a rocker and agitating for 30 minutes at room temperature (20–22°C).

iii) A dilution of 1/200 of test sera and of each of the controls is added to the appropriate trough. The strips must be completely submerged and facing upwards, and maintained in that position during the whole process.

iv) Strips are incubated for 60 minutes on a rocker at room temperature.

v) Liquid is removed from the trays, and each test strip is washed three times with washing solution (50 mM Tris/HCl, pH 7.5; 150 mM NaCl; and 0.2% Tween 20) by agitation for 5 minutes.

vi) The alkaline-phosphatase-conjugated rabbit anti-bovine solution is added to each test well, and the strips are incubated with shaking for 60 minutes at room temperature.

vii) The liquid is removed from the trays and each test strip is washed three times with washing solution as above.

viii) Substrate solution (0.015% bromochloroindolylphosphate/0.03% nitroblue tetrazolium) is prepared in substrate buffer (100 mM NaCl; 5 mM $MgCl_2$; and 100 mM Tris/HCl, pH 9.3), and is added to each test well.

ix) Strips are incubated by placing the test tray on the orbital mixer and agitating until the cut-off control shows five distinct, discernible bands. Strips are washed with running deionised water and air-dried.

x) *Interpreting the results:* The EITB may be scanned with a densitometer but visual reading, although more subjective, is considered suitable as well. Individual control sera are tested that exhibit minimal but consistent staining for each of the five antigens. A test sample is considered positive if antigens 3ABC, 3A, 3B and 3D (±2C) demonstrate staining densities equal to or higher than that of their appropriate controls. A sample is considered negative if two or more antigens demonstrate densities below their control sera. Test samples not fitting either profile are considered indeterminate.

C. REQUIREMENTS FOR VACCINES

The control of FMD is a national and regional responsibility and, in many countries, the vaccine may be used only under the control of the Veterinary Authority.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements. Varying requirements relating to quality, safety and efficacy apply in particular countries or regions in order for manufacturers to obtain ~~an authorisation or licence~~ regulatory approval for a veterinary vaccine. Where possible, manufacturers should seek to obtain such ~~a license or authorisation~~ regulatory approval for their FMD vaccines as independent verification of the quality of their product.

FMD vaccine production facilities should operate under appropriate biosecurity and containment procedures and practices as outlined in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*.

Routine vaccination against FMD is used in many countries or zones recognised as free from foot and mouth disease with vaccination and in countries where the disease is endemic. In contrast, a number of disease-free countries have never vaccinated their livestock but have preferred the use of strict movement controls and culling of infected and contact animals when outbreaks have occurred. Nevertheless, many disease-free countries maintain the option to vaccinate and have their own strategic reserves of highly concentrated inactivated virus preparations. Such antigen reserves offer the potential of supplying formulated vaccine in an 'emergency' at short notice. (See also Chapter 1.1.10 *Vaccine banks*.)

Traditional FMD vaccines may be defined as a fixed formulation containing defined amounts (limits) of one or more chemically inactivated cell-culture-derived preparations of a seed virus strain blended with a suitable adjuvant/s and excipients. See chapter 1.1.8 for biotechnology-derived vaccines such as recombinant or peptide vaccines.

Antigen banks may be defined as stockpiles of antigen components, registered or licensed according to the finished vaccine, and which can be stored under ultra-low temperatures for a long time for subsequent formulation into vaccine as and when required.

The vaccines are formulated for their specific purpose and in the case of vaccines destined for use in cattle, both aluminium hydroxide saponin adjuvanted and oil adjuvanted vaccines may be used. For use in swine, double oil emulsions are preferred due to their efficacy.

FMD vaccines may be classified as either 'standard' or 'higher' potency vaccines. Standard potency vaccines are formulated to contain sufficient antigen and appropriate adjuvant to ensure that they meet the minimum potency level required (recommended 3 PD_{50} [50% protective dose]; 75% EPP (expected percentage of protection) or 12 protected out of 16 vaccinated and challenged in PGP [protection against generalised foot infection] test) for the duration of the shelf life claimed by the manufacturer. This kind of vaccine is usually suitable for use in routine vaccination campaigns. For vaccination in naïve populations to control FMD outbreaks, higher potency vaccines (e.g.> 6 PD_{50} for the duration of the shelf life claimed by the manufacturer) are recommended for their wider spectrum of immunity as well as their rapid onset of protection.

Because of the presence of multiple serotypes of the virus, it is common practice to prepare vaccines from two or more different virus serotypes. In certain areas, it may be advisable to include more than one virus strain per serotype to ensure broad antigenic coverage against prevailing viruses.

1. Seed virus management

1.1. Characteristics of the seed virus

Selection of master seed viruses (MSVs) should ideally be based on their ease of growth in cell culture, virus yield, antigenic stability at industrial level and broad antigenic spectrum. Isolates to prepare MSVs should be characterised and distributed, preferably by the WOAH FMD Reference laboratories; they should be selected in accordance with the regional epidemiological importance of each strain.

The exact source of the isolate should be recorded and should include details such as the location, species and the type of material from which the virus was derived. Unique nomenclature should be used to identify the FMDV strain. The *in-vitro* passage history of the virus and details of the ingredients should be recorded in accordance with chapter 1.1.8. The passage level of the seed virus should be kept to a minimum to avoid antigenic or genetic changes.

1.2. Method of culture

Methods of culture shall comply with the chapter 1.1.8. Where no suitable established vaccine strain exists, new vaccine strains are derived through the establishment of MSVs from local field isolates by adapting them to growth in suspension or monolayer cells by serial passages. In order to remove the risk of contaminating lipid-enveloped viruses, it is recommended that putative MSVs undergo a validated organic solvent treatment prior to, or during, adaptation.

1.3. Validation as a vaccine strain

MSVs must be antigenic and genetically well characterised and proven to be pure and free from extraneous agents in accordance with Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use* and those listed by the appropriate ~~licensing~~ regulatory authorities. Homology should be established with the original candidate isolates and effectiveness against the circulating strains from which they were developed should be proven. This often encompasses a number of methods, the most reliable being *in-vivo* protection assays. Alternatively, *in-vitro* tests (preferably virus neutralisation) can also be used, which require the availability of post-vaccination sera against these master seeds (see Section D of this chapter).

Seed viruses may be stored at low temperature (e.g. –70°C) or freeze-dried. Working seed viruses may be expanded in one or a few more passages from the master seed stock and used to infect the final cell culture.

Consideration should also be given to minimising the risk of transmission of transmissible spongiform encephalopathy agents (TSEs) by ensuring that TSE risk materials are not used as the source of the virus or in any of the media used in virus propagation.

1.4. Emergency procedures for provisional acceptance of new MSV, and subsequent release of formulated vaccines

In the case of incursion in a region of a new strain to which protection is not elicited by existing vaccine strains, it may be necessary to develop a new vaccine strain from a representative field isolate. Before the new MSV can be accepted, full compliance should be demonstrated with the relevant guidelines to demonstrate freedom from all extraneous agents listed by the appropriate ~~licensing~~ regulatory authorities using both general and specific tests, and to establish homology to the original candidate isolates. The time taken to raise the specific antisera necessary to neutralise the new strain for use in the general tests for detection of extraneous agents and to conduct other specific tests that require specialised techniques may be lengthy. Therefore, in emergency situations where there is insufficient time to complete full testing of the MSV, provisional acceptance of the new strain should be based on a risk analysis of the possibility of contamination of the antigen produced from the new MSV with extraneous agents. This risk assessment should take into account that the virus is inactivated using a chemical inactivant with first order kinetics. Further assurance is provided by the requirement for the kinetics of inactivation to be monitored and recorded for each production batch.

2. Method of manufacture

The recommended method of virus propagation for antigen production is the growth of FMDV in large-scale suspension cultures or monolayers using cell lines under sterile conditions.

A suitable strain of the virus is used to infect a suspension or monolayers of an established cell line, such as BHK-21. Such cell cultures should be proven to be free from contaminating microorganisms.

When the virus is expected to have reached its maximum yield, the culture is clarified, often by centrifugation and/or filtration. The virus is subsequently inactivated by addition of an inactivant of first order, usually ethyleneimine (EI) in the form of binary ethyleneimine (BEI) (Bahnemann, 1990). It is important that the necessary safety precautions for working with BEI/EI are fully observed. The BEI is added to a virus suspension, to give a predetermined final concentration. Inactivation must be duly validated and documented to show the inactivation kinetic and the results of the inactivation controls. The time period for BEI treatment and temperature used for inactivation must be validated for the actual conditions and equipment used. To decrease the likelihood of live virus failing to contact the inactivant, e.g. BEI/EI, it is essential to transfer the vessel contents immediately to a second sterile vessel where inactivation is allowed to go to completion according to the validated inactivation kinetic and taking into account possible regulatory requirements for additional waiting times.

During inactivation, the virus titre is monitored by a sensitive and reproducible technique. After inactivation any residual BEI/EI in the harvest should be neutralised, for example by adding excess sodium thiosulphate solution to a final concentration of 2%.

The inactivated virus is concentrated by ultrafiltration. Concentrated inactivated virus may be purified further by procedures such as chromatography. These concentrated and purified antigens can be formulated into vaccines or stored at low temperatures for many years, and made into vaccine when required by dilution in a suitable buffer and addition of adjuvants (Doel & Pullen, 1990).

Conventional FMD vaccines are usually formulated as oil adjuvanted or aqueous. Oil-adjuvanted vaccines are usually formulated as water-in-oil emulsion using mineral oils. The mineral oil is usually premixed with an emulsifying agent before the addition of a proportion, or all, of the aqueous phase of the vaccine, and emulsified by use of a colloid mill or continuous mechanical or flow ultrasonic emulsifier. More complex double emulsions (water/oil/water) may be produced by emulsifying once more in an aqueous phase containing a small amount of detergent such as Tween 80. Ready-to-use oil adjuvants are now available commercially for different types of emulsion.

Aqueous vaccine is prepared by adsorbing the virus on to aluminium hydroxide gel, one of the adjuvant constituents of the final vaccine blend. The final blend of the vaccine may include other components, such as antifoam, lactalbumin hydrolysate, tryptose phosphate broth, amino acids, vitamins, buffer salts and other substances. An adjuvant such as saponin is also usually incorporated, as well as preservatives.

When using novel components, including adjuvants or preservatives, in any vaccine it is important to take into account that its status, with regard to residues in products derived from food-producing species, must be assessed to ensure that adequate assurance can be giving to ~~licensing~~ regulatory authorities in relation to safety for consumers. This requirement limits considerably the choice of adjuvants and preservatives for use in food-producing species.

3. In-process control

In general, virus titres reach optimum levels between 18 and 24 hours of the cell culture being infected, depending on the serotype. The time chosen to harvest the culture may be based on a number of assays; for instance cell death. Virus concentration may be assessed by an infectivity test, sucrose or CsCl density gradient or serological techniques. It is preferable to use more than one method as they may complement one another.

3.1. Inactivation kinetics

During inactivation of the virus, timed samples should be taken at regular intervals for the purpose of monitoring the rate and linearity of the inactivation process. Virus titres in the samples are determined by inoculation of cell cultures proven to be highly susceptible to FMDV, e.g. BHK. Such cultures permit the testing of statistically meaningful samples under reproducible conditions. The $\log_{10}$ infectivity of the timed samples are plotted against time, and the inactivation procedure is not considered to be satisfactory unless at least the latter part of the slope of the line is linear and extrapolation indicates that there would be less than one infectious particle per 10^4 litres of liquid preparation at the end of the inactivation period.

3.2. Inactivation control

The test for innocuity is an in-process test that should be carried out for every batch of antigen. Cells used to test for absence of residual live virus should undergo a sensitivity test to prove that they are suitable for virus replication. Following inactivation, a sample of each batch of inactivated antigen

representing at least 200 doses of vaccine antigen should be tested for freedom from infectious virus by inoculation of sensitive monolayer cell cultures, preferably of the same origin as those used for the production of antigen. It may be necessary to concentrate the antigen to do this, in which case it must be shown that the concentrated material does not interfere with the sensitivity or reading of the assay. The cell sheets are examined daily over a period of 2–3 days, after which the spent medium is transferred to fresh monolayers and the original monolayers are replenished with fresh medium. Using this method, traces of live virus can be amplified by the passage procedure and detected on the basis of CPE observed. Three passages of the original virus preparation are commonly used. A variant on this method is to freeze–thaw the old monolayers to release intracellular virus, which can be detected by further passage.

4. Final product batch tests

4.1. Innocuity testing

The bulk inactivated antigen and the final formulated product should undergo innocuity test to prove absence of infectious virus. In the final product, antigen must be extracted from adjuvant following an appropriate validated method. A sample representing at least 200 doses of vaccine (including all product presentations) must be used for testing for freedom of infectious virus by inoculation of sensitive cell culture monolayers. After elution, antigen may be concentrated for inoculation in cell monolayers. Test procedure is as described in Section C.3.2 *Inactivation control*.

4.2. Sterility testing

The bulk inactivated antigen, concentrated antigen and the final formulated product should undergo sterility testing. Guidelines on techniques and culture media, which allow the detection of a wide range of organisms, are described in Chapter 1.1.9.

4.3. Identity testing

The bulk inactivated antigen, concentrated antigen and the final formulated product should undergo identity testing to demonstrate that the relevant strains are present. No other FMD virus serotype registered on the manufacturing site should be present in the vaccine, to be assured by appropriate tests such as serotype-specific RT-PCR.

4.4. Purity testing

Purity relates to the level of FMD NSPs in the final product, which should not induce antibodies that would interfere with serological tests used for sero-surveillance of virus circulation in vaccinated populations. Products claiming to be purified from NSPs have to demonstrate their level of purification. Lack of reactivity has to be demonstrated in the final product (see Section C.5. *Requirements for registration of vaccine*). In cases where consistency of purification is demonstrated and approved in the registration dossier, and the production process is approved for consistency in accordance with the standard requirements referred to in chapter 1.1.8, the Veterinary Authority may agree to omit the test in the final product.

Confirmation of vaccine purity may be shown by testing sera from animals vaccinated at least twice with the batch for absence of antibodies to NSPs.

4.5. Safety testing

The safety of the final product should be proven batch to batch. The safety testing is conducted to detect any abnormal local or systemic adverse reactions. In cases where consistent safety of the product is demonstrated and approved in the registration dossier and the production process is approved for consistency in accordance with the standard requirements referred to in chapter 1.1.8, Veterinary Authority may agree to omit the test in the final product.

Safety could be checked in animals used for the potency test. Animals are inoculated by the recommended route of administration with the recommended dose of vaccine. When potency is assessed by PGP or EPP, all animals are observed for local and systemic reactions to vaccination for the 30 days duration of the potency test. When PD_{50} test is used, at least two healthy sero-negative target animals inoculated as above are observed for local and systemic reactions to vaccination for no fewer than 14 days. Any undue reaction attributable to the vaccine should be assessed and may prevent acceptance of the batch.

4.6. Potency testing

Potency is examined on the final formulated product, or alternatively for antigen banks on a representative batch of vaccine prepared from the same bulk inactivated antigen.

The potency testing standard is the vaccination challenge test. However, indirect tests can also be used for practicability and animal welfare considerations, as long as correlation has been validated to expectancy of protection in the target animal. Frequently indirect potency tests include antibody titration after vaccination of target species. Alternative methods could be used if suitably validated.

Ideally, indirect tests are carried out for each strain for one species and each formulation of vaccine to establish correlation between the indirect test results and the vaccine efficacy.

4.6.1. Expected percentage of protection (EPP) (Maradei *et al.*, 2008; Periolo *et al.,* 1993)

The EPP estimates the likelihood that cattle would be protected against a challenge of 10,000 bovine infective doses after a single vaccination.

i) Individual sera collected 30–60 days post-vaccination using a full dose of the vaccine are required from a group of either 16 or 30 18–24 month-old cattle.

ii) This panel of sera and sera of two control cattle are tested for antibody titres to the homologous FMD vaccine strain in an LPB-ELISA or VN test (see Sections B.2.1 *Virus neutralisation* and B.2.3 *Liquid-phase blocking enzyme-linked immunosorbent assay*).

iii) The antigens used in the ELISA may be inactivated using BEI.

iv) The EPP is determined by reference to predetermined tables of correlation between serological titres and clinical protection[2] (Maradei *et al.,* 2008; Periolo *et al.,* 1993).

v) Batches with at least 75% EPP (with 16 vaccinated cattle) or at least 70% EPP (with 30 vaccinated cattle) are satisfactory for potency.

The presence of more than one serotype in a vaccine does not diminish the induction of antibodies against another serotype or the correlation of antibody titre with protection.

4.6.2. Other methods for evaluating protection

Other tests were published using different ELISA methods and VNT methods to indirectly evaluate the protection given by vaccines. Their results could be accepted only if a valid correlation with protection in relation to the vaccine strain being tested and the serological method being used has been scientifically demonstrated.

5. Requirements for registration of vaccine

5.1. Manufacturing process

For registration of vaccine, all relevant details concerning manufacture of the vaccine and quality control testing (see Sections C.1–4) should be submitted to the National Veterinary Authority. This information shall be provided from three consecutive vaccine batches with a volume not less than 1/3 of the minimum allowed industrial batch volume from the country of origin.

5.2. Safety

For the purposes of gaining regulatory approval, a trial batch of vaccine should be tested for local and systemic toxicity by each recommended route of administration in an *in-vivo* test in at least eight animals of each target species. Double dose (e.g. two injections) and repeat single dose (after 14 days) tests using vaccines formulated to contain the maximum permitted payload and number of antigens are recommended to be conducted. In total animals receive three injections. The animals are observed for local and systemic reactions to vaccination for no fewer than 14 days after each injection. Any undue reaction attributable to the vaccine should be assessed and may prevent acceptance of the vaccine by the National Veterinary Authority.

5.3. Efficacy

[2] The WOAH Reference Laboratory in Brazil can provide tables upon request

Vaccine efficacy is estimated in vaccinated animals directly, by evaluating their resistance to live virus challenge or indirectly through *in-vitro* testing using well established correlations. The uncertainty of measurement in tests should be taken into account when interpreting its significance (Goris *et al.,* 2008). Vaccine efficacy should be established for every strain to be ~~authorised~~ approved for use in the vaccine.

Live reference FMD viruses corresponding to the main vaccine virus strains used in the region are available under certain conditions from WOAH Reference Laboratories for FMD in the region and from the National Veterinary Authority. These reference viruses are stored at ultralow temperatures and are sent, in strict accordance with shipping regulations.

The stock of challenge virus to be aliquoted is prepared from lesions collected from at least two cattle above 6 months of age that have been recognised as free from FMDV antibodies. These animals are tranquillised, then inoculated intradermally in the tongue with the suspension in about 20 sites of 0.1 ml each. The vesiculated tongue tissue or other body parts and vesicular fluid is harvested at the peak of the lesions, approximately 2 days later.

Harvested tissue is macerated and a 2% suspension is prepared, filtered through a 0.2 µm filter, aliquoted and quickly frozen in the gas phase of liquid nitrogen; this constitutes the challenge virus stock. The infective titres of this stock are determined both in cell culture ($TCID_{50}$) and in two cattle (BID_{50}) (50% bovine infectious dose). Two tranquilised cattle are injected intradermally in the tongue with tenfold dilutions (1/10 through 1/10,000), using four sites per dilution. The cattle titrations are read 2 days later. Titres are usually above 10^6 $TCID_{50}$ for 0.1 ml and above 10^5 BID_{50} for 0.1 ml, calculated using the Spearman–Kärber method. The dilution for use in the cattle challenge test is 10 000 BID_{50} in a total volume of 2 × 0.1 ml and is injected intradermally in the upper surface of the tongue both for the PD_{50} test and PGP test.

5.3.1. PD_{50} test

The number of protective doses in a vaccine is estimated from the resistance to infectious virus challenge of animal groups receiving different amounts of vaccine. Cattle of at least 6 months of age, obtained from areas free from FMD that have not previously been vaccinated against FMD and are free from antibodies to FMDV should be used. Three groups of no fewer than five cattle per group should be vaccinated by the route recommended by the manufacturer. The vaccine should be administered at different doses per group by injecting different volumes of the vaccine. For example, if the label states that the injection of 2 ml corresponds to the administration of 1 dose of vaccine, a 1/4 dose of vaccine would be obtained by injecting 0.5 ml, and a 1/10 dose would be obtained by injecting 0.2 ml. These animals and a control group of two unvaccinated animals are challenged either 3 weeks (aqueous) or up to 4 weeks (oil) after vaccination with a suspension of bovine virus that is fully virulent and appropriate to the virus types in the vaccine. The challenge test is done by inoculating the equivalent of a total of 10,000 BID_{50} intradermally into two sites on the upper surface of the tongue (0.1 ml per site). Animals are observed for at least 8 days. Vaccinated animals are considered unprotected if they show FMD lesions on at least 1 foot within 7 days after inoculation. ~~Unprotected animals will show lesions at sites other than the tongue.~~ Control animals must develop lesions on at least three feet. From the number of animals protected in each group, the PD_{50} content of the vaccine is calculated. There are a variety of methods for calculating PD_{50} but procedures based on the Kärber (1931) method are generally preferred when interpreting PD_{50} estimates calculated in this way. The vaccine should contain at least 3 PD_{50} per dose for cattle.

5.3.2. PGP test (protection against generalised foot infection)

For this method, a group of 16 FMD-seronegative cattle of 18–24 months of age, with the same characteristics described for the PD_{50} test, are vaccinated with a full bovine dose by the route and in the volume recommended by the manufacturer. These animals and a control group of two unvaccinated animals are challenged 4 weeks or more after vaccination with a suspension of bovine virus that is fully virulent and appropriate to the virus types in the vaccine. The challenge test is performed by inoculating the equivalent of a total of 10,000 BID_{50} intradermally into two sites on the upper surface of the tongue (0.1 ml per site). Animals are observed for 7–8 days. Vaccinated animals are considered unprotected if they show FMD lesions on at least 1 foot within 7 days after inoculation. ~~Unprotected animals will show lesions on the feet within 7 days after inoculation.~~ Control animals must develop lesions on at least three feet. For routine prophylactic use, the vaccine should protect at least 12 animals out of 16 vaccinated. This test does not provide an estimate of how many protective doses are in a single vaccine dose but comparison between tests suggested that 12 protected out of 16 vaccinated and challenged animals correlates with 3 PD_{50} (Vianna Filho et al., 1993).

5.3.3. Efficacy estimated by indirect tests

When direct challenge tests are not available to estimates efficacy, National Veterinary Authority may decide to use indirect tests (such as VN or LPB-ELISA), provided there is a correlation determined between antibody level and protection against challenge with 10,000 BID_{50}.

5.3.4. Efficacy in other species

Efficacy tests in other target species, such as sheep, goats, pigs or buffalo are either different or not yet standardised. In general, a successful test in cattle is considered to be sufficient evidence of the quality of a vaccine to endorse its use in other species. Under circumstances where a vaccine is produced for use primarily in a species other than cattle, it may be more appropriate to potency test the vaccine in that same species. With respect to sheep, goats and African (*Syncerus caffer*) or Asiatic buffalo (*Bubalus bubalis*), due to the often inapparent nature of the disease in these species, potency results from a cattle test may be a more reliable indicator of vaccine quality than attempting a potency test reliant on the detection of clinical signs in these other species.

5.4. Purity: testing for antibody against NSP

Viral circulation within a defined population can be assessed by testing for presence of antibodies against NSPs. Furthermore, the WOAH *Terrestrial Animal Health Code* stipulates that a criterion for regaining FMD free status following an outbreak, if vaccine is used, is to test the vaccinated animals for antibodies against NSP. Likewise, countries wishing to be recognised as FMD free with vaccination must demonstrate the absence of virus circulation by showing that vaccinated animals are free from antibody to NSPs arising as a result of infection. Consequently, FMD antigens used to formulate vaccines that may be used in these circumstances should be purified to reduce the NSP content. In addition to providing supporting documentation on the processes involved in such purification, manufacturers should demonstrate lack of immunogenicity against NSPs as part of the licensing procedure in order to make such a claim on their product literature. When purity tests include the use of full vaccine doses, at least eight cattle should be booster vaccinated at 28–30 days after first vaccination and tested for purity 28–30 days later. Up to one reactive animal at 28–30 days post-booster vaccination could be accepted. If more than two animals are reactive at 28–30 days post-booster vaccination, the batch should be rejected. If two animals are reactive, the manufacturers have the option to ask for a batch retest. A recommended test method that can be used is to vaccinate not less than 8 naïve cattle with a full dose of the vaccine containing the maximum number of strains and amounts of antigen permitted on the authorisation. Cattle should be vaccinated at least twice at 21- to 30-day intervals and then tested before each revaccination and 30–60 days after the last vaccination for the presence of antibody to NSPs using the tests described in Section B.2.4 *Nonstructural protein* (*NSP*) *antibody tests*. Negative results in NSP assays may support claims that the vaccine does not induce antibody to NSPs for the number of injections tested. These cattle may be the same as those used for the safety test described in Section C.5.2 *Safety*.

5.5. Duration of immunity

The duration of immunity (D.O.I) of an FMD vaccine will depend on the efficacy (formulation and antigen payload). As part of the ~~authorisation~~ regulatory approval procedure, the manufacturer should be required to demonstrate the D.O.I. of a given vaccine by either challenge or the use of a validated alternative test, such as serology using the same test and animals described in Section C.5.3 *Efficacy* tested at the end of the claimed period of protection, in compliance with Section C.5.3. D.O.I. studies should be conducted in each species for which the vaccine is indicated or the manufacturer should indicate that the D.O.I. for that species is not known. Likewise, the manufacturer should demonstrate the effectiveness of the recommended booster regime in line with these guidelines, usually by measuring the magnitude and kinetics of the serological response observed.

5.6. Stability

The stability of all vaccines, including oil emulsion vaccines, should be demonstrated as part of the shelf-life determination studies for ~~authorisation~~ regulatory approval.

The shelf life of conventional FMD vaccines is usually 1–2 years at 2–8°C. Vaccines should never be frozen or stored above the target temperature.

Stability should be tested using the same methods described in Section C.5.3 *Efficacy*, but vaccinating the animals at the end of the shelf life of the product.

5.7. Precautions (hazards)

Current FMD vaccines are innocuous and present no toxic hazard to the vaccinators. Manufacturers should provide adequate warnings that medical advice should be sought in case of self-injection of a vaccine.

6. Storage and monitoring of concentrated antigens

Chapter 1.1.10 provides international standards for vaccine banks.

The process of storing concentrated antigens at ultra-low temperature for later formulation into FMD vaccine as described in Section C.2, is a well established procedure for building stocks of immunogenic material ready to be formulated into vaccines in case of need. It not only forms the basis for the storage of antigens in a strategic reserve for emergency purposes, but allows the manufacturer immediate access to many different antigen strains that can be rapidly formulated and dispatched to the customer (Lombard & Fussel, 2007). Such stockpiling minimises delays subsequent to an order, particularly where a multivalent vaccine is requested. Another advantage of this procedure is that much of the quality testing can be completed well in advance of shipment. It is necessary to state that the concentrated antigens have to be controlled using standards indicated in Sections C.1–4.

6.1. Storage conditions

6.1.1. Facilities

It is important that all aspects of the storage of concentrated antigens conform fully to internationally accepted requirements such as those referred to in chapter 1.1.8. Housing, facilities and procedures should ensure the security of the stored antigen and prevent tampering, contamination or damage.

6.1.2. Containment of stored antigens

The dose numbers or volumes stored are an important consideration, particularly where a reserve is shared between WOAH Members Countries and there is variation in number of doses perceived to be needed by each Member in an emergency. Where the requirement is for a large stockpile of a particular vaccine strain that can only be produced from several separate production runs, vaccine bank managers must consider the need to either formulate each lot into a representative final blend for testing purposes or mixing the individual batches, at some convenient point, for ease of formulating and/or testing.

The type of container used to hold antigen concentrate is important. Under ultra-low temperature conditions it is important to use containers made from materials that do not become brittle or fragile at a temperature range allowing for heat sterilisation and cold storage.

6.1.3. Labelling of stored antigens

The concentrated antigens do not need to be labelled according to final or finished vaccine requirements and may be labelled as “in process” materials. Under ultra-low temperature conditions, the method of labelling must be of a durable nature. From experience, wire tagging bottles is the most preferred option using a metal/plastic tag sizeable enough to allow the necessary detail. Such detail should include the antigen/vaccine strain, batch number, date received and should also include an individual container or stock number. This information should be clear to read and marked on the tag using an indelible marker pen. Storage records and positions of containers should be carefully maintained.

6.2. Monitoring of stored concentrated antigens

It is vitally important that antigen concentrates are optimally maintained and routinely monitored in order to have some assurance that they will be efficacious when needed. Therefore arrangements should be made to monitor these antigen concentrates on a routine basis and to include where necessary, and at appropriate time intervals, a testing regime to ensure integrity of the antigen component or acceptable potency of the final product.

146S quantification, vaccination serology or vaccination challenge studies can be used for monitoring FMD antigen banks. It is recommended to carry out these tests on receipt (year 0) and every 5 years thereafter.

To support these testing requirements for depositories of antigen, concentrates should include a number of small samples that are representative of the larger stock. Small aliquots/stocks of FMD antigen have usually consisted of a volume representing approximately one milligram of antigen. These aliquots should be stored side by side with the bulk antigen,

7. Emergency release of vaccines prepared from concentrated antigens

In situations of extreme urgency and subject to agreement by the National Veterinary Authority, a batch of vaccine may be released before completion of the tests and the determination of potency if a test for sterility has been carried out on the bulk inactivated antigen and all other components of the vaccine and if the test for safety and the determination of potency have been carried out on a representative batch of vaccine prepared from the same bulk inactivated antigen. In this context, a batch is not considered to be representative unless it has been prepared with not more than the amount of antigen or antigens and with the same formulation as the batch to be released.

D. VACCINE MATCHING TESTS

1. Introduction

Appropriate vaccine strain selection is an important element in the control of FMD and is necessary for the application of vaccination programmes in FMD-affected regions as well as for the establishment and maintenance of vaccine antigen reserves to be used in the event of new FMD incursions. The decision to change or include new strains in vaccine formulations is a multifaceted process and, among other issues, experimental, epidemiological and field observations should be considered.

Vaccination against one serotype of FMDV does not cross-protect against other serotypes and may also fail to protect fully or at all against other strains of the same serotype. The most direct and reliable method to measure cross-protection is to vaccinate relevant target species and then to challenge them by exposure to the virus isolate against which protection is required. This will take account of both potency and cross-reactivity.

However, such an approach requires the use of live FMDV and appropriate biosecurity procedures and practices must be used. The facility should meet the appropriate level of biocontainment, determined by risk analysis as outlined in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*. In addition to the safety concerns, this procedure is slow and expensive and requires specific expertise that is best available in WOAH Reference laboratories. The use of animals for such studies should be avoided where possible by the use of *in-vitro* alternatives.

A variety of *in vitro* serological methods can be used to quantify antigenic differences between FMDV strains and thereby estimate the likely cross-protection between a vaccine strain and a field isolate. Genetic characterisation and antigenic profiling together with epidemiological observations can also reveal the emergence of new strains for which vaccine matching may be required and, conversely, may indicate that an isolate is similar to one for which vaccine matching information is already available. Such tests should be carried out in laboratories that work according to the standard specified in chapter 1.1.4 and Chapter 1.1.5 *Quality management in veterinary testing laboratories*, preferably WOAH Reference Laboratories for FMD.

Shipping of samples should be in accordance with Chapter 1.1.3 *Transport of biological materials*.

Vaccine potency and booster doses may contribute to the range of antigenic cover provided by a vaccine. A highly potent vaccine that stimulates a strong immune response may give protection to heterologous viruses. Furthermore, booster doses of vaccine can increase efficacy and the subsequent breadth of antigenic cover provided by a given vaccine, although the onset of full protection may be delayed (Pay, 1984).

2. Selection of field viruses for vaccine matching

For vaccine matching, preferably, more than one representative isolate should be evaluated from any outbreak.

Viruses should be selected based on epidemiological information, for instance isolation at different stages of an outbreak, from different geographical locations, or from different hosts (Alonso *et al.,* 1987). Field evidence for a suspected lack of vaccine quality as shown by reduced apparent protection, is an important criterion for vaccine matching, but other reasons for apparent vaccine failure (inadequate coverage, inadequate cold chain, insufficient boosting, lack of complementary control measures, etc.) should be also thoroughly explored.

The serotype of the field isolate is usually determined by ELISA ~~or CFT~~ using type-specific serological reagents, although methods based on MAbs or genetic typing may also be used. If the number of viruses exceeds the capacity

of the laboratory to carry out methods described in Section D.4 *Vaccine matching tests*, a pre-selection of isolates should be done.

In order to minimise the risk of missing a relevant sample, the pre-selection should be carried out using all the isolates received by the laboratory. The recommended approach is to engage in serological validated antigenic profiling methods using MAb ELISA ~~or CFT~~. VP1 sequencing should be used to verify the genetic homogeneity of the virus isolate population and its genetic distance with respect to the available vaccine strains.

The emergence of a new virus strain may be characterised by rapid dissemination with many outbreaks, and isolates from such epidemics are a priority for vaccine matching. Furthermore, isolates showing prevalence within the outbreak are the best candidates for vaccine matching tests. Isolates showing important differences with vaccine strains and not prevalent within the outbreak should be monitored closely through active surveillance in the field.

3. Selection of vaccine strains to be matched

The serotype of the virus, the region of origin and any information on the characteristics of the field isolate and, as appropriate, the vaccine strain used in the region of origin, may give indications as to the vaccine strains to be selected for vaccine matching tests. The availability of reagents for matching to particular vaccine strains may limit the extent of testing that is possible. To avoid this problem, it is expected that vaccine manufactures should provide, upon request of the vaccine purchaser and WOAH Reference Laboratories, post-vaccination sera produced during final product batch potency testing. It is also recommended that WOAH Reference Laboratories guarantee the availability of reference post-vaccination sera produced with relevant vaccine strains. Vaccine matching approaches have two purposes: first, to guide the selection of the most effective vaccine strain for use in a particular field circumstance, either routine prophylactic vaccination or emergency use, for which matching requirements are not necessarily the same; and second, to monitor, on an ongoing basis, the suitability of vaccine strains maintained in strategic antigen reserves.

4. Vaccine matching tests

The serological relationship between a field isolate and a vaccine virus ('r' value) can be determined by VNT~~,~~ or ELISA ~~or CFT~~. One way testing is recommended (r_1) with a vaccine antiserum, rather than two way testing (r_2), which also requires an antiserum against the field isolate to be matched. *In-vitro* neutralisation may be more relevant to predict *in-vivo* protection by the vaccine than other measures of virus-antibody interaction. VNT using the chequer-board titration method or other layout can be undertaken based in the laboratory experience. ELISA depends on the availability of trapping and detector antibodies suitable to the field strains, but it is more reproducible than VNT and can be carried out with inactivated virus. ~~CFT can be used as a screening method to select strains to be tested by VNT or ELISA.~~ The reproducibility of VNT results can be improved by incorporating multiple virus dilutions into the test so that the virus titre can be determined accurately by logistical regression.

For either VNT or ELISA, post-vaccination sera should be derived from at least five cattle 21–30 days after primary vaccination and/or 21–30 days after booster vaccination. The titre of antibody to the vaccine strain is established for each serum. Sera are used individually or pooled, after excluding low responders (Mattion *et al.,* 2009).

4.1. Relationship between the field isolate and the vaccine strain

The recommended standard test is the VNT. The ELISA can also be used if suitable reagents are available or as a screening test. ~~The CFT is suitable as a screening test to select strains to be analysed by VNT or ELISA.~~

4.1.1. Vaccine matching by two-dimensional (chequerboard) neutralisation test

This test uses antiserum raised against a vaccine strain. The titres of this serum against 100 $TCID_{50\%}$ of the homologous vaccine strain and the same dose of a field isolate are compared to estimate the immunological coverage of the vaccine strain in relation to the field virus. For the test each 100 µl of virus/serum mixture contains 100 $TCID_{50}$ of test virus.

4.1.1.1. Test procedure

The procedure is similar to that of the VNT (see Section B.2.1 *Virus neutralisation*).

Additional biological reagents are: monovalent 21–30 day post-vaccination bovine sera (inactivated at 56°C for 45–60 minutes), the homologous vaccine strain; and the test virus, a field isolate of the same serotype as the vaccine strain.

i) Field isolates are passaged on cell cultures until adapted to give 100% CPE in 24 hours. Passages should be kept to a minimum. When adapted, determine the virus titre (log_{10} $TCID_{50}$/ml) by end-point titration.

ii) For each test and vaccine virus a chequerboard titration is performed of virus against vaccine serum along with a back-titration of virus. Cells are added and incubated at 37°C for 48–72 hours after which time CPE is assessed.

iii) Antibody titres of the vaccine serum against the vaccine strain and field isolate for each virus dose used are calculated using the Spearman–Kärber method. The titre of the vaccine serum against 100 $TCID_{50}$ of each virus can then be estimated by regression. The relationship between the field isolate and the vaccine strain is then expressed as an 'r' value as:

$$r_1 = \frac{\text{reciprocal arithmetic titre of reference serum against field virus}}{\text{reciprocal arithmetic titre of reference serum against vaccine virus}}$$

4.1.1.2. Interpretation

Interpretation of the results of cross-reactivity tests: It has been generally accepted that in the case of neutralisation, r_1 values greater than 0.3 indicate that the field isolate is sufficiently similar to the vaccine strain so that use of a vaccine based on this strain is likely to confer protection against challenge with the field isolate. However, protection depends on both the cross-reactivity of antibodies elicited by the vaccine and the strength of the antibody response. The latter will be influenced by the potency of the vaccine and the number of doses given.

When deciding whether or not to use vaccines for which r_1 values lower than 0.3 are observed, factors to be considered include the availability of better matching vaccine strains, the potency of the vaccine and the potential for this to be increased to provide heterologous responses, the possibility of using additional booster doses, and the extent to which control of disease will be complemented by other zoosanitary measures or will be dependent on vaccination. The combined impact of potency and match can be estimated from the serological titre of the vaccine antiserum against the field virus, although correlating this precisely to protection requires a cross-protection test.

Alternatively, a suitable field isolate could be adapted to become a new vaccine strain.

Tests should always be repeated more than once. The confidence with which 'r' values can be taken to indicate differences between strains is related to the number of times that the examination is repeated. In practice, a minimum of at least three repetitions is advised.

4.1.2. Vaccine matching by expectancy of protection (EPP) determined by one-dimensional neutralisation test

Vaccine matching based on EPP values is widely used in South America where correlation tables are available for the vaccine strains used in the Region. This test uses antiserum raised against a vaccine strain (primo and booster vaccination). The titres of sera against 100 $TCID_{50}$/100 µl of sera/virus mixtures of the homologous vaccine strain and the same dose of a field isolate are compared to estimate the immunological coverage of the vaccine strain in relation to the field virus.

4.1.2.1. Test procedure

The procedure is similar to that of the VNT (see Section B.2.1 *Virus neutralisation*).

Additional biological reagents are: monovalent 21–30 day post-vaccination, and/or 21–30 day post-booster vaccination bovine sera (inactivated at 56°C for 45–60 minutes); the homologous vaccine strain; and the test virus, a field isolate of the same serotype as the vaccine strain. For regions where multivalent vaccines are used, it is advisable to use sera panels raised against the vaccine commonly used in the vaccination programme.

i) Field isolates are passaged on cell cultures until adapted to give 100% CPE in 24 hours. Passages should be kept to a minimum. When adapted, determine the virus titre (log_{10} $TCID_{50}$/ml) by end-point titration.

ii) For each test and vaccine virus, a titration of antibodies is performed against a fixed amount of virus (100 $TCID_{50}$ of virus/100 µl of mixture sera/virus), along with a back-titration of working virus. Sera/virus mixtures and virus back titration are incubated at

37°C for 60 minutes and then inoculated on to microplates with preformed cell monolayers. Each serum dilution is inoculated in four wells and at least six sera dilutions are used. Microplates are incubated at 37°C for 48 hours under CO_2 atmosphere after which time CPE is assessed.

iii) Antibody titres of the vaccine serum against the vaccine strain and field isolate are calculated using the Spearman–Kärber method. The titre of the vaccine serum against 100 $TCID_{50}$ of each virus can then be estimated and expressed per ml. Individual EPP values are determined for each individual neutralisation titre using predefined correlation tables, and then the mean of the EPP is calculated for each group of sera (vaccinated and booster vaccinated). The immunological coverage of the vaccine strain is expressed by the EPP value.

4.1.2.2. Interpretation of the results

To interpret the results it is necessary to have a correlation defined between *in-vitro* titres and *in-vivo* challenge protection against 10,000 BID_{50} of vaccine virus. In the PANAFTOSA experience with FMDV control and eradication programmes in South America, a mean EPP value of 75% in booster vaccinated animals indicates that the vaccine strain is suitable to be used together with appropriate field measures to control outbreaks with the field strain under test (correlation tables for O1, A24 and C3 are available upon request to PANAFTOSA).

4.1.3. Vaccine matching by ELISA

Similarly, the liquid-phase blocking ELISA described in Section B.2.3 is recommended for vaccine matching calculating the r_1 or EPP value.

4.1.3.1. Test procedure

The procedure is similar to that of the LPBE (see Section B.2.3 *Liquid-phase blocking enzyme-linked immunosorbent assay*).

Additional biological reagents are: monovalent 21–30 day post-vaccination and/or 21–30 day post-booster vaccination bovine sera (inactivated at 56°C for 45–60 minutes); the homologous vaccine strain; and the test virus, a field isolate of the same serotype as the vaccine strain. For regions where multivalent vaccines are used, it is advisable to use sera panels raised against the vaccine commonly used in the vaccination programme.

4.1.3.2. Interpretation of the results

Interpretation of r1 results: it has been proposed that in the case of LPBE, r_1 values greater than 0.4 indicate that the field isolate is sufficiently similar to the vaccine strain so that use of a vaccine based on this strain is likely to confer protection against challenge with the field isolate. However, protection depends on both the cross-reactivity of antibodies elicited by the vaccine and the strength of the antibody response. The latter will be influenced by the potency of the vaccine and the number of doses given. When deciding whether or not to use vaccines for which r_1 values lower than 0.4 are observed, factors to be considered include the availability of better matching vaccine strains, the potency of the vaccine and the potential for heterologous responses to be increased, the possibility of using additional booster doses, and the extent to which control of disease will be complemented by other zoo sanitary measures or will be dependent on vaccination.

Interpretation of EPP results: to interpret the results it is necessary to have a correlation defined between *in-vitro* titres and *in-vivo* challenge protection against 10,000 BID_{50} of vaccine virus. In the PANAFTOSA experience with FMDV control and eradication programmes in South America, a mean EPP value of 75% in booster vaccinated animals indicates that the vaccine strain is suitable to be used together with appropriate field measures to control outbreaks with the field strain under test (correlation tables for O1, A24 and C3 are available upon request to PANAFTOSA).

~~4.1.4. Vaccine matching by CFT~~

~~The CFT preferably performed in a tube, can be used as a screening test to select those strains to be tested by VN or ELISA. The test is performed as described in Section B.1.2.4. Additional biological reagents are guinea pig hyper immune sera against vaccine strains. Sera antibody titre is determined against homologous virus and field strains. The r_4 value is calculated as previously described:~~

~~r_1 = reciprocal arithmetic titre of reference serum against field virus / reciprocal arithmetic titre of reference serum against vaccine virus~~

~~*Interpretation of the results:* it is generally accepted that r_1 values equal or greater to 0.25 indicate that the field isolate is sufficiently similar to the vaccine strain. Studies by VNT or ELISA need to be performed with selected strains to confirm suggested CFT classification.~~

4.2. Testing the fitness for purpose of a vaccine

The "r" value should not be used in isolation to select the most appropriate vaccine strain to be used in the field. Particularly when it suggests an insufficient match of a certain vaccine strain, the suitability of a vaccine based on such a vaccine strain could be demonstrated by a heterologous cross-protection challenge test carried out as described in Section C.4.3 *Identity testing* in animals vaccinated with a known vaccine and challenged with the (heterologous) field virus. Vaccinate at least seven cattle without FMD antibodies, with a commercial dose of the current vaccine to be used in the region. Between 28 and 30 days later, boost all these animals with a second commercial dose in the same conditions and vaccinate a second group of at least seven animals with the same vaccine dosage and same route. Challenge the two groups and two control animals (not vaccinated) 30 days later with the equivalent of a total of 10,000 BID 50% of the new field strain duly titrated. The results are valid if each of the two control animal shows podal lesions on at least three feet. Final results are reported either as the number of protected animals (without podal lesion) over the total number of animal per group, or by percentage of protection where 100% is the total number of animals used per group. If results in the group of once vaccinated cattle indicate a protection level under 50%, and in the group of twice vaccinated cattle, protection under 75%, the change for a more appropriate vaccine strain is recommended.

The use of the EPP method is possible when correlation studies have been performed for the vaccine strain. The EPP method proved to be useful in some regions of the world applied together with epidemiological observations and active surveillance in the field. This method measures the reactivity of a panel of post-vaccination antisera using either VNT or ELISA and relates the serological titres to the probability of protection, established through correlation tables associating antibody titres with protection against challenge of 10000 BID_{50} of the homologous vaccine strain.

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NB: There are WOAH Reference Laboratories for foot and mouth disease (please consult the WOAH web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3). Please contact WOAH Reference Laboratories for any further information on FMD diagnostic tests, reagents and vaccines.

NB: FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2022.

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CHAPTER 3.1.9.

HEARTWATER

SUMMARY

***Description and importance of the disease**: Heartwater (also known as cowdriosis) is an acute, fatal, non-contagious, infectious, tick-borne rickettsial disease of ruminants caused by* Ehrlichia ruminantium (*formerly* Cowdria ruminantium) *and transmitted by* Amblyomma *ticks. It occurs in nearly all sub-Saharan countries of Africa, in its neighbouring islands, and also in the Caribbean. The disease can cause high mortality (up to 90%) in susceptible domestic ruminants. Goats and sheep are more susceptible than cattle, and European breeds are generally more susceptible than indigenous African breeds.*

Clinically, the disease most commonly presents as an acute form characterised by a sudden high fever, depressed demeanour, nervous signs and a high mortality. Hydropericardium and hydrothorax and lung oedema are commonly associated post-mortem lesions. Acute and peracute clinical forms of the disease occur: in the former, there are high death rates without many clinical manifestations and, in the latter, there is a higher recovery rate.

Recovered animals become carriers of infection. Certain wild animals can play a role as reservoir; Rusa deer, white-tailed deer, and springbok are susceptible to this infection and can experience high mortality.

***Identification of the agent:** The specific diagnosis of heartwater is based on the specific detection of the pathogen during the febrile phase or by observation of colonies of* E. ruminantium *in capillary endothelial cells of the brain.*

In the absence of a molecular diagnostic ~~tools~~ capability, a piece of cerebellum can easily be removed with a curette through the foramen magnum after cutting off the head, while a sample of cerebral cortex can be obtained through a hole made in the skull with a hammer and a large nail. Brain smears are prepared by crushing to a paste and spreading thinly a small piece of cerebral or cerebellar cortex between two microscope slides. The capillaries are spread out in a single cell layer by drawing one slide across the other. The smears are air-dried, fixed with methanol and stained with Giemsa. The colonies (clusters) of E. ruminantium *are reddish-purple to blue, and very often close to the nucleus of the infected endothelial cell. They can be scanty and difficult to find, particularly in peracute cases, but they are always present in the brain of a ruminant that died from heartwater, if not treated with drugs. Colonies are not likely to be detected in animals that were treated with antibiotics. The colonies are still visible 2 days after death in a brain that has been stored at room temperature (20–25°C) and up to 34 days in a brain that has been stored in a refrigerator at 4°C.*

Ehrlichia ruminantium *can be isolated from the blood of an infected host using cultivation on ruminant endothelial cells. When a cytopathic effect consisting of plaques of cell lysis appears, the presence of characteristic morulae is confirmed by staining the cell monolayer with Giemsa or RAL555 or by immunofluorescence.*

Molecular tools such as nested polymerase chain reaction (PCR) and real-time PCR targeting E. ruminantium*-specific genes are currently available for the detection of the presence of*

E. ruminantium *in the blood of animals with clinical signs, in organs from dead animals* (*confirming clinical cases of heartwater*), *and also in the tick vectors. They could not however, detect* E. ruminantium *in asymptomatic carriers. Two multi-pathogen methods, including* E. ruminantium *detection, have been developed allowing differential diagnosis for tick-borne diseases to be undertaken. Apart from diagnosis, molecular methods are widely used for research on the* E. ruminantium *genome and for epidemiological studies, including* E. ruminantium *tick prevalence*. No commercial molecular kits targeting *E. ruminantium* are currently available.

Serological tests: *Two enzyme-linked immunosorbent assays* (*ELISAs*) *have been evaluated: an indirect ELISA and a competitive ELISA targeting major antigenic protein 1* (*MAP1*) *antibodies.*

The current indirect ELISA uses a recombinant antigen expressed as a partial fragment of the MAP1 antigens – the MAP1-B ELISA – which gives improved specificity over earlier methods. However, the assay still detects cross-reacting antibodies to other Ehrlichial organisms including Ehrlichia chaffeensis, E. canis *and Panola Mountain* Ehrlichia. *Hence, definitive proof of heartwater must rely on epidemiological evidence and additional molecular testing indicating the presence of the organism. This ELISA can help to monitor experimental infections and to measure the immune response of immunised animals, whose pre-immunisation serological history is known or for epidemiological studies. Serology has very limited diagnostic use as clinically infected animals remain sero-negative during the febrile reaction and sero-convert after recovery.*

Serology is also not an ~~effective import test~~ efficacious test for health certification prior to international movement. Prior to importation of animals from a heartwater endemic region, it is important to study the epidemiological data to try to establish that the herd and the resident ticks are not infected. Repeated serology on the herds and PCR on tick samples in targeted herds can be carried out to demonstrate that they are free of E. ruminantium.

Requirements for vaccines: *Immunisation against heartwater by the 'infection and treatment' method using infected blood is still in use in some countries. A first-generation vaccine consisting of inactivated purified elementary bodies of* E. ruminantium *emulsified in an oil adjuvant has given promising results in experimentally controlled conditions and has demonstrated significant protection in the field, but only for homologous challenges. Further improvement of the method to produce the inactivated vaccine using bioreactors, antigen storage conditions, and different adjuvant has demonstrated good efficiency in controlled conditions. An additional isolate, Welgevonden, has been attenuated and shown to confer good protection in controlled conditions, and significant protection has also been obtained using DNA vaccination. However, none of these experimental vaccines has been fully validated under field conditions. Field trials and studies on genetic characterisation of strains have revealed the presence of a high number of* E. ruminantium *strains in restricted areas. Thus, antigenic diversity is important in formulating effective vaccines, and further investigations are critical for the delivery of any vaccine in the field.*

A. INTRODUCTION

Heartwater (cowdriosis) is a rickettsial disease of domestic and wild ruminants caused by *Ehrlichia ruminantium* (formerly *Cowdria ruminantium*) and transmitted by *Amblyomma* ticks (Marcelino *et al.,* 2016). *Ehrlichia ruminantium* is classified in the order Rickettsiales and in the family Anaplasmataceae, together with the genus *Anaplasma*. Although ruminants remain the primary target of the pathogen, in South Africa a possible canine *E. ruminantium* infection has been reported, and *E. ruminantium* has been strongly suspected in several cases of rapidly fatal encephalitis in humans. However, in all cases, evidence of *E. ruminantium* infection was based on molecular detection. Isolation and characterisation of the infectious agent is necessary before *E. ruminantium* can be considered an emerging pathogen in species other than ruminants and especially in humans. Since then, no other clinical human case associated with heartwater has been observed.

Heartwater is an important tick-borne disease of livestock in Africa occurring in nearly all the sub-Saharan countries where *Amblyomma* ticks are present and in the surrounding islands: Madagascar, Reunion, Mauritius, Zanzibar, the Comoros Islands and Sao Tomé. The disease is also reported in three Caribbean islands (Guadeloupe, Marie-Galante and Antigua) (Marcelino *et al.,* 2016). All domestic and wild ruminants can be infected, but the former appear to be the most susceptible. Indigenous domestic ruminants are usually more resistant to the disease. Wild animals could play a role as reservoir, but Rusa deer, white-tailed deer, springbok, chital, timor deer, which are used in wildlife farming, seem to be the main wild ruminant species in which heartwater can have a significant economic impact.

The average natural incubation period is 2–3 weeks, but can vary from 10 days to 1 month. In most cases, heartwater is an acute febrile disease, with a sudden rise in body temperature, which may exceed 41°C within 1–2 days after the onset of fever. It remains high for 4–5 weeks with small fluctuations and drops shortly before death.

Fever is followed by inappetence, sometimes listlessness, diarrhoea, particularly in cattle, and dyspnoea indicative of lung oedema. Nervous signs develop gradually. The animal is restless, walks in circles, makes sucking movements and stands rigidly with tremors of the superficial muscles. Cattle may push their heads against a wall or present aggressive or anxious behaviour. Finally, the animal falls to the ground, pedalling and exhibiting opisthotonos, nystagmus and chewing movements. The animal usually dies during or following such an attack.

Subacute heartwater with less pronounced signs, and peracute heartwater with sudden death, can also occur, according to the breed of ruminant and the strain of *E. ruminantium* involved.

The most common macroscopic lesions are hydropericardium, hydrothorax, pulmonary oedema, intestinal congestion, oedema of the mediastinal and bronchial lymph nodes, petechiae on the epicardium and endocardium, congestion of the brain, and moderate splenomegaly (Marcelino *et al.,* 2016).

A tentative diagnosis of heartwater is based on the presence of *Amblyomma* vectors, nervous signs, and presence of transudates in the pericardium and thorax on post-mortem examination. When making a diagnosis based on clinical signs, the following other diseases should be considered: bovine cerebral babesiosis and theileriosis, anaplasmosis, botulism, haemonchosis in small ruminants, rabies and poisoning.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for diagnosis of heartwater and their purpose

Method	Purpose					
	Population freedom from infection[(a)]	Individual animal freedom from infection prior to movement[(b)]	Contribute to eradication policies	Confirmation of clinical cases[(c)]	Prevalence of infection – surveillance[(a)]	Immune status in individual animals or populations post-vaccination[(b)]
Identification of the agent						
In-vitro bacterium isolation	–	–	–	+	–	–
Real-time PCR	–*	–	–	+++	–	–
Nested PCR	–*	–	–	+++	–	–
Multi- pathogen real-time PCR	–*	–	–	+++	–	–
Detection of immune response						
ELISA	++	+	–	–	+++	++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
*can be used to screen tick populations, in parallel with serology on hosts.
PCR = polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay.
[(a)]See Appendix 1 of this chapter for justification table for the scores giving to the tests for this purpose.
[(b)]See Appendix 2 of this chapter for justification table for the scores giving to the tests for this purpose.
[(c)]See Appendix 3 of this chapter for justification table for the scores giving to the tests for this purpose.

1. Identification of the agent

During the febrile reaction, *E. ruminantium* can be readily isolated in culture from blood or plasma; however, it is difficult to detect these organisms in a blood smear. Typical colonies of *E. ruminantium* can be observed in brain smears made after death and this represents a definitive diagnosis for heartwater.

Opening the cranium is not necessary. An alternative method is to cut off the head in front of the first cervical vertebra. Then, introduce a curette through the foramen magnum, between the medulla and the meninges. The curette is turned over towards the brain and removed with a piece of cerebellum. Another method consists of making a hole in the skull with a hammer and a large nail and aspirating a sample of brain cortex with a needle attached to a syringe. These methods also lessen the danger to the operator in cases where the nervous signs have been caused by rabies.

In the live animal, a brain biopsy may be obtained aseptically after local anaesthesia, although with difficulty; appropriate restraint must be used especially with large and horned animals. Colonies of *Ehrlichia* are observed during the febrile period. This method is useful for experimental studies, but not suitable for routine diagnosis.

Colonies of *E. ruminantium* are still present 48 hours after death in a brain that has been stored at room temperature (20–25°C) and for up to 34 days in a brain that has been stored in a refrigerator at 4°C.

A small fragment of grey matter (approximately the size of a match head) is placed on a microscope slide, crushed to a paste consistency by another slide and, while maintaining pressure, the slides are drawn over each other lengthwise to produce a single layer of cells. The slides are air-dried, fixed in methanol, and stained with eosin and methylene blue or Giemsa. They are then examined under a microscope at a low magnification (×10 objective) to locate the cerebral capillaries and with a magnification of at least ×50 to identify the colonies of rickettsiae. *Ehrlichia ruminantium* are reddish-purple to blue coccoid organisms in the cytoplasm close to the cell nucleus which is coloured in pink (Marcelino *et al.,* 2016). Experience is required as *E. ruminantium* colonies must be differentiated from other haemoparasites (*Babesia bovis*), certain blood cells (thrombocytes, granulocytes), normal subcellular structures (mitochondria, mast cell granules), or stain artefacts (stain precipitates).

Ehrlichia ruminantium colonies are formed from clusters of granules (0.2–0.5 µm), sometimes arranged in the shape of a ring or a horseshoe (1–3 µm), that are placed close to the nucleus inside the endothelial cell. The granules can be scanty, particularly in peracute cases, but they are always present in the brain of an animal that died from heartwater. However, if the animal has been treated with doxycyclin or oxytetracyclin 48 hours before, the granules of *Ehrlichia* tend to fuse, making the diagnosis very difficult, and sometimes impossible. Transmission electron microscopy has been used to demonstrate that the *E. ruminantium* organisms develop inside a vacuole-like structure, which is surrounded by a membrane in the endothelial cell's cytoplasm. Each organism is enclosed by a double membrane. Within the vacuole-like structure, *E. ruminantium* electron-dense forms (elementary bodies), as well as intermediate reticulate forms, are identified.

1.1. Isolation of *Ehrlichia ruminantium* using *in-vitro* culture

Although numerous cell lines have been shown to support the growth of *E. ruminantium,* isolation is not the test of first choice for rapid diagnosis of heartwater, as it is labour intensive and time-consuming. For a rapid diagnosis, molecular methods are preferred. However, *E. ruminantium* isolation should be encouraged for typing the strains present in a region for the purpose of vaccination programmes. *Ehrlichia ruminantium* can be isolated from the blood of reacting animals by cultivation on ruminant endothelial cells. Endothelial cells from umbilical cord, aorta, or the pulmonary artery of different ruminant species (cattle, goat, sheep) are used most often for isolation, although other endothelial cell types (brain capillaries, circulating endothelial cells, etc.) have been described for the routine culture of the microorganism. Endothelial cell lines from sable, eland, buffalo, kudo and bush pig can also be used to grow *E. ruminantium*. No standard cell line has yet been designated for isolation.

1.1.1. Isolation procedure

i) The blood of the clinically affected animal (optimally on the second or third day of febrile reaction) is collected in anticoagulant (heparin or sodium citrate, not ethylene diamine tetra-acetic acid) and diluted 1/2 in the complete culture medium consisting of ~~Glasgow~~ high glucose (4.5 g/litre) Dubelco minimal essential medium (DMEM) supplemented with 10% inactivated fetal bovine serum, 200 mM L-glutamine, and antibiotics if necessary (penicillin 100 U/ml, streptomycin 100 µg/ml).

ii) The culture medium is poured off the endothelial cell monolayer, and sampled blood (approximately 2 ml for a 25 cm^2 flask) is added. The flask is incubated at 37°C (if possible with 5% CO_2) on a rocking platform for 2 hours.

iii) After incubation, the blood is poured off and the monolayer is gently washed three times with culture medium prewarmed at 37°C. Fresh complete culture medium (5 ml per 25 cm^2 flask) is added and the flask is incubated at 37°C with 5% CO_2. The medium is changed every 2 days.

(The *use of plasma instead of blood is more efficient* when taken from an animal with a febrile reaction >41°C. In this case, steps ii and iii above may be replaced with the following:

a) Seed 4 ml of plasma (smaller inoculum can be used if there is a limited amount of plasma available) on to a susceptible endothelial cell culture and incubate for 1 hour at 37°C on a rocking platform.

b) Wash plasma three times with culture medium prewarmed at 37°C. and then add 5 ml of complete culture medium (per 25 cm^2 flask) and observe for development of ~~cytopathic effect~~ cell lysis.

iv) The monolayer is inspected regularly for the appearance of small plaques of cell lysis. The first plaques generally appear after about 2 weeks. Passaging on uninfected cell monolayers is performed when the lysis reaches 80% of the cell layer. The remaining cells are stained with eosine and methylene blue or Giemsa and examined microscopically for the presence of *E. ruminantium* morulae (Marcelino *et al.,* 2016).

2. Molecular methods

2.1. Detection of *Ehrlichia ruminantium* using nested polymerase chain reaction

Two nested polymerase chain reaction (PCR) assays have been developed to enhance detection of low levels of rickettsemia (Martinez *et al.,* 2004; Semu *et al.,* 2001). Both use the *pCS20* region as the target sequence. The Semu *et al.* assay uses two external primers: U24 (5'-TTT-CCC-TAT-GAT-ACA-GAA-GGT-AAC-3') and L24 (5'-AAA-GCA-AGG-ATT-GTG-ATC-TGG-ACC-3'), and then the AB 128 (5'-ACT-AGT-AGA-AAT-TGC-ACA-ATC-TAT-3') and AB 129 (5'-TGA-TAA-CTT-GGT-GCG-GGA-AAT-CCT-T-3') for the nested reaction. The analytical sensitivity of detection of this assay is one organism per reaction. The other nested PCR assay (Martinez *et al.*, 2004) uses a pair of external primers that comprise AB128/AB130 (5'-ACT-AGC-AGC-TTT-CTG-TTC-AGC-TAG 3') followed by a second amplification based on AB128/AB129 primers. The final PCR product is 278 pb long. The nested PCR shows a hundred-fold improvement in sensitivity compared with the simple AB128/AB129 PCR, and an average detection limit of 6 organisms per reaction.

Lack of amplification with conventional *pCS20* nested PCR was observed due to single nucleotide polymorphisms (SNPs) on primer hybridisation areas of the *pCS20* fragment DNA. Thus, AB128/129 and 130 primers modified using universal nucleotides allowed the detection of a wider range of *E. ruminantium* strains: AB128' (5'-ACT-AGT-AGA-AAT-TGC-ACA-ATC-YAT-3'), AB130' (5'-RCT-DGC-WGC-TTT-YTG-TTC-AGC-TAK-3') and AB129' (5'-TGA-TAA-CTT-GGW-GCR-RGD-ART-CCT-T-3'). This *pCS20* nested PCR ~~is~~ was used ~~routinely~~ at the WOAH Reference Laboratory for diagnostic purposes on blood samples from clinical cases and for tick screening (Adakal *et al.,* 2009; 2010a; Cangi *et al*., 2016) just before validation of the pCS20 real time PCR (see further). The *pCS20* nested PCRs allow detection in organs (lung and brain) from infected dead animals, blood from infected animals during hyperthermia, and ticks fresh, frozen or preserved in 70% ethanol. The detection of *E. ruminantium* by nested PCR is possible in the blood of animals 1 or 2 days before hyperthermia and during the hyperthermia period but not on asymptomatic animals.

A nested PCR targeting the entire *map*1 polymorphic gene has been developed in parallel in order to type the strains by restriction fragment length polymorphism (RFLP) or sequencing of the amplification fragment directly from the pathological samples testing positive in the *pCS20* nested PCR (Martinez *et al.*, 2004). The *map*1 nested PCR performs well although with a slightly lower sensitivity than the *pCS20* nested PCR with 60 copies per sample. This tool is useful for genetic characterisation of *E. ruminantium* but not for diagnosis due to the polymorphic property of *map1* targeted genes.

The genetic characterisation and structure of the *E. ruminantium* population at the regional level is essential for the selection of potential vaccinal strains. The genetic typing of strains was previously done using RFLP on the *map1* polymorphic gene after PCR amplification (Adakal *et al.,* 2010b; Faburay *et al.,* 2007). However, multi-locus methods adapted to *E. ruminantium* have been validated such as multi-locus sequence typing (Adakal *et al.,* 2009) and multi-locus variable number of tandem repeat sequence analysis (Pilet *et al.,* 2012). These tools are being used on field tick samples for molecular epidemiological studies to better characterise the genetic structure of *E. ruminantium* strains (Adakal *et al.,* 2010a; Cangi *et al.,* 2016). However, these genetic characterisations are not associated with clusters of protection.

2.2. Detection of *Ehrlichia ruminantium* using real-time PCR

Several real-time PCR tests targeting *map1*, *map1-1* and *pCS20* region genes have been developed for the detection of *E. ruminantium* organisms. SYBR Green real-time PCRs targeting *map1* and *map1-1* were used to detect and quantify *E. ruminantium in vitro* during mass antigen production in a bioreactor and in experimentally infected sheep during the hyperthermia period (Marcelino *et al.,* 2005; 2007;

Peixoto *et al.,* 2005; Postigo *et al.,* 2002). They were tested on a limited number of strains (up to six strains) and therefore they are not recommended for diagnostic purposes due to the polymorphic characteristics of *map1* multigenic family.

A real-time PCR assay targeting the *pCS20* region using a fluorescent-labelled probe has been developed to detect *E. ruminantium* in livestock blood and ticks from the field, and has a sensitivity similar to the nested PCR with seven copies per sample. The sequences of primers and probes are: CowF (5'-CAA-AAC-TAG-TAG-AAA-TTG-CAC-A-3'), CowR (5'-TGC-ATC-TTG-TGG-TGG-TAC-3') and Cow probe (5'-FAM-TCC-TCC-ATC-AAG-ATA-TAT-AGC-ACC-TAT-TA-XT-PH-3'). Unfortunately, this assay displayed cross reaction with *E. canis* and *E. chaffeensis*. It successfully detected 15 different *E. ruminantium* strains (Steyn *et al.,* 2008). As shown previously for the *pCS20* nested PCR, the presence of SNPs on hybridisation regions could inhibit strain detection. Testing more strains is necessary to further validate the method.

The method of choice for detecting *E. ruminantium* in animal blood during clinical phase or directly in ticks is a more recently new real-time PCR targeting another *pCS20* region that has been developed and demonstrated a good reproducibility, sensitivity and specificity, with a limit of detection of 6 copies per sample. It can be used with appropriate fluorescent probes. Primers and probes are: Sol1F (ACA-AAT-CTG-GYC-CAG-ATC-AC), Sol1R (CAG-CTT-TCT-GTT-CAG-CTA-GT) and Sol1^{TqM} (6-FAM-ATC-AAT-TCA-CAT-GAA-ACA-TTA-CAT-GCA-ACT-GG-BHQ1). It detects 16 *E. ruminantium* strains from different geographical areas and there is no cross protection with *Anaplasma marginale, A. phagocytophilum, A. platys, Babesia bovis*, *B. bigemina, E. canis*. *E. muris* and *Rickettsia felis* and *parkeri* (Cangi *et al*., 2017). It has been tested on 700 tick field samples from Mozambique and ~~will be~~ it is used routinely in the WOAH Reference Laboratory for diagnostic use and tick screening.

Although nested and real-time PCR methods have proved highly effective in detecting infection in ticks or in animal samples during the clinical phase of the disease or after death, they could not allow detection of *E. ruminantium* in healthy carrier ruminants. A useful technique for confirming the status of a suspected carrier animal, whose blood is PCR negative, is to feed batches of naive ticks on the animal and then test the ticks by *pCS20* nested or real-time PCR. It is not known whether ticks act simply by concentrating circulating organisms, or by amplifying their number or even by inducing release of micro-organisms in the circulation during feeding.

2.3. Detection of *E. ruminantium* using multi-pathogen real-time PCR

A single FRET-real-time PCR has been developed to differentiate eight species in four distinct groups in a single reaction: *E. canis, E. chaffeensis, E. ewingii, E. muris, E. ovina, Ehrlichia* sp. *BOV2010,* Panola Mountain *Ehrlichia* and *E. ruminantium* (Zhang *et al.,* 2015). It is based on 16S recombinant RNA amplification using two fluorescent probes; different dissociation curves are detected depending on the species. *Ehrlichia ruminantium* could be clearly differentiated from other species with the lowest melting temperature. The limit of detection is five copies per sample and simultaneous detection is possible with a mix of 300 copies of each species. However, only four *E. ruminantium* strains were tested. Even if the lack of amplification is limited due to the targeted conserved gene 16S recombinant RNA, validation on more *E. ruminantium* strains is necessary.

On the other hand, Sayler *et al.* developed a dual-plex Taqman real-time PCR targeting the groEL gene of Panola Mountain *Ehrlichia* and *E. ruminantium* (Sayler *et al.,* 2016). This assay allows Panola Mountain *Ehrlichia,* which is endemic in the USA, to be differentiated from *E. ruminantium,* which is currently not present. The limit of detection is 10 copies per sample, and 27 *E. ruminantium* strains from 11 countries were detected. It seems therefore to be a promising method for differential diagnosis between the two species.

2.4. Detection of *Ehrlichia ruminantium* using the reverse line blot technique

The reverse line blot technique (RLB) has been used for the simultaneous detection and identification of *Anaplasma* and *Ehrlichia* species known to occur in ruminants, on the basis of differences in the small subunit rRNA gene (Bekker *et al.,* 2002). Primers 16S8FE and B-GA1B-new were designed from conserved domains and used to amplify a 492–498 bp fragment of the 16S rRNA gene spanning the variable V1 region. Species-specific oligonucleotide probes were designed in this V1 loop to allow species-specific detection of *E. ruminantium*, *E. ovina*, *Ehrlichia* sp. strain Omatjenne, *Anaplasma marginale*, *A. centrale*, *A. bovis*, *A. ovis* and *A. phagocytophilum*. One oligonucleotide probe cross-reactive with all species (catch-all probe) was also designed to serve as a control in case a PCR product does not hybridise to any of the species-specific probes. In the method, the species-specific probes are covalently linked to the hybridisation membrane, which is hybridised with the PCR product obtained using primers 16S8FE and B-GA1B-new. PCR products obtained from all above-mentioned microorganisms were shown to bind with specific oligonucleotide probes only. No PCR product was detected and no

hybridisation occurred when the PCR-RLB was applied to *Theileria annulata*, *Babesia bigemina* or mammalian DNA. Similarly, negative control ticks were always negative in the RLB assay whereas it was possible to detect *Ehrlichia ruminantium* infection in 15–70% of ticks fed on experimentally infected or long-term carrier sheep. In Mozambique, *E. ruminantium* could also be detected in the blood of 12 sentinel small ruminants placed in the field with the infected animals; mixed infection was detected in five of the infected sentinel animals, thus demonstrating the usefulness of the method for detecting multiple infections. The RLB has been used recently in several studies in Western Kenya and in Nigeria to evaluate the prevalence of tick-borne diseases in cattle (Lorusso *et al.,* 2016; Njiiri *et al.,* 2015) however, they obtained very low prevalence of *E. ruminantium*. It was suggested by the authors that sequences of the primers and probes of RLB were not adapted to Kenya *E. ruminantium* strains.

2.5. Reading the results

As *E. ruminantium* is an obligate intracellular bacterium that cannot be cultivated in acellular media and its isolation is complex and takes several weeks, molecular tools are the best methods for the diagnosis of heartwater. Nested and real-time PCRs prove to be easier to perform and more sensitive than RLB. With all PCRs, however, care must be taken to ensure that no cross-contamination occurs between samples. Negative and positive controls must be included in each test. For each PCR assay, nested or real-time, positive and negative extraction controls (from experimentally infected and uninfected blood or organs, infected or uninfected ticks) should be included allowing detection of a default in the extraction procedures. The absence of inhibitor products in each sample should be proven by targeting a housekeeping gene from the vector or the host such as 16S or 18S ribosomal DNA (for tick screening, Cangi *et al*., 2017). As heartwater serology has several limitations (see Section B.3), the PCR could be used to help confirm if seronegative animals, originating from an endemic area, are not infected, prior to translocating them to a heartwater-free area that has the risk of becoming infected, because of the presence of potential vectors. Screening of ticks by PCR, along with serology on targeted herds over time could be used to establish the herd status before any movement of animals from this endemic area to a free area. However, *E. ruminantium* cannot be detected in asymptomatic carriers by molecular methods. The results obtained with nested PCRs, the RLB assay and real-time PCR, show that the direct detection of *E. ruminantium* in the blood is only reliable during and around the febrile phase of the disease. PCR-based methods appear to be more reliable in detecting infection in ticks, and this could have epidemiological value in determining the geographical distribution of *E. ruminantium*. In addition, when necessary in endemic areas, the inclusion of testing (originally naive) ticks fed on a suspect animal would greatly improve the sensitivity of carrier detection when serology and PCR on blood have failed. The procedure is nevertheless not suitable for routine diagnostic laboratories as it requires the maintenance of tick colonies and the capacity to experimentally infect animals.

3. Serological tests

To minimise the problem of cross-reactions with *Ehrlichia* spp., two enzyme-linked immunosorbent assays (ELISAs) based on a recombinant MAP1 antigen have been developed. The first is an indirect ELISA that uses an immunogenic region of the MAP1 protein (called MAP1-B) and gives far fewer cross-reactions with *Ehrlichia* spp. (MAP1-B ELISA) (Semu *et al.,* 2001). The second is a competitive ELISA that uses the *map*1 gene cloned in a baculovirus and monoclonal antibodies (MAbs) raised against the MAP1 protein (MAP1 C-ELISA) (Mondry *et al.,* 1998). Both tests have dramatically improved specificity, but they still show some reactivity with high titre sera against *E. canis*, *E. chaffeensis* and Panola Mountain *Ehrlichia*. Cross reaction of serum from Panola Mountain *Ehrlichia*-infected goats has been observed with *E. ruminantium* MAP1-B antigen and, conversely, serum from heartwater-infected sheep with MAP1-B of Panola Mountain *Ehrlichia,* thus preventing their use in the detection of *E. ruminantium* introduction on the American mainland (Sayler *et al.,* 2016). The MAP1-B ELISA has been the most extensively used and will be described in detail. Serology as a diagnostic tool for detecting individual animals exposed specifically to *E. ruminantium* is therefore unreliable. Serology should be considered at the herd level taking into consideration the epidemiological environment and, if necessary, be complemented by molecular techniques.

3.1. MAP1-B enzyme-linked immunosorbent assay (Mondry *et al,* 1998 and Semu *et al*., 2001)

Using the vector pQE9, the PCR fragment MAP1-F2R2, which encodes the amino acids 47–152 of the MAP1 protein including the immunogenic region MAP1-B, is expressed in *Escherichia coli* M15[pREP4] as a fusion protein containing six additional histidine residues. The recombinant MAP1-B is purified using Ni^{2+}-NTA agarose (nitrilotriacetic acid agarose) under denaturing conditions as described by the manufacturer. The antigen is preserved at 4°C and each batch is titrated.

The antigen is diluted at 0.5 µg/ml in 0.05 M sodium carbonate buffer, pH 9.6, and immobilised onto polystyrene plates by incubation for 1 hour at 37°C, and stored at 4°C until use. However, in initial trials,

an antigen concentration of 2 µg/ml reduced background noise and improved specificity (data not shown: Semu *et al.*, 2001).

3.1.1. Test procedure

i) Plates are blocked for 30 minutes by adding 100 µl per well of 0.1 M phosphate buffered saline (PBS), pH 7.2, supplemented with 0.1% Tween 20 (PBST) and 3% non-fat dry milk (PBSTM).

ii) The plates are washed three times with PBS supplemented with 0.1% Tween 20 (PBST) and twice with distilled water.

iii) 100 µl of test serum diluted 1/100 in PBSTM is added in duplicate to wells, which are then incubated for 1 hour at 37°C.

iv) Plates are washed three times in PBST and twice in distilled water.

v) Horseradish-peroxidase-conjugated anti-species IgG optimally diluted in PBSTM is added at 100 µl per well and the plate is incubated for 1 hour at 37°C.

vi) After washing as in step iv, each well is filled with 100 µl of 0.1 M citrate buffer, pH 5.5, containing 0.5 mg/ml orthophenylene-diamine and 3 µl/ml of 9% H_2O_2.

vii) The reaction is stopped after 30 minutes of incubation at room temperature (20–25°C) by adding 50 µl of 2 N H_2SO_4. Absorbance is read at 495 nm. Positive and negative controls are included in each plate.

3.2. MAP1 competitive enzyme-linked immunosorbent assay (Mondry *et al.*, 1998)

Recombinant MAP1 antigen is prepared as follows: 8-day-old *Trichoplusia ni* insect larvae are infected by a baculovirus expressing the *map1* gene and moribund larvae are homogenised (10% [w/v]) in PBS supplemented with 0.001% (v/v) Triton X-100.

Anti-MAP1 MAb is prepared as follows: spleen cells of BALB/C mice previously inoculated with larval homogenate are fused to SP2/0 cells. Supernatant fluids from hybridoma cell cultures are screened for reactivity with MAP1 by immunoblotting and immunoperoxidase methods. A reactive cell culture is subcloned, isotyped and subsequently used for MAb production.

After a further 1/800 (v/v) dilution in PBS, the antigen is immobilised on to polystyrene plates (Nunc-Immuno Plates PolySorp) by incubation overnight at 4°C, and stored at –70°C.

3.2.1. Test procedure

i) Prior to use, the plates are blocked for 30 minutes by adding 100 µl per well of PBS, pH 7.2, supplemented with 0.05% Tween 20 and 5% nonfat dry milk.

ii) Plates are washed three times with PBS/Tween, 50 µl/well of test serum diluted 1/50 in PBS supplemented with 0.05% Tween 20 and 1% nonfat dry milk (PBSTM) is added in duplicate and the plates are incubated for 30 minutes at 37°C.

iii) Without an intervening washing step, 75 µl/well of the MAb diluted 1/4000 (v/v) in PBSTM is added and the plates are incubated for another 30 minutes at 37°C.

iv) Plates are washed three times in PBS/Tween and horseradish-peroxidase-conjugated anti-mouse IgG optimally diluted in PBSTM is added at 50 µl per well. The plate is incubated for 1 hour at 37°C.

v) After three washings as before, 100 µl of 0.1 M citrate buffer, pH 5.5, containing 0.5 mg/ml O-phenylene diamine and 3 µl/ml of 9% H_2O_2 are added to each well. After 30 minutes of incubation at room temperature in the dark, the reaction is stopped by adding 50 µl of 2 N H_2SO_4 and the absorbance is read at 495 nm. Positive and negative controls are included in each plate.

3.3. Reading the results

Both the MAP1-B ELISA and the MAP1 C-ELISA have shown a high specificity after evaluation in 3000 ruminant sera (goat, sheep and cattle) collected from 14 *A.-variegatum*-infested islands of the Lesser Antilles, among which only three are known to be infected by *E. ruminantium* (Mondry *et al.*, 1998). Overall specificity calculated from the 11 heartwater-free islands was 98.5% and 99.4% for the MAP1 C-ELISA and the MAP1-B ELISA, respectively. In another study undertaken in the Caribbean, ELISA MAP1-B positive samples were found in four of six islands free of heartwater (Kelly *et al.,* 2011).

Moreover, high seroprevalence in vector-free areas of Zimbabwe or South Africa has also been reported although not explained (it may be caused by a cross-reacting agent not transmitted by *Amblyomma*) and should be kept in mind when interpreting the results (Kakono *et al.,* 2003).

Evaluating the sensitivity of the tests is more problematic as it would require knowledge of the exact status of a high number of animals sampled in the field. As mentioned before there is currently no simple technique available to confirm if an animal is infected. Experimentally, the sensitivity of the C-ELISA in goats was reported to be 91.6–95.4% for the MAP1-B ELISA, and 96.3–96.9% for the MAP1 C-ELISA (Mondry *et al.,* 1998). However, in another study the sensitivity averaged 95% for cut-off values set at 31% and 26.6% of the positive control serum for sheep and goat sera, respectively (Mboloi *et al.,* 1999). Indeed, calculations are based on a limited number of experimentally inoculated animals in a period of time soon after inoculation, when almost all the animals are still positive. Sensitivity in cattle is even lower and several reports show that after infection most of the animals become seronegative again in less than 6 months and some animals never seroconvert (Mahan *et al.,* 1998b; Semu *et al.,* 2001). This observation is in line with the difference in antibody prevalence observed between small ruminants and cattle in epidemiological surveys that cannot be explained by a lower risk of infection of the latter. For example, in Zimbabwean farms situated in endemic areas, more than 90% of goats presented antibodies in their serum compared with only 33% of cattle maintained in the same conditions (Mahan *et al.,* 1998b). Similar observations were made in the Caribbean.

Serological tests are useful for the assessment of heartwater antibody response in vaccinated animals. The tests should not be used to screen animals for importation into heartwater-free areas. Antibodies are maintained at detectable levels in naturally infected domestic ruminants for a few months only and circulating antibodies disappear more rapidly in cattle than in small ruminants. It is thus possible that serologically negative animals may be carriers of infection. Serology should therefore only be considered as a diagnostic method to be applied at the herd level and not at the individual animal level (Peter *et al.,* 2001). When interpreting diagnostic serology results, other epidemiological parameters must be considered.

3.4. MAP1-B pan-species indirect ELISA

More recently, the WOAH Reference Laboratory has developed a pan-species ELISA validated in domestic cattle, sheep and goats (Rodrigues *et al.*, data not yet published). This ELISA is also based on MAP1-B antigen recognition, and therefore has the same analytical specificity as the MAP1-B ELISA described in Section B.3.1. The test could also be used for other mammals (except felines) and wildlife for large-scale seroprevalence studies to improve heartwater control and surveillance in the concerned areas. In the case of species other than domestic cattle, sheep or goats, results should be interpreted with care, possibly with the assistance of the WOAH Reference Laboratory.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

No commercial vaccines are available at present. The only method of immunisation against heartwater remains the "infection and treatment" method using infected blood followed by treatment of reacting animals with tetracycline. This method is still in use in several areas, but it is likely to be replaced by preparations using attenuated or inactivated organisms, which have given promising research results.

1. Inactivated vaccine preparations

Inactivated vaccine based on *E. ruminantium* elementary bodies chemically inactivated or lysed, emulsified in oil adjuvant, conferred good protection against homologous and field challenges (Adakal *et al.,* 2010b; Mahan *et al.,* 1998a; Marcelino *et al.,* 2015a; Martinez *et al.,* 1996). However, it does not prevent vaccinated animals from developing clinical signs, and morbidity is observed after virulent challenge.

The development of a large-scale production process and optimisation of storage conditions for the inactivated vaccine has led to a decrease in the cost of a vaccinal dose to 0.11 euros (Marcelino *et al.,* 2007; Peixoto *et al.,* 2005). In 2015, Marcelino *et al.* developed a ready-to-use inactivated vaccine that could be easily used in the field. It was shown that even after breaking the cold-chain of 3 days at 37°C, mimicking field conditions, the vaccine was still efficient (Marcelino *et al.,* 2015a). This study showed the robustness of the vaccine under field conditions.

In Zimbabwe, field trials of the inactivated vaccine emulsified in oil adjuvant have also demonstrated protection of sheep against natural tick challenge (Mahan *et al.,* 1998a). In larger field trials conducted in eastern and southern Africa, a significant reduction in mortality has been achieved in cattle, goats and sheep using either a prototype strain from Zimbabwe (Mbizi strain) or a local strain from the experimental sites (Mahan *et al.,* 2001). However, in three out of four sites, the vaccine prepared from the local isolate was less effective than the prototype Mbizi

vaccine, strongly suggesting an inadequate coverage of the antigenic repertoire of isolates present in each site. Vaccination trials in Burkina Faso showed a significant increase in the protective effect of inactivated vaccine when a local strain was added to the Gardel vaccinal strain (Adakal *et al.,* 2010b).

Lack of cross-protection between *E. ruminantium* isolates due to genetic or antigenic diversity is well established, but the complexity of the *E. ruminantium* population structure in the field has been underestimated. A large *E. ruminantium* genetic diversity has been observed throughout Africa, the Caribbean islands and the Indian Ocean, which raises the problem of the protective effect of the vaccinal strain against field strains (Adakal *et al.,* 2010a; Cangi *et al.,* 2016; Raliniaina *et al.,* 2010). Even if genetic characterisation is defined, there is a lack of a genetic marker associated with protection; moreover, it is essential to isolate *in-vitro* field strains to know their capacity for protection against heterologous strains and be able to mix several strains in the inactivated vaccine in order to cover widely the genetic diversity of field strains.

Inactivated vaccine is being developed commercially in South Africa. These inactivated vaccines do not prevent infection but do prevent or reduce death of vaccinated animals when exposed to live virulent challenge. The advantage however is that several field strains can be incorporated to make the vaccine more widely cross-protective.

A major challenge remains the identification of *E. ruminantium* genetic markers associated with protection in order to identify the vaccinal strains to include in the inactivated vaccine adapted to a region.

2. Attenuated vaccine preparations

Infection of ruminants with live *E. ruminantium* strains induces a strong long-lasting protection against a homologous isolate. This is the basis for the "infection-and-treatment" method using virulent isolates. Isolates of attenuated virulence that do not require the treatment of animals would be ideal, but a limited number of such attenuated isolates are available. An attenuated Senegal isolate has been obtained and shown to confer 100% protection against a homologous lethal challenge, but very poor protection against a heterologous challenge. The Gardel isolate, which gives a significant level of cross-protection with several isolates (although far from complete), has also been attenuated (Marcelino *et al.,* 2015b). A third isolate named Welgevonden from South Africa has been attenuated and shown to confer complete protection against four heterologous isolates under experimental conditions (Zweygarth *et al.*, 2005). However, it has not been tested in field conditions. More recently, an attenuated Welgevonden strain vaccine inoculated by the intramuscular route has demonstrated good levels of safety and protection against virulent homologous intravenous challenges and homologous infected tick challenges in domestic cattle, goats and sheep. The advantage of this method is that it avoids the need for antibiotic treatment to protect animals (Latif *et al.*, 2020). The main drawback of attenuated vaccines is their extreme lability, which necessitates their storage in liquid nitrogen and their distribution in frozen conditions. In addition, they have to be administered intravenously. Moreover, there is also a possible reversion to virulence and, as it is a live vaccine, it could not be used in heartwater free areas. Despite the recent efforts to understand the mechanism of virulence and attenuation (Marcelino *et al.,* 2015a), these are still largely unknown independently from the strain.

3. Recombinant vaccine preparations

Several reports show partial protection of mice using *map1* DNA vaccination and an improvement of protection by vaccination following a prime (plasmid) – boost (recombinant MAP1) protocol (Nyika *et al.,* 2002). However, protection of ruminants has never been demonstrated using this strategy. ~~In opposition~~ However, significant protection of sheep was reported against homologous and heterologous experimental challenge following plasmid vaccination using a cocktail of four ORFs (open reading frames) from the 1H12 locus in the *E. ruminantium* genome (Collins *et al.,* 2003). No further results have been described since then. Recombinant vaccines will probably not be available in the near future. A prime DNA/boost recombinant protein vaccine has been developed (Pretorius *et al.,* 2008). An efficient protective effect was obtained using a cocktail of four open reading frames (ORFs) against homologous challenge, but the vaccine did not give satisfactory results during field tick challenge. Moreover, simple intramuscular immunisation is not sufficient to induce protection. The use of a gene gun is necessary for prime DNA injection, which is not suitable for a large vaccination campaign. A polymorphic gene was identified as an efficient component of a recombinant vaccine against heartwater using the prime/boost method (Pretorius *et al.*, 2010). However, as this gene is polymorphic, a recombinant vaccine should include at least three different genotypes.

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619 **NB:** There is a WOAH Reference Laboratory for heartwater (please consult the WOAH Web site:
620 https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3)
621 Please contact the WOAH Reference Laboratories for any further information on
622 diagnostic tests, reagents and vaccines for heartwater

623 **NB:** FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2018.

Appendix 1: Heartwater
Intended purpose of test: purpose 1 – population freedom from infection, prevalence of infection/epidemiological surveillance

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
MAP-1B indirect ELISA Bovine, Caprine, Ovine: ++ and +++, respectively	Serum or plasma Antibodies against MAP1-B subunit of *Ehrlichia ruminantium*	For cattle, goat and sheep: Specificity = 99.4% Sensibility = 96.5%	Positive control: Cattle: serum from a naturally infected animal Goat and sheep: sera from animals infected with a virulent culture supernatant Validation samples: 2418 negative sera from heartwater-free lesser Antilles 37 positive sera from controlled conditions vaccinated animals	See reference	Easy to set up in a local laboratory	Present limitations for population freedom from infection because of the limited persistence of antibodies in animals Coating antigen not commercially available	Mondry *et al.* (1998)
Real-time PCR: =	Ticks (*Amblyomma* species)	Three copies by reaction	26 different strains of *E. ruminantium* detected 15 other *Ehrlichia*, *Babesia* and *Anaplasma* undetected, (even Panola Mountain *Ehrlichia*) nine uninfected *Amblyomma variegatum* samples	See reference	Can be performed to screen tick population collected on animals, as a complement to the serological test on hosts. Specific and rapid test.	Expensive and fragile equipment requires	Cangi *et al.* (2017)

627 **Appendix 2: Heartwater**

628 **Intended purpose of test: purpose 2 and purpose 5 – individual animal freedom from infection prior to**

629 **movement; immune status of individual animals; population post-vaccination studies**

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
MAP1-B indirect ELISA: + and ++, respectively	Serum or plasma Antibodies against MAP1-B subunit of *Ehrlichia ruminantium*	For cattle, goat and sheep: Specificity = 99,4% Sensibility = 96,5%	Positive control: Cattle: serum from a naturally infected animal Goat and sheep: sera from animals infected with a virulent culture supernatant Validation samples: 2418 negative sera from heartwater-free lesser Antilles 37 positive sera from controlled conditions vaccinated animals	See reference	Easy to set up in a local laboratory	Present limitations for population freedom from infection because of the limited persistence of antibodies in animals Coating antigen not commercially available	Mondry *et al.* (1998)

630 **Appendix 3: Heartwater**
631 **Intended purpose of test: purpose 4 – confirmation of clinical cases**

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
Real-time PCR: +++	Fresh blood collected on EDTA or 2/3 blood + 1/3 ultrapure ethanol for longer conservation *Ehrlichia ruminantium* pCS20 gene	See above			Method of choice for diagnostic during febrile phase		
Nested PCR: +++	Fresh blood collected on EDTA or 2/3 blood + 1/3 ultrapure ethanol for longer conservation Brain, lung *Ehrlichia ruminantium* pCS20 gene	Six elementary bodies of *E. ruminantium* per reaction Diagnostic sensibility: 2 log_{10} higher than Map1 PCR Specificity = as high as the Map1 PCR	Ticks and ruminant blood Comparison between Map1 nested PCR and pCS20 nested PCR	See reference	Need only a conventional PCR equipment	High risk of contamination because of two successive amplifications	Cangi *et al.* (2016)
In-vitro bacterium isolation: +	Blood collected on heparin and treated within 4 hours after collection Isolation of live *E. ruminantium*	Bacterial loads in blood, organs and ticks are not yet formally known.	Isolation could be performed on blood from animal in clinical phase, in organs up to 2 days after death and on ticks during all cycle life	See reference	Formal proof of the presence of the pathogen	Suitable but in very limited circumstances: Long, laborious, risky. Need a well-trained cell culture laboratory technician; risks of culture lost and false negative results	Marcelino *et al.* (2016)

Annexe 17. Chapitre 3.1.21. Peste bovine (infection par le virus de la peste bovine)

RÉUNION DE LA COMMISSION DES NORMES BIOLOGIQUES
Paris, 9–13 septembre 2024

CHAPTER 3.1.21.

RINDERPEST (INFECTION WITH RINDERPEST VIRUS)

SUMMARY

In the past, classical rinderpest was an acute, viral disease of domestic cattle, yaks, wild African buffaloes (Syncerus caffer) *and Asian water buffaloes* (Bubalus bubalis *and* B. arnee). *It was characterised by high morbidity and mortality rates. ~~Sheep, goats, pigs and wild ungulates~~ Other species of domestic and wild cloven-hooved animals might also be affected, but are not considered epidemiologically significant. Between 2002 and 2011, there were no reported field cases of rinderpest. The eradication campaign concluded in 2011 with an international declaration of global freedom from rinderpest.*

Existing collections of virulent and attenuated rinderpest viruses will remain under sequestration in approved research, diagnostic and vaccine manufacturing laboratories. To guard against the accidental release of virus from laboratory sources, FAO[1] and WOAH are collaborating in ~~establishing the principle of~~ maintaining international oversight and regulation of facilities holding rinderpest virus (RPV). All diagnostic testing that uses RPV-containing material should be performed by an RPV WOAH Reference Laboratory. Vaccine production and research activities that use live RPV or RPV-containing materials should be performed in an FAO-WOAH approved Rinderpest Holding Facility (RHF) conditional on receiving explicit authorisation from FAO and WOAH.

Rinderpest remains a notifiable disease and adequate surveillance systems must be maintained for the early detection of clinical cases should there be any accidental escape of the virus. WOAH (with FAO) will ensure the permanent availability of educational materials demonstrating the range of signs associated with rinderpest cases in live animals.

***Description of the disease:** Clinical recognition of classical rinderpest is based on the finding of an individual dead animal or small groups of extremely sick animals showing two or more of the following signs: pyrexia, inappetance, depression, emaciation, shallow erosions of the upper and lower lip and gum, erosions or blunting of the cheek papillae, serous or mucopurulent ocular and/or nasal discharges, diarrhoea or terminal recumbency. It is more than likely that the group will contain a number of dead animals with such lesions. The introductory section of this chapter provides a more detailed description.*

***Detection and identification of the agent:** Laboratory confirmation is required, and is based on demonstrating the presence of the virus, ~~viral~~ virus-specific RNA or viral antigen in samples from the spleen, tonsils, lymph nodes, white blood cells, ocular or nasal secretions of acutely infected animals.*

***Serological tests:** Antibodies to RPV can be detected in serum from animals that have been infected with field virus or received rinderpest vaccine. This ~~could~~ can be done ~~using estimation~~ by determination of neutralising antibody; ~~from the results of~~ it could previously also be done using a competition enzyme-linked immunosorbent assay (C-ELISA), but no such test is currently available. Any test used must be highly specific for RPV. Such*

[1] FAO: Food and Agriculture Organization of the United Nations

tests can currently only be carried out in FAO-WOAH approved ~~Rinderpest Holding Facilities~~ RHFs, as the neutralisation tests require the use of live RPV ~~(neutralisation tests) or antigen derived from live virus (C-ELISA)~~.

Requirements for vaccines: *~~A live attenuated rinderpest cell culture vaccine is available.~~ Currently, two live attenuated rinderpest cell culture vaccines, described in this chapter, are available. Under the terms of the* Guidelines for Rinderpest Virus Sequestration*, of Resolution No. 21* (*adopted by the WOAH Assembly, May 2017*) *governing the post-eradication era, it is not permitted to inoculate an animal with a rinderpest vaccine without prior permission from WOAH and FAO.*

In order to prepare for the possibility of a RPV re-emergence or release, FAO and WOAH, in collaboration with member countries, have developed a Global Rinderpest Action Plan for the post-eradication era that includes an international contingency plan, designation of a minimum number of Reference Centres/Reference Laboratories and an operational framework for emergency vaccine repositories to maintain preparedness. The retention and further manipulation of vaccine seed viruses is regulated jointly by FAO and WOAH.

A. INTRODUCTION

In the past, classical rinderpest was an acute, viral disease of domestic cattle, yaks, wild African buffaloes (*Syncerus caffer*) and Asian water buffaloes (*Bubalus bubalis* and *B. arnee*). It was characterised by high morbidity and mortality rates. ~~Sheep, goats, pigs and wild ungulates~~ Other species of domestic and wild cloven-hooved animals might also be affected (Taylor & Barrett, 2007). Rinderpest is not a zoonotic disease, but the virus or virus-containing materials must be handled in accordance with strict biocontainment procedures as described in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*, and in conformity with the Guidelines for Rinderpest Virus Sequestration.

Between 2002 and 2011 there were no reported field cases of rinderpest. Further, in the period leading up to January 2011, the WOAH Scientific Commission for Animal Diseases scrutinised a comprehensive world-wide list of applications (evidence-based and historical) for national recognition of rinderpest-freedom. This process concluded in 2011 with an international declaration of global freedom from rinderpest. For the immediate future, existing collections of virulent and attenuated rinderpest viruses will remain under sequestration in FAO-WOAH designated Rinderpest Holding Facilities (RHF) ~~research and approved vaccine manufacturing laboratories~~. To guard against the accidental release of virus from laboratory sources, FAO and WOAH are collaborating in establishing the principle of international oversight and regulation of facilities holding rinderpest virus (RPV) based on minimising the number of repositories. All diagnostic testing, vaccine development and research activities that use live RPV or other RPV-containing materials~~s~~[2] should be performed in an FAO-WOAH approved RHF and after approval of the activity by WOAH and FAO.

Rinderpest remains a notifiable disease and adequate surveillance systems must be maintained for the early detection of clinical cases should there be any accidental escape of the virus. WOAH and FAO will ensure the permanent availability of educational materials demonstrating the range of signs associated with rinderpest cases in live animals. A recent account of the history of rinderpest, its eradication and its socio-economic impact is available (Roeder & Rich, 2009).

~~Rinderpest is caused by~~ RPV is a negative-strand RNA virus of the *Morbillivirus* genus within the family *Paramyxoviridae*. The virus has a single serotype with at least three geographically restricted clades: African Lineages 1 and 2 and Asian Lineage 3, which cross-protect fully and are only differentiated by molecular characterisation. Although some strains of rinderpest evolved into a mild, nonfatal, infectious disease of cattle, all strains retain two very dangerous attributes. The first is an almost certain ability to undergo virulence modulations. The second is an ability to infect wild animal species and, in African buffaloes, eland, giraffe, lesser kudu and warthog, to cause an acute infection associated with high mortality.

An illustrated description of the disease is given in the WOAH Atlas of Transboundary Animal Diseases (Fernandez & White, 2010). Classical rinderpest has an incubation period of between 1 and 2 weeks. A peracute form is characterised by high pyrexia and sudden death in newborn or young animals. The acute disease is characterised by an acute febrile attack within which prodromal and erosive phases can be distinguished. The prodromal period lasts approximately 3 days, during which affected animals develop a pyrexia of between 40 and 41.5°C together with partial anorexia, constipation, congestion of visible mucosae, serous ocular and nasal discharges, depression and drying of the muzzle. However, it is not until the onset of the erosive phase, and the development of necrotic mouth lesions, that a tentative clinical diagnosis of rinderpest can be made. At the height of fever, flecks of necrotic epithelium appear on the lower lip and gum and in rapid succession may appear on the upper gum and dental pad, on the underside of the tongue, on the cheeks and cheek papillae and on the hard palate. Through the enlargement of existing lesions and the development of new foci, the extent of the oral necrosis can increase dramatically over the following 2–3 days. Much of the necrotic material works loose giving

[2] See chapter 8.16 of the WOAH *Terrestrial Code* for definition of Rinderpest virus containing material~~s~~.

rise to shallow, non-haemorrhagic mucosal erosions. Necrotic lesions may also be found on the nares, vulva, vagina and preputial sheet.

Diarrhoea is another characteristic feature that develops 1–2 days after the onset of mouth lesions. The diarrhoea is usually copious and watery at first, but later on may contain mucus, blood and shreds of epithelium and it may be accompanied, in severe cases, by tenesmus. Anorexia develops, the muzzle dries out completely, the animal is depressed and emaciated, the breath is fetid and mucopurulent ocular and nasal discharges develop.

Deaths will occur but, depending on the strain involved, the breed of cattle infected and environmental conditions, the mortality rate may vary from 100% (acute strains in European breeds), to 20–30% (acute strains in zebu cattle), to zero (mild strains in zebu cattle). With both acute and mild strains, the mortality rate may be expected to rise as the virus gains progressive access to large numbers of susceptible animals. In the terminal stages of the illness, animals may become recumbent for 24–48 hours prior to death. Some animals die while showing severe necrotic lesions, high fever, emaciation and diarrhoea, others after a sharp fall in body temperature, often to subnormal values. In survivors, the pyrexia may remit slightly in the middle of the erosive period and then, 2–3 days later, return rapidly to normal accompanied by a quick resolution of the mouth lesions, a halt to the diarrhoea and an uncomplicated convalescence.

In cases where rinderpest is suspected, post-mortem examinations should pay particular attention to the abomasum, which may be highly engorged or show a grey discoloration; to the Peyer's patches, which may show lymphoid necrosis; and to the development of linear engorgement and blackening of the crests of the folds of the caecum, colon and rectum. Typically the carcass of the dead animal is dehydrated, emaciated and soiled. The nose and cheeks bear evidence of mucopurulent discharges, the eyes are ~~is~~ sunken and the conjunctiva congested. In the oral cavity, there is often extensive desquamation of necrotic epithelium, which always appears sharply demarcated from adjacent areas of healthy mucosa. The lesions frequently extend to the soft palate and may also involve the pharynx and the upper portion of the oesophagus; the rumen, reticulum and omasum are usually unaffected, although necrotic plaques are occasionally encountered on the pillars of the rumen. The abomasum, especially the pyloric region, is severely affected and shows congestion, petechiation and oedema of the submucosa. Epithelial necrosis gives the mucous membrane a grey colour. The small intestine is not commonly involved except for striking changes to the Peyer's patches where lymphoid necrosis and sloughing leaves the supporting architecture engorged or blackened. In the large intestine changes involve the ileocaecal valve, the caecal tonsil and the crests of the longitudinal folds of the caecal, colonic and rectal mucosae. The folds appear highly engorged in acute deaths or darkly discoloured in long-standing cases; in either event the lesions are referred to as 'zebra striping'.

The principal differential diagnoses in cattle are bovine viral diarrhoea/mucosal disease complex and malignant catarrhal fever; differentiation of these diseases requires the use of appropriate laboratory tests. Definitive diagnosis of rinderpest can currently only be undertaken in WOAH rinderpest Reference Laboratories.

In the mild form of rinderpest, which was associated with African lineage 2 strains of the virus that were found in endemic areas of eastern Africa, the incubation period could be between 1 and 2 weeks and the ensuing clinical disease little more than a subacute febrile attack in cattle. The fever was not invariable; it was short-lived (3–4 days) and low (38–40°C). The depression that characterised more acute forms of rinderpest was absent from mildly affected animals and, as a result, cattle often did not lose their appetite, and continued to graze, water and trek as well as unaffected animals. Diarrhoea, if present, was not marked. On close examination there might be some slight congestion of the visible mucous membranes and small, focal areas of raised, whitish epithelial necrosis might be found on the lower gum – sometimes no larger than a pin head – along with a few eroded cheek papillae. Some animals totally escaped the development of such erosions, the appearance of which was fleeting. Other animals might show a slight, serous, ocular or nasal secretion but, in contrast to the more severe forms of the disease, these did not progress to become mucopurulent.

Even though infections with mild rinderpest could pass unnoticed in cattle, the virus remained highly infectious for wildlife species, and among those generally regarded as highly susceptible (tragelaphine species such as lesser kudu and eland, African buffalo, and giraffe) it caused fever, a nasal discharge, typical erosive stomatitis, gastroenteritis, and death. Kock (2006) observed that, in addition, African buffaloes infected with lineage 2 showed enlarged peripheral lymph nodes, plaque-like keratinised skin lesions and keratoconjunctivitis. Lesser kudus were similarly affected but, whereas blindness – caused by a severe keratoconjunctivitis – was common, diarrhoea was unusual. Eland also showed necrosis and erosions of the buccal mucosa together with dehydration and emaciation. Therefore, in these circumstances, a diagnosis of rinderpest in any of these species points to the likelihood of the simultaneous transmission of the virus, even at a subclinical level, in neighbouring cattle and possible dissemination of infection through live animal trade.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for rinderpest diagnosis and their purpose

Method	Purpose					
	Population freedom from infection[a]	**Individual animal freedom from infection prior to movement[b]**	**Contribute to eradication policies[c]**	**Confirmation of clinical cases[d]**	**Prevalence of infection – surveillance[e]**	**Immune status in individual animals or populations post-vaccination[f]**
Detection and identification of the agent[g]						
Virus isolation	–	–	+	+ ~~++~~	–	–
~~Antigen detection (AGID)~~	~~–~~	~~–~~	~~±~~	~~±~~	~~±~~	~~–~~
Conventional RT-PCR	~~±~~	+++	+++	+++	~~±~~	~~±~~
Real-time RT-PCR	–	+++	+++	+++	+	–
Detection of immune response						
~~AGID~~	~~±~~	~~±~~	~~±~~	~~–~~	~~±~~	~~±~~
~~C-ELISA~~	~~++~~	~~–~~	~~++~~	~~–~~	~~++~~	~~++~~
VN	++ ~~±~~	–	++ ~~±~~	–	++ ~~±~~	++ ~~±~~

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
~~AGID = agar gel immunodiffusion;~~ RT-PCR = reverse-transcriptase polymerase chain reaction;
~~C-ELISA = competitive enzyme-linked immunosorbent assay;~~ VN = virus neutralisation.
[a] See Appendix 1 of this chapter for justification table for the scores given to the tests for this purpose.
[b] See Appendix 2 of this chapter for justification table for the scores given to the tests for this purpose.
[c] See Appendix 3 of this chapter for justification table for the scores given to the tests for this purpose.
[d] See Appendix 4 of this chapter for justification table for the scores given to the tests for this purpose.
[e] See Appendix 5 of this chapter for justification table for the scores given to the tests for this purpose.
[f] See Appendix 6 of this chapter for justification table for the scores given to the tests for this purpose.
[g] A combination of agent identification methods applied on the same clinical sample is recommended.

Special Post-Eradication Note: *there are no diagnostic tests for RPV or antibodies to RPV for which there is a positive control that does not come within the FAO-WOAH definition of a Rinderpest Virus Containing Material (RVCM). Continued storage of RVCM requires approval of the laboratory through the FAO-WOAH Rinderpest secretariat as an FAO-WOAH ~~Holding Facility~~ RHF; use of RVCM for any purpose, including validation of diagnostic tests, requires explicit permission of FAO and WOAH.*

Suspect cases, that is animals with clinical signs similar to those seen in the case of infection with RPV, will still arise, and need to be tested to ensure that any future re-emergence or escape of RPV is detected in a timely manner. For the initial testing of samples from suspect cases, laboratories that are not FAO-WOAH-approved ~~Rinderpest Holding Facilities~~ RHFs are recommended to use (gel-based or real-time) reverse-transcriptase polymerase chain reaction (RT-PCR) using the established primer sets. The test can be run without a RPV positive control; parallel tests using (vaccine or wild type) peste des petits ruminants virus (PPRV) and published primer sets for PPRV can be used as a control for most of the stages of the assay (RNA extraction, reverse transcription and PCR reagents); alternatively the bovine actin primers can be used in parallel as an internal control reaction. For definitive diagnosis, samples should be sent to one of the ~~FAO~~-WOAH ~~approved Rinderpest Holding Facilities~~ rinderpest Reference Laboratories.

There are no circumstances where tests for anti-RPV antibodies will be required unless there is a re-emergence or escape of the virus.

1. Detection and identification of the agent

Any suspicion of rinderpest must be viewed as a potential threat to international biosecurity and must be rapidly confirmed or differentiated. RT-PCR is the most rapid and specific test. If RPV is confirmed, back-tracing measures must be immediately instigated. In addition, samples must be sent to a WOAH Reference Laboratory for rinderpest for final confirmation of the diagnosis, and the virus origin should be identified by sequencing and comparison with known RPV genomic data. If possible, the virus should be isolated (Anderson *et al.,* 1996), though this should only be attempted in a WOAH Reference Laboratory ~~an FAO-WOAH approved Rinderpest Holding Facility~~.

1.1. Virus isolation

RPV can be cultured from the leukocyte fraction of whole blood that has been collected into heparin or EDTA (ethylene diamine tetra-acetic acid) at final concentrations of 10 international units (IU)/ml and 0.5 mg/ml, respectively. Samples should be thoroughly mixed and transferred to the laboratory on ice, but never frozen. On average, the onset of viraemia slightly precedes the onset of pyrexia, and may continue for 1–2 days after pyrexia begins to wane. Consequently, animals showing a pyrexia are probably viraemic and therefore the best source of blood with which to attempt virus isolation. However, as occasional febrile animals may no longer be viraemic, samples from several febrile animals should be collected for submission. Virus can also be isolated from samples of the tonsil, spleen, prescapular or mesenteric lymph nodes of dead animals; these samples may be frozen for transportation. Transportation must be under biosecure conditions in compliance with international transport regulations described in Chapter 1.1.2 *Collection, submission and storage of diagnostic specimens*, Chapter 1.1.3 *Transport of biological materials* and with the Guidelines for Rinderpest Virus Sequestration.

To isolate the virus from blood, uncoagulated blood is centrifuged at 2500 ***g*** for 15 minutes to produce a buffy coat layer at the boundary between the plasma and erythrocytes. This is removed as cleanly as possible, mixed in 20 ml physiological saline and recentrifuged in a washing procedure designed to remove any neutralising antibody present in the plasma. The resulting cell pellet is suspended in cell culture maintenance medium and 2 ml aliquots are distributed onto established monolayers of primary calf kidney, B95a marmoset lymphoblastoid, *Theileria*-transformed bovine T lymphoblast or African green monkey kidney (Vero) cells, preferably Vero cells expressing morbillivirus receptor SLAM. These cells may be cultured in roller tubes, culture flasks or multiwell plates.

Alternatively, 20% suspensions (w/v) of post-mortem tissue may be used. These should be made by macerating the solid tissues in serum-free culture maintenance medium using standard grinding or shearing techniques and inoculating monolayers as before. The release of virus from solid tissue can be achieved in several ways. Perhaps the easiest is with a pestle and mortar, but this technique requires the use of sterile sand as an abrasive. Alternatively, tissue may be ground without an abrasive using all-glass grinders, for example, a Ten Broeck grinder. Shearing techniques are equally applicable using laboratory blenders. Virus-containing suspensions are clarified by low-speed centrifugation. The volume of the inoculum is not critical; a working volume is between 1 and 2 ml. Commonly used antibiotics are penicillin and streptomycin in combination, ~~each~~ at a concentration of 100 IU/ml or 100 µg/ml, respectively. A similar broad-spectrum cover can be obtained using neomycin at 50 µg~~µl~~/ml. Amphotericin B should be included at 2.5 µg/ml.

The inoculum should be removed after 1–2 hours and replaced with fresh medium. Thereafter, the culture maintenance medium should be decanted and replaced every 2 or 3 days and the monolayer observed microscopically for the development of cytopathic effects (CPE). These are characterised by refractility, cell rounding, cell retraction with elongated cytoplasmic bridges (stellate cells) and/or syncytium formation. The speed with which the CPE develops varies by substrate and probably by strain of virus also. Up to 12 days should be allowed in primary cells, a week in Vero and 2–5 days in B95a cells or Vero cells expressing SLAM. Blind passages may be attempted before declaring an important sample negative. Isolates of virus can be ~~partially identified by the demonstration of morbillivirus-specific precipitinogens in infected cell debris~~, or completely identified by ~~either~~ RT-PCR using RPV-specific primers (see below) ~~or the demonstration of specific immunofluorescence using a RPV-specific monoclonal antibody~~.

~~1.2. Antigen detection by agar gel immunodiffusion~~

~~The agar gel immunodiffusion (AGID) tests may be conducted in Petri dishes or on glass microscope slides (Foreman *et al.*, 1983). In either instance the surface should be covered with agar to a depth of about 4 mm using a 1% aqueous solution of any high quality agar or agarose. Wells are usually cut in a hexagonal pattern of six peripheral wells around a single central well. For slides, wells should be 3 mm in diameter and 2 mm apart. For Petri dishes, the wells can be increased to 4 mm in diameter and the distance between wells to 3 mm. The closer the wells are placed from each other, the shorter the reaction time.~~

~~Using a small volume pipette, rinderpest hyperimmune rabbit serum should be placed in the central well. In the absence of a rinderpest-containing positive control, PPRV (e.g. preparations of vaccine virus) can be used as the control, which should be placed in alternate peripheral wells (i.e. one, three and five). Negative control antigen is placed in well four. Test antigens are obtained as exudates from the cut surface of spleen or lymph nodes submitted for testing; if no exudate can be obtained a small portion of the sample should be ground with a minimum of saline. Ocular exudates may be squeezed directly from the swabs or, alternatively, by compression in a microtip (the cotton wool should be cut off the swab and placed into the wide end of a plastic 50–250 µl pipette tip; the stem of the swab may then be used to compress the cotton wool and force a small volume of exudate out of the narrow end of the tip). Test samples are added to wells two and six. Tests are best developed at 4°C or low ambient temperatures. The reaction area should be inspected from 2 hours onwards for the appearance of clean, sharp lines of precipitation between the wells forming a line of identity with the controls. Tests should be discarded after 24 hours if no result has been obtained. The result is not acceptable unless precipitation reactions are also obtained giving a line of identity with the control positive antigen preparation.~~

~~Although the test is neither highly sensitive nor highly specific, it is robust and adaptable to field conditions. A positive reaction from a large domestic ruminant should be treated as if it were rinderpest. From a small ruminant, a positive result should be treated as having been derived from a case of peste des petits ruminants (PPR) although further testing is recommended, given the lack of specificity in this test.~~

1.2~~3~~. Nucleic acid detection and characterisation methods

RT-PCR techniques based on the amplification of parts of the N or F protein genes have been developed for the specific diagnosis of RPV (Forsyth & Barrett, 1995). This technique is extremely sensitive, specific and can detect RPV in cattle as early as two days post-infection with the advantage that results are obtained in 5 hours, including the RNA extraction. The two most commonly used protocols are given in some detail below. The PCR products are analysed on a 1.5% (w/v) agarose gel along with a suitable DNA size marker to identify the specific DNA product.

A real-time RT-PCR assay for RPV diagnosis was described by Carrillo *et al.* (2010). This assay has been shown to be sensitive, to detect isolates representative of all known phylogenetic lineages of the virus, and to clearly differentiate RPV from PPRV and other clinically similar diseases (foot and mouth disease virus, bovine viral diarrhoea virus, bovine herpes virus, vesicular stomatitis virus). Comparison of samples from experimentally infected animals showed that white blood cells and conjunctival swabs are the sample of choice for this test, allowing the preclinical detection of the disease by 2–4 days post-infection. In the event of a RPV outbreak, this single-tube format real-time RT-PCR has the capability of preclinical diagnosis, thus aiding efforts to prevent further transmission of disease. It should be noted, however, that the assay was developed after the last RPV case, and was never used in practice for RPV diagnosis, and appropriate diagnostic controls are not generally available.

In general, any molecular diagnostic methods, including RT-PCR, require appropriate positive and negative controls during their execution. Careful consideration is essential when interpreting results in the absence of proper control reactions. ~~Laboratories, other than the WOAH Reference Laboratories, that wish to carry out their own testing of suspect cases are advised to carry out gel based RT-PCR using the available controls.~~ For both gel-based and real-time PCR methods, a positive control such as PPRV (with its specific primers) or bovine actin, and an appropriate negative control ~~using sterile distilled water~~ instead of test RNA, must be included. Positive reactions with an RPV-specific primer set should be confirmed either by using additional RPV-specific primer sets or by sequence analysis of the DNA product.

1.3.1. Extraction of RNA from field samples

Viral RNA can be purified from lymph ~~node~~ nodes or ~~tonsil~~ tonsils (ideal), peripheral blood lymphocytes (PBLs), swabs from eyes or mouth lesions, or from spleen (not ideal because of its high blood content). Tissue samples should be extracted with acidified guanidinum thiocyanate phenol (Forsyth & Barrett, 1995) using one of the commercial preparations available. Solid tissues (0.5–1.0 g) are minced and homogenised with 10 ml reagent, eye and mouth swabs are extracted with 1.0 ml, and purified PBLs (from 5 ml whole blood) are homogenised with 1.0 ml; RNA is then purified according to the manufacturer's procedure. For PBLs or swabs, RNA extraction spin columns are also suitable. The resulting RNA is stored at –70°C or –20°C until required.

The cDNA synthesis and PCR are carried out using a one-tube combined reaction. Suitable reagents are available from a number of manufacturers ~~in addition to that given in the example protocol~~. The PCR products are analysed on a 1.5% (w/v) agarose gel along with a suitable DNA size marker to identify the specific DNA products. An internal positive control such as the beta-actin primers should be included to

validate the RNA extraction step and the RT-PCR reagents; if possible a parallel extraction of PPRV should be carried out and the viral RNA identified using PPRV-specific primers (Chapter 3.8.8, Section 2.4). A negative control using sterile distilled water instead of RNA must be included in each set of reactions. Positive reactions with either RPV-specific primer set should be confirmed by sequence analysis of the DNA product. In addition, positive samples should be sent to the WOAH Reference Laboratory in the United Kingdom (UK) for confirmatory testing. It is important to use more than one set of primers for the PCR step when testing for the presence of RNA viruses, as their nucleotide sequences can vary significantly and a mismatch of the primer at the 3'-end or within the primer sequence may result in failure of the primers to amplify the DNA. The ~~FAO World Reference Laboratory[3] in the UK, which is also a WOAH Reference Laboratory for rinderpest, and the~~ WOAH Reference Laboratory~~-~~ies for rinderpest in France and the UK[4]~~)~~, can advise on use of the technique for field sample analysis.

1.3.2. RT-PCR for the diagnosis of RPV based on the amplification of parts of the N and/or F genes

N and F gene amplification is based on the initial protocol described by Forsyth & Barrett (1995), reformulated as a one-step RT-PCR method. The test described requires the following materials: a commercial one-step RT-PCR kit, distilled water and primers, and a suitable PCR machine. Facilities for DNA agarose gel electrophoresis are also required.

i) Sequences of primers used:

Gene	Product size	Primer	Sequence (5' → 3')
RPV N	297 bp	B2A	ATC-CTT-GTC-GTT-RTA-TGC-TCT-YRG
		B12A	CAA-GGG-RRT-GAG-ACC-CAG-MAC-AR
RPV F	448bp	F3B	AGT-ATA-AGA-GGC-TGT-TGG-GGA-CAG-T
		F4D	TGG-GTC-TCT-GAG-GCT-GGG-TCC-AAA-T
β-Actin	275bp	BA1	GAG-AAG-CTG-TGC-TAC-GTC-GC
		BA2	CCA-GAC-AGC-ACT-GTG-TTG-GC

ii) Prepare each primer dilution by adding 5 µl of the primer stock solution (100 µM) to 45 µl of distilled water. A primer concentration of 10 µM is obtained with a final volume of 50 µl.

iii) For each test gene, prepare a PCR master mix ~~containing 0.6 µM~~ so that the final concentration of primers in the reaction is 0.6 µM; 45 µl of master mix are required per reaction.

iv) Add 5 µl of RNA template to 45 µl of each master mix. Distilled water (5 µl) is added in place of RNA to provide a negative control which has to be included in each set of tests.

v) The full thermal cycler program will depend on the machine and reagents used in a given laboratory and must be optimised. For the PCR step, 40 cycles with an annealing temperature of 55°C are recommended as an initial point for optimisation. Fully tested protocols can be obtained from either of the WOAH rinderpest Reference Laboratories.

~~Thermal cycler conditions are as follows:~~

~~50°C for 30 minutes~~	~~1 cycle~~	~~Reverse transcription step~~
~~95°C for 15 minutes~~	~~1 cycle~~	~~Inactivates RT and activates polymerase~~
~~94°C for 30 seconds~~ ~~55°C for 30 seconds~~ ~~72°C for 1 minute~~	~~40 cycles~~	~~PCR amplification of the cDNA~~
~~72°C for 5 minutes~~	~~1 cycle~~	~~Final extension~~
~~4°C (indefinite)~~	~~–~~	~~–~~

vi) Ten microlitres of each reaction are analysed by electrophoresis on a 1.5 % agarose gel. For all positive results, the remainder of the final product may be directly used for sequencing.

~~[3] http://www.fao.org/docrep/004/X2096E/X2096E09.htm~~
[4] https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3

1.3.3. Real-time RT-PCR for the diagnosis of RPV

The real-time RT-PCR assay is carried out essentially as described in Carrillo *et al.,* 2010. It is typically performed as a 20 µl reaction. Several suitable reagents for one step real-time RT-PCR are available, and the exact reaction conditions should be altered to fit with the reagents and the real-time PCR machine being used. For detailed advice on this test, including appropriate control reactions, contact the WOAH Reference Laboratories.

2. Serological tests

2.1. Competitive enzyme-linked immunosorbent assay (ELISA)

There is currently no competitive ELISA available for the detection of rinderpest antibodies. Any old reagents retained in RHFs should be destroyed, as they will have expired and the assay will no longer be valid. A new assay is under development.

~~A competitive ELISA is useful for the detection of rinderpest antibodies in the serum of animals of any species previously exposed to the virus. The test is based on the ability of positive test sera to compete with a rinderpest anti-H protein MAb for binding to rinderpest antigen. The presence of such antibodies in the test sample will block binding of the MAb, producing a reduction in the expected colour reaction following the addition of enzyme-labelled anti-mouse IgG conjugate and a substrate/chromogen solution. As this is a solid-phase assay, wash steps are required to ensure the removal of unbound reagents.~~

~~The rinderpest antigen is prepared from Madin–Darby bovine kidney cell cultures infected with the attenuated Kabete 'O' strain of rinderpest virus and inactivated at 56°C for 2 hours. The viral antigen is extracted from the infected cells by repeated cycles of sonication and centrifugation. The MAb was obtained by fusing the splenocytes of hyperimmunised mice with the NSO myeloma cell line, and then shown to be rinderpest H protein specific; this MAb has now been designated as C1. Kits will continue to be available commercially.~~

~~2.1.1. Test procedure~~

~~i) Reconstitute the freeze-dried rinderpest antigen with 1 ml of sterile water and further dilute it to the manufacturer's recommended working dilution using 0.01 M phosphate buffered saline (PBS), pH 7.4.~~

~~ii) Immediately dispense 50 µl volumes of the diluted antigen into an appropriate number of wells of a flat-bottomed, high protein-binding ELISA microplate using two wells per test serum. Tap the sides of the microplate to ensure that the antigen is evenly distributed over the bottom of each well and, having sealed the plate, incubate it on an orbital shaker for 1 hour at 37°C. Wash the wells three times with 0.002 M PBS, pH 7.4.~~

~~iii) Add 40 µl of blocking buffer (0.01 M PBS, 0.1% [v/v] Tween 20 and 0.3% [v/v] normal bovine serum) to each test well followed by 10 µl volumes of all test sera.~~

~~iv) Follow the manufacturer's recommendations to prepare a working dilution of the MAb in blocking buffer, and add 50 µl of this to each test well. Seal the plates and re-incubate on an orbital shaker for 1 hour at 37°C.~~

~~v) Follow the manufacturer's recommendations to prepare a working dilution of rabbit anti-mouse immunoglobulin horseradish peroxidase conjugate in blocking buffer and add 50 µl to each test well. Seal the plates and re-incubate on an orbital shaker for 1 hour at 37°C.~~

~~vi) At the end of this period the plates are washed as before and immediately refilled with 50 µl volumes of substrate/chromogen mixture (1 part 3% H_2O_2 to 250 parts OPD), and incubate at room temperature for 10 minutes without shaking. Then add 50 µl of a stopping solution consisting of 1 M sulphuric acid.~~

~~vii) The test system must include known rinderpest positive and negative serum samples, a MAb control and a conjugate control.~~

~~viii) Measure the resulting absorbance values on an ELISA reader with a 492 nm interference filter and express the test results as percentage inhibition values compared with the value obtained using the MAb control. Inhibition values of 50% or more are considered to be positive and values below 50% are considered to be negative.~~

~~Lowering the positive/negative threshold to 40% or less increases the sensitivity of the test, but inevitably affects specificity by increasing the proportion of false-positive test results encountered. In practise, the 50% value is recommended by GREP at which level sensitivity is at least 70% and specificity exceeds~~

~~99%. The sensitivity needs to be taken into account when designing sampling frames for serosurveillance.~~

~~2.2. Antibody detection by agar gel immunodiffusion (AGID)~~

~~The AGID test can be used for screening bovine sera where there is suspected rinderpest disease and where PPRV is not circulating. As noted in section 1.2, the test does not distinguish between PPRV and RPV, so antibodies to either virus will give a positive reaction. Set up the AGID as described in 1.2, except that the central well contains a suspension of PPRV vaccine, while the outer wells contain known anti-RPV antisera (positions 1, 3 & 5), negative control serum (e.g. commercial bovine serum) in position 4 and test sera in positions 2 and 6. Antibodies to RPV will cross-react with the PPRV antigens, giving rise to precipitin lines.~~

2.1~~3~~. Virus neutralisation

The virus neutralisation test (VNT) is performed in roller-tubes or culture flask cultures of primary calf kidney cells following the method of Plowright & Ferris (1961) or in 96-well microplates (Taylor & Rowe, 1984); both tests have been validated in experimentally infected cattle.

In the roller tube procedure, sera, that have not been heat inactivated, are diluted at intervals of 1 in 10 and then, starting with undiluted serum, mixed with an equal volume of $10^{3.0}$ $TCID_{50}$ per ml of an attenuated vaccine strain virus. Mixtures are held overnight at 4°C, after which 0.2 ml volumes are inoculated into each of five roller tubes, immediately followed by 1 ml of dispersed indicator cells suspended in growth medium at a rate of 2×10^5 cells per ml. Tubes are incubated at 37°C, sloped for the first 3 days, after which they are replenished with maintenance medium and placed on a roller apparatus. They are examined regularly for virus-specific cytopathology and positive tubes recorded and securely discarded; the final examination takes place on day 10. For calculating end-points, the virus dose is regarded as satisfactory if the final dilution falls within the range $10^{1.8}$ to $10^{2.8}$ $TCID_{50}$/tube. Under these circumstances, the presence of any detectable antibody in the 1/2 final serum dilution is considered to indicate previous infection with RPV.

In the microplate method, sera are heat-inactivated for 30 minutes at 56˚C before use. An initial serum dilution of 1/5 is further diluted at twofold intervals. Thereafter, 50 µl volumes of serum are incubated with 50 µl volumes of virus diluted to contain between $10^{1.8}$ and $10^{2.8}$ $TCID_{50}$ (Taylor & Rowe, 1984). Following a 45-minute to overnight incubation, 50 µl RPV-susceptible cells (between 1 and 2×10^5 primary calf or lamb kidney cells, 5×10^3 Vero or Vero-SLAM cells, or 5×10^4 B95a cells) are added as indicators. Tests are terminated after 6 or 7 days. Such tests may give indications of nonspecific neutralisation at high serum concentrations. There appear to be factors in some normal (with respect to prior rinderpest exposure) sera that bring about the failure of the virus to penetrate and replicate in indicator cells. In the tube test, these factors were probably removed during changes in maintenance medium; in the microplate method, they remain present the whole time. If the most concentrated final serum dilution is limited to 1/10, the effect disappears.

It should be noted that, since this test requires the manipulation of live vaccine virus, the VNT can currently only be undertaken in FAO-WOAH approved ~~Rinderpest Holding Facilities~~RHFs with specific permission to carry out the procedure.

C. REQUIREMENTS FOR VACCINES

1. Background

1.1. Rationale and intended use of the product

The live attenuated tissue culture rinderpest vaccine (TCRV) described in previous editions of the *Terrestrial Manual* (Plowright, 1962) was developed in Kenya through the serial passage in primary bovine calf kidney cells of RBOK (rinderpest bovine old Kabete, or “Kabete O”), a virulent bovine rinderpest field strain isolated in 1910. While the modern division of rinderpest viruses into four lineages (Africa 1 and 2 and an old African one which includes Kabete O, and Asian) was unknown until 1995 (Wamwayi *et al.,* 1995), the Plowright vaccine virus undoubtedly cross-protects against all strains of all lineages. Since its development, the Plowright vaccine seed was widely distributed and hundreds of millions of doses of it were used on the Indian subcontinent, the Middle and Near East, and Africa in the control and eradication of rinderpest. A curated seed stock of this vaccine is currently stored at the FAO-WOAH-approved RHF in France[4], and can be provided for production of rinderpest vaccine for emergency use, following explicit permission of FAO and WOAH.

Other ~~currently active~~ TCRV strains, LA (Nakamura & Miyamoto, 1953) and LA-AKO (Furutani *et al.,* 1957a), were established from a previously developed lapinised vaccine strain, Nakamura III (alternatively known as L strain; Nakamura *et al.,* 1938), by repeated passages in rabbits and chick embryos. The parental Nakamura III was widely used to control the disease in East and South-East Asia. LA and LA-AKO are reported to be far less virulent than the parental strain, especially in highly susceptible cattle in Eastern Asia such as Japanese black and Korean yellow. Currently, LA-AKO is being used, at an FAO-WOAH-approved ~~Rinderpest Holding Facility~~ RHF, for production of rinderpest vaccine for emergency use.

2. Outline of production and minimum requirements for conventional vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

i) Plowright (RBOK) vaccine

The vaccine strain was developed by 90 passages in primary calf kidney cells, and shown to be safe, effective and to resist reversion to virulence during 7 back passages in cattle (Plowright, 1962). The vaccine sequence has been published (Baron & Barrett, 1995) and deposited in the public databases. Seed lots used in the manufacture of Plowright TCRV must produce a cell-culture vaccine that is similarly safe and that confers an immunity in cattle lasting at least 5 years. The immunogenicity of seed virus was demonstrated up to the 122nd BK passage level, which should not be exceeded. Therefore, vaccine seed must be maintained in a seed lot system between passage levels 90 and 120. Seed lot virus must be preserved in a freeze-dried state at a temperature of -20°C or lower. The virus must be cultured in Vero cells or primary or serially cultivated kidney cells derived from a normal bovine fetus or a very young calf. Serially cultivated cells may not be more than ten passages removed from the primary cultivation.

~~The seed virus produces a vaccine that is safe to use in a variety of European, African and Indian cattle breeds. Its safety and efficacy have never been assessed in Chinese or Japanese cattle breeds.~~

ii) LA-AKO vaccine

The master seed virus (LA-AKO) was established from the lapinised "Nakamura III" vaccine strain (at the 897th rabbit passage level) by repeated passages in rabbits (29 passages) and chick embryos (456 passages). LA-AKO does not cause any clinical signs except slight hyperthermia in highly susceptible animals such as Japanese black cattle. It should be noted however that the virus induces marked enlargement of the spleen in inoculated chick embryos (Furutani *et al.,* 1957b). The whole genome sequence of LA-AKO, and its strain of origin, Nakamura III, have been registered in the public database (Fukai *et al.,* 2011; Takamatsu *et al.,* 2015).

Seed lots should be lyophilised or frozen and stored at a temperature of –20°C or lower until use.

2.1.2. Quality criteria

i) Special considerations

Due to the fact that RPV has been eradicated worldwide, special consideration needs to be made in regards to animal inoculation used to assess safety and efficacy. It is recommended to sequence the full genome of a candidate vaccine virus and compare with reference strains of RPV to assess similarities that would negate the need to inoculate animals.

Subject to the above~~, for both Plowright and LA-AKO strains,~~ seed lots should be shown to be:

a) Pure

Free from contamination with viruses, bacteria, fungi or mycoplasmas.

b) Safe

Inducing no abnormal clinical reaction on inoculation into rinderpest-susceptible cattle.

c) Efficacious

Inducing an immunity to rinderpest in rinderpest-susceptible cattle.

These tests have already established the safety and efficacy of the master seed stock of LA-AKO and that of the Plowright vaccine held at the RHF in France.

2.2. Method of manufacture

2.2.1. Procedure

Individual vaccine batches are prepared by infecting cell cultures and, after an appropriate incubation period, harvesting either the overlying media or the media and infected cells together. Virus should be harvested from cultures not more than 7 days (LA-AKO) or 10 days (Plowright) after the date that these cultures were infected. The decision to harvest should be based on the development of extensive characteristic CPEs within the cell monolayer.

To constitute a batch, infected cultures must have been inoculated with the same seed virus and incubated and harvested together.

To form a bulk suspension, the harvest should be clarified by low-speed centrifugation or by filtration before mixing with cryoprotectant.

Multiple harvests are permissible from the same set of cultures and may be pooled to form a single bulk suspension. For long-term storage and cold chain distribution, bulk suspensions are freeze dried.

Written records must accompany all stages of vaccine manufacture.

2.2.2. Requirements for substrates and media

i) Cells

Plowright vaccine may be grown in primary kidney cells from BVDV-free bovine embryos or calves, or cells derived by up to ten serial subcultures from such primary cells ~~either of these sources~~. In ~~addition~~ preference, the vaccine ~~may~~ should be manufactured in ~~approved~~ validated and tested continuous cell lines; Vero cells have been used for this purpose. The master seed stocks of LA-AKO are normally prepared in embryonated SPF chick eggs~~.~~, while Vero cells are used for the production of working/production seed stocks or vaccine ~~of LA-AKO~~. In all cases, the cells should be shown not to be infected with adventitious viruses including bovine viral diarrhoea virus (BVDV), bovine leukaemia virus (BLV), bovine rotavirus and bluetongue virus (BTV), and should be maintained in a seed lot system.

ii) Media

In all cases the bovine (fetal or new-barn calf) serum used must come from countries that are FMD-free and with a negligible risk of bovine spongiform encephalopathy; it must be shown to be free from BVDV and any other adventitious viruses; the use of gamma-irradiated serum is recommended.

Calf kidney cells are grown and maintained in Earle's Balanced Salts Solution or Eagle's Minimum Essential Medium [MEM] supplemented with 0.5% lactalbumin hydrolysate and 0.1% yeast extract together with 5% new-born calf serum ~~that must come from rinderpest-susceptible animals and originate from countries with negligible risk of bovine spongiform encephalopathy~~.

Vero cells are grown in Eagle's MEM supplemented with 10% heat-treated fetal calf serum ~~and~~, 0.295% tryptose phosphate broth (TPB), and ~~with~~ antibiotics as required. Other formulations of medium have been used, e.g. Glasgow Modified Eagle's medium (GMEM) supplemented with 14% (v/v) TPB and 6% (v/v) non-heat-treated ~~(rinderpest antibody free)~~ bovine serum, with antibiotics as required. ~~All serum must come from rinderpest susceptible animals and originate from countries with negligible risk of bovine spongiform encephalopathy.~~

iii) Cryoprotectant

For lyophilisation, the bulk suspension of virus is mixed with an equal volume of a solution containing either 5% lactalbumin hydrolysate and 10% sucrose, or 1% sodium glutamate, 0.3% polyvinylpyrolidone and 10% sucrose.

2.2.3. In-process controls

To ensure the properties of a master seed stock, a marker test should be undertaken where possible. A virus titration must be undertaken on each batch of a bulk suspension, and on the final bulk suspension itself, using tenfold virus dilutions in a microplate or roller tube system and employing four to ten replicates per dilution. Each batch of the final bulk suspension, or the final bulk suspension itself, should also be examined for adventitious viral contamination by relevant assays, including one or more of the following:

i) Samples are mixed with a neutralising titre of rabbit anti-rinderpest antiserum, added to continuous cultures of Vero cells, bovine kidney or testicular cells, and incubated at 37°C for 7 days. These cells must not develop any CPE within the incubation period.

ii) Samples are inoculated onto an African monkey-derived embryonic kidney cell line, MA-104, which is reported to be highly susceptible to Simian rotavirus (Smith *et al.,* 1979). Inoculated MA-104 cells must not develop CPE.

iii) A 10 ml of the sample from the batch of suspension clarified harvest or bulk suspension is mixed with a neutralising titre of rabbit anti-rinderpest antiserum and inoculated into a bovine leukemia virus (BLV)-susceptible sheep via an intramuscular route. The sera obtained from the sheep at 2 and 3 months after inoculation should be examined for the presence of BLV antibodies by an agarose gel immunodiffusion test.

The batch of a clarified harvest or bulk suspension may also be subjected to a marker test if available. LA-AKO vaccine induces a marked increase in the size of the spleen in inoculated chick embryos. 15 µl of 10- and 100-fold dilutions of a sample from a final bulk suspension are inoculated into a blood vessel of more than ten eggs each on day 11 to 12 after laying. Inoculated eggs are incubated at 38°C for 5 days. Spleens of inoculated chick embryos which are still alive after incubation are collected and weighed. These spleens become heavier than 15 mg in weight.

Checks for adventitious viral contamination should be undertaken on at least two uninfected control cell cultures prepared from the cell suspension used in batch production, after having been maintained using the same media and incubation conditions as the rinderpest-infected cells. They must be subjected to frequent in-process microscopic observations with negative results. After virus harvesting, the control cultures should be washed to remove bovine serum and re-incubated for 10 days in media containing bovine serum substitutes during which period they are again subject to frequent microscopic observations for evidence of cytopathic change. At the end of this period at least one culture should be examined for the presence of noncytopathic BVD virus using an immunofluorescence or immunoperoxidase test or RT-PCR.

The control cultures may also be examined for haemadsorption activity. The uninfected cultures should be washed to remove bovine serum, and divided into two groups. Each group is overlaid with 0.1% suspension of guinea-pig or goose red blood cells (RBCs) for 1 hour, then subjected to microscopic observation. The control cultures must not adsorb RBCs from either of those species.

Prior to lyophilisation, the batch of a clarified harvest or bulk suspension may be held for not more than 5 days at 4°C, but considerably longer storage is achievable if frozen at –20°C to –80°C.

2.2.4. Final product batch tests

i) Sterility and purity

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9.

The final batch product consists of the freeze dried vials produced from a single bulk suspension; a batch may contain several filling lots. The contents of one container from each filling lot must be exposed to neutralisation by rabbit rinderpest antiserum, using a varying virus/constant serum method, and inoculated into primary bovine kidney or other susceptible cells. The identity of the product is established if no rinderpest-specific CPE develops.

ii) Safety and efficacy

~~Procedures may present slight variations depending on the country and system of production.~~ For the established ~~virus~~ vaccine seed stocks (Section 2.1.1), animal-based testing of safety and

efficacy ~~may be deemed~~ is unnecessary. For any other candidate vaccine that differs at the sequence level from the reference vaccines, animal tests for assessing the safety and efficacy of the vaccine will be required but may only be conducted with the prior approval of the FAO-WOAH Rinderpest Secretariat.

Animals used in these procedures should be kept in isolation from other rinderpest-susceptible animals. At the end of the procedures they must be killed and the carcases disposed of securely. Using rinderpest-susceptible cattle, the contents of one to five randomly selected vials are pooled and used to inoculate each of two or three cattle with a volume equivalent to a single cattle field dose (where a field dose is taken to be ≥ 1000 $TCID_{50}$). In addition, one bovine may be inoculated with a volume equivalent to 100 cattle field doses. These animals are maintained in a biologically secure animal facility for the following 2–3 weeks. During this period the animals are subjected to daily temperature recording and frequent clinical inspections. At the end of this period, the cattle are examined for the presence of rinderpest neutralising serum antibodies (Section B.2.2). The vaccine is considered safe and efficacious if it does not induce any abnormal clinical reaction except for slight hyperthermia and if all vaccinated animals show a RPV-neutralising titre of 1/10 or greater.

In general terms, the safety of the Plowright vaccine has been widely demonstrated in both European and Indian breeds of cattle and Dwarf West African breeds. It has not been tested in Japanese or Chinese breeds and its safety in such animals cannot be guaranteed. The LA-AKO vaccine has been tested for ~~the~~ safety in a highly susceptible breed, Japanese black, as well as in the Holstein breed.

iii) Batch potency

The close relationship between immunising potency and infectivity allows the latter to be used as the basis for potency estimations. Infectivity titrations are undertaken using cells of an approved continuous line or cells grown from each of three different bovine calf or embryonic kidneys. The number of estimates of the virus titre, and the number of vials pooled for each estimate, should be determined depending on the batch size and the local reproducibility of the assay. The sensitivity of the cells used in each working session must be measured using a standard laboratory RPV preparation of an approved facility. The final titre is the geometric mean of all estimates, each undertaken using tenfold dilutions and four to ten observations per dilution. Potent vaccine should contain ≥ 100 field doses per vial.

2.3. Requirements for ~~authorisation~~ regulatory approval

2.3.1. Safety requirements

i) Target and non-target animal safety

Plowright vaccine causes no clinical signs in rinderpest susceptible cattle or Asian water buffaloes. LA-AKO vaccine causes no clinical signs except slight pyrexia in rinderpest-susceptible cattle. Neither spread by contact transmission to rinderpest susceptible cattle housed in close proximity to vaccinates.

ii) Reversion to virulence

Plowright vaccine virus retains its attenuated characteristics during at least five back passages in cattle and lacks the ability to spread by contact. Any sub-strain of the Plowright or LA-AKO strains used in the manufacture of rinderpest vaccine must be identifiable by written historical records that trace its origins to either of these vaccine strains.

iii) Environmental considerations

There are no environmental considerations with respect to either the manufacture or application of rinderpest vaccine.

2.3.2. Efficacy requirements

i) For animal production

Both vaccines protect vaccinated animals from clinical disease caused by virulent RPV infection.

ii) For control and eradication

For eradication purposes the object should be to use vaccine to immunise all susceptible animals in and around the vicinity of an outbreak in as short a period of time as possible (Taylor *et al.,* 2002).

2.3.3. Stability

Both the Plowright and LA-AKO strains of TCRV are highly stable when correctly freeze-dried and will keep for long periods at either +4 or –20°C provided the product retains a vacuum or is filled with nitrogen gas. The rate of degradation of lyophilised TCRV can be altered by the choice of cryoprotectant and by variations in the drying cycle. Good results have been obtained with the use of (a) a 5% lactalbumin hydrolysate/10% sucrose stabiliser, a 72–74 hour drying cycle under reduced vacuum (≤ 13 Pa), initial drying for 16 hours at –30°C, and a final shelf temperature of 35°C, or (b) a 1% sodium glutamate/0.3% polyvinylpyrolidone/10% sucrose stabiliser, a 48 hour drying cycle under reduced vacuum (≤ 10 Pa), initial drying for 24 hours at –45°C, a final shelf temperature of 22°C, and filling the vial with nitrogen gas.

Following reconstitution in either normal saline or 1M magnesium sulphate, the virus becomes much more thermolabile. The period for field distribution of reconstituted vaccine should not exceed its half-life, but as this parameter is temperature dependent and varies between 8 and 24 hours over a range from 4°C to 37°C, a universal period of 4 hours can be recommended.

3. Vaccines based on biotechnology

No biotechnology-based vaccines have so far been approved.

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678 **NB:** There are WOAH Reference Laboratories for rinderpest (please consult the WOAH Web site:
679 https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
680 Please contact the WOAH Reference Laboratories for any further information on
681 diagnostic tests, reagents and vaccines for rinderpest

682 **NB:** FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2018.

683 **Appendix 1: Rinderpest**
684 **Intended purpose of test: population freedom from infection**

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
Virus neutralisation ++ Any species of wild or domestic *Artiodactyla*	Serum Antibodies to RPV capable of preventing infection of cultured cells	Virus neutralisation is the reference test for other serological tests	See reference	See reference	Only currently available technique for determination of serum antibody that neutralises RPV Is the reference test that other tests for serum antibody are measured against	Very high titres of PPRV-neutralising antibody can cross-react (although such titres would be rare in cattle) Is not suitable for screening large numbers of samples, so using it for population surveillance is not recommended, although currently it is the only option Requires cell culture facilities and staff with specific experience in the lab doing such a test Requires use of live RPV, so can only be carried out in FAO/WOAH-approved RHFs with specific permission	Rossiter & Jessett (1982)

685 **Appendix 2: Rinderpest**
686 **Intended purpose of test: individual animal freedom from infection prior to movement**

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
Conventional RT-PCR +++	Anti-coagulant-treated blood (EDTA better than heparin); ocular or nasal swabs can also be used. Virus specific RNA (N or F gene)	This and real-time RT-PCR are the reference tests against which other tests are measured	Samples from infected and uninfected cattle (References 1 & 2) or cell-culture-grown virus (Reference 3)	See references	Highly sensitive Widely available technology Can use simple-to-obtain samples	Only indirect controls for the assay function are available Proper positive controls for the assays are only available in FAO/WOAH-approved RHFs which have specific permission for their use	1. Forsyth & Barrett (1995) 2. Forsyth *et al.* (2003) 3. Couacy-Hymann *et al.* (2006)
Real-time RT-PCR +++	Anti-coagulant-treated blood (EDTA better than heparin); ocular or nasal swabs can also be used. Virus specific RNA (N or F gene)	This and conventional RT-PCR are the reference tests against which other tests are measured	Samples from infected and uninfected cattle	See reference	Highly sensitive Widely available technology Can use simple-to-obtain samples Real-time assay is even more sensitive than gel-based assay	Proper positive controls for the assays are only available in FAO/WOAH-approved RHFs which have specific permission for their use There is a lack of experience of use of the real-time assay in diagnosis	Carrillo *et al.* (2010)

Appendix 3: Rinderpest
Intended purpose of test: contribute to eradication policies

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
Virus isolation ±	Anti-coagulant-treated blood (EDTA or heparin); ocular or nasal swabs; lymph nodes, tonsils or spleen from acutely infected animals Live RPV	No data; old technique that was rarely applied once RT-PCR and ELISAs became available	No data; old technique that was rarely applied once RT-PCR and ELISAs became available	None	Isolation and preservation of strain of live virus which can be used for other studies	Requires good cell culture facilities and experienced staff Takes a long time (> 1 week) Has to be combined with RT-PCR anyway to confirm virus is RPV Entirely unsuitable for anything but small numbers of samples	
Conventional RT-PCR +++	Anti-coagulant-treated blood (EDTA better than heparin); ocular or nasal swabs can also be used. Virus specific RNA (N or F gene)	This and real-time RT-PCR are the reference tests against which other tests are measured	Samples from infected and uninfected cattle (References 1 & 2) or cell-culture-grown virus (Reference 3)	See references	Highly sensitive Widely available technology Can use simple-to-obtain samples	Only indirect controls for the assay function are available Proper positive controls for the assays are only available in FAO/WOAH-approved RHFs which have specific permission for their use	1. Forsyth & Barrett (1995) 2. Forsyth *et al.* (2003) 3. Couacy-Hymann *et al.* (2006)
Real-time RT-PCR +++	Anti-coagulant-treated blood (EDTA better than heparin); ocular or nasal swabs can also be used. Virus specific RNA (N or F gene)	This and conventional RT-PCR are the reference tests against which other tests are measured	Samples from infected and uninfected cattle	See reference	Highly sensitive Widely available technology Can use simple-to-obtain samples Real-time assay is even more sensitive than gel-based assay	Proper positive controls for the assays are only available in FAO/WOAH-approved RHFs which have specific permission for their use There is a lack of experience of use of the real-time assay in diagnosis	Carrillo *et al.* (2010)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
Virus neutralisation ++ Any species of wild or domestic *Artiodactyla*	Serum. Antibodies to RPV capable of preventing infection of cultured cells	Virus neutralisation is the reference test for other serological tests	See reference	See reference	Only currently available technique for determination of serum antibody that neutralises RPV Is the reference test that other tests for serum antibody are measured against	Very high titres of PPRV-neutralising antibody can cross-react Is not really suitable for screening large numbers of samples so using it for population surveillance is not recommended, although currently it is the only option Requires cell culture facilities in lab doing such a test Requires use of live RPV, so can only be carried out in FAO/WOAH-approved RHFs with specific permission	Rossiter & Jessett (1982)

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Appendix 4: Rinderpest
Intended purpose of test: confirmation of clinical cases

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
Virus isolation ±	Anti-coagulant-treated blood (EDTA or heparin); ocular or nasal swabs; lymph nodes, tonsils or spleen from acutely-infected animals Live virus	No data; old technique that was rarely applied once RT-PCR and ELISAs became available	No data; old technique that was rarely applied once RT-PCR and ELISAs became available	None	Isolation and preservation of strain of live virus which can be used for other studies	Requires good cell culture facilities and experienced staff Takes a long time (> 1 week) Has to be combined with RT-PCR anyway to confirm virus is RPV Entirely unsuitable for anything but small numbers of samples	
Conventional RT-PCR +++	Anti-coagulant-treated blood (EDTA better than heparin); ocular or nasal swabs can also be used. Virus specific RNA (N or F gene)	This and real-time RT-PCR are the reference tests against which other tests are measured	Samples from infected and uninfected cattle (References 1 & 2) or cell-culture-grown virus (Reference 3)	See references	Highly sensitive Widely available technology Can use simple-to-obtain samples	Only indirect controls for the assay function are available Proper positive controls for the assays are only available in FAO/WOAH-approved RHFs which have specific permission for their use	1. Forsyth & Barrett (1995) 2. Forsyth *et al.* (2003) 3. Couacy-Hymann *et al.* (2006)
Real-time RT-PCR +++	Anti-coagulant-treated blood (EDTA better than heparin); ocular or nasal swabs can also be used. Virus specific RNA (N or F gene)	This and conventional RT-PCR are the reference tests against which other tests are measured	Samples from infected and uninfected cattle	See reference	Highly sensitive Widely available technology Can use simple-to-obtain samples Real-time assay is even more sensitive than gel-based assay	Proper positive controls for the assays are only available in FAO/WOAH-approved RHFs which have specific permission for their use There is a lack of experience of use of the real-time assay in diagnosis	Carrillo *et al.* (2010)

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Appendix 5: Rinderpest
Intended purpose of test: prevalence of infection – surveillance

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
Conventional RT-PCR ±	Anti-coagulant-treated blood (EDTA better than heparin); ocular or nasal swabs can also be used. Virus specific RNA (N or F gene)	This and real-time RT-PCR are the reference tests against which other tests are measured	Samples from infected and uninfected cattle (References 1 & 2) or cell-culture-grown virus (Reference 3)	See references	Highly sensitive Widely available technology Can use simple-to-obtain samples	Only indirect controls for the assay function are available Proper positive controls for the assays are only available in FAO/WOAH-approved RHFs which have specific permission for their use	1. Forsyth & Barrett (1995) 2. Forsyth *et al.* (2003) 3. Couacy-Hymann *et al.* (2006)
Real-time RT-PCR ±	Anti-coagulant-treated blood (EDTA better than heparin); ocular or nasal swabs can also be used. Virus specific RNA (N or F gene)	This and conventional RT-PCR are the reference tests against which other tests are measured	Samples from infected and uninfected cattle	See reference	Highly sensitive Widely available technology Can use simple-to-obtain samples Real-time assay is even more sensitive than gel-based assay	Proper positive controls for the assays are only available in FAO/WOAH-approved RHFs which have specific permission for their use There is a lack of experience of use of the real-time assay in diagnosis	Carrillo *et al.* (2010)
irus neutralisation ++ Any species of wild or domestic *Artiodactyla*	Serum Antibodies to RPV capable of preventing infection of cultured cells	Virus neutralisation is the reference test for other serological tests	See reference	See reference	Testing for antibody is superior to testing for virus RNA or antigen in surveillance due to the long period during which antibody can be detected after infection Only currently available technique for determination of serum antibody that neutralises RPV Is the reference test that other tests for serum antibody are measured against	Very high titres of PPRV-neutralising antibody can cross-react Is not suitable for screening large numbers of samples, so using it for population surveillance is not recommended, although currently it is the only option. Requires cell culture facilities in lab doing such a test Requires use of live RPV, so can only be carried out in FAO/WOAH-approved RHFs with specific permission	Rossiter & Jessett (1982)

Appendix 6: Rinderpest

Intended purpose of test: Immune status in individual animals or populations post-vaccination

Test with score and species	Sample type and target analytes	Accuracy	Test population used to measure accuracy	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
Virus neutralisation ++ Any species of wild or domestic *Artiodactyla*	Serum. Antibodies to RPV capable of preventing infection of cultured cells	Virus neutralisation is the reference test for other serological tests	See reference	See reference	Only currently available technique for determination of serum antibody that neutralises RPV Is the reference test that other tests for serum antibody are measured against	Very high titres of PPRV-neutralising antibody can cross-react Is not suitable for screening large numbers of samples, so using it for population surveillance is not recommended, although currently it is the only option Requires cell culture facilities in lab doing such a test Requires use of live RPV, so can only be carried out in FAO/WOAH-approved RHFs with specific permission	Rossiter & Jessett (1982)

Annexe 18. Chapitre 3.2.4. Infestation des abeilles mellifères par le petit coléoptère des ruches (*Aethina tumida*)

RÉUNION DE LA COMMISSION DES NORMES BIOLOGIQUES
Paris, 9–13 septembre 2024

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CHAPTER 3.2.4.

INFESTATION OF HONEY BEES WITH *AETHINA TUMIDA* (SMALL HIVE BEETLE)

SUMMARY

***Description and importance of the disease**: The small hive beetle,* Aethina tumida ~~*(Murray 1867)*~~ (Coleoptera: Nitidulidae), *is a parasite and predator of honey bees. Adults and larvae of small hive beetles feed on honey bee brood, honey and pollen. While feeding on food stores the remaining honey is fermenting and the comb is destroyed. The beetles can promote structural collapse of the nest and cause the adult honey bees to abscond from severely infested colonies. The extent of beetle-associated damage depends on climate, colony strength and other conditions. Small hive beetles tend to be more problematic in areas with warm temperatures and high humidity. The small hive beetle can be a serious problem in honey-extracting facilities where stored comb, honey and wax cappings are potential feeding and breeding areas. Beetle development from egg to adult requires 3–12 weeks, depending on humidity, temperature and food availability. The flying adult beetles actively infest honey bee colonies of all strengths and sizes.*

***Detection and identification of the agent:** An infestation by the small hive beetle can be recognised either indirectly via colony-wide damage associated with the beetle or directly via eggs, larvae and adults. An early diagnosis can be made after opening the colony and finding adult beetles under the colony lid, on the bottom board, or hiding in the combs (especially peripheral combs). In zones at risk of new introductions, it is recommended to install sentinel apiaries that consist of fully functional queenright honey bee colonies to attract and trap adult beetles. Different trap models can help to detect and capture A. tumida. Definitive diagnosis at the laboratory is based on morphological examination under a stereomicroscope. Confirmatory testing can be done by real-time polymerase chain reaction.*

***Serological tests:** Serological tests are not applicable.*

***Requirements for vaccines:** No vaccines are available.*

A. INTRODUCTION

The small hive beetle (hereafter referred to as "beetle"), *Aethina tumida,* order *Coleoptera,* family *Nitidulidae* ~~(Murray, 1867)~~, is native to sub-Saharan Africa ~~(Hepburn & Radloff, 1998)~~ but has been found in various regions of the world over the past ~~few~~ decades. *Aethina tumida* was first detected in the United States of America in 1996. Since then it has spread to Canada and a number of countries in South and Central America. *Aethina tumida* has also been found in Australia, Egypt, Italy, ~~Korea and the Philippines~~ in several Asian countries and in the Mascarene region of the Indian Ocean (WOAH WAHIS Interface, database accessed on ~~20/06/2017~~ 02/01/2024; Lee *et al.,* 2017; Liu *et al.,* 2021).

Aethina tumida is originally a pest of the honey bee *Apis mellifera.* However, since its introduction in Asia, several studies have shown that it can maintain and multiply in colonies of the Asian honey bee species *Apis cerana* (Cervancia *et al.,* 2016; Liu *et al.,* 2021). The beetle can also infest other social pollinators such as bumblebees (Hymenoptera: Apidae, Bombini) and stingless bees (Hymenoptera: Apidae, Meliponini), which are found in tropical and subtropical regions of America, Africa and Australia (Neumann *et al.,* 2016). Finally, certain species of solitary bees, which have

small reserves of nectar and pollen, might constitute occasional hosts, for example in the absence of wild or domestic social bees (Gonthier *et al.*, 2019).

1. Life cycle

The infesting small hive beetle adults mate in the honey bee colony and the female beetles oviposit several eggs in typical clutches in small cracks or within capped brood cells (~~Cuthbertson *et al.*, 2013;~~ Ellis, 2005; Lundie, 1940). In some situations, more than 1000 adult beetles may occur within a colony ~~(Elzen *et al.*, 1999)~~. Adult beetles can survive up to 12 months (records indicate up to 16 months in laboratories~~; Somerville [2003]~~), but females ~~die quickly~~ usually have shorter lives when ~~ovipositing~~ oviposition occurs on a daily basis (Neumann *et al.*, 2016). Females ~~can~~ may oviposit about 1000 eggs in their lifetime (Lundie, 1940), ~~though Hood (2004) suggested~~ the upper limit may be 2000 eggs. Successful egg emergence is correlated with relative humidity, with fewer eggs hatching at a relative humidity of <50%. The larvae emerge from the eggs after 1–6 days (most within 3 days) and feed on pollen, honey and bee brood (Lundie, 1940~~; Schmolke, 1974~~). Adult beetles can be fed by worker bees via trophallaxis, especially while confined in bee-guarded "prisons" (Ellis, 2005). Larval development usually takes about 2 weeks (8–29 days depending on food availability and temperature; ~~de Guzman & Frake, 2007;~~ Ellis *et al.*, 2002; Lundie, 1940~~; Schmolke, 1974~~). Following this, the larvae reach the wandering phase and leave the colony to pupate in the soil surrounding the colony (Lundie, 1940). Pupation takes about 2–12 weeks depending on temperature and soil moisture ~~(Ellis *et al.*, 2004)~~. Emerging adults leave the soil and can fly to search for new host colonies, thereby completing their life cycle.

In laboratory conditions, the small hive beetle can survive and reproduce on ripe or rotten fruits ~~(Buchholz *et al.*, 2008)~~. Flowering plants might also constitute an alternative food resource for *A. tumida* in the absence of hosts, and thus contribute to its survival during a biological invasion (Gonthier *et al.*, 2019). Wild bees and feral honey bee colonies can act as a reservoir of *A. tumida*.

2. Impact of the pest

Small hive beetle is seldom a serious problem for beekeeping in Sub-Saharan Africa. The reasons for the apparent difference in its impact on colonies within its native range and those in its new ranges are not well understood ~~(Ellis & Hepburn, 2006)~~. They may include quantitative behavioural differences between African and European honey bee subspecies, different beekeeping techniques, climatic differences, or escape from natural enemies, among other plausible hypotheses ~~(Hood, 2004; Neumann & Elzen, 2004)~~.

While bee colony damage due to adult beetles is relatively minor, the adults can cause colonies to abscond (i.e. the adult bees completely abandon the nest~~; Ellis *et al.*, 2003~~). If not prevented, larval feeding behaviour is often associated with fermentation of stored honey, causes severe damage to combs and often results in the full structural collapse of the nest (Lundie, 1940). Economic losses also can be associated with beetle infestations in the honey-extracting facility. Environmental conditions generally associated with extracting facilities, such as high temperatures and humidity, provide optimal conditions for beetle development. Cryptic low-level reproduction may ~~also~~ occur either in the debris or underneath hive inserts without any signs of colony damage (Spiewok & Neumann, 2006).

The impact of the small hive beetle on *Apis cerana* is poorly documented. In some cases, *A. cerana* apiaries can be heavily infested, leading to significant colony losses (Liu *et al.*, 2021) and also absconding behaviour has been observed (Cervancia, 2016).

*C*ertain species of stingless bees are reared for honey production or pollination services. Knowledge regarding the impact of *A. tumida* on stingless bees is still limited. Healthy colonies seem to be able to defend themselves, for example by covering the adult beetles with propolis (Greco *et al.*, 2010).

Little is known about the effects of *A. tumida* on wild honey bees and bumblebees.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of infestation with Aethina tumida and their purpose

Method	Purpose					
	Population freedom from infestation[(a)]	**Individual animal or bee nest freedom from infestation prior to movement[(a)]**	**Contribute to eradication policies[(a)]**	**Confirmation of clinical cases[(a)]**	**Prevalence of infestation – surveillance[(a)]**	**Immune status in individual animals or populations post-vaccination**
Detection and identification of the agent						
Visual inspection of colonies	++	++	++	+++ (adults) +++ (larvae)	+++ (sentinel colonies or apiaries)	=
Morphology	+++	+++	+++	+++ (adults) + (larvae)	+++	–
Real-time PCR	++	++	++	++	+	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose. PCR = polymerase chain reaction.
[(a)]See Appendix 1 of this chapter for justification table for the scores given to the tests for this purpose.

1. Field detection

1.1. Adult beetles

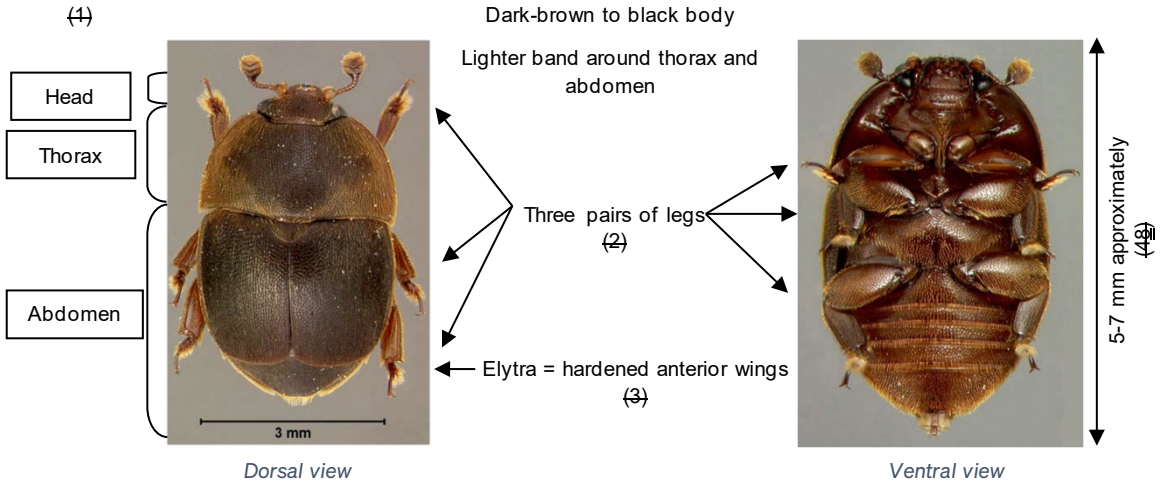

Fig. 1. Small hive beetle, Aethina tumida. Photographs by Lyle Buss (left) and Josephine Ratikan (right), University of Florida.

The first sign of an infestation by the small hive beetle is the occurrence of adult beetles (Figure 1). Adult beetles are ~5 mm long and ~3 mm wide, with females being slightly longer than males ~~(Ellis *et al.*, 2001)~~. The adults are dark brown to black (lighter brown shortly after ~~eclosion~~ emergence). During inspections, adults avoid sunlight, try to hide, and ~~can~~ might be observed while running for cover into corners or similarly over the combs. Adults can be confused with other Nitidulid beetles, which can also be associated with colonies (see Section 2.2.3 below for details; also Ellis *et al.* [2008], Marini *et al.* [2013] and Neumann & Ritter [2004]).

1.2. Beetle eggs, larvae and pupae

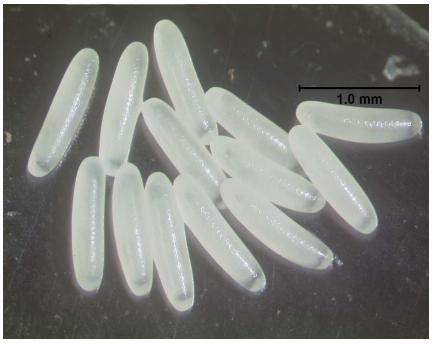

Fig. 2. Small hive beetle eggs. Photograph by Josephine Ratikan, University of Florida.

Small hive beetle eggs (Figure 2 are white, ~1.4 × 0.26 mm (length × width); ~2/3 of the size of a honey bee egg, and are deposited in clutches in cracks, on the bottom board, on the combs and underneath the cappings of sealed brood cells. Larvae (Figure 3) are whitish, up to ~1 cm long (wandering phase), have three pairs of legs, and have dorsal spikes. Larvae can be found mining in the wax combs (Lundie, 1940) or in colony debris (Spiewok & Neumann, 2006). Larval infestations are typically associated with a rotten smell due to death of honey bee brood or fermentation of the stored honey. Wandering larvae often leave smear trails (or "slime") inside and outside the colony (Figure 4). Once in the ground, the larvae excavate small pupation chambers (Figure 5) 1–20 cm deep in the soil ~~(Pettis & Shimanuki, 2000)~~, develop into pupae (Figure 6, whitish to dark brown depending on age, ~5 mm long and 3 mm wide) and then into adults. Most larvae tunnel into soil that is <180 cm from the colony ~~(Pettis & Shimanuki, 2000)~~.

Fig. 3. Dorsal (left) and ventral (right) view of a small hive beetle larva. Photographs by Josephine Ratikan, University of Florida.

Fig. 4. Comb damage attributed to the feeding/crawling habits of small hive beetle larvae. Notice the 'slime' on the frame (i.e. the wax comb looks 'wet' and it 'glistens'). This is caused by the fermentation of honey, which is moved around the comb by crawling larvae. Beetle larvae can be seen in cells in the centre of the comb, where brood was present originally. Photograph credit, University of Georgia.

Fig. 5. Small hive beetle larvae that has tunnelled into the soil and hollowed out a chamber in which to pupate. Photograph credit, University of Georgia.

Fig. 6. Small hive beetle pupa (ventral view). Photograph by Lyle Buss, University of Florida.

It is difficult to find beetle eggs in a colony, especially at low levels of infestation. Cracks or crevices around the nest should be investigated or capped brood cells that have small holes in the cappings, possibly indicating that a female beetle has punctured the capping and oviposited within the cell.

Small hive beetle pupae can be found by sifting the soil around the colony and looking for the pupal chambers or the pupae themselves.

1.3. Visual inspection of colonies

When monitoring honey bee colonies for the presence of small hive beetles, an examination of the hive may provide an early indication of infestation. In countries still free of *A. tumida*, it is recommended to monitor sentinel apiaries in zones at risk of an introduction, to detect an infestation at an early enough stage to eradicate it (Chauzat *et al.,* 2016). Furthermore, if the intent is to eradicate *A. tumida,* sentinel colonies should be placed in the location of infested apiaries that have been previously sanitised. Such sentinel colonies act as a bait to attract free-flying beetles and must be in place immediately after all colonies are destroyed, as adult beetles staying outside colonies might survive the eradication and spread further to other hosts nearby (Schäfer *et al.,* 2019). The use of sentinel colonies can also be an effective and timesaving approach to monitoring the presence and spread of small hive beetle in an infested territory (Formato *et al.*, 2021). Sentinel hives should be small to allow rapid visual inspection. They may consist of queenright honey bee colonies composed of one hive-box with three frames of brood and two frames with honey and pollen, and equipped with traps that can be quickly checked. They are inspected every 2–3 weeks (Formato *et al.*, 2021).

It is important to adapt the method of visual inspection to limit the expected spread of *A. tumida*, as colony manipulation might induce disorder and robbing (where bees steal honey from other colonies) or adult small hive beetles may occasionally leave the colonies during manipulation. The currently used visual inspection methods in infested areas are most feasible in the field and easy to carry out for anyone trained to manipulate a beehive (EFSA, 2015; Neumann *et al.,* 2016). Colony manipulation requires a certain minimum training and awareness of small hive beetle biology, behaviour and morphology, to correctly inspect field-colonies and to quickly detect and recognise damage caused by the different life stages of *A. tumida* (Cornelissen & Neumann, 2018). A colony inspection begins right at the entrance of the hive and relies on the rapid but meticulous examination of the lid, the inner cover, the frames and the bottom board. The following recommendations are from the EFSA scientific opinion (EFSA, 2015).

1.3.1. Colony inspection method (EFSA, 2015)

i) Remove the lid and check for the presence of adult beetles running away.

ii) Remove the inner cover and check both sides. Check also the top of the frames for running adults.

iii) Remove the frames from the hive one by one. Each side of the frame should be quickly observed to check the presence of adult beetles, larvae, eggs and damage. The first frame can be left outside the body of the hive to make it easier to handle the other frames. Subsequent frames should be put back into the body or super (the part of the hive in which bees store honey) to prevent robbing in the apiary during the examination.

iv) Beetles can hide inside the cells of combs. It is also important to examine the lid, the bottom board, the side faces, corners, interstices of the hive and hive components.

If robbing is unlikely, the super can be examined by placing it on the inverted lid of the hive in a sunny spot. Adults will escape from the sunlight and retreat down into the lid. After about 10 minutes, the presence of adult beetles in the lid can be checked by lifting the super ~~(Zawislak, 2014)~~. If there is a risk of robbing, the super should be inspected in the same way as the body of the hive, i.e. comb by comb, by replacing each frame in the box after its examination. During the examination of the body, the super can be placed on a reversed lid, so that no bees or beetles can escape (Spiewok *et al.,* 2007).

To improve the sensitivity of the visual inspection, the hive can first be removed from its original position, then opened and replaced by an empty hive (~~Neumann & Hoffmann, 2008;~~ Spiewok *et al.,* 2007). Each frame is then removed and examined for beetles for the first time. The bees are then shaken into an empty box and the comb is inspected for a second time for beetles, this time in the absence of bees, before being placed into the new hive. Once all the frames have been examined, the original hive box and bottom board are inspected. However, this method is more time-consuming, and requires additional beekeeping equipment and therefore is not suitable for routine monitoring of small hive beetle infestation in large apiaries. It is however, recommended for health certification to demonstrate the absence of *A. tumida* infestation in a colony.

A method, originally described in Canada, uses a white 12-litre bucket fitted with a wire-mesh screen (about 6 mm) fitted halfway down the depth of the bucket. The bottom of the bucket is covered with a thin layer of vegetable oil. The frames are shaken inside the bucket and bees are stopped unharmed by the wire-mesh

whereas beetles fall into the vegetable oil. Field data suggest that this method is more sensitive than simple visual inspection when the infestation level is low[4].

Another method for colony examination is described below. The method can be used to search for beetle adults and larvae, if larval infestations are moderate to high (~~Ellis *et al.*, 2002a;~~ Ellis & Delaplane, 2006), but should not be used if eradication is planned, as it might increase the number of free-flying beetles.

Fig. 7. Inspecting a colony for adult small hive beetles. The inspector on the right has shaken the bees onto a piece of plywood. Both faces of the framed comb were then bounced onto the wood to dislodge the beetles from the cells. The inspector on the left is shifting through the adult bees and using a mouth aspirator to collect the beetles. Photograph by Keith Delaplane, University of Georgia.

Notes:

i) This procedure is best accomplished with two people, one to work the colony and the second to collect the beetles if quantification is desired. Only one person is needed if beetle detection is the sole desired outcome.

ii) Some beetles inevitably fly away or hide from view during this procedure. The number of beetles that escapes is presumed to be low (<5%).

iii) This procedure is best used for qualification of adult beetles. However, larval beetles can be found this way as well.

a) Place a sheet of opaque plastic (~2 × 2 m, preferably white or light in colour) or plywood in front of the colony which you want to inspect for beetles.

b) Lightly smoke the colony.

c) Remove the lid from the colony and bounce the lid on the plywood. This should be done to dislodge all adult bees and beetles adhering to the lid.

d) A second individual (the beetle collector) should comb through the bees (this can be done with the hand or using a small stick) and collect all adult beetles seen using an aspirator. All bees on the plywood should be inspected as beetles can easily be concealed by clusters of bees (Figure 7).

e) Remove the outermost frame in the uppermost super (i.e. the uppermost “box” containing bees) and shake the bees from the frame onto the plywood.

f) The beetle collector should repeat step d.

g) Once the bees have been shaken from the frame, the frame should be turned onto its face and bounced against the plywood to dislodge adult beetles from the comb. This step should be repeated two-to-three times for both sides of the frame.

h) The beetle collector should repeat step d.

i) The individual working the colony should repeat step g to all frames in the uppermost super and then bounce the empty super on the plywood. This step should be repeated for all supers, all frames, and the bottom board of the colony.

The latter two described colony inspection methods are time-consuming and there is a very high risk of inducing disorder and robbing in the apiary (EFSA, 2015) and a risk of making the beetles fly away.

1.4. Colony examination using traps

[4] ~~http://www.omafra.gov.on.ca/english/food/inspection/bees/2011-shb-report.htm~~

The use of traps for small hive beetle detection has been described in the Guidelines for the surveillance of the small hive beetle (*Aethina tumida*) infestation (updated version: April 2016) developed by the European Union reference laboratory for honey bee health (Chauzat *et al.,* 2016).

The principle of most small hive beetle traps is to offer shelter from bee aggression by providing a passage that is large enough for beetles but too small for bees to enter. In their attempt to get away from the bees that chase them, *A. tumida* adults will enter the trap in which oil or veterinary medicines may be used as a killing agent. Sometimes this principle is combined with the use of bait that can increase trap efficacy. The position of the trap inside the hive is important and has to be adjusted to the hive type and to climatic conditions as beetles may hide on the bottom boards or in the periphery of the colony if climatic conditions are warm but tend to stay within the clustering bees when temperatures are low. Therefore, traps are available for all positions in the hive and all of them should be checked regularly during apiary visits.

Traps that are placed between frame top-bars consist of small containers that are covered by a grid. These kinds of traps are usually filled with vegetable oil ~~(diatomaceous earth was successfully tested in the laboratory; Cribb *et al.,* 2013) and they were shown to be effective in North American conditions (Bernier *et al.,* 2015)~~. The trap is placed between the top-bars of two frames, close to the brood nest or the winter cluster. When visiting the colony, traps are examined for the detection of any beetles. If the container is transparent, this observation is easy and straightforward. It was shown using these kinds of traps that bees might seal the openings with propolis thereby reducing their efficacy ~~(Bernier *et al.,* 2015)~~. Care must also be taken to prevent any oil spill.

In the warm season, traps placed on the bottom boards or modified bottom boards could be used for the detection of adult small hive beetles. Modified bottom boards usually consist of an oil filled tray that is placed underneath a grid or a mesh-screen. If the tray is covering the whole bottom, the hive must be levelled, but some of these traps cover only parts of the bottom board. Although these traps work well, they require hive-modification and therefore are mainly feasible for stationary beekeeping.

Fig. 8. Corrugated plastic insert used to detect adult small hive beetles. The plastic insert contains square flutes (left) in which adult beetles hide when inserted onto the bottom board of a colony, through the colony entrance (right). The insert must be used in conjunction with a traditional solid bottom board rather than a screened bottom board. Photographs by James Ellis (left) and Stephanie Kimball (right), University of Florida.

Many traps were invented for use on the bottom board. One example without any bait and killing agent is a 4 mm corrugated plastic strip (Figure 8). It was shown to be effective in field trials in Australia and the USA. The corrugated plastic suits the thigmotactic behaviour of *A. tumida* as it consists of square flutes, big enough for the beetle to get in, but too small for the bees to enter (approx. 4 × 4 mm). The hive does not need to be opened as traps are placed inside the hive through the entrance. It is important to properly place the trap in contact with the solid floor of the hive. If not, beetles can seek refuge in the space located between the trap and the floor. For optimum use, traps should be left in hives for a minimum of 48 hours before they are checked. The trap should preferably be made of a transparent material so that beetles can quickly be detected ~~(Schäfer *et al.,* 2008)~~. Other bottom board traps bring the beetle in contact with killing agents inside the traps. Chemicals can have high efficacy but there is always a risk that resistant strains may develop, residues might accumulate in honey or other hive products or that the chemicals could spread to the bees leading to adverse side effects on them. An alternative to the use of chemicals is diatomaceous earth or traps that just use adhesive film.

A biomechanical way of trapping small hive beetle inside the hive is the placement of ~~kitchen wipes or similar material~~ non-woven microfibre wipes (such as those commonly used in the household) on top of the frames. The bees ~~shred~~ chew this material, which becomes fuzzy and fray to the point where ~~into fibres in which the~~ beetles become entangled in this fibre. This control method is very simple to use and economical ~~control method has the advantage of functioning without any lethal substance, but fibres might also end up in the honey~~. However, Buteler *et al.* (2023) demonstrated that the fibres of these wipes contaminate the honey and the bees with microplastic. Although the acute toxicity of these microfibres for bees is low, chronic effects could be observed in the event of repeated exposure (for example, impact on immunity, on the diversity of the microbiome, and more generally on mortality). The microplastic may also present a risk to public health, in connection with the consumption of honey.

In low infested areas it is especially recommended to always undertake a combination of visual inspections and traps to increase the sensitivity of detection. Depending on the seasonal conditions, it may be decided to use either visual observation or traps, but, whenever possible, combining them both is best. In apiaries where inspections are frequently undertaken (sentinel apiaries), surveillance traps may be used. For single inspections, visual inspections may be best because of the higher detection sensitivity and to avoid a return visit to check the trap.

For a more detailed description of different traps refer to EFSA (2015) and Neumann *et al.* (2016).

2. Laboratory identification

Rapid and reliable diagnosis is crucial for the implementation of sanitary measures and to avoid spread in non-infested territories. Suspect field specimens should be sent to official laboratories for confirmation of *A. tumida* identification. Morphological identification is fast and inexpensive, and does not require sophisticated equipment. Confirmatory testing can be done by molecular methods (polymerase chain reaction [PCR]), and is particularly useful for larval identification or when specimens are damaged.

2.1. Special precautions required for sample handling

The specimens to be identified are collected in or near honey bee hives (for example, in colonies, beekeeping equipment or queen cages).

Suspect specimens should be killed before submission to the laboratory e.g. in 70% ethanol. Denatured ethanol should not be used where molecular methods are to be ~~used~~ performed because of possible PCR inhibition. Alternatively, specimens can be stored overnight at –20°C to kill the specimens.

On arrival at the laboratory packages should be opened in containment conditions. If the specimens are found to be alive on arrival, the submission should be placed at –80°C for approximately 1 hour before any work can be done with them. This procedure immobilises the specimens, which can subsequently be stored in 70% ethanol.

2.2. Morphological identification of adults and larvae

The test method aims to identify *A. tumida* by examining the external appearance of adults or larvae specimens in the laboratory. It consists of the visual examination of specimens noting morphological characteristics specifically selected to differentiate *A. tumida* from other nitidulid beetles and wax moth larvae, commonly found in honey bee colonies, queen cages or beekeeping equipment.

2.2.1. Equipment and reagents

Classical entomological materials are required for the morphological identification of *A. tumida*, including a stereomicroscope (or a magnifier), entomological tweezers, evaporating dishes (glass, plastic or porcelain) or Petri dishes, capped tubes for specimen storage, 70% ethanol (not denatured ethanol).

2.2.2. Test procedure

A general observation of the specimens should be made by placing them in a dish and checking for homogeneity (using a magnifier or stereomicroscope as necessary). If they are of uniform type, the samples can be processed further. If they are not uniform (i.e. multiple species may be present) then samples should be taken of each type present for further identification. When possible, select undamaged samples for further analysis, using entomological tweezers.

Microscopic examination should be done at different magnifications to visualise the critical identification criteria (see Section 2.2.3 below). Before observation, allow the ethanol to evaporate so that the colour of the specimens can be properly assessed.

The size of the specimens should be ~~measured~~ evaluated. The length of adult beetles is measured from the caudal to the cranial end of the specimens without taking into account the antennae and the mouthparts, which may possibly protrude from the body of the insect. The width of the body is measured at the widest part of the pronotum.

Samples can be compared with reference specimens if available.

After examination, beetles are stored in 70% ethanol.

2.2.3. Guidelines for the identification of *Aethina tumida*

Differentiation should be made between *A. tumida and* other non-pest nitidulid beetles that can be found in honey bee hives, for example: *Cychramus luteus*, found in Europe, that mainly feeds on pollen (Neumann & Ritter, 2004), *Carpophilus lugubris*, found in hives in Italy (Marini *et al.,* 2013), and *Glischrochilus fasciatus, Lobiopa insularis, Carpophilus dimidiatus* and *Epuraea corticina* found in hives in the United States (Ellis *et al.,* 2008).

The larvae of *A. tumida* can also be mistaken for larvae of the lesser wax moth, *Achroia grisella,* or the honeycomb (greater wax) moth, *Galleria mellonella*. These ~~lepidoptera~~ lepidopteran are generally found in colonies and on beekeeping equipment.

2.2.3.1. Adult form

Identification of adult *A. tumida* is based on the following morphological criteria: (Figures 1~~,~~ ~~and~~ 9 and 10)

1. Body divided in three parts: head, thorax and abdomen.
2. Three pairs of legs.
3. Presence of elytra: elytra are sclerotised (i.e. thickened) forewings covering the hind wings at rest in beetles and some other insects.
4. Elytra not covering the entire abdomen: some abdominal segments are apparent in dorsal view.
5. Overall uniform body colour (no spots), light brown to black when beetles are fully mature.
   Note: The colour may change with environmental conditions and conservation of the specimens.
6. Ends of antennae with compact, almost rounded clubs[2]. The three terminal articles of the antennae, corresponding to the "clubs", are narrowed between them. The transversal size of the first segment of the clubs (proximal article) is slightly larger than the other two segments (distal articles).
7. ~~Sharp~~ Posterio-lateral angles of the pronotum appear sharp (dorsal view)[3].
8. Dimensions: length: ~~5–7~~ 3–8 mm; width: ~~3–4.5~~ 2–4 mm ~~(approximately)~~
9. ~~Colour: reddish-brown when newly hatched, turning dark-brown to black in adulthood~~
   ~~Presence of a lighter band around thorax and abdomen (optional criterion)~~
   ~~Note: The colour may change with environmental conditions and conservation of the specimens~~
10. ~~Club-shaped antennae~~
11. ~~Sharp posterio-lateral angles of the pronotum~~

Notes:

i) The size of *A. tumida* can vary if the beetle is in a retracted (defensive) or "extended" position, and according to sex (Ellis *et al.,* 2002; Menier & Jouan, 2003). Factors such as food availability, climate or soil type could also influence size.

ii) *Aethina tumida* has a lighter border around the pronotum and elytra (row of fine yellow bristles). This characteristic is not always observable on dead specimens preserved in ethanol.

[2] In the Nitidulidae, the terminal articles of the antennae are larger and club-shaped.
[3] The pronotum corresponds to the dorsal part of the first segment of the thorax.

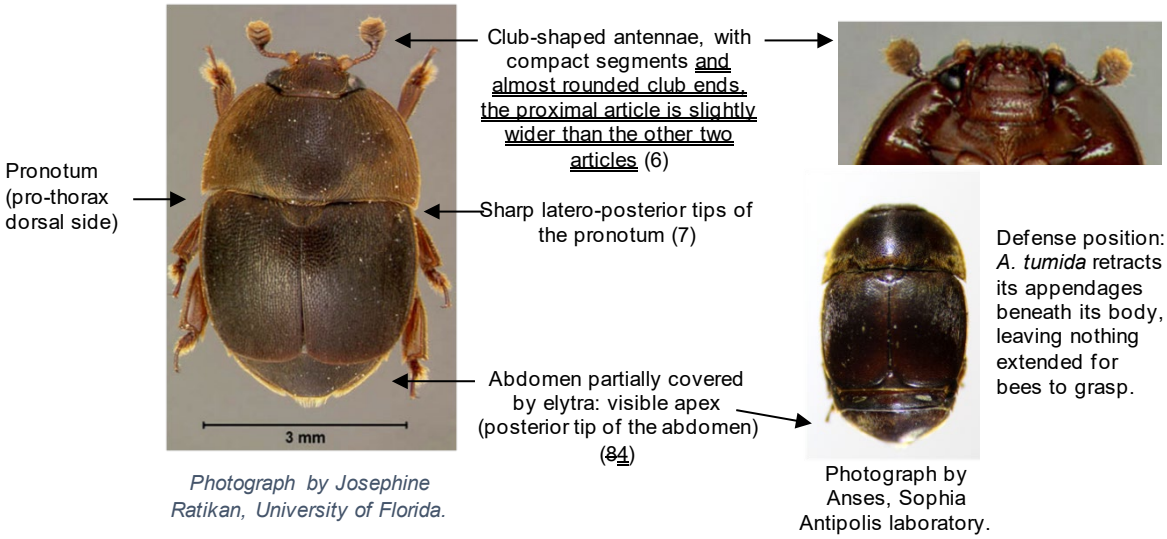

Fig. 9. Small hive beetle, Aethina tumida Murray.

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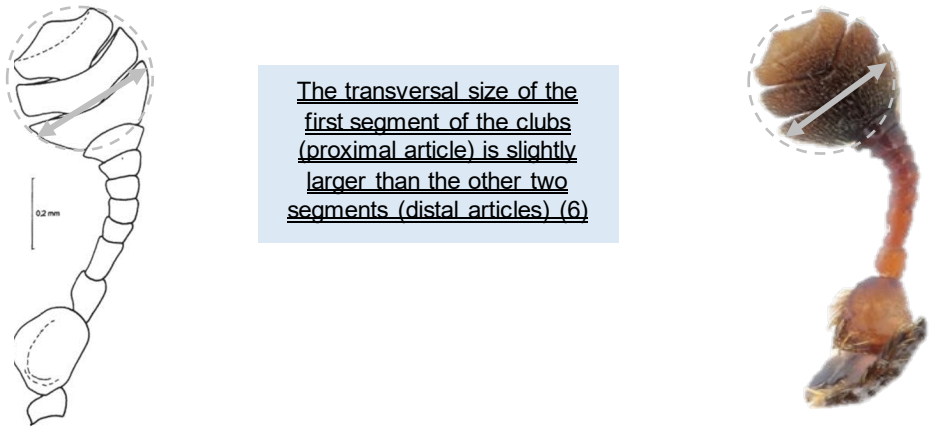


Fig. 10. Aethina tumida antenna (Photograph: Lee et al., 2017; Drawing: Menier & Jouan, 2003).

335 For differential diagnosis, *Cychramus luteus* is shown below with the following features (Figure ~~10~~
336 11; Neumann & Ritter, 2004):

337 elytra completely covers the abdominal apex;
338 antennal clubs are looser with detached segments;
339 latero-posterior tips of the pronotum are not sharp;
340 colour of the body is light-brown.

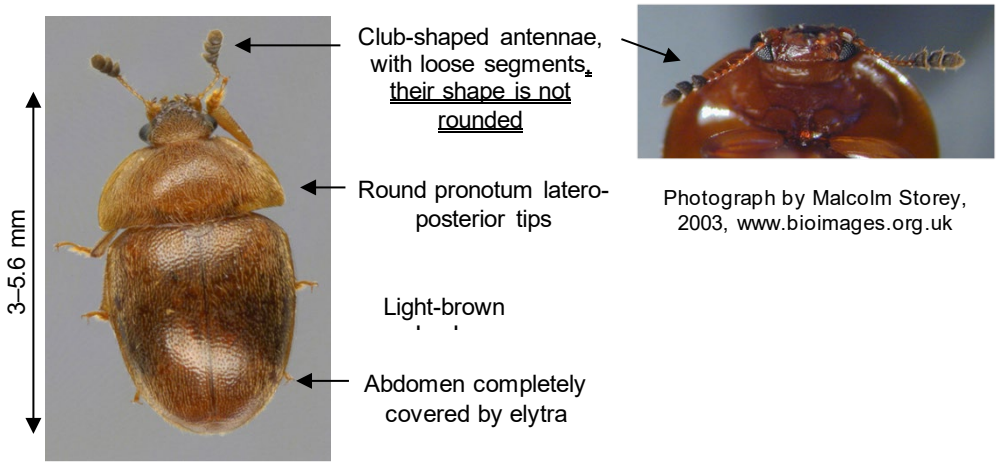


Fig. ~~10~~ 11. Cychramus luteus (Neumann & Ritter, 2004).

Carpophilus lugubris has the following characteristics: (Figure ~~11~~ 12; Marini *et al.,* 2013):

body is brown;
elytra have orange regions;
legs and antennae are orange (antennal clubs are dark orange);
body length: 3.3–4.5 mm.
~~However, as for *A. tumida:*~~
elytra do not cover the entire abdomen;
club-shaped antennae have compact segments, but their shape is oval rather than rounded and the proximal article is narrower that the distal ones;
latero-posterior tips of the pronotum are sharp.

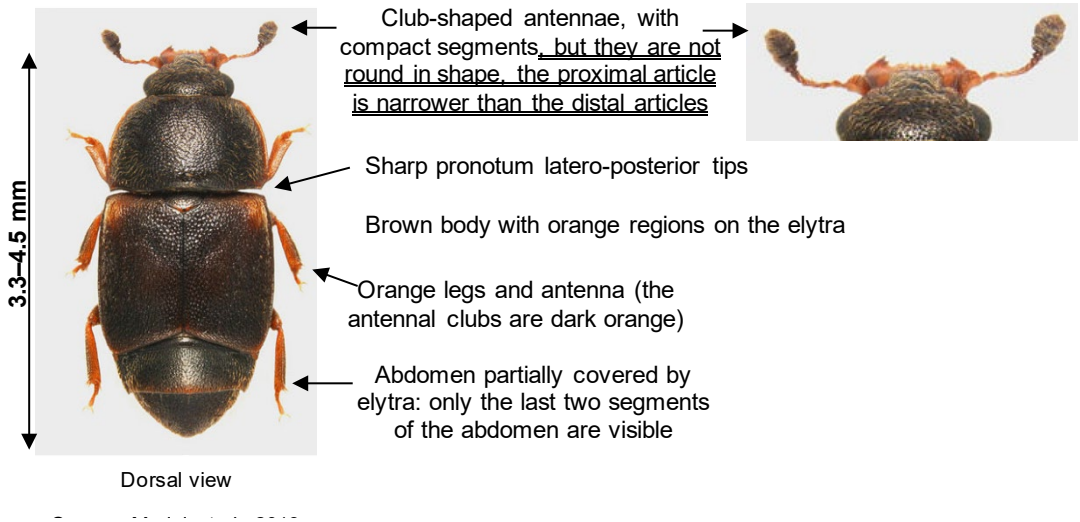


Fig. ~~11~~ 12. Carpophilus lugubris (Marini et al., 2013).

2.2.3.2. Larval form

Larvae of *A. tumida* have a light beige body. The cephalic capsule (head of the larva) is brown. The colour may change with environmental conditions and conservation of the specimens. The body length at maturity is about 1 cm (1.2 cm maximum) depending on feeding. The width is about 1.6 mm.

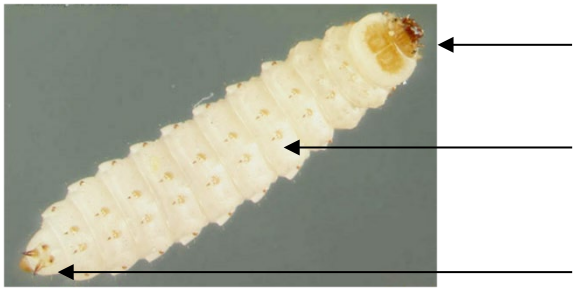
Larva identification is based on the following morphological criteria: (see Figure ~~12~~ 13 and 14).

1. Three pairs of legs, one on each of the anterior (thoracic) segments

2. All the posterior leg segments are bare and have no false legs (also called pseudopods) on their ventral part. ~~Two dorsal spines on each segment (these spines are thicker on the last segment)~~

3. From the mesothorax[4], presence on each segment, of two dorsal tubers on either side of the midline. These tubers are finished with a short fine silk. They look like 'spines'. ~~No false legs (pseudopods or prolegs) on the ventral side of the posterior abdominal segments.~~

Note: The dorsal tubers are pigmented. They are terminated by a short fine silk and preceded by two short spines. The last segment of the larva abdomen has two dorsal tubers and two urogomphi[5] (Figure 13).

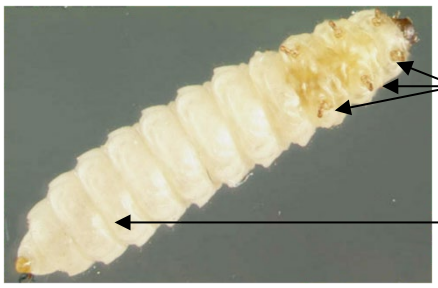


Light-beige body; Light brown cephalic capsule

Two rows of dorsal tubers ('spines') on each segment starting from the mesothorax) (~~2~~ 3)

Two ~~thick~~ dorsal tubers ('spines') and two urogomphs on last segment (~~2~~ 3)

Larva of *A. tumida*, dorsal view.



Three pairs of legs, one on each of the anterior segments (1)

No pseudopods on the ventral side of the posterior abdominal segments (~~2~~ 3)

Larva of *A. tumida*, ventral view.

Fig. ~~12~~ 13. Larva of Aethina tumida. Photographs by Josephine Ratikan, University of Florida.

[4] The mesothorax corresponds to the second thoracic segment of the larva. It has the second pair of legs. The prothorax corresponds to the first thoracic segment; it does not have a tuber, its dorsal part (tergum) is sclerified.

[5] Urogomphus is an extension, fixed or mobile, attached to one of the last segments of the abdomen of certain larvae.

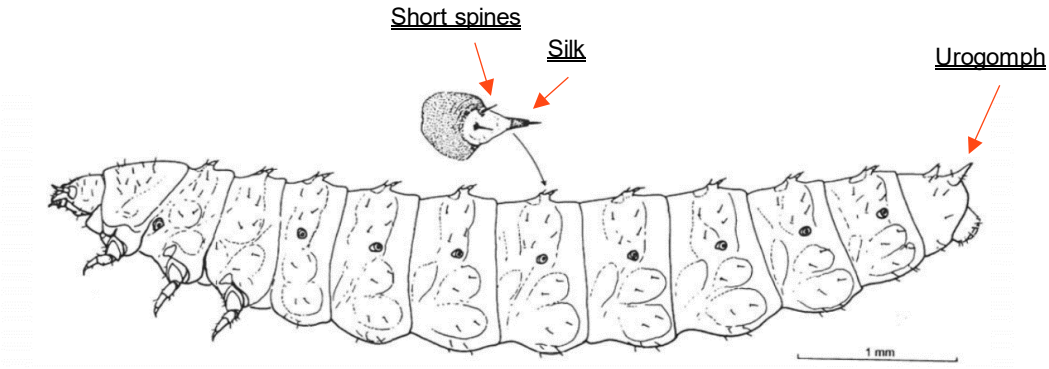


Fig. 14. Detail of dorsal tuber (Menier & Jouan, 2003)

To distinguish *A. tumida* larvae from Lepidoptera larvae (lesser wax moth, *A. grisella* and honeycomb/greater wax moth, *G. mellonella*), frequently present in honeybee hives:

The Lepidoptera larvae have pseudopods on the ventral side of the abdominal segments.

There are two bare segments between the last segment with legs and the first segment with pseudopods (Figure ~~13~~15).

The Lepidoptera larvae can make a silky web, cocoons, and have dark faeces (these webs and faeces may be observed in the sample containers received by the laboratory).

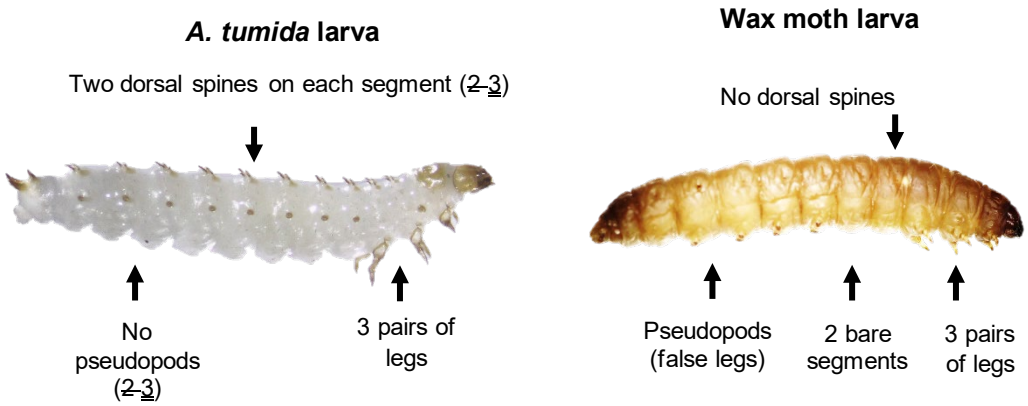


Fig. ~~13~~ 15. Differentiation of A. tumida from wax moth larvae. Photographs by Nicolas Cougoule. Anses, Sophia Antipolis laboratory.

Certain larvae of other species of Nitidulidae are morphologically very similar to *A. tumida*. If there is any doubt about identification, a confirmatory PCR analysis is recommended.

2.2.4. Interpretation of results

2.2.4.1. Adult form

i) If all the criteria 1 to 8 are confirmed for *A. tumida*, the result is "positive". The identification of *A. tumida* is confirmed. Confirmatory testing by PCR is advised.

ii) If certain fundamental morphological characteristics of *A. tumida* are not present (i.e. at least one out of the criteria 1 to 8), the result is "negative". The identification of *A. tumida* is not confirmed.

iii) Where the positive or negative status of the sample cannot be assessed (for example, a damaged sample makes it impossible to rule on the presence or absence of certain morphological criteria), the result is 'inconclusive' ~~Where definitive morphological criteria cannot be determined (e.g. damaged sample), the result is "inconclusive"~~. Molecular identification is essential for confirmation.

2.2.4.2. Larval form

i) If all the criteria 1 to 3 are confirmed, the result is "*A. tumida* suspected". PCR testing is essential for final confirmation and confidence in the diagnosis.

ii) If at least one out of the criteria 1 to 3 is not confirmed, the result is "negative". The suspicion of *A. tumida* is not confirmed.

iii) Where the positive or negative status of the sample cannot be assessed (for example, a damaged sample makes it impossible to rule on the presence or absence of certain morphological criteria), the result is 'inconclusive' ~~Where definitive morphological criteria cannot be determined (e.g. damaged sample), the result is "inconclusive"~~. Molecular identification is essential for confirmation.

2.3. Molecular identification

The morphological identification of the small hive beetle is increasingly confirmed by molecular methods ~~using real-time PCR~~, especially for the examination of larvae where morphology is ~~less~~ not clear ~~cut~~. Various real-time PCR-based methods have been reported in the literature (Li *et al.*, 2018; Silacci *et al.*, 2018; Ward *et al.*, 2007). The real-time PCR method described below was ~~has been~~ developed by Ward *et al.* (2007) ~~and~~ is based on the amplification of a partial sequence of the mitochondrial gene of *A. tumida* that encodes cytochrome oxidase I (COI). This method was validated in accordance with Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals*. The primers SHB207F and SHB315R can amplify a fragment of 109 base pairs, specific to *A. tumida*. This fragment is visualised in real-time due to a 5'-labelled probe. To take into account the two haplotypes identified by bioinformatic analysis by Ward *et al.* (2007), the SHB207F primer includes a degenerate base in the position 228 (A/G) (GenBank No. AF227645). However, due to the emergence of genetic variations following its worldwide dispersal, the Ward *et al.* (2007) method was adapted by adding a modified forward primer (SHB207F-M) to increase the level of detection of certain specimens from countries in Asia and the Indian Ocean region (Del Cont *et al.*, 2024; Liu *et al.*, 2020). The adapted method was also validated in accordance with Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals*.

2.3.1. Sample preparation, equipment and reagents method

The samples are typically adults or larvae kept in >90% non-denatured alcohol, or kept dry. Alcohol-preserved specimens should be left on tissue paper allowing the alcohol to evaporate or rinsed three times in a large volume of phosphate buffer (50 ml tube, for example). The specimen is then transferred to a 1.5 ml microtube where it is ground manually using a disposable pellet pestle. The volume depends on the size of the sample (for example: one adult beetle in 1 ml; one larva: in 200 µl). Samples can be stored at ≤ –16°C.

Genomic DNA from suspected specimens is extracted using a method that ensures the quality of the nucleic acids extracted (example in Franco *et al.*, 2022). There are a number of specialised methods for particular types of samples and tissues, some of which are now commercially available either as manual or automated systems for robotic workstations. Regardless of the DNA extraction method used, it is recommended to add a positive extraction control, to ensure the effectiveness of the extraction, and a negative extraction control to ensure absence of contamination.

A real-time PCR detection system and the associated data analysis software are required to perform the test. Several proprietary systems for real-time PCR are available. The method described below uses one such system, but the precise parameters of the method should be validated according to the system in use in a particular laboratory. Because of the high sensitivity of the method, appropriate measures are required to avoid DNA contamination. All materials and methods used for the test should comply with the standards set out in the Chapter 2.1.2 *Biotechnology advances in the diagnosis of infectious diseases*, including measures to prevent contamination of DNA in the specimen.

2.3.2. Preparation of reagents

The real-time PCR reaction mixture is usually provided as a ready to use 2× concentration. The manufacturer's instructions should be followed for use and storage. Working stock solutions for the primers and probe are prepared with nuclease-free TE buffer at the concentration of 20 µM and 50 µM respectively. The stock solutions are stored at ≤ –16°C ~~–20°C~~ and the probe should be protected from light. Single-use aliquots can be prepared to reduce the number of freeze–thawing cycles and to increase the shelf life of the primers and probes.

2.3.3. Real-time PCR test procedure

Primer/probe name	Sequence
SHB207F	5'-TCT-AAA-TAC-TAC-TTT-CTT-CGA-CCC-ATC-(A/G)-3'
SHB207F-M	5'-CCT-AAA-TAC-TAC-TTT-CTT-TGA-TCC-ATC-(A/G)-3'
SHB315R	5'-TCC-TGG-TAG-AAT-TAA-AAT-ATA-AAC-TTC-TGG-3'
SHB245T probe	5'-(6-FAM)-ATC-CAA-TCC-TAT-ACC-AAC-ACT-TAT-TTT-GAT-TCT-TCG-GAC-(TAMRA)-3'*

*To increase the signal, it is possible to modify the fluorochrome and the quencher. However, it is recommended to verify the PCR performance.

Positive and negative extraction controls, as well as reagent controls, should be included in each PCR test. To minimise the risk of contamination by the positive control, a dilution resulting in a Ct value of about 30 should be used. A suitable control would be crushed *A. tumida* beetles diluted to 10 times the detection limit of the method (LD_{method}). Alternatively, a plasmid containing the target sequence may be added diluted to 10 times the detection limit of the PCR (LD_{PCR}). For the negative extraction control, it is recommended to use the buffer used for crushing the specimens. An internal positive control (IPC) is highly recommended to check the absence of PCR inhibitor in the extract analysed.

Appropriate thermocycler conditions should be determined and validated for the equipment and reagents in use in the particular laboratory.

PCR reagent mixtures are added in a clean room (no pathogens or amplification products should be handled), for example:

	Final concentration	Volume for one tube (µl)
Nuclease free H_2O	/	~~4.1~~3.7
Real-time PCR reaction mixture (2×)	1×	12.5
SHB207F (20 µM)	320 nM	0.4
SHB207F-M (20 µM)	320 nM	0.4
SHB315R (20 µM)	320 nM	0.4
245 probe (50 µM)	100 nM	0.05
10× IPC Mix	1×	2.5
50× IPC DNA	0.1×	0.05
Mix total volume		20

Add 5 µl of the DNA template (unknown sample or plasmid DNA) or positive or negative control to the reagent mixture to a final volume of 25 µl. DNA samples are prepared and added to the PCR mix in a separate area.

The thermocycler programme will depend on the equipment used and the real-time PCR reaction mixture, for example:

Step	Cycle	Temperature (°C)	Time (minutes)
Polymerase activation	1	95	3:00
PCR	40	95	0:10
		60	0:30

2.3.4. Interpretation of results

The threshold for the analysis of the amplification curves (determined by the background noise associated with the detection system) is usually set according to the manufacturer's instructions for the software used. It can be performed on confirmed negative specimens (e.g. larvae of wax moth *Galleria melonella* or adult beetles of the genus *Meligethes*).

A result identifying *A. tumida* by real-time PCR is considered valid only if the positive extraction and PCR controls are positive (Ct ≤ 35) and if the negative extraction and PCR controls are negative (Ct = N/A).

A positive result is recorded for any sample with a Ct value <35. Negative results are for any sample with a Ct value >35 or which presents no Ct value. Samples giving negative results should be checked for the absence of PCR inhibitor in the extract analysed through the result of the IPC. PCR inhibitors can lead to false negative results. Inhibition may be overcome by dilution of the sample for example to 1/10.

3. Serological tests

Serological tests are not appropriate or relevant to bee colony infestations.

C. REQUIREMENTS FOR VACCINES

There are no vaccines available.

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FURTHER READING

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* *

NB: There are WOAH Reference Laboratories for infestation with *Aethina tumida* (small hive beetle) (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3). Please contact the WOAH Reference Laboratories for any further information on diagnostic tests and reagents for infestation with *Aethina tumida* (small hive beetle)

NB: FIRST ADOPTED IN 2008. MOST RECENT UPDATES ADOPTED IN 2018.

609 **Appendix 1: Infestation of honey bees with *Aethina tumida* (small hive beetle)**
610 **Intended purposes of test: population freedom from infestation; individual animal freedom from infestation prior to movement; contribute to eradication**
611 **policies; confirmation of clinical cases; prevalence of infestation – surveillance**

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
Visual inspection of colonies: +++ for the purposes: 'confirmation of clinical cases' and 'prevalence of infestation – surveillance' ++ for the purposes: 'population freedom of infestation', 'individual animal freedom from infestation prior to movement' and 'contribute to eradication policies' *Apis mellifera*	*Sample type: Apis mellifera* colonies (including sentinel colonies) *Target analytes:* adult SHB beetles and beetle larva specimens	Spiewok *et al.*, 2007: *Reference test:* visual inspection of six SHB-free colonies in which specific numbers (42, 88, 98, 112, 135 and 172) of SHB adults (unknown to the investigator) were introduced and given 1 hour to disperse inside the colonies before the inspection started *Results:* average of 9 (8; 10) SHB were not found during the visual inspection, corresponding to failure rate of 8.4% (6.4; 11.5) Neumann & Hoffmann (2008): *Reference test:* dissection after killing the bees and the pest to assess the number of adult SHB remained undetected during visual inspection *Results:* 14.06 ± 10.53% of the adult SHB remained undetected during visual inspections - No data reported on the detection limit and/or sensitivity of this diagnostic method (EFSA, 2015)	Spiewok *et al.*, 2007: 42, 88, 98, 112, 135 and 172 adult SHB were introduced into six *A. mellifera* colonies in Maryland, USA in July/August 2005. Neumann & Hoffmann (2008): 30 *A. mellifera* colonies, naturally infested with SHB, in Langstroth hives with two boxes, arranged in three different apiaries in New South Wales, Australia. Experiments conducted in November 2005 (i.e. local springtime)	See references	*A. mellifera* colonies are very attractive to *A. tumida*. Such *A. mellifera* colonies can be used as sentinels in at-risk areas and for surveillance. The method is easy to learn for persons that can deal with honey bees. If done properly, the method is very accurate	The screenings are labour-intensive and time-consuming (depending on colony size and experience of the inspectors). The level of detail of the inspection determines its accurateness. To obtain reliable results, training is required. As beehive inspection implies its opening, it is not possible to apply it during winter or bad weather conditions (e.g. low temperatures, rain or snow). Visual inspection of colonies can be used in combination with traps to increase its sensitivity, particularly in these type of periods. Opening a colony for long time might induce robbing behaviour between colonies	Spiewok *et al.* (2007) Neumann &, Hoffmann (2008) EFSA (2015)
Morphology +++ for all purposes *Apis* spp.	*Sample type:* Adult beetle and beetle larva specimens *Target analyte: Aethina tumida*	*Reference test:* real-time PCR and sequencing *Results:* DSp = 100% DSe = 100%	41 specimens of *A. tumida* (18 larvae and 23 adults) from different geographical areas (South Africa, United States, Canada, Mexico, Italy, France/Réunion Island, Mauritius, England/FERA experimental production).	Validation carried out by Anses Sophia Antipolis Laboratory. The robustness of the method was assessed by the	Rapid (less than 1 hour) Does not require sophisticated equipment, or reagents Inexpensive method	Requires a certain amount of experience and technical skill to handle specimens and identify morphological criteria. The differential diagnosis between *A. tumida* larvae and those of other Nitidulidae can	Franco *et al.* (2022)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
			82 negative specimens (67 adults and 15 larvae) corresponding to samples received for analysis (and collected in beehive environment) or to Nitidulidae species collected on plant crops	way of comparative laboratory testing organised with EU National Reference Laboratories (see Franco *et al.* [2022])	recommended for first intention diagnosis	be tricky. PCR is highly advisable if there is any doubt about identification. If *A. tumida* is detected in an area presumed to be free of the disease, it is recommended that identification be confirmed by PCR	
Real-time PCR ++ for all purposes except for 'prevalence of infestation – surveillance': + *Apis* spp.	*Sample type:* Adult and immature beetle specimens *Target analyte:* *Aethina tumida*	*Reference test:* sequencing *Results:* DSp = 100% DSe = 100%	39 specimens of *A. tumida* from different geographical areas (South Africa, United States, Canada, Mexico, Italy, France/Réunion Island, Mauritius, England/FERA experimental production). 28 negative specimens corresponding to samples received for analysis (and collected in beehive environment) or to Nitidulidae species collected on plant crops	Validation carried out by Anses Sophia Antipolis Laboratory (validation report submitted to WOAH in 2016, completed with new data in 2023). The robustness of the method was assessed by the way of comparative laboratory testing organised with EU National Reference Laboratories (see Franco *et al.* [2022])	High specificity and sensitivity (generally more than morphology) Method recommended for second intention diagnosis Could be applied on immature stages as eggs and pupae	Requires reagents and equipment for real-time PCR detection and analysis system More expensive than the morphology Decrease in the level of detection in the case of new haplotypes	Franco *et al.* (2022)

Annexe 19. Chapitre 3.2.5. Infestation des abeilles mellifères par *Tropilaelaps* (*Tropilaelaps* spp.)

RÉUNION DE LA COMMISSION DES NORMES BIOLOGIQUES
Paris, 9–13 septembre 2024

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CHAPTER 3.2.5.

INFESTATION OF HONEY BEES WITH *TROPILAELAPS* SPP.

SUMMARY

***Description and importance of the disease**: The mites in the genus* Tropilaelaps *are parasites of honey bee brood. Feeding on bee larvae and pupae causes brood malformation, death of bees and subsequent colony decline or absconding. Development requires about 1 week, and the mites are dispersed on bees. Four species of the* Tropilaelaps *genus have been described so far~~: There are at least four species in the genus Tropilaelaps~~; T. mercedesae, T. clareae, T. koenigerum *and* T. thaii. *Each species tends to be originally associated with a particular giant honey bee in Asia. Two species* (T. clareae *and* T. mercedesae) *are damaging pests of the European honey bee* Apis mellifera. *~~The other two species~~ (~~T. koenigerum *and* T. thaii~~) ~~appear to be harmless to~~ A. ~~mellifera.~~*

***Detection and identification of the agent:** ~~Molecular and morphological methods are available for identifying each species.~~ An infestation by* Tropilaelaps *can be recognised either visually on bees and brood combs or by examining hive debris. Irregular brood pattern, dead or malformed immatures, bees with malformed wings that crawl at the hive's entrance, and especially the presence of fast-running, red-brown, elongated mites on the combs, are diagnostic for the presence of ~~T. clareae and/or~~* T. mercedesae or T. clareae*. An early diagnosis can be made after opening brood cells and finding immature and adult mites therein. The hive (colony) may be treated with various chemicals that cause the mites to drop off combs and bees. Sticky boards on the bottom of the colony can be used to examine hive debris and mites. Alternatively, the "bump test" can be used for rapid screening. Definitive diagnosis at the laboratory is based on morphological examination under a microscope. Confirmatory testing can be done by conventional polymerase chain reaction and sequencing.*

***Serological tests:** Serological tests are not applicable.*

***Requirements for vaccines:** No vaccines are available.*

A. INTRODUCTION

Tropilaelaps ~~spp. Mites belong to the class *Arachnida*, subclass *Acari*, superorder *Parasitiformes*, order *Mesostigmata* and~~ is a genus of mites from the *Laelapidae* family (Anderson & Roberts, 2013), which reproduces in bee brood. *Tropilaelaps* should not be confused with *Varroa*, another ectoparasitic mite of honey bees with similar characteristics in terms of life cycle and pathogenicity, and which share much of its range with *Tropilaelaps* (see Chapter 3.2.6 *Varroosis of honey bees* [*infestation of honey bees with* Varroa *spp.*]). ~~They should not be confused with the mite *Varroa* destructor, a parasite that is well established in Europe. *Tropilaelaps clareae* occurs in Asia where it is a parasite of the native honey bee *Apis dorsata breviligula*. It is also a parasite of the introduced honey bee species *A. mellifera* in the Philippines and the native honey bee species *A. dorsata binghami* on Sulawesi Island in Indonesia. *Tropilaelaps mercedesae*, which was formerly mistaken for *T. clareae*, together with *T. koenigerum*, are parasites of the native *A. dorsata dorsata* in mainland Asia and Indonesia (except Sulawesi Island). *Tropilaelaps mercedesae* is also a parasite of the introduced *A. mellifera* in these and surrounding regions~~

and, with another species, *T. thaii*, also parasitises *A. laboriosa* in mountainous Himalayan regions (Anderson & Morgan, 2007).

1. Nature and classification of the pathogen

Tropilaelaps spp. are mites belonging to the Class Arachnida, Subclass Acari, Superorder Parasitiformes, Order Mesostigmata and Family Laelapidae (Anderson & Roberts, 2013). Based on genetic and morphological differences, four species of *Tropilaelaps* have been described: *T. mercedesae*, *T. clareae*, *T. koenigerum* and *T. thaii* (Anderson & Morgan, 2007).

Tropilaelaps spp. are originally parasites of the Asian giant honey bees (*Apis dorsata*, *Apis laboriosa*, and *Apis breviligula*). *Tropilaelaps koenigerum* is a parasite of *A. dorsata* in Sri Lanka, mainland Asia, and Indonesia apart from Sulawesi and Borneo. *Tropilaelaps thaii* has only been observed infesting *A. laboriosa* in Vietnam. *Tropilaelaps clareae* parasitises *A. dorsata* and *A. breviligula* in the Philippines and Indonesia. *Tropilaelaps mercedesae* was originally found in *A. dorsata* in mainland Asia and Indonesia, and *A. laboriosa* in Himalayas (Chantawannakul *et al.*, 2018).

Amongst the four species of *Tropilaelaps*, *T. clareae* and *T. mercedesae* have successfully adapted to the European honey bee *Apis mellifera* and can reproduce in the brood cells of this new host (de Guzman *et al.*, 2017). *Tropilaelaps mercedesae*, which was formerly mistaken for *T. clareae*, has a wider geographical range. *Tropilaelaps clareae* has indeed only been reported in *A. mellifera* in the Philippines (except Palawan Islands), whereas *T. mercedesae* is regionally widespread within mainland Asia, Palawan Islands and extreme East of Europe (i.e. Krasnodar and Rostov regions, Russia), and found to infest *A. mellifera* in both tropical and temperate zones (Brandorf *et al.*, 2024). Anthropogenic movements contribute to its spread. Climate change is probably also a factor, creating favorable conditions for its establishment (Chantawannakul *et al.*, 2018).

2. Life cycle

The ~~colonising~~ *Tropilaelaps* female (or females; as many as a dozen may occur within a single cell) enters the brood cell shortly before the cell is capped and lays from one to four eggs on a mature bee ~~larva shortly before the brood cell is capped~~. In its adapted host, *A. mellifera*, *Tropilaelaps* appears to prefer ~~The~~ drone brood ~~is preferred by *Tropilaelaps*~~ over that of workers ~~and may be almost 100% parasitised~~ (Burgett *et al.,* 1983). The mite progeny, usually one male and several females, feed on and seriously damage the bee brood. Development of the mite requires about 1 week. The adults, including the foundress female, emerge with the adult bee and search for new hosts (de Guzman *et al.,* 2017) (Fig. 1).

~~The short life-cycle, as well as a very brief stay on adult bees, explains why populations of *T. clareae* increase faster than those of *Varroa* mites. When both *T. clareae* and *Varroa destructor* infest the same colony, the former may out-compete the *Varroa* mite (Burgett *et al.,* 1983; Ritter & Schneider-Ritter, 1988). It has been reported that when both mite species are in the same cell, the reproduction of both mites declines (Rath *et al.*, 1995).~~

Phoretic survival on adult bees is quite short (~~only 1–2 days~~ up to 3 days) likely because of lack of morphological adaptation for attachment and their apparent inability to feed on adult bees, linked to the fact that *Tropilaelaps* cannot pierce their integument ~~of adult bees~~ (de Guzman *et al.*, 2017).

The short life cycle, as well as a very brief stay on adult bees and the possibility of deuterotoky (ability of virgin females to lay both male and female eggs) (de Guzman *et al.*, 2018), explains why populations of *Tropilaelaps* increase faster than those of *Varroa* mites. Buawangpong *et al.* (2015) showed that when both *T. mercedesae* and *Varroa destructor* infest the same colony, the former may out-compete the *Varroa* mite.

Survival time in beehive products is up to 3 days in dry pollen, royal jelly and honey, and up to 6 days in empty honeycombs thereby suggesting a sufficient time window for the potential introduction of *T. mercedesae* into mite-free countries via import of these hive products (Khongphinitbunjong *et al.*, 2019; Pettis *et al.*, 2019).

~~The phoretic time for *Tropilaelaps* spp. is important in understanding the life cycle, and recent research suggests the period can be as long as 5–10 days (Wilde, 2000a; 2000b). Gravid female mites will die within 2 days unless they deposit their eggs (Woyke, 1987).~~ The dispersal of *Tropilaelaps* is achieved by worker bees robbing colonies of stored and accidental drifting of infested bees. The mites can also jump between host species when uninfested and infested foragers visit the same flowers simultaneously. Beekeeping practices, such as colony divisions, transferring brood frames from one colony to another or migratory beekeeping, can spread the parasite. The trade in queen bees and package bees also contributes to the spread of *Tropilaelaps* spp. throughout the world. Accidental introductions can occur via infested swarms on ships (de Guzman *et al.*, 2017).

~~Like *Varroa*, *Tropilaelaps* can act as a potential vector for honey bee viruses, such as deformed wing virus (DWV) (Forsgren *et al.*, 2009). DWV has been reported to replicate in *T. mercedesae*, suggesting that the mite may act as a biological vector~~

~~of DWV (Dainat *et al.*, 2009). The impact of the mite–virus complex is not fully understood. Some data indicate that the major impact of *Tropilaelaps* infestation could be caused by the mite itself, reducing bee host immune responses (Khongphinitbunjong *et al.*, 2015).~~

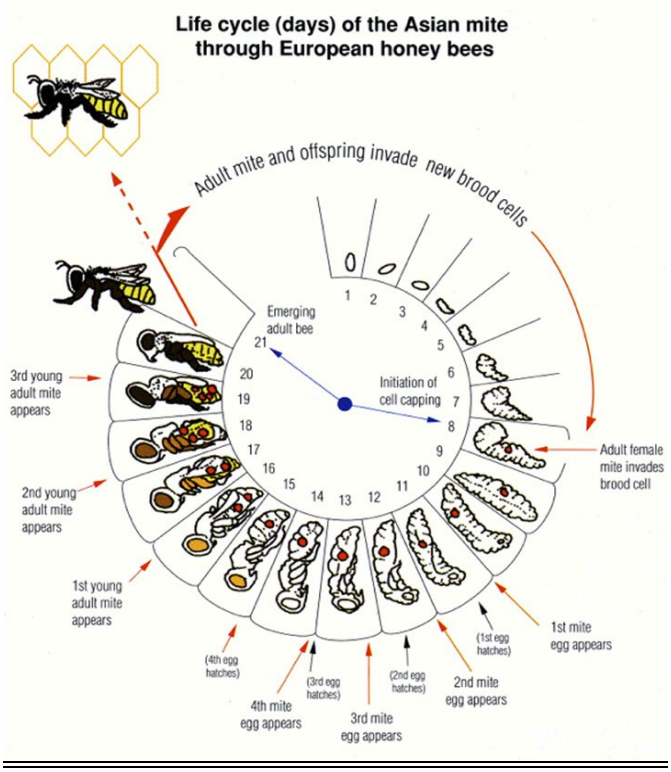

Fig. 1. *Life cycle of* Tropilaelaps *mite on European honey bee* (*Anderson & Roberts, 2013*).

3. Description and impact of the disease

Infestation by *Tropilaelaps* causes the death of many bee larvae (up to 50%), resulting in an irregular brood pattern and of which the cadavers that may partially protrude from the cells. Many malformed bees occur, with ~~distorted~~ shortened abdomens, ~~stubby~~ distorted and stubby wings and deformed or missing legs~~, probably resulting from DWV associated infection~~. Some of the affected bees crawl at the hive's entrance (Atwal & Goyal, 1971). In addition, perforated cappings are seen, the result of sanitation activities by the worker bees, which evict the infested bee pupae or young adults. In highly infested *A. mellifera* colonies, bald brood, a condition where the capping over pupal bees has been removed by workers, can be observed (Pettis *et al.*, 2013). Some infested colonies abscond, carrying the mites to a new location.

The behavioral responses of honey bees to *T. mercedesae* depend on the *Apis* species. *A. cerana* and *A. dorsata* ~~(the natural host of *T. mercedesae*)~~ showed a higher behavioral resistance than *A. mellifera* (Khongphinitbunjong *et al.*, 2012; Shrestha, *et al.*, 2020). In *A. mellifera*, *T. mercedesae* infestation significantly reduced honey bee lifespan and emergence weight, and had negative impacts on olfactory learning, flight ability and homing ability~~; it also promoted DWV levels and associated clinical signs~~ (Gao *et al.*, 2021; Khongphinitbunjong *et al.*, 2016)~~ and could cause severe damage for colonies~~. Heavy infestations can lead to reduced honey yields and colony losses.

Some of the clinical signs observed can be attributed to viral infection. Indeed, like *Varroa*, *Tropilaelaps* can act as a potential vector for honey bee viruses, such as deformed wing virus (DWV), black queen cell virus (BQCV), acute bee paralysis virus (ABPV), Israeli acute paralysis virus (IAPV) and sacbrood virus (SBV) (Chanpanitkitchote *et al.*, 2018; Forsgren *et al.*, 2009; Truong *et al.*, 2023). The effect of the mite–virus complex is not fully understood. Some data indicate that the major impact of *Tropilaelaps* infestation could be caused by the mite itself, reducing bee host immune responses and inflicting multiple wounds on developing host bees when feeding (Khongphinitbunjong *et al.*, 2015). While in *V. destructor*, the mother mite establishes one feeding site that she shares with her offspring, *T. mercedesae* uses multiple feeding sites on its host (Ling *et al.*, 2023). Moreover, the puncture wound derived from mite feeding certainly allows for mechanical vectoring as it is an entry point for several bee pathogens, including viruses (Chantawannaku *et al.*, 2018).

4. Zoonotic potential and biosafety and biosecurity requirements

Tropilaelaps infestation is not zoonotic. Being exotic in many areas, biosecurity measures must be implemented to avoid its dispersal. Suspect mites must be dead when sent to the laboratory (see Section B.2.1).

5. Differential diagnosis

Tropilaelaps should not be confused with other honey bee ectoparasites such as *Varroa* spp. (in particular with the two species *V. destructor* and *V. jacobsoni*, which are described in *A. mellifera)* and *Braula coeca* flies. A differential diagnosis must also be made with other *Laelapidae* mites living in debris of honey bee hives, such as *Melittiphis alvearius* (Cook & Bowman, 1983), the Ameroseiidae mite *Neocypholaelaps apicola* (Delfinado-Baker & Baker, 1983; Kontschán *et al.*, 2015) (Fig. 2).

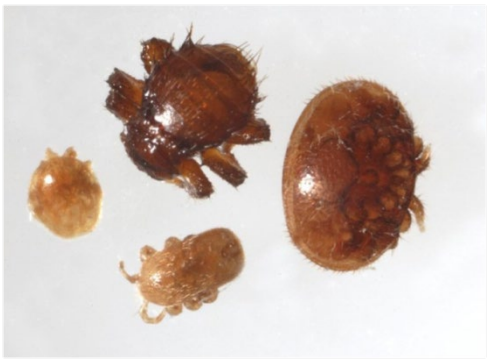

Fig. 4-2. Braula coeca (above), Varroa destructor (right), Tropilaelaps spp. (below centre) and *Melittiphis alvearius* (left) (dorsal view).
Photo supplied by APHA Bee Unit, York. UK Crown Copyright.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of infestation of honey bees with *Tropilaelaps* spp. and their purpose

Method	Purpose					
	Population freedom from infestation[a]	Individual animal or bee nest freedom from infestation prior to movement[a]	Contribute to eradication policies[a,b]	Confirmation of clinical cases[a]	Prevalence of infestation – surveillance[a]	Immune status in individual animals or populations post-vaccination
Detection and identification of the agent						
Bee examination ('ice sugar roll' method)	++	++	++	++	++	=
Capped brood examination	+++	+++	+++	+++	+++	=
"Bump test"	±	±	±	++	++	=
Debris examination	++	++	++	++	++	=
Morphology	+++	+++	+++	+++	+++	–

Method	Purpose					
	Population freedom from infestation[(a)]	Individual animal or bee nest freedom from infestation prior to movement[(a)]	Contribute to eradication policies[(a,b)]	Confirmation of clinical cases[(a)]	Prevalence of infestation – surveillance[(a)]	Immune status in individual animals or populations post-vaccination
Conventional PCR	++	++	++	++	+	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose. PCR = polymerase chain reaction.
[(a)]See Appendix 1 of this chapter for justification table for the scores given to the tests for this purpose.
[(b)]A combination of morphology and PCR applied on the same specimen is recommended in case of first detection in a free area.

1. Field detection of the mite

The first sign of an infestation by *Tropilaelaps* species is often the occurrence of red-brown, elongated mites on the combs or on adult bees (Figs. ~~2~~ 3 and ~~3~~ 4).

Fig. ~~2~~ 3. *Tropilaelaps* on *Apis dorsata* larvae. Photo by D. Anderson.

The body length depends on the species and varies between the male and the female. *Tropilaelaps koenigerum* is the smallest member of the genus with a body length of < 0.7 mm for females and ~0.575 mm for males. Female *T. mercedesae*, *T. clareae* and *T. thaii* are much longer at ~0.95–0.99 mm, ~0.87–0.885 mm and ~0.89 mm respectively, while the body lengths of male *T. mercedesae* and *T. clareae* are slightly smaller than their respective females at 0.907–0.927 mm and 0.852–0.858 mm, respectively. Males of *T. thaii* have yet to be discovered (Anderson & Roberts, 2013).

In the brood cells, the different developmental stages of *Tropilaelaps* can be observed: larva, protonymph, deutonymph, young adult (Fig. 4).

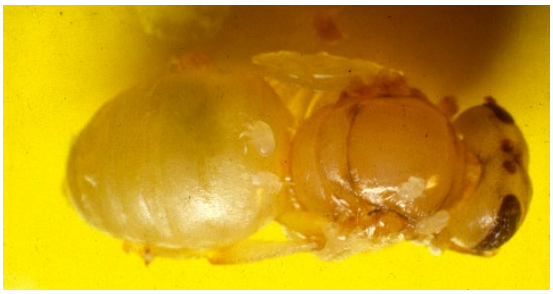

Fig. ~~3~~ 4. *Tropilaelaps* offspring on *Apis mellifera* pupae. Photo by W. Ritter.

Tropilaelaps can easily be separated from the *Varroa* mite using a ~~×10~~ magnifying glass. The body of the *Varroa* mite is wider than it is long and it moves slowly, whereas the body of *Tropilaelaps* is elongated (Fig. ~~1~~ 5), and it is a fast-moving mite.

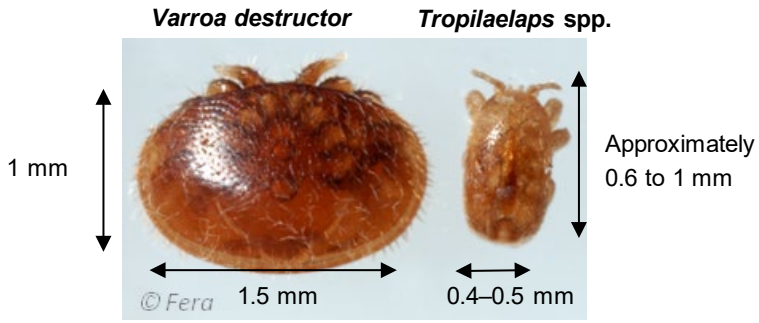

Fig. ~~1~~ 5. Varroa destructor and Tropilaelaps spp. (dorsal view). Photo supplied by APHA Bee Unit, York. UK Crown Copyright.

~~*Tropilaelaps* should also not be confused with other honey bee ectoparasites such as *Braula* flies, or other *Laelapidae* mites living in debris of honey bee hives such as *Mellitiphis alvearius* (Cook & Bowman, 1983) (Fig. 4) or the Ameroseiidae mite *Neocypholaelaps apicola* (Delfinado-Baker & Baker, 1983; Kontschán *et al.*, 2015).~~

1.1. ~~Mite collection~~ Bee examination

Collecting mites from adult bees is a quick and simple method for *Tropilaelaps* detection. Mites are dislodged from bees by washing the bees in ethanol, soapy water or by treating them by the 'icing sugar roll' method (Anderson & Roberts, 2013; Gill *et al.*, 2024; Pettis *et al.*, 2013).

Collect approximately ~~100–200~~ 200–300 bees in a wide-mouthed jar with lid. Scrape the bees into the jar or use a modified vacuum to suck them in. *Tropilaelaps* mites are usually only present in low numbers on adult bees. To increase the likelihood of collecting mites, a frame with emerging brood can be selected, allowing mites to be collected as they emerge with the bees (Anderson & Roberts, 2013).

Knock the bees to the bottom of the jar with a sharp blow~~; there should be about a 2.5–5 cm layer of bees on the bottom~~. Remove the lid and ~~spray a 2-second burst with ether starter fluid. Alternatively,~~ use enough 70% ~~alcohol~~ ethanol or soapy water to cover the bees. Secure the lid, agitate the jar vigorously for 1 minute and then strain out the bees with a mesh strainer, a sieve or a mesh large enough for *Tropilaelaps* to pass through. Mites will be in the liquid.

Alternatively, ~~; or~~ add around 25 g ~~powdered~~ of icing sugar ~~(or flour)~~, secure the lid and roll the bees for one minute. Then leave the jar to stand out of direct sunlight for 1 minute. ~~If using ether replace the lid and agitate or roll the jar for about 10 seconds; mites should stick to walls. If using soap or alcohol, agitate and then strain out the bees with a coarse hardware cloth or mesh strainer; mites will be in the liquid. If using sugar or other powder,~~ Put screening material (such as mesh strainer, sieve or hardware cloth) on top of the jar and shake the mites on to white ~~paper~~ tray to assess for the presence of mites. ~~;~~ Repeat the process after 2 minutes. ~~For a more accurate count, finish with an alcohol or soapy water wash to collect all the mites.~~ The 'icing sugar roll' offers non-destructive, quick, inexpensive and easily deployable detection method, and appears to be more effective than 'ethanol wash' for *Tropilaelaps* detection (Gill *et al.*, 2024).

1.2. ~~Colony and~~ Capped brood examination

When monitoring honey bee colonies for the presence of *Tropilaelaps* (or *Varroa*), an examination of both drone and worker brood may provide an early indication of infestation.

Remove the wax cappings from large number of brood cells, preferably by using fine-nose forceps (instead of honey uncapping fork) to avoid damaging *Tropilaelaps* mites, which are more fragile than *Varroa* (Gill *et al.*, 2024).

Remove the immature honey bee (larva and pupae) from each cell and inspect them carefully for mites. Also, inspect the inside of the cells, as *Tropilaelaps* mites tend not to be present on pupae at certain stages of bee development (between larval instar/day 9 to pharate pupal stage/day 13, and after pink eyed stage/day 18) (Gill *et al.*, 2024). ~~Mites can be observed inside capped bee brood by using a honey scratcher (with fork-like tines) to pull up capped pupae. The mites are clearly visible.~~ The younger mite stages are whitish and may be almost motionless while feeding on their hosts' bodies, as their mouthparts and front legs are fixed to the cuticle of the bee host (Ritter & Schneider-Ritter, 1988). The use of a headlamp and a 40× magnification hand lens can aid mite discovery.

To collect the *Tropilaelaps* mites use a fine paintbrush wetted in honey, ethanol or water, or with a pair of fine tweezers (Anderson & Roberts, 2013). Place the mites into small vials containing 70% undenatured ethanol for storage.

The extent of ~~parasitisation~~ parasitism can be estimated by opening a predetermined number of brood cells (for example 100) ~~(Pettis, 2013)~~; infestation rates are then calculated as per cent of capped brood containing live mites ~~(Burgett & Kitprasert, 1990~~ Pettis, 2013).

According to Gill *et al.* (2024), the 'brood uncapping' method is the most effective technique for *Tropilaelaps* field detection.

1.3. "Bump test"

Another rapid and simple technique is the "bump test" (Pettis *et al.*, 2013). The method consists of firmly rapping a honey bee brood frame over a collecting pan. First, all adult bees are removed from one comb containing capped brood by shaking the frame over the colony. Once adult bees are cleared away, frames are firmly bumped over a white metal pan by hitting one end of the frame on the side of the pan, turning the frame, re-bumping the frame, and repeating the process once more for a total of four bumps. This process dislodges mites on the surface of the comb, which then can be counted ~~(Pettis *et al.*, 2013)~~.

However, Gill *et al.* (2024) showed that this method could lead to significant brood mortality and was found to be ineffective for detecting *Tropilaelaps*.

1.4. ~~Sticky board~~ Hive debris examination

~~A precise~~ Diagnosis of infestation can be made using a sticky board covered with a mesh, such as fly screen, that prevents the bees from removing the dislodged mites. The mesh must be large enough for mites to pass through.

Make a sticky board with poster board, cardboard or other white, stiff paper coated with petroleum jelly or other sticky substance (Koeniger *et al.*, 2002; Ostiguy & Sammataro, 2000~~; Sammataro *et al.*, 2000~~), or use a sheet of sticky shelf paper. Cut the paper to fit the bottom board of a hive. Cut a piece of hardware cloth or screen to fit on top of the sticky board. To keep the bees from cleaning off the board, fold under the outside edges of the screen to raise it off the board, and staple or tape in place.

Leave the board in the colony for up to 3 days, collecting and examining the debris for mites. Examination of the debris should preferably be carried out indoors, in a laboratory for example. Indeed, variable light conditions and the need to wear veils made examination of floor inserts in the field an unreliable method for detecting *Tropilaelaps* (Gill *et al.*, 2024). Use a headlamp and a magnifying glass to spot *Tropilaelaps* among the debris and other mites that may be present.

Sieving hive debris and then floating mites in ethanol (as sometimes practiced for *Varroa* detection) is not recommended, as it seems to be ineffective for *Tropilaelaps* (Gill *et al.*, 2024).

Acaricides ~~are sometimes~~ can be used to knock mites off bees and will appear on the sticky boards.

2. Laboratory identification of the mite

Rapid and reliable diagnosis is crucial to enable implementation of sanitary measures and to avoid spread in non-infested territories. In case of field suspicion, specimens suspected to belong to the genus *Tropilaelaps* should be sent to official laboratories to confirm the diagnosis. Morphological identification should be carried out for primary diagnosis. This method is fast and cheap, not requiring sophisticated equipment. Confirmatory testing may be done using polymerase chain reaction (PCR) for molecular identification of *Tropilaelaps* species.

2.1. Special precautions required for sampling

The specimens to be identified are collected in honey bee hives, for example, in colonies, in batches of bees, or on queen bees~~) or on bumble bees~~.

Suspect specimens should be killed before submission to the laboratory e.g. in 70% ethanol. Denatured ethanol should not be used where molecular methods are to be used because of possible inhibition of the ~~polymerase chain reaction (~~PCR~~)~~. Alternatively, samples can be stored overnight at –20°C to kill the specimens.

On arrival at the laboratory, packages should be opened in containment conditions. If the specimens are found to be alive on arrival, the submission should be placed at least at ~~–8~~ 70°C for approximately one hour before opening fully. This procedure immobilises the specimens, which can subsequently be stored in 70% ethanol.

2.2. Morphological identification of *Tropilaelaps* spp.

The method is based on the visual examination of adult mites only, taking into account the morphological characteristics of the adult *Tropilaelaps* mite compared with those of other mite genera commonly found in bee colonies (particularly *V. destructor*). The visual examination described is not sufficient to differentiate amongst the four species of *Tropilaelaps* as they are morphologically very similar (Anderson & Morgan, 2007; Tangjingjai *et al.*, 2003). Species identification can be carried out by PCR and molecular sequencing (see Section B.2.3.).

2.2.1. Equipment and reagents

Classical entomological equipment and materials are required:

- Stereomicroscope
- Compound microscope (1000×)
- Hot plate
- Dishes: glass Petri dishes, porcelain ceramic dishes, watch glass or similar
- Micro-dissecting needle holders equipped with minutien pins and with pins made of fishing line (with the extremity crushed in order to obtain a spoon-like shape)
- Fine-tipped tweezers
- Glass microscope slides (classic and concave) and cover slips
- Hermetically sealed vials
- Lactic acid
- Mounting medium (e.g. Hoyer's medium) and clear nail polish for the long-term conservation of microscopic slides
- Ethanol 70% (avoid denatured ethanol).

2.2.2. Test procedure

All the specimens are placed in a dish and checked for homogeneity using a stereomicroscope. If the specimens are not homogeneous, then each type present is examined separately. ~~Samples for examination should be selected from undamaged mites.~~ Samples are taken using fine-tipped tweezers or needle holders and placed in a dish for further study.

Under the stereomicroscope, the mites are checked for the three primary identification criteria of *Tropilaelaps* spp. (see Table 2 below). If none of the three criteria are met, then further microscopic examination is not pursued.

Note: Evaluate the ratio of mite body length to width by measuring the specimen in dorsal view:

- Use the same magnification to measure length and width
- Measure where the body is widest/longest
- For length, do not include mouthparts and antennae
- For width, do not include legs.

For microscopic examination, the soft tissues must be cleared to reveal the morphological characteristics. Deposit a few drops of lactic acid on a microscope slide (using concave slides for larger specimens). Place the selected specimens on the slide in lactic acid with the needle holders (fishing line equipped) (or with extra-fine tweezers). Using two holders (minutien pin equipped), position the specimens so as to have a ventral view. Place a cover glass over the microscope slide without crushing the mite, avoiding the formation of air bubbles. If possible, carefully press on the cover glass with a tweezer in order to spread open the legs, which are usually curled up beneath the body. Place the slide on a heating plate at approximately 50°C) and wait for the lactic acid to have effect (approximately 30 minutes). NB: the liquid should not boil on the slide as this would destroy the specimen.

Examine the slide(s) under the compound microscope at 100×, 200×, and then 400× magnification in order to observe fully the various diagnostic criteria as detailed in Table 2. Comparative observations should be carried out with reference slides or specimens if available. The depth of field viewed may need to vary according to the thickness of the mite's body.

Specimens can be stored at room temperature in a hermetically sealed vial with 70% ethanol. Slides may be kept long term by mounting the mites in Hoyer's medium, allowing to dry for 2–4 weeks at room temperature (depending to room conditions) ~~2 weeks at 50°C~~, then sealing the cover slip with clear nail varnish. Drying can be accelerated by using a hot plate (heating at 50°C for 1–2 weeks). For further information on storage and mounting of mites see Dietemann *et al.* (2013).

2.2.3. Identification criteria for adult *Tropilaelaps* spp.

Tropilaelaps spp. are visible to the naked eye. It is approximately between 0.6 mm and 1.0 mm long and between 0.4 to 0.5 mm wide. *Tropilaelaps* is smaller than *V. destructor* (Figs. ~~1~~ 2 and ~~4~~ 5). If all the morphological characteristics of the adult mite are confirmed (criteria 1 to 9 in Table 2), the result is "positive" confirmation of the identification of *Tropilaelaps* genus. If one or more of the fundamental morphological characteristics (criteria 1 to 9) of *Tropilaelaps* spp. are not present the result is "negative" and the identification of *Tropilaelaps* genus is not confirmed. If the presence or absence of all nine criteria cannot be determined (e.g. due to a damaged sample), the result is "inconclusive" and molecular methods should be used for confirmation.

Table 2. Criteria for recognising Tropilaelaps spp. (Anderson & Roberts 2013; Delfinado & Baker 1961; Smiley 1991~~; University of Michigan 2014~~)

The following features should be examined:

a) The stigmata are tracheal openings;

b) The coxa is the first leg segment and connects the leg and the body;

c) The peritremes are tubular structures running on from stigmata. They could have a role in respiration;

d) The tritosternum is a bristle-like Y-shaped sensory organ located caudally to the gnathosoma (the gnathosoma is the body part of *Acari* that includes the mouthparts and oral aperture);

e) Reticulated means that it has broken eggshell or fish scale pattern.

	Stereomicroscope	***Compound microscope***
1. *Tropilaelaps* has four pairs of legs AND the first pair is vertically aligned, resembling antennae (Fig. ~~5~~ 6). □ Class *Arachnida*	X	
2. The body is unsegmented, with a single visible region, due to the fusion of the prosoma (the equivalent of the cephalothorax) and the opisthosoma (or abdomen) into a single mass (Fig. ~~5~~ 6). □ Subclass *Acari*	X	
3. The body is longer than wide (as opposed to *V. destructor*) (Figs. ~~1~~ 2 and ~~4~~ 5). The ratio of length to width is greater than 1.3.	X	
4. It has a pair of latero-ventral stigmata between coxa III and IV (Figs. ~~7~~ 8). □ Order Parasitiforms		400×
5. Presence of elongated peritremes (Fig.~~7~~ 8). ~~Presence of a tritosternum (Fig.7) (optional criterion, difficult to observe).~~ □ Suborder *Mesostigmata*		200×
6. Elongated epigynial plate, posteriorly rounded or sharp. Triangular-shaped ventrianal plate (Figs. ~~5~~ 6 and ~~6~~ 7). Note: the point of the "triangle" is cranially oriented □ Family *Laelapidae*		100×
7. Elongated epigynial plate, at least twice as long as the ventrianal plate (Figs. ~~5~~ 6 ~~and 6~~).		100× or 200×
8. Reticulated sternal plate (Fig. ~~7~~ 8).		400×
9. Opisthosoma with coarse bristles, thick at the base, on the apical half of the ventral side (Figs. ~~5~~ 6 and ~~6~~ 7).		200×
Note: Criteria for distinguishing between males and females: the mobile digit of the male's chelicerae is filiform (spermodactyls) ~~(Fig. 8)~~. The epigynial plate is shorter in the male than in the female (Fig. ~~8~~ 9). (Anderson & Morgan, 2007)		200×

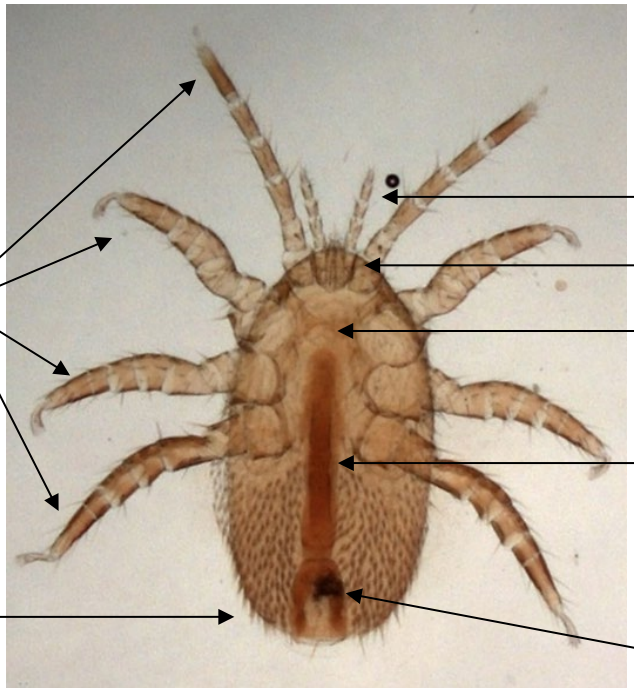

Fig. ~~5~~ 6. Tropilaelaps clareae, female (ventral view). Photo by S. Franco, Anses, Sophia Antipolis laboratory.

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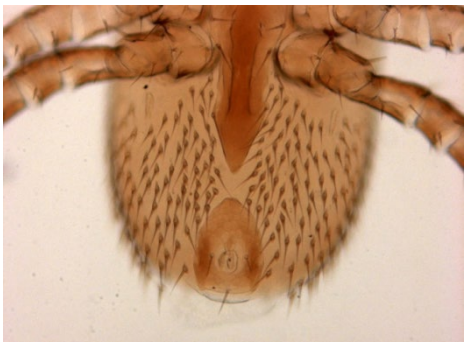
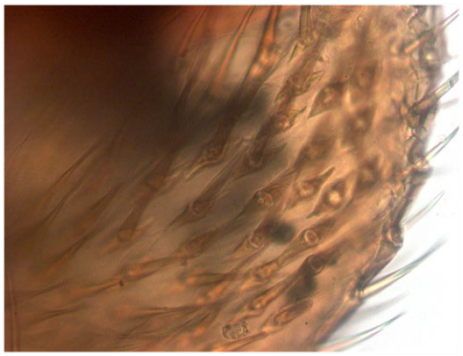


Magnification 100×



Magnification 400×

Fig. ~~6~~ 7. Tropilaelaps sp. (ventral view). Opisthosoma, coarse apical bristles, thick at their base. Photos by S. Franco, Anses, Sophia Antipolis laboratory.

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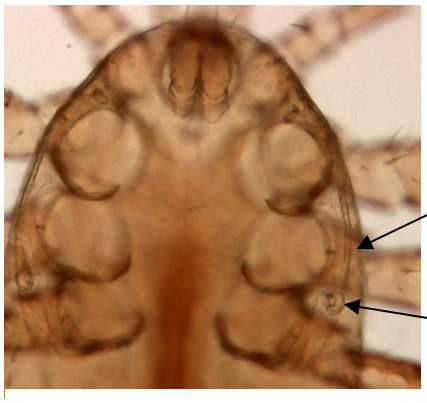
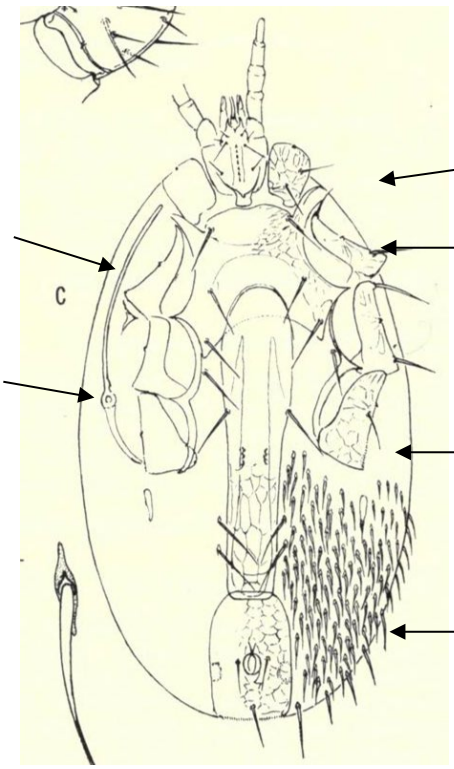
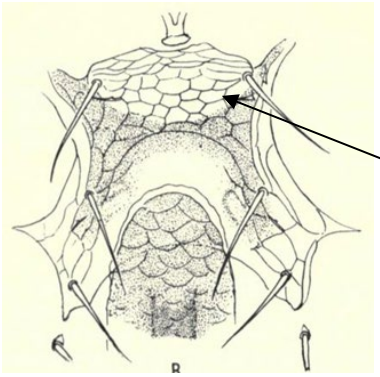


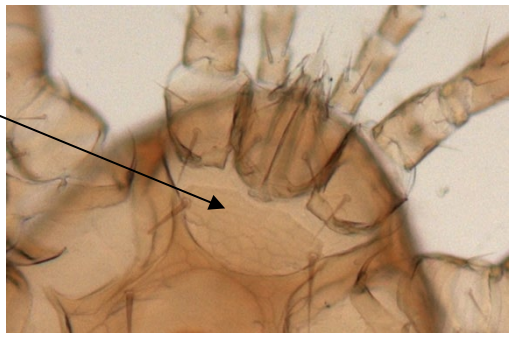
T. clareae – Magnification 100×



T. clareae, female (ventral view)

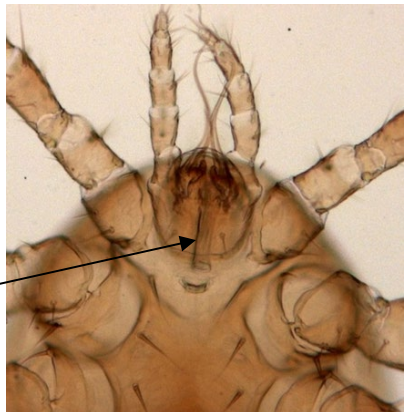


Sternal plate and epigynial plate (ventral view)



T. clareae – Magnification 200×

Gnathosoma (ventral view)



T. clareae – Magnification 200×

Fig. ~~7~~ 8. Tropilaelaps clareae, anatomy.
Source of the figures: Delfinado & Baker, 1961. Photos by S. Franco, Anses, Sophia Antipolis laboratory.

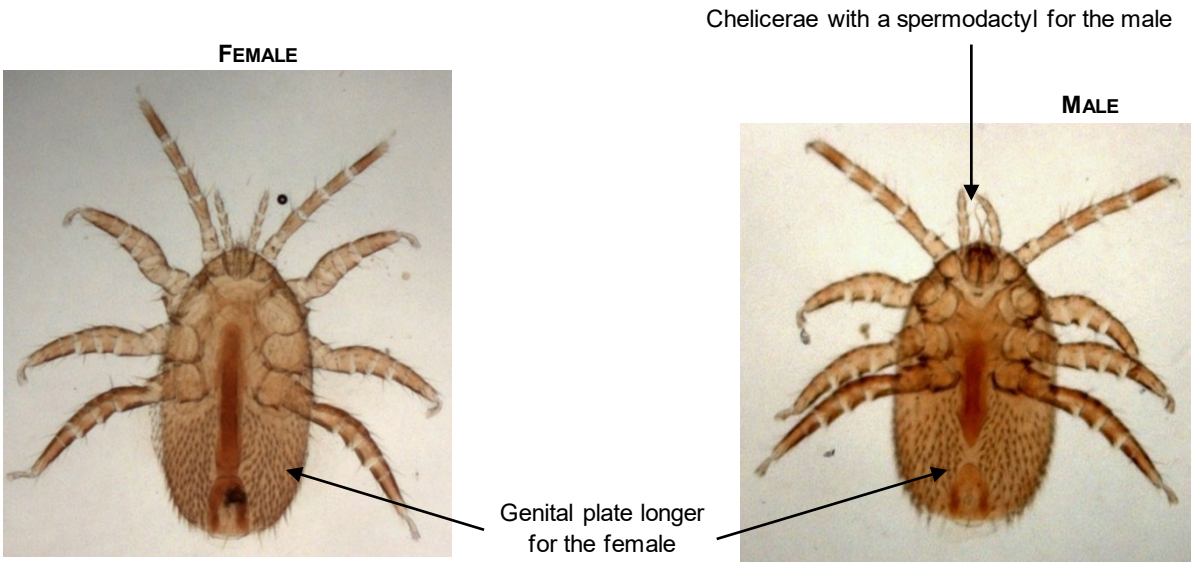


Fig. ~~8~~ 9. Tropilaelaps clareae, male and female (ventral view). Photos by S. Franco, Anses, Sophia Antipolis laboratory.

2.3. Molecular identification

The morphological identification of *Tropilaelaps* spp. is complicated because of their resemblance to other mites that may be found in hives. In addition, some samples may be difficult to identify using morphological methods due to loss of integrity, and these methods are not applicable to immature stages. As a result, various molecular methods have been developed (Anderson & Morgan, 2007; Del Cont *et al.*, 2021; Tangjingjai *et al.*, 2003) and ~~PCR methods~~ are increasingly used to confirm the suspicion of infestation. The conventional PCR method described below is based on the amplification of a partial sequence of the mitochondrial gene of *Tropilaelaps* spp. which encodes cytochrome oxidase I (COI) (Anderson & Morgan, 2007). The primers COI-TCF1 and COI-TCR2 amplify a fragment of 580 base pairs. The size of PCR products is determined by ~~agarose gel electrophoresis~~ a system separating DNA fragments in comparison with a DNA ladder (molecular weight marker). The primers ~~are not specific~~ could have a limited specificity to *Tropilaelaps* spp. and amplification of the COI gene from other parasites can occur, so it is ~~necessary~~ recommended to sequence the DNA when a PCR product of the expected size is found.

2.3.1. Sample preparation, equipment and reagents

The samples tested are typically about 10 adult mites, kept in > 95% non-denatured alcohol or kept dry. If stored in alcohol they should first be rinsed three times in a large volume of phosphate buffer (50 ml/tube) or simply dry for several minutes on tissue paper before the DNA extraction step. The mites are then transferred to a 1.5 ml microtube. This step is important to avoid inhibition. The mites are ground in 200 µl phosphate buffer using a disposable pellet pestle in the 1.5 ml microtube. Ground samples can be stored frozen at ≤ –16°C.

A conventional PCR detection and analysis system is required. Any suitable methods or kits can be applied for the extraction of DNA, amplification in a thermocycler, followed by electrophoresis on agarose gel. All equipment and materials should be validated for use in the individual laboratory. The usual measures are required to avoid DNA contamination, and procedures should follow the standards set out in Chapter 2.1.2 *Biotechnology advances in the diagnosis of infectious diseases*. Positive and negative controls must be used for all stages.

2.3.2. PCR procedure

The primers developed by Anderson & Morgan (2007) are as follows:

Name	Sequence
COI-TCF1	5'-CTATCCTCAATTATTGAAATAGGAAC-3'

COI-TCR2	5'-TAGCGGCTGTGAAATAGGCTCG-3'

The PCR reaction mixture should follow the manufacturer's instructions for use and storage. Working stock solutions for the primers are prepared with nuclease-free low ethylene diamine tetra-acetic acid (EDTA) TE buffer at the concentration of 20 µM. The stock solutions are stored at –20°C.

The PCR reaction mixtures are prepared in a separate laboratory room. All reagents, except the DNA samples, are mixed prior to distribution in each reaction tube. In each PCR test, appropriate controls must be included, including at least a template control (NTC, reagents only), negative controls (i.e. 1 per 10 samples tested) and a positive control (plasmid DNA solution including the sequence to be amplified, added to diluted 10 times the detection limit of the PCR [LD_{PCR}]). The amplifications are carried out in a total volume of 20 µl.

PCR reagent mixtures are added in a clean room (no pathogens or amplification products should be handled). The conditions above have been defined in the PCR validation steps. Other conditions could be applied after an optimisation.

	Final concentration	**Volume for one tube (µl)**
Nuclease free H_2O	/	12.6
Taq DNA pol (5 U/µl)	0.5 U/µl	0.2
Taq DNA pol buffer (10×)	1×	2.0
$MgCl_2$ (50 mM)	3.5 mM	1.3
dNTP mix (10 mM)	450 µM	0.9
COI-TCF1 (20 µM)	500 nM	0.5
COI-TCR2 (20 µM)	500 nM	0.5
Mix total volume		18

Add 2 µl of the DNA template (unknown sample or plasmid DNA) or negative control to the reagent mixture to a final volume of 20 µl. DNA samples are prepared and added to the PCR mix in a separate area.

An example thermocycler programme is as follows:

Step	**Cycle**	**Temperature (°C)**	**Time (minutes)**
Initial denaturation	1	95	5:00
PCR	35	94	0:30
		58	0:30
		72	0:45
Final extension	1	72	7
Hold		10	∞

Optimisation of PCR should be carried out according to the Mastermix and PCR machine used, especially testing with different annealing temperatures.

~~Detection of amplified products:~~

~~i) Prepare a 1.2% agarose gel in 1× TAE (Tris-acetate-EDTA) with the appropriate number of wells.~~

~~ii) 2 µl of 6× loading buffer are added to 10 µl of PCR products.~~

~~iii) Load 10 µl of the samples into the wells.~~

~~iv) To control the size of the amplified products, a 100 bp ladder is recommended.~~

~~v) Run the gel.~~

~~vi) Analyse by UV illumination after staining with a suitable DNA stain.~~

The interpretation of the results is based on the presence or absence of the amplified product: the size of the expected PCR product is 580 bp including the two primers. However, the presence of a PCR product of the right size is not sufficient to identify the *Tropilaelaps* genus and species. A sequencing step is required.

2.3.3. Sequencing of PCR products

If a 580 bp band is detected the PCR product must be sequenced. ~~The method is not described here, and can be outsourced.~~ A panel of COI sequences available on NCBI Genbank ~~(EF025423 to EF025468 and HQ533148 to HQ533159 [Luo *et al.*, 2011])~~ is included in the analysis to construct the phylogenetic tree and to identify the species of *Tropilaelaps*. An outgroup COI sequence from *Varroa destructor* ~~(EF025469, 253947435)~~ is included.

3. Serological tests

Serological tests are not appropriate or relevant to bee colony infestations.

C. REQUIREMENTS FOR VACCINES

There are no vaccines available.

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NB: There is a WOAH Reference Laboratory for infestation of honey bees with *Tropilaelaps* spp. (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3http://www.oie.int/). Please contact the WOAH Reference Laboratory for any further information on diagnostic tests and reagents for infestation of honey bees with *Tropilaelaps* spp.

NB: FIRST ADOPTED IN 2004. MOST RECENT UPDATES ADOPTED IN 2018.

Appendix 1: Infestation of honey bees with *Tropilaelaps* spp.

Intended purposes of test: population freedom from infestation; individual animal freedom from infestation prior to movement; contribute to eradication policies; confirmation of clinical cases; prevalence of infestation – surveillance

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
Field detection of the mite *Apis mellifera* Bee examination ('wash method' using 'icing sugar roll'): ++ for all purposes Brood examination: +++ for all purposes "Bump test": + for 'population freedom from infestation', 'individual animal or bee nest freedom from infestation prior to movement' and 'contribute to eradication policies'; ++ for 'confirmation of clinical cases' and 'prevalence of infestation – surveillance' Debris	Sample type: *Apis mellifera* colonies Target analytes: *Tropilaelaps* spp.	Pettis *et al.* (2013) study aimed to compare the different methods commonly used in the field to detect *Tropilaelaps* infestation The test sensitivity was calculated with two different standards: i) Infestation verified (i.e. at least one sampling method detected mites); ii) Universal infestation assumed (i.e. it was assumed that every colony in an apiary with some mites is infested assuming universal infestation of all hives in an infested apiary) Bee examination (ethanol wash): i) DSe = 7.8% ; ii) DSe = 5.2% Worker brood examination (100 cells examined): i) DSe = 75.7%; ii) DSe = 56.7% Drone brood examination (20 cells examined): i) DSe = 32%; ii) DSe = 24.7%	Pettis *et al.* (2013) study: Ten *Apis mellifera* apiaries in Chiang Mai Thailand (236 colonies examined). Note: for debris examination ("drop method") and bee examination ("wash method"), experiments carried out in only three apiaries. Experiments conducted in September 2009 Gill *et al.* (2024) study: 60 colonies of *A. mellifera*, sourced from six apiaries in Chiang Mai province Thailand. Each colony was assessed using all four of the field protocols (brood examination, 'bump test', hive debris examination (natural drop) and adult honey bee examination). Period of experiments unknown	See references	Debris examination ("drop method"): high sensitivity according to Pettis *et al.* (2013) Worker brood examination: high sensitivity (Pettis *et al.*, 2013), most effective method for *Tropilaelaps* detection according to Gill *et al.* (2024) Adult bee examination based on 'icing sugar roll': non-destructive method, quite efficient, rapid and easily applicable in the field (Gill *et al.*, 2024) "Bump test" method: least time-consuming, can be carried out in the field, appropriate for large field surveys	Debris method: time consuming (required at least two visits on consecutive days to each apiary and careful examination of sticky board insertion), difficult to practice for large-scale screening Worker brood examination (100 brood cell): time-consuming, induce brood destruction Adult bee examination (ethanol wash): low sensitivity (Gill *et al.*, 2024; Pettis *et al.*, 2013) "Bump test" method: induce brood destruction but less limited than brood examination (Pettis *et al.*, 2013), ineffective for Tropilaelaps detection (Gill *et al.*, 2024)	Pettis *et al.* (2013) Gill *et al.* (2024)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
examination ("drop method"): ++ for all purposes		"Bump test": i) DSe = 50%; ii) DSe = 36.3% Debris examination ("Drop method"): i) DSe = 81.3%; ii) DSe = 54.2% The sensitivities were also evaluated at different infestation rates: minimal, medium, high. The "brood examination" was used to categorise the infestation level of the colonies. See detailed results in Pettis *et al.* (2013) Gill *et al.* (2024) assessed the efficacy of different field and laboratory methods for *Tropilaelaps* detection based on brood examination, 'bump test', hive debris examination (natural drop) and adult honey bee examination. Results indicated that uncapping infested brood with tweezers, catching mite drop using sticky traps and rolling adult bees in icing sugar were significantly more likely to detect *Tropilaelaps* than methods using an uncapping fork on infested brood, or the brood 'bump' method. Uncapping brood with tweezers appeared to be the more robust technique.					

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
Morphology: +++ for all purposes *Apis* spp.	Adult mite specimens	Reference tests were conventional PCR, sequencing and morphological analysis by acarologists from other reference laboratories. DSp = 100% DSe = 100%	Tropilaelaps mites from different species and localities (*n* = 14): *Three samples of *T. clareae* collected in different localities in the Philippines (Luzon island); *11 samples of *T. mercedesae* from Indonesia (New Guinea), Sri Lanka and Nepal Mites different from *Tropilaelaps* spp. detected in honeybee hives in France during apiary inspections (*n* = 17): *Varroa destructor, Varroa jacobsoni, Braula coeca, Neocypholaelaps apicola, Macrocheles* spp., *Androlaelaps casalis, Stratiolaelaps scimitus, Parasitellus* sp. **Note:** Due to the limited availability of reference material to validate the method, the data was consolidated through a literature review	Validation carried out by Anses Sophia Antipolis Laboratory (validation report submitted to WOAH in 2016) and supplemented with additional data	Rapid (less than 1 hour). Does not require sophisticated equipment, or reagents Inexpensive Method recommended for first intention diagnosis	Requires a certain amount of experience and technical skill to handle specimens and identify morphological criteria Differential diagnosis between the four species of *Tropilaelaps* not possible PCR is highly advisable if there is any doubt about identification If *Tropilaelaps* is detected in an area presumed to be free of the disease, it is recommended that identification be confirmed by PCR	Data not published at present
Conventional PCR: ++ for all purposes except for 'prevalence of infestation –	Adult and immature mite specimens	Reference test was morphology, HRM-PCR or sequencing DSp = 100% DSe = 100%	Tropilaelaps mites from different species and localities (*n* = 22): *Three specimens of *T. clareae* from Philippines *19 specimens of *T. mercedesae* from	Validation carried out by Anses Sophia Antipolis Laboratory (validation report	High specificity and sensitivity (generally more than morphology) Enables *Tropilaelaps* species to be identified after sequencing, HRM-PCR	Requires reagents and equipment for conventional PCR detection and analysis system More expensive and longer than the	Data not published at present

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
surveillance (+) *Apis* spp.			Pakistan, Indonesia, Sri Lanka, Vietnam and Nepal Mites and pathogens different from *Tropilaelaps* spp. detected in honeybee hives (n = 17) during apiary inspections (i.e. *Varroa destructor, Braula coeca, Neocypholaelaps apicola, Macrocheles* spp., *Androlaelaps casalis*)	submitted to WOAH in 2016) and supplemented with additional data	Method recommended for second intention diagnosis	morphology (> 1 day of analysis) Need for sequencing to identify species (specific equipment and competence or subcontracting)	

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SECTION 3.3.

AVES

CHAPTER 3.3.1.

AVIAN CHLAMYDIOSIS

SUMMARY

Description and importance of the disease: *Avian chlamydiosis* (*AC*) *is caused by a* Chlamydia *species in birds. The taxonomy of the family* Chlamydiaceae *was recently revisited. The genus* Chlamydia *currently includes ~~11~~ 15 recognised species, and among them* C. psittaci, C. avium, C. abortus, C. buteonis *and* C. gallinacea *have been isolated from birds. Among the avian species,* C. psittaci *is the only one with proven zoonotic potential.*

Outbreaks of AC in psittacine birds and domestic poultry farms cause considerable economic damage. The infection can lead to systemic and occasionally fatal disease in birds. The clinical signs are generally nonspecific and vary greatly in severity, depending on the species and age of the bird and the virulence of the Chlamydia *strain, but respiratory distress is mostly involved. Many birds, especially older psittacine birds and poultry, may show no clinical signs; nevertheless, they may often shed the agent for extended periods.*

Special laboratory handling as determined by biological risk analysis (*see Chapter 1.1.4* Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities) is *recommended and, ~~even obligatory~~ in many countries, mandatory for* C. psittaci *strains because ~~avian chlamydial strains~~ this species can cause serious illness* (*pneumonia*) *and death in humans ~~when~~ if left untreated.*

Detection and identification of the agent: *Due to the challenges associated with strain isolation (requirements for well preserved samples, cell culture, secure laboratory), the recommended diagnostic method is polymerase chain reaction (PCR), which provides rapid, sensitive and specific diagnosis. ~~The preferred method for the identification of AC is no longer isolation of the organism. Considering the time involved, the need for high quality samples, the fact that some strains will never grow~~ in vitro ~~and the hazard to laboratory personnel, nucleic acid amplification tests (NAATs) are currently recommended for quick, sensitive and specific diagnosis. These methods include conventional and real-time polymerase chain reaction (PCR), DNA microarray-based detection and DNA sequencing. Isolation,~~ Cytological staining of smears of exudate or faeces, and of impression smears of tissues, immunohistochemical staining of cytological and histological preparations ~~and antigen capture enzyme-linked immunosorbent assays (ELISA) can be used if NAATs are not available~~ if PCR cannot be performed.*

Serological tests: *Serology alone is not particularly useful in diagnosing a current chlamydial infection in birds because of the high prevalence of this infection in birds and the long-term* (*up to several months*) *persistence of anti-chlamydial antibodies. In most bird species, there is a high*

background rate of anti-chlamydial antibodies. Thus, to determine if a single bird is infected, serology should always be used in conjunction with gene or antigen detection, or paired sera should be examined. A positive test is evidence that the bird was infected by the bacterium but does not necessarily indicate an active infection. False negative results can occur in birds with acute infections that are sampled before seroconversion. Treatment with antibiotics may also delay or diminish the antibody response.

The main serological methods that are used for detecting chlamydial antibodies are: (1) ~~various~~ agglutination methods ~~of elementary body agglutination (EBA)~~, (2) the complement fixation test and (3) enzyme-linked immunosorbent assays (ELISA) is highly sensitive and specific when using recombinant proteins/peptides ~~as antigen targets~~ and ~~it~~ can detect IgM, IgG and IgA, but host specific conjugates have to be used.

Requirements for vaccines: *There are no commercial vaccines available for avian chlamydiosis ~~control in poultry~~.*

A. INTRODUCTION

1. Description and impact of the disease

Avian chlamydiosis (AC) is caused by infection with a *Chlamydia* species in birds. *Chlamydia psittaci* is the most important aetiological agents with a proven pathogenic and zoonotic potential. While it was considered to be the only chlamydial agent in birds for a long time, a number of novel species have been identified in recent years, such as avian *C. abortus*, *C. avium*, *C. buteonis* and *C. gallinacea*. ~~In 2015, the taxonomy of the family *Chlamydiaceae* was revisited by Sachse *et al.* (Sachse *et al.*, 2015). The genus *Chlamydia* currently includes 11 recognised species, namely *C. abortus* (sheep, goats, cattle), *C. caviae* (guinea-pigs), *C. felis* (cats), *C. muridarum* (mouse, hamster), *C. psittaci* (birds and others), *C. pecorum* (sheep, cattle), *C. pneumonia* (human and others), *C. suis* (swine), *C. trachomatis* (human) and two recently established species isolated from birds, *C. avium* and *C. gallinacea* (Sachse *et al.*, 2014). While most of these organisms are highly host specific, *C. pneumonia* and *C. psittaci* have a broader host range. The latter has been reported to occur not only in birds and humans, but also in cattle, sheep, swine, horses and other animals. Until very recently, *C. psittaci* was considered to be the sole causative agent of the disease in birds. Originally called psittacosis, the term ornithosis was introduced later to differentiate the disease in domestic and wild fowl from the disease in psittacine birds. The two syndromes are currently considered to be the same (Andersen & Vanrompay, 2008). Their earlier separation was based on the assumption that in humans, ornithosis was a milder disease than psittacosis. However, it should be noted that the disease in humans contracted from turkeys and ducks is often as severe as that contracted from psittacine birds.~~

Infection of birds with *C. psittaci* is common all over the world ~~and has been found in about 465 avian species (Kaleta & Taday, 2003)~~. Outbreaks ~~of AC~~ in psittacine birds and domestic poultry farms cause considerable economic damage. The infection can lead to systemic and occasionally fatal disease in birds. The clinical signs are generally nonspecific and vary greatly in severity, depending on the species and age of the bird and the chlamydia strain. *C. psittaci* ~~AC~~ can produce lethargy, hyperthermia, abnormal excretions, nasal and eye discharges, and reduced egg production. Mortality rates will vary greatly. In pet birds, the most frequent clinical signs are conjunctivitis, anorexia and weight loss, diarrhoea, yellowish droppings, sinusitis, biliverdinuria, nasal discharge, sneezing, lachrymation and respiratory distress. Many birds, especially older psittacine birds and poultry, may show no clinical signs; nevertheless, they may often shed the agent for extended periods. Necropsy of affected birds will often reveal multifocal hepatic necrosis, spleen and liver enlargement, fibrinous airsacculitis, pericarditis and peritonitis (Andersen & Vanrompay, 2008; Vanrompay, 2013). Histological lesions are suggestive of infection but are non-pathognomonic unless there are identifiable chlamydiae present.

~~Until recently, nine different genotypes based on the ompA gene coding for the major outer membrane protein (MOMP) were distinguished within C. psittaci strains. Seven of these genotypes are thought to occur predominantly in a particular order or class of Aves and two in non-avian hosts, i.e. genotype A in psittacine birds, B in pigeons, C in ducks and geese, D in turkeys, E in pigeons, ducks and others, E/B in ducks, turkeys and pigeons, F in parakeets, WC in cattle, and M56 in rodents. Most of the avian genotypes have also been identified sporadically in isolates from cases of zoonotic transmission to humans, particularly A, B and E/B (Heddema et al., 2006; Vanrompay et al., 2007). Meanwhile, subgroups for three of the more heterogeneous genotypes have been introduced, i.e. A-VS1, A-6BC, A8455, EB-E30, EB-859, EB-KKCP, D-NJ1, D-9N, and provisional genotypes to cover the strains that were previously non-typable have been suggested (Sachse et al., 2008).~~

Of the other *Chlamydia* species detected in birds, *C. gallinacea* appears to be fairly widespread in poultry, primarily chickens and turkeys, although its presence in other birds is suspected and anecdotally documented. *Chlamydia gallinacea* infections are mainly subclinical, although reduced weight gain has been observed in infected chickens

(Guo *et al.*, 2016; Heijne *et al.*, 2021). *Chlamydia avium* has been detected in psittacines and pigeons, and can cause respiratory disease, enteritis, anaemia, emaciation and sudden death without prior signs (Sachse *et al.*, 2014). Co-infection with other *Chlamydia* spp. or pathogens may exacerbate the course of the infection. *Chlamydia buteonis* has been associated with conjunctivitis, respiratory disease, and death in addition to subclinical carriage in raptors (Laroucau *et al.*, 2019). Recently, avian *C. abortus* has been detected in wild birds and poultry, in most cases without overt disease, but with suspected zoonotic transmission.

Antibiotics are the only current treatment. *Chlamydia* is susceptible to a number of antibiotics: the drug of choice varies from country to country. Chlortetracycline, doxycycline, and other tetracyclines are the most commonly used. Treatment must be maintained for an extended period of time. For pet birds, 45 days is often recommended (Vanrompay, 2013).

2. Nature and classification of the pathogen

Members of the *Chlamydiaceae* family are obligate intracellular bacteria with a biphasic life cycle that includes extracellular infectious elementary bodies (EBs) and intracellular metabolically active reticulate bodies (RBs). Replication takes place within a membrane-bound vacuole.

Classical genotyping of *C. psittaci* is based on the *omp*A gene, which encodes for the major outer membrane protein (MOMP). The majority of genotypes are thought to occur predominantly in a particular Order or Class of aves and two in non-avian hosts, i.e. genotype A in psittacines, B in pigeons, C in ducks and geese, D in turkeys, E in pigeons, ducks and others, E/B in ducks, turkeys and pigeons, F in parakeets, WC in cattle, and M56 in rodents. Most of the avian genotypes have been associated with human cases, particularly A, B, C and E/B (Heddema *et al.*, 2006). Recently, an analysis of the genomic sequences of *C. psittaci* strains has identified eight major groups that roughly correspond to the *omp*A-based genotypes, and a rapid single nucleotide polymorphism (SNP)-based typing tool now makes it possible to rapidly identify the genotype and therefore the likely origin of contamination (Vorimore *et al.*, 2021).

A multi-locus sequence typing (MLST) scheme based on the partial sequences of seven housekeeping genes is available to evaluate the population genetic structure of chlamydiae and the diversity between and within these species (Pannekoek *et al.*, 2010; https://pubmlst.org/).

~~Evidence suggests that other chlamydial species, such as *C. abortus*, *C. pecorum*, *C. trachomatis*, *C. suis* and *C. muridarum* can also be harboured by birds (Guo *et al.*, 2016; Pantchev *et al.*, 2009), as well as by the avian species *C. avium* and *C. gallinacea* described by Sachse *et al.*, in 2014. Their epidemiological importance is still unclear, however *C. avium* and *C. gallinacea* appear to be quite widespread in pigeons and psittacines or poultry birds, respectively. The pathogenicity of these two newly introduced species has yet to be systematically investigated. In surveys reported to date, no clinical signs have been observed in chickens carrying *C. gallinacea* (Guo *et al.*, 2016; Laroucau *et al.*, 2009), nor in most of the *C. avium* carriers among pigeons. However, it seems likely from currently available data that *C. avium* is able to cause respiratory disease in parrots and pigeons (Sachse *et al.*, 2014). It is now recommended to do a differential diagnosis and use diagnostic methods that are capable of differentiating between *C. psittaci* and the other species that can be hosted by birds. To date only molecular methods can make this distinction.~~

~~1~~ 3. Zoonotic risk and biosafety requirements

The *C. psittaci* strains ~~of avian chlamydiae~~ can infect humans and should be handled with appropriate biosafety and containment procedures (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*). ~~Risk assessment and management are essential when performing diagnosis of AC.~~ Adequate information, communication, and health surveillance by an occupational physician are recommended.

Most infections occur through inhalation of infectious aerosols. While the disease from psittacine birds is best known, the infection in poultry is of particular concern as transmission to humans is common during handling and slaughter of the birds (Dickx *et al.,* 2010; Lagae *et al.*, 2014). Post-mortem examinations of infected birds and handling of cultures should be done in certified Class II ~~laminar flow hoods~~ biosafety cabinet whenever possible or with proper protective equipment. Appropriate zoonotic agent decontamination procedures should be followed because human infection can result from transient exposures. The incubation period is usually 5–14 days; however, longer incubation periods are known. Human infections vary from inapparent to severe systemic disease with interstitial pneumonia and encephalitis. The disease is rarely fatal in properly treated patients; ~~therefore~~ so awareness of the danger and early diagnosis are important. Infected ~~humans~~ individuals typically develop headache, chills, malaise and myalgia, with or without signs of respiratory involvement. Pulmonary involvement is common. However, auscultatory findings~~, however,~~ may appear ~~to be~~ normal or ~~to~~ may underestimate the extent of involvement. ~~Diagnosis can be difficult and in the past was usually established through testing paired sera for antibodies to chlamydia by the complement fixation test (CFT). However, some patients remain seronegative though~~

~~hospitalised with psittacosis; serology~~ Serological diagnosis is now ~~therefore~~ being replaced by ~~nucleic acid amplification tests (NAATs)~~ polymerase chain reaction (PCR), which also allows the ~~bird~~ avian source to be traced. In humans, tetracycline, doxycycline, or azithromycin are usually the drugs of choice unless contraindicated. The length of treatment ~~will vary~~ varies with the drug, but should be continued for at least 14 days for tetracycline.

4. Differential diagnosis

The differential diagnosis can be salmonellosis (see Chapter 3.10.3 *Salmonellosis*) in the case of diarrhoea and mycoplasma infections (see Chapter 3.3.5 Avian mycoplasmosis [*Mycoplasma gallisepticum, M. synoviae*]) in the case of rhinitis. As multiple species can be involved in avian chlamydiosis, it is recommended to use diagnostic methods that are capable of differentiating between *C. psittaci* and the other species. To date only molecular methods can make this distinction.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of avian chlamydiosis and their purpose

Method	Purpose					
	Population freedom from infection[(a)]	Individual animal freedom from infection prior to movement[(b)]	Contribute to eradication policies	Confirmation of clinical cases[(c)]	Prevalence of infection – surveillance[(d)]	Immune status in individual animals or populations post-vaccination
Detection and identification of the agent						
Conventional PCR	–	–	–	++	+	–
Real-time PCR	–	–	–	+++	++	–
~~DNA microarray~~	~~–~~	~~–~~	~~–~~	~~++~~	~~+~~	~~–~~
Cytological staining	–	–	–	+	–	–
Isolation in cell culture or embryonated eggs	–	–	–	++	+	–
IHC on fixed tissue	–	–	–	++	–	–
Detection of immune response						
ELISA	++	+	=	+	++	=
CFT	+	+	–	+	+	–
~~ELISA~~	~~++~~	~~+~~	~~–~~	~~+~~	~~++~~	~~–~~

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; IHC = immunohistochemistry; CFT = complement fixation test;
ELISA = enzyme-linked immunosorbent assay.
[(a)] See Appendix 1 of this chapter for justification table for the scores given to the tests for this purpose.
[(b)] See Appendix 2 of this chapter for justification table for the scores given to the tests for this purpose.
[(c)] See Appendix 3 of this chapter for justification table for the scores given to the tests for this purpose.
[(d)] See Appendix 4 of this chapter for justification table for the scores given to the tests for this purpose.

1. Detection and identification of the agent

The preferred method for the identification of AC is no longer isolation of the organism. Considering the time involved, the need for high-quality samples, ~~the fact that some strains will never grow *in vitro* and~~ the hazard to laboratory personnel, ~~NAATs~~ molecular techniques are currently recommended for quick, sensitive and specific diagnosis. These include conventional and real-time polymerase chain reaction (PCR), ~~DNA microarray based detection~~ and DNA sequencing. Isolation, cytological staining of smears of exudate or faeces, and of impression smears of tissues, immunohistochemical staining of cytological and histological preparations ~~and antigen-capture enzyme-linked immunosorbent assays (ELISA)~~ can be used if ~~NAATs are not available~~ PCR methods cannot be performed.

The ~~samples~~ specimens to be collected ~~will~~ depend on the ~~disease~~ signs ~~in evidence~~ of the disease. Specimens from acute cases should include inflammatory or fibrinous exudates in or around organs ~~that display~~ showing lesions, ocular and nasal exudates, impression smears of liver, whole blood, and tissue samples from kidney, lung, pericardium, spleen, and liver. In cases ~~with~~ of diarrhoea, ~~colon~~ intestinal contents or ~~excrement should~~ faeces can be ~~used~~ tested. In live birds, cloacal, ~~the preferred samples are~~ pharyngeal and nasal swabs are the preferred samples. ~~Intestinal excrement, cloacal swabs~~ Faeces, conjunctival scrapings, and peritoneal exudates can also be ~~taken~~ collected.

1.1. Molecular methods ~~– detection of nucleic acids~~

PCR techniques have replaced isolation for the detection of chlamydiae from animal tissues.

Chlamydia psittaci can be ~~identified~~ detected and ~~subtyped~~ genotyped using: (1) species-specific conventional PCR; (2) real-time PCR ~~(reviewed in Sachse *et al.*, 2009)~~; (3) *omp*A sequencing (Sachse *et al*., 2008); (4) multi-locus sequence typing (MLST) (Pannekoek *et al*., 2010) or (5) PCR-HRM (high resolution melting) genotyping (Vorimore *et al*., 2021)~~; and (5) DNA microarray (Sachse *et al.*, 2005, 2008)~~.

As mentioned above, *C. psittaci* is not the only chlamydial agent encountered in birds. If a *Chlamydiaceae*-specific PCR or immunohistochemistry test is positive, but a species-specific test for *C. psittaci* is negative, the newly described *Chlamydial agents* species ~~described by Sachse *et al.* in 2014 have to be taken into consideration~~ should be considered. Species-specific real-time PCRs are available for their specific detection (Zocevic *et al.*, 2013 [*C. avium*], Laroucau *et al.*, 2015 [*C. gallinacea*], Laroucau *et al.*, 2019 [*C. buteonis*]) as well as for the detection of avian *C. abortus* strains (Aaziz *et al.*, 2023). ~~when a given avian sample is positive in a general chlamydial test, e.g. *Chlamydiaceae*-specific PCR or immunohistochemistry, but negative in a species-specific test for *C. psittaci*. In such a case, partial or complete sequencing of the *omp*A gene and the rRNA operon or alternative species-specific PCR assays or whole genome sequencing, will reveal the identity of the strain. The occurrence of *Chlamydia* strains that are phylogenetically in between *C. psittaci and C. abortus* has also been described (Pannekoek *et al*., 2010; Van Loock *et al*., 2003), and should likewise be considered as a possible differential diagnosis.~~

~~Reagents designed to stabilise the DNA should be considered when a delay in processing the sample is anticipated (DeGraves *et al.*, 2003).~~ DNA samples can be prepared using inexpensive reagents or commercially available kits. Risk of infection to laboratory staff is avoided by inactivating the sample prior to testing or by handling under a Class II biosafety cabinet.

1.1.1. Conventional polymerase chain reaction

~~PCR techniques have been replacing isolation for the detection of chlamydiae from animal tissues. Infection risks to laboratory staff are avoided by inactivation of the sample prior to testing. The sensitivity of conventional PCR assays will usually exceed that of isolation. Current~~ Conventional PCR ~~tests~~ assays for the detection of *C. psittaci* target the 16S–23S rDNA or the *omp*A gene (reviewed in Sachse *et al.,* 2009), but sensitivity and specificity vary depending on ~~sample preparation and~~ the PCR test used~~, but are considered inferior when compared with quantitative real-time PCR assays.~~ Sensitivity ~~is~~ can be increased by targeting ~~a relatively~~ short DNA ~~segment~~ fragments or using a nested procedure. However, there is the risk of contamination if ~~extreme care is~~ strict precaution are not taken when manipulating the ~~reactions~~ PCR products (see Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals*).

No conventional PCR assays have been developed to detect the newly described avian species.

1.1.2. Real-time PCR

Real-time PCR ~~has become~~ is the preferred method in diagnostic laboratories ~~for~~ because of its speed, ~~its rapidity,~~ high throughput, potential for quantification and ease of standardisation (Sachse *et al.,* 2009). This technology requires a fluorescent-labelled probe and a special equipment, which increases costs. Its sensitivity can be equivalent to that of the nested system, but contamination problems and labour are reduced as it is based on ~~one~~ a single reaction in a closed system.

A hierarchical approach is recommended for the detection and identification of *C. psittaci* DNA. Such an approach includes a *Chlamydiaceae*-specific screening PCR based on the sequences of 23S-rRNA in positive cases (~~DeGraves *et al.*, 2003;~~ Ehricht *et al.,* 2006~~; Everett *et al.,* 1999~~), followed by ~~a~~ *C. psittaci*-specific PCR assays based on sequences of the outer membrane protein (OmpA) (Pantchev *et al.,* 2009) or on a *C. psittaci*-specific gene sequence allowing a clear

separation from phylogenetically close *C. abortus* strains (Angen *et al.*, 2021). This latter PCR system has been shown to have a higher specificity than the *omp*A-based system ~~or of the incA gene (Ménard *et al.*, 2006). Minor groove binding (MGB) probes are used to rule out cross-reactions with *C. abortus*~~.

Commercial PCR kits for the detection of *C. psittaci* for human and veterinary samples are available.

Table 2. Examples of validated and published real-time PCR assays for screening and species identification of C. psittaci

Reference	Ehricht *et al.* (2006)	Pantchev *et al.* (2009)	Angen *et al.* (2021)
Specificity	*Chlamydiaceae*	*C. psittaci*	*C. psittaci*
Target	23S rRNA	*omp*A	CDS CPSIT_RS03505
Amplicon size	111 bp	76 bp	91 bp
Primer forward 5'–3'	CTG-AAA-CCA-GTA-GCT-TAT-AAG-CGG-T	CAC-TAT-GTG-GGA-AGG-TGC-TTC-A	CGA-GGC-ATC-TTG-TAG-TAG-AGA-A
Primer reverse 5'–3'	ACC-TCG-CCG-TTT-AAC-TTA-ACT-CC	CTG-CGC-GGA-TGC-TAA-TGG	ATT-ATC-GGC-ACT-ACT-TCT-AAC-A
Probe 5'–3'	FAM-CTC-ATC-ATG-CAA-AAG-GCA-CGC-CG-TAMRA	FAM-CGCTACTTGGTGTGAC-BHQ1 (MGB)	FAM-AAA-CTA-CCC-TCC-TCA-GGG-ACC-ACT-TAMRA
Cycling conditions	95°C/10 minutes 45 × (95°C/15 seconds, 60°C/60 seconds)	95°C/10 minutes 45 × (95°C/15 seconds, 60°C/60 seconds)	95°C/10 minutes 45 × (95°C/15 seconds, 60°C/60 seconds)

For greater sensitivity, real-time PCR protocols are recommended. However, a conventional PCR method, originally developed for improved detection of *C. abortus* in ruminants, has also been shown to detect *C. psittaci* strains. This method targets the pmp gene family, producing amplification products of approximately 300 bp. The primers used are: CpsiA: 5'-ATG-AAA-CAT-CCA-GTC-TAC-TGG-3, CpsiB: 5'-TTG-TGT-AGT-AAT-ATT-ATC-AAA-3'. The PCR cycle involves denaturation at 94°C for 30 seconds, annealing at 50°C for 1 minute, and extension at 72°C for 2 minutes. A final incubation step at 72°C for 10 minutes is then performed (Laroucau *et al.*, 2007).

~~The protocol of the *omp*A based *C. psittaci* specific assay (Pantchev *et al.*, 2009) is given in more detail below. The assay is conducted as a duplex amplification that includes an internal amplification control (IAC). A detection limit of 2 inclusion forming units per reaction mix was determined.~~

~~i) The *C. psittaci* specific oligonucleotides are primers CppsOMP1-F (5'-CAC-TAT-GTG-GGA-AGG-TGC-TTC-A-3') and CppsOMP1-R (5'-CTG-CGC-GGA-TGC-TAA-TGG-3'), as well as MGB® probe CppsOMP1-S (FAM-CGC-TAC-TTG-GTG-TGA-C-TAMRA). The IAC system includes primers EGFP1-F (GAC-CAC-TAC-CAG-CAG-AAC-AC) and EGFP10-R (CTT-GTA-CAG-CTC-GTC-CAT-GC), as well as TaqMan probe EGFP-HEX (HEX-AGC-ACC-CAG-TCC-GCC-CTG-AGC-A-BHQ1). Plasmid IC2 (available commercially) serves as the IAC template.~~

~~ii) The amplification is conducted in 96-well microtitre plates on an Mx3000P thermocycler or comparable equipment. Each 25 µl reaction contains 12.5 µl of 2 × universal real-time PCR Master Mix. The final concentration is 0.8 µM for each *C. psittaci* primer, 0.4 µM for each IAC primer, and 0.2 µM for each probe.~~

~~iii) IAC template DNA (500 copies) is added to each reaction before the final volume is made up with water.~~

~~iv) The following cycling parameters are used: initial heating cycle at 95°C for 10 minutes (single denaturation step), 45 cycles of 95°C for 15 seconds and 60°C for 1 minute (annealing and extension).~~

~~v) The cycle threshold value (Ct = Cq quantification cycle) automatically calculated by the software should be used. Cq values of 35 or lower are considered as positive. Cq values higher than 35 should be treated with caution as they may represent cross-reaction with~~

~~related microorganisms. In such cases, the samples should be re-examined, preferentially by alternative tests using different genomic targets (Ménard *et al.*, 2006; Opota *et al.*, 2015). Samples can also be re-tested in the real-time PCR. In this case, only repeatedly positive samples are judged as true positive.~~

~~Other *omp*A based real-time PCR protocols were developed to differentiate between genotypes of *C. psittaci* (Geens *et al.* 2005, Heddema *et al.*, 2015). The latter PCR is also validated for use on human samples in case of a zoonotic infection and thus helpful to trace chains of zoonotic transmission.~~

~~Real-time PCR protocols are available for the specific detection of *C. avium* (Zocevic *et al.*, 2013) and *C. gallinacea* (Laroucau *et al.*, 2015) species.~~

~~1.1.3. DNA microarray~~

~~DNA microarray technology was shown to be a powerful tool in the diagnosis of chlamydial infections (Sachse *et al.*, 2005). The assay for detection and identification of *Chlamydiaceae* spp. is based on PCR amplification of the 23S rRNA gene and subsequent identification of *C. psittaci* and the other avian agents *C. avium* and *C. gallinacea* by hybridisation with species-specific probes. It has been validated and proved suitable for routine diagnosis (Borel *et al.*, 2008). This methodological approach enables detection of mixed chlamydial infections and identification of unexpected chlamydial species directly from clinical samples. An extended version of the DNA microarray allows for *omp*A based genotyping of *C. psittaci* strains and clinical samples (Sachse *et al.*, 2008).~~

1.2. Direct visualisation – cytological staining

Chlamydiae can be detected in smears of cloacal or conjunctival swabs and in impression smears of tissues (lung, liver, spleen, kidney and airsacs if enough material is available) by cytological staining such as Giemsa, Giménez, modified Giménez, Ziehl–Neelsen and Macchiavello's stains (Campbell ~~*et al.*~~, 2015). The modified Giménez technique is most often used (Andersen & Vanrompay, 2008). However, none of the stains specifically detects chlamydia. They are all less sensitive than antibody-based antigen detection methods or specific ~~NAATs~~ molecular techniques. Therefore, use of a cytological staining is losing popularity.

1.2.1. Modified Giménez staining

i) Reagents

a) Solution 1

Distilled H_2O (450.0 ml) and phenol (5.0 ml) added to basic fuchsin (2.5 g) and 95% ethanol (50.0 ml). Incubate at 37°C for 48 hours. Filter and store in the dark at room temperature.

b) Solution 2

Na_2HPO_4 (11.65 g); $Na_2HPO_4.H_2O$ (2.47 g); distilled H_2O, pH 7.5 (to 1.0 litre).

c) Solution 3

Solution 1 (20.0 ml); and solution 2 (25.0 ml). Let stand for 10 minutes, filter and use.

d) Solution 4

0.5% citric acid.

e) Solution 5

Fast green (0.2 g); distilled H_2O (100.0 ml); and glacial acetic acid (0.2 ml).

f) Solution 6

Solution 5 (20.0 ml); and distilled H_2O (50.0 ml).

ii) Procedure for smears

a) Fix in methanol for 5 minutes.

b) Stain in solution 3 for 10 minutes and rinse in tap water.

c) Counterstain in solution 6 for 2 minutes.

d) Rinse in tap water and air-dry.

iii) Procedure for paraffin sections

a) Deparaffinise and hydrate with distilled H_2O.

b) Stain in solution 3 for 10 minutes and rinse in tap water.

c) Dip in solution 4 until no more red runs out of the section. Rinse in tap water.

d) Counterstain in Solution 6 for 20 dips.

e) Dip in two changes of 95% alcohol, for five dips each. Dehydrate, clear, and mount.

Note: a shorter procedure with “ready to use” carbol fuchsin (1/10 in distilled water), acetic acid (0.1%,) and malachite green counterstain (0.8%) is also available (Vanrompay *et al.,* 1992). Chlamydiae will appear red against a green background.

1.3. Isolation in cell culture

1.3.1. Treatment of samples for isolation

Proper handling of clinical samples is necessary to prevent loss of infectivity of chlamydiae during shipping and storage. A special medium consisting of sucrose/phosphate/glutamate (SPG) was developed for rickettsiae and has proven to be satisfactory for transport of chlamydial field samples. The medium as recommended for chlamydiae consists of SPG buffer: sucrose (74.6 g/litre); KH_2PO_4 (0.52 g/litre); K_2HPO_4 (1.25 g/litre); L-glutamic acid (0.92 g/litre), and bovine serum albumin – fraction V (1 g/litre), which can be sterilised by filtering. Added to this are streptomycin, vancomycin, (25-100 µg/ml), amphotericin B and gentamicin (50 µg/ml each). The addition of antibiotics reduces the effect of contamination, even when samples are shipped at ambient temperatures. The organism remains viable for several days even in the absence of refrigerative storage. This medium can also be used as a laboratory diluent and for freezing of chlamydiae.

Contaminated samples must be pre-treated before being used to inoculate cell cultures. There are three basic methods: treatment with antibiotics, treatment with antibiotics together with low-speed centrifugation (Andersen & Vanrompay, 2008), and treatment with antibiotics with filtration (Andersen & Vanrompay, 2008). A number of antibiotics that do not inhibit chlamydia can be used. Samples are homogenised in phosphate buffered saline (PBS), pH 7.2, containing a maximum of the following: streptomycin, vancomycin (100 µg/ml each), and gentamicin (50–200 µg/ml). Amphotericin B or nystatin (50 µg/ml each) can be added to control yeast and fungal growth. Penicillin, tetracycline and chloramphenicol should be avoided as these inhibit the growth of chlamydiae. ~~In some cases, for example porcine faecal samples, treatment with penicillin G (500 IE/ml) can been useful.~~

When contamination is light, samples should be homogenised in the antibiotic solution prior to inoculation into tissue cultures. Samples are often left to stand in the antibiotic solution for 24 hours at 5°C before inoculation. Heavily contaminated samples, such as faecal samples, should be homogenised in antibiotics and then centrifuged at 500 ***g*** for 20 minutes. The surface layer and the bottom layer are discarded. The supernatant fluid is collected and recentrifuged. The final supernatant fluid is used for inoculation. Samples should be passed through a filter of 450–800 µm average pore size if contamination persists.

Cell cultures are the most convenient method for the isolation of *C. psittaci*. The most common cell lines are buffalo green monkey (BGM), McCoy, HeLa, African green monkey kidney (Vero), and L cells (Vanrompay *et al.,* 1992). The cells are grown as monolayers using standard tissue culture media containing 5–10% fetal calf serum and antibiotics that are not inhibitory to chlamydia (as described previously).

When selecting cell culture equipment, it is important to remember that:

i) Chlamydiae can be identified by PCR, direct or indirect immunofluorescence, or some other appropriate staining technique;

ii) The inoculum is usually centrifuged on to the monolayer to enhance its infectivity;

iii) The sample may need to be blind passaged at 4–5 days to increase sensitivity of isolation;

iv) The sample will need to be examined from two to three times during any one passage; and

v) Chlamydiae can be infectious to humans.

Small flat-bottomed vials, such as 1-dram (3.7 ml, 15 × 45 mm) shell vials or bottles containing cover-slips that are 12 mm in diameter, will meet these requirements. A number of vials, often four to six, are inoculated with each sample to permit fixing and staining at various intervals, and to permit repassaging of apparently negative samples 6 days after inoculation. When testing multiple samples, 24-well multiwell dishes can also be used as they have a labour-saving advantage. However, it should be noted that cross-contamination between samples can be a problem.

Chlamydiae can be isolated from cells that are replicating normally, but the use of non-replicating cells is preferable as these may provide increased nutrients for the growth of chlamydiae. Suppressed cells can also be observed for longer periods. Host cell division can be suppressed by cytotoxic chemicals, such as cycloheximide, which can be added to the medium at the rate of 0.5–2.0 µg/ml at the time of inoculation of the monolayer (Andersen & Vanrompay, 2008). A similar cytostatic effect that will enhance the growth of most chlamydial strains is reached by the use of serum-free tissue culture medium.

Attachment of chlamydia to cells is increased by centrifuging the inoculum on to the monolayer at 2000–3500 ***g*** for 30–90 minutes at 37°C. After a 2-hour incubation period at 37°C and 5% CO_2, the inoculum is removed and replaced with serum-free or cycloheximide-containing tissue culture medium, and cultures are incubated at 37–39°C. Cultures must be examined for chlamydiae at regular intervals using an appropriate staining method. This is usually done on day 2 or 3, as well as on day 4 or 5. Cultures that appear to be negative at the fifth day are harvested and repassaged. When repassaging chlamydiae, cells and culture media should be passaged without using freeze–thawing to disrupt cells, as this will destroy the chlamydiae.

Before staining the cultures, the medium is first removed, the cultures are washed with PBS and fixed with acetone or methanol for 2–10 minutes. The fixation time will depend on the tissue culture vessel used. As acetone will soften most plastics, the use of a mixture of 50% acetone and 50% methyl alcohol may be preferable. A number of staining methods can be employed to demonstrate chlamydial inclusions. The preferred method is direct immunofluorescence (Andersen & Vanrompay, 2008). A chlamydial fluorescein-conjugated antiserum is applied to the infected cells and incubated in a humid chamber for 30 minutes at 37°C. The cover-slips are then washed three times with PBS, mounted immediately, and examined. Chlamydial inclusions fluoresce in green. Commercial conjugate preparations using monoclonal antibodies (MAbs) are available and are highly specific. Conjugates may also be prepared from polyclonal sera, but it is important to obtain specific, high-titred antisera. Polyclonal antisera can be prepared in rabbits, guinea-pigs, sheep or goats. Conjugates are then prepared using standard techniques (Andersen & Vanrompay, 2008). Chlamydial inclusions can also be demonstrated by indirect fluorescent antibody and immunoperoxidase techniques (Andersen & Vanrompay, 2008). Direct staining can be done with Gimenez (see Section B.1.2.1), Giemsa, Ziehl–Neelsen, or Macchiavello's stains. Except for immunofluorescence, all these stains have the advantage that standard light microscopes can be used.

1.4. Isolation in embryonated eggs

Chicken embryos are still used for the primary isolation of chlamydiae. Samples should be handled and pre-treated with antibiotics as described in Section B.1.3. The standard inoculation procedure is to inject up to 0.5 ml of inoculum into the yolk sac of a specific pathogen free 6- to 7-day-old embryo (Andersen & Vanrompay, 2008). The eggs are then incubated in a humid atmosphere at 39°C, rather than at 37°C, as multiplication of chlamydiae is greatly increased at the higher temperature. Replication of the organism usually causes the death of the embryo within 3–10 days. If no deaths occur, two additional blind passages are usually made before designating any sample as negative. Chlamydial infections will give rise to a typical vascular congestion of the yolk sac membranes. These are harvested and homogenised as a 20% (w/v) suspension in SPG buffer, and can be frozen to preserve the strain, or inoculated into eggs or on to cell cultures.

The organism can be identified ~~by preparing an antigen from an infected yolk sac and testing it~~ by PCR, direct staining of smears using appropriate stains ~~or by using the antigen in a serological test~~ or by immunofluorescence. Cell culture monolayers can be inoculated with the yolk sac suspension and examined by direct immunofluorescence 48–72 hours later for the presence of chlamydial inclusions. Typical inclusions are intracytoplasmic round or hat-shaped bodies. With some virulent strains, the inclusions rapidly break up and the chlamydial antigen is dispersed throughout the cytoplasm.

1.5. Antigen detection

1.5.1. Immunohistochemical staining

Immunohistochemical staining can be used to detect chlamydiae in cytological and histological preparations and is an indispensable tool to show the association of chlamydial agents and pathological lesions in tissues. The technique is more sensitive and specific than histochemical staining. Antigen detection can be performed using commercially available anti-*Chlamydia* antibodies directed against LPS or MOMP (major outer membrane protein).

The selection of the primary antibody is very important. Both polyclonal and monoclonal antibodies have been used. Because formalin affects chlamydial antigens, it is recommended that polyclonal antibodies be made to purified formalin-inactivated chlamydiae. The chlamydial strain used is not important, as the antibodies will be reactive mainly to the group-specific antigens. MAbs should also be selected for reactions to formalin-fixed chlamydiae. A pool of group-reactive MAbs can be used.

~~**1.5.2. Enzyme-linked immunosorbent assays**~~

~~The ELISA has been extensively promoted in kit format for use in the diagnosis of human chlamydiosis. These test kits detect the lipopolysaccharide (LPS) antigen (group reactive) and will detect all species of *Chlamydiaceae*. A number of these kits have been tested for use in detecting chlamydiae in birds (Vanrompay *et al.*, 1994), but none of the kits has been licensed for detection of *C. psittaci*. One problem with some of these tests is that the chlamydial LPS shares some epitopes with other Gram-negative bacteria, and these epitopes can cross-react, resulting in a high number of false-positive results. This problem has been reduced or eliminated in more recently developed kits by careful selection of the MAbs used. These kits, however, still lack sensitivity because a few hundred organisms are still needed to give a positive reaction. Most diagnosticians believe that a diagnosis of AC can be made when a strong positive ELISA reaction is obtained from birds with signs of psittacosis. Because of the number of false-positive results, a positive in an individual bird without signs of disease is not considered to be significant, but indicates the need for further testing using different methods.~~

2. Serological tests

Serology alone is not particularly useful in diagnosing a current chlamydial infection in birds because of the high prevalence of this infection in birds and the long-term (up to several months) persistence of antichlamydial antibodies. In most bird species, there is a high background rate of antichlamydial antibodies in birds. Thus, to determine if a single bird is infected, serology should always be used in conjunction with antigen or gene detection, or paired sera should be examined (Vanrompay, 2013). However, obligatory examination of paired sera removes serology from immediate clinical relevance. A positive test is evidence that the bird was infected by the bacterium but does not necessarily indicate an active infection. False negative results can occur in birds with acute infections that are sampled before seroconversion. Treatment with antibiotics also may delay or diminish the antibody response. The main serological methods that are being used for detecting chlamydial antibodies are: (1) ~~various methods of elementary body~~ agglutination ~~(EBA)~~, (2) the enzyme-linked immunosorbent assay (ELISA) ~~CFT~~ and (3) ~~enzyme-linked immunosorbent assay (ELISA)~~ CFT.

~~EBA~~ Agglutination tests detect primarily IgM antibodies and thus can detect early infections. A negative result does not guarantee that a bird is free of infection because the sensitivity of the test is rather low. The direct CFT detects avian IgG but not IgM, so recent infections can be 'missed'. Its disadvantages are that: (1) the test antigens are not commercially available, (2) the test cannot be used for testing sera from avian species whose immunoglobulins do not fix complement, (3) it is only relatively sensitive, (4) it cannot be used to differentiate between IgG and IgM antibodies, and (5) it is fairly laborious when there is a large number of samples to be tested. The modified CFT is more sensitive but has the same disadvantages as the CFT.

The CFT is ~~more and more~~ being replaced by highly sensitive and specific ELISAs based on the use of recombinant proteins (Verminnen *et al.*, 2006) or peptide antigens (Sachse *et al.*, 2009). ELISAs can detect avian IgM, IgG and IgA as long as the correct isotype-specific conjugate is used.

2.1. Recombinant major outer membrane protein ELISA

The recombinant major outer membrane protein (MOMP) ELISA (Verminnen *et al.*, 2006) can be performed on chicken and turkey sera pretreated with kaolin to remove background activity. MOMP-specific antibody titres are determined using a standard ELISA protocol and micro-well plates coated with recombinant MOMP. Serum, diluted 1/100, is added to the coated wells. Recombinant MOMP is produced in pcDNA4::MOMPHis transfected COS7 cells (Vanrompay *et al.*, 1999). Briefly, COS7 cells are cultured in Dulbecco's modified Eagle's medium supplemented with 3.7 g of sodium bicarbonate/litre,

1 mM L-glutamine, and 10% fetal calf serum. Transfection with plasmid DNA is performed by the diethylaminoethyl dextran method. Forty-eight hours post-transfection recombinant MOMP production is monitored by an indirect immunofluorescence staining using serovar and genus-specific MAbs. His-tag labelled recombinant MOMP is purified by affinity chromatography and the protein concentration is determined by the bicinchoninic acid protein assay. For the determination of antibody titres, 1/2000 and 1/4000 dilutions of biotinylated anti-chicken/turkey IgG (H+L) antibody and peroxidase-conjugated streptavidin are used, respectively. The results are positive if the absorbance exceeds the cut-off value of the mean of the negative control sera plus three times the standard deviation.

No commercial ELISA kits are available for detection of antibodies against *C. psittaci* and other avian chlamydiae.

2.2. Modified direct complement fixation test for *Chlamydia*

For AC, a modified direct CFT method is used. This method differs from the direct CFT in that normal, unheated chicken serum from chickens without chlamydial antibody is added to the complement dilution. The normal serum increases the sensitivity of the CFT procedure so that it can be used to test sera from avian species whose antibodies do not normally fix guinea-pig complement.

2.2.1. Test procedure

i) Serum to test is heat-inactivated at 60°C for 30 minutes prior to use.

ii) Serum is then diluted 1/5 in veronal (barbiturate) buffer saline (VBS)

iii) Twofold dilutions of the diluted serum are prepared in 96-well round bottom microtitre plates.

iv) Guinea-pig complement is diluted in VBS prior to the addition of the antigen and 2 complement haemolytic units are used.

v) Sera complemented with 5 % of fresh chicken serum, complement and antigen are reacted in the plates and incubated for 1 hour at 37°C (and alternate acceptable procedure is overnight incubation at 4°C).

vi) A 2–4% suspension of sensitised washed red blood cells is added.

vii) Plates are incubated for 30 minutes at 37°C, and the centrifuged for 5 minutes at 600 g.

When using ~~commercially available CFT antigens and~~ ready-to-use CFT reagents, the manufacturers' instructions should be applied.

Recommended controls to verify test conditions:

i) Positive control: a control serum that gives a positive reaction;

ii) Negative control serum: a control serum that gives a negative reaction;

iii) Anti-complementary control (serum control): diluent + inactivated test serum + complement + haemolytic system;

iv) Antigen control: diluent + antigen + complement + haemolytic system;

v) Haemolytic system control: diluent + haemolytic system;

vi) Complement control: diluent + complement titration + antigen + haemolytic system.

vii) The absence of anti-complementary activity must be checked for each serum; anticomplementary sera must be excluded from analyses.

A sample that produces 100%haemolysis at the 1/5 dilution is negative and a sample that produces 25–100% haemolysis is positive.

2.2.2. CFT antigen preparation

The simplest methods start with the growth of chlamydiae in cell culture. The two methods described below produce antigens that can be used in the micro-CFT. The procedures are quite similar: both include the growth of chlamydiae in cell culture, the inactivation of the chlamydiae, partial purification of the antigen, mechanical disruption, and dilution into the appropriate buffer. The method selected will depend on the equipment available.

The first procedure (Grimes, 1985) starts with the chlamydiae and cell culture debris harvested when cytopathic effects are noted. The culture is inactivated by the addition of phenol to a final concentration of 1.0%, incubated for 24 hours at 37°C, and concentrated by centrifugation at

10,000 ***g*** for 1 hour. The sediment is reconstituted to 10% of the original volume using VBS, pH 7.2, containing 1.0% phenol and 1.0% glycerol.

The sediment is then homogenised in an omnimixer at top speed for three 1-minute periods while cooled in ice water. The homogenate is centrifuged for 15 minutes at 100 ***g*** to remove debris. Some procedures suggest heating the antigen for 30 minutes in a boiling water bath at this time. The supernatant is saved and diluted to the desired concentration.

In the second procedure for the production of antigen for the CFT (Bracewell & Bevan, 1986), antigen is prepared from L cells infected with a psittacine strain. The cell culture medium is discarded, and the cells are heated for 40 minutes at 56°C. The cells are lysed in distilled water, the chlamydiae are disrupted by ultrasonication, and then made isotonic in VBS. The antigen is tested against a standard sheep convalescent serum and used at 2 units in the micro- CFT.

There are a number of procedures for preparing the antigen from infected yolk sacs, some of which are quite elaborate. However, with the following procedure it is relatively easy to prepare a crude infected yolk sac antigen that works well in the modified direct CFT. An egg-adapted strain of *Chlamydia* is used to inoculate 6–7-day-old embryonated chicken eggs via the yolk sac. The yolk sacs are harvested from embryos that die between 3 and 7 days post-inoculation. The yolk-sac harvest is diluted 1/3 in PBS, Tris buffer, or cell culture medium, and then autoclaved for 20 minutes. The suspension is cooled and then homogenised thoroughly. The use of a high-speed tissue homogeniser for 3–5 minutes is recommended. After homogenisation, phenol is added to make a final concentration of 0.5% phenol (prepare a 5% phenol stock solution and add 1 ml for every 9 ml of antigen). The antigen preparation is prepared, held for 3 days, and then the supernatant is used after centrifugation for 20 minutes at 1000 ***g***. The antigen can be stored for long periods of time at 4°C.

2.3. Other tests

Other tests include the agar gel immunodiffusion test, the latex agglutination (LA) test, ~~the EBA test (Grimes & Arizmendi, 1996)~~ and the micro-immunofluorescence test (MIFT). Immunodiffusion is less sensitive than the CFT. The LA test will detect antibodies to *C. psittaci*, and is easy and rapid to perform (Grimes *et al.,* 1993). ~~Latex beads are coated with purified chlamydial antigen, mixed thoroughly with the test serum on a glass plate, and rotated for 2 minutes to enhance agglutination. The test is read against a dark background. Sera giving positive reactions should be retested with uncoated beads to eliminate possible nonspecific agglutination. The LA and direct CFTs correlate in 72.5% of tests with paired sera. The LA test has a sensitivity of 39.1% and a specificity of 98.8% relative to the direct CFT (Grimes *et al.,* 1993).~~ The test detects both IgM and IgG, but it is best at detecting IgM. It has been suggested for use in detecting recent or active infections. ~~The EBA test detects only IgM, and it is indicative of a current infection.~~ The MIFT is rapid and easy to perform; however, fluorescence-conjugated anti-species sera are not always available.

C. REQUIREMENTS FOR VACCINES

To date, no commercial vaccines ~~against avian chlamydiosis~~ are available for avian chlamydiosis. Several vaccine candidates have been evaluated in recent decades. For example, a recombinant DNA plasmid containing the *C. psittaci ompA* gene provided significant (partial) protection in experimental studies in specific pathogen-free (SPF) turkeys and budgerigars (Harkinezhad & Schautteet, 2009; Verminnen *et al.*, 2010). Recent research has explored the use of recombinant proteins, such as the OmpA in combination with the polymorphic membrane protein G. Studies have shown that the combination of multiple proteins confers superior protection compared to single-valence vaccines in experimental avian models (Li *et al.*, 2022). Another promising vaccine candidate is based on the plasmid Pgp3 protein, a virulence factor of *Chlamydia*. This protein has demonstrated robust immunogenicity and efficacy in conferring immunity to *C. psittaci* infection in mouse models (Wang *et al.*, 2023). ~~although vaccination with a recombinant DNA plasmid containing the *C. psittaci ompA* gene provided significant (partial) protection in experimentally infected specified pathogen free (SPF) turkeys (Verminnen *et al.*, 2010) and budgerigars (Harkinezhad & Schautteet, 2009). DNA vaccination has the advantage that it can be used in the presence of maternal antibodies (Van Loock *et al.*, 2004) and the antigen is processed in the same way as during a natural infection, resulting in humoral and cell-mediated immune responses.~~

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701 **NB:** There are WOAH Reference Laboratories for avian chlamydiosis (please consult the WOAH Web site:
702 https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3.
703 Please contact the WOAH Reference Laboratories for any further information on
704 diagnostic tests and reagents for avian chlamydiosis

705 **NB:** FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2018.

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Appendix 1: Avian chlamydiosis
Intended purpose of test: population freedom from infection

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
ELISA ++ birds	Serum Antibodies against *C. psittaci*	N/A	N/A	See references	High sensitivity Rapid and simple	No commercial test Limited availability of avian conjugates	Verminnen *et al.* (2006) Sachse *et al.* (2009)
CFT ± birds	Serum Antibodies against *C. psittaci*	N/A	N/A	See references	Inexpensive	Low sensitivity and specificity Limited commercial availability of antigen	Grimes (1985) Bracewell & Bevan (1986)

Appendix 2: Avian chlamydiosis

Intended purpose of test: individual animal freedom from infection prior to movement

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
ELISA + birds	Serum Antibodies against *C. psittaci*	N/A	N/A	See references	High sensitivity Rapid and simple	No commercial test Limited availability avian conjugates	Verminnen *et al.* (2006) Sachse *et al.* (2009)
CFT + birds	Serum Antibodies against *C. psittaci*	N/A	N/A	See references	Inexpensive	Low sensitivity and specificity Limited commercial availability of antigen	Grimes (1985) Bracewell & Bevan (1986)

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Appendix 3: Avian chlamydiosis
Intended purpose of test: confirmation of clinical cases

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
Conventional PCR ++ birds	Cloacal, pharyngeal, ocular swabs from live birds Spleen, liver, lungs, intestine from dead birds *C. psittaci* DNA	N/A	N/A	See reference	High specificity	Intermediate sensitivity Time consuming	Laroucau *et al.* (2007)
Real-time PCR +++ birds	Cloacal, pharyngeal, ocular swabs from live birds Spleen, liver, lungs, intestine from dead birds *C. psittaci* DNA	N/A	N/A	See references	High sensitivity and specificity Rapid	Needs expensive laboratory equipment	Pantchev *et al.* (2009) Angen *et al.* (2021)
Cytological staining ± birds	Cloacal, pharyngeal, ocular swabs from live birds Spleen, liver, lungs, intestine from dead birds *C. psittaci* bacteria	N/A	N/A	N/A	Simple and cheap	Low to intermediate sensitivity Depends on experienced staff	
Isolation in cell culture or embryonated eggs ++ birds	Cloacal, pharyngeal, ocular swabs from live birds Spleen, liver, lungs, intestine from dead birds *C. psittaci* isolate	N/A	N/A	N/A	Gold standard Obtaining isolates for further characterisation	Low to intermediate sensitivity Cumbersome and long laboratory procedures Depends on experienced staff	
Antigen detection by IHC ++ birds	Cloacal, pharyngeal, ocular swabs from live birds Spleen, liver, lungs, intestine from dead birds *C. psittaci* antigen	N/A	N/A	N/A	Enables association of *Chlamydia* presence with tissue lesions	Intermediate sensitivity	
ELISA ± birds	Serum Antibodies against *C. psittaci*	N/A	N/A	See references	High sensitivity Rapid and simple	No commercial test Limited availability of avian conjugates	Verminnen *et al.* (2006) Sachse *et al.* (2009)
CFT ± birds	Serum Antibodies against *C. psittaci*	N/A	N/A	See references	Inexpensive	Low sensitivity and specificity Limited commercial availability of antigen	Grimes (1985) Bracewell & Bevan (1986)

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Appendix 4: Avian chlamydiosis
Intended purpose of test: prevalence of infection – surveillance

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
Conventional PCR ± birds	Cloacal, pharyngeal, ocular swabs from live birds Spleen, liver, lungs, intestine from dead birds *C. psittaci* DNA	N/A	N/A	See reference	High specificity	Intermediate sensitivity Time consuming	Laroucau *et al.* (2007)
Real-time PCR ++ birds	Cloacal, pharyngeal, ocular swabs from live birds Spleen, liver, lungs, intestine from dead birds *C. psittaci* DNA	N/A	N/A	See references	High sensitivity and specificity Rapid	Needs expensive lab equipment	Pantchev *et al.* (2009) Angen *et al.* (2021)
Isolation in cell culture or embryonated eggs ± birds	Cloacal, pharyngeal, ocular swabs from live birds Spleen, liver, lungs, intestine from dead birds *C. psittaci* isolate	N/A	N/A	N/A	Gold standard Obtaining isolates for further characterisation	Low to intermediate sensitivity Cumbersome and long laboratory procedures Depends on experienced staff	
ELISA ++ birds	Serum Antibodies against *C. psittaci*	N/A	N/A	See references	High sensitivity Rapid and simple	No commercial test Limited availability of avian conjugates	Verminnen *et al.* (2006) Sachse *et al.* (2009)
CFT ± birds	Serum Antibodies against *C. psittaci*	N/A	N/A	See references	Inexpensive	Low sensitivity and specificity Limited commercial availability of antigen	Grimes (1985) Bracewell & Bevan (1986)

Annexe 21. Chapitre 3.3.2. Bronchite infectieuse aviaire

RÉUNION DE LA COMMISSION DES NORMES BIOLOGIQUES
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CHAPTER 3.3.2.

AVIAN INFECTIOUS BRONCHITIS

SUMMARY

Description and importance of the disease: *Avian infectious bronchitis* (*IB*) *is caused by the* Gammacoronavirus *infectious bronchitis virus* (*IBV*). *It has no zoonotic relevance. The virus causes infections mainly in chickens and is a significant pathogen of commercial meat and egg type birds. IB is an acute, contagious disease characterised primarily by respiratory signs in growing chickens. In hens, decreased egg production and quality are often observed. Several strains of the virus are nephropathogenic and may produce interstitial nephritis and mortality. The severity of IBV-induced respiratory disease is enhanced by the presence of other pathogens, including bacteria, leading to chronic complicated airsacculitis. Diagnosis of IB requires detection of the virus by virus isolation, antigen staining techniques or demonstration of viral nucleic acid from diseased flocks. Demonstration of a rising serum antibody response may also be useful. The widespread use of live and inactivated vaccines may complicate both the interpretation of virus detection and serology findings. The occurrence of antigenic variant strains may overcome immunity induced by vaccination.*

Detection and identification of the agent: *For the common respiratory form, IBV is most successfully isolated from upper respiratory tract, tracheal mucosa and lung several days to one week following infection. For other forms of IB, or in a more chronic phase of the infection, kidney, oviduct or the caecal tonsils of the intestinal tract tissues may be better sources of virus depending on the pathogenesis of the disease. Reverse-transcriptase polymerase chain reaction* (*RT-PCR*) *is increasingly being used to identify the spike* (*S*) *glycoprotein genotype of IBV field strains. Genotyping using primers specific for the S1 subunit of the S gene or sequencing of the same gene generally provides similar but not always identical findings to serotyping by haemagglutination inhibition* (*HI*) *or virus neutralisation* (*VN*) *tests. Supplementary tests include electron microscopy, the use of monoclonal antibodies, VN, immuno-histochemical or immunofluorescence tests, and immunisation–challenge trials in chickens. ~~Specific~~ Specified pathogen free chicken embryonated eggs or chicken tracheal organ cultures* (*TOCs*) *from embryos may be used for virus isolation. Following inoculation of the allantoic cavity, IBV produces embryo stunting, curling, clubbing of the down, or urate deposits in the mesonephros of the kidney, often within three serial passages. Alternatively, VN or HI tests using specific antiserum may be used to identify the serotype.*

Serological tests: *Commercial enzyme-linked immunosorbent assays* (*ELISA*) *kits are often used for monitoring serum antibody responses. The antigens used in the kits are broadly cross-reactive among serotypes and allow for general serological monitoring of vaccinal responses and field challenges. The HI test is used for identifying serotype-specific responses to vaccination and field challenges especially in young ~~growing chickens~~ rearing hens. Because of multiple infections and vaccinations, the sera of breeders and layers contain cross-reactive antibodies and the results of HI and VN test testing cannot be used with a high degree of confidence for serotyping the infection.*

Requirements for vaccines: *Both live attenuated and oil emulsion inactivated vaccines are available. Live vaccines, usually attenuated by serial passage in chicken embryos, confer better local immunity of the respiratory tract than inactivated vaccines. The use of live vaccines carries a risk of residual pathogenicity associated with vaccine back-passage in flocks. However, proper mass application will generally result in safe application of live vaccines.*

Inactivated vaccines are injected and a single inoculation does not confer significant protection unless preceded by one or more live IBV priming vaccinations.

A. INTRODUCTION

Avian infectious bronchitis (IB) was first described in the United States of America (USA) in the 1930s as an acute respiratory disease mainly of young chickens. A viral aetiology was established, and the agent was termed avian infectious bronchitis virus (IBV). The virus is a member of the genus *Gammacoronavirus*, subfamily ~~*Coronavirinae*~~ *Orthocoronavirinae*, family *Coronaviridae*, in the order *Nidovirales*. IBV and other avian coronaviruses of turkeys and pheasants are classified as gammacoronaviruses, with mammalian coronaviruses comprising *Alpha* and *Betacoronaviruses*. ~~Novel~~ Related coronaviruses have been discovered in wild birds and pigs and have been designated *Deltacoronaviruses* (Woo *et al*., 2012), interestingly the avian *Deltacoronaviruses* have a different genomic order and show no close relationship to the *gammacoronaviruses*. Coronaviruses have a non-segmented, positive-sense, single-stranded RNA genome.

IB affects chickens of all ages (Britton & Cavanagh, 2007; Cavanagh *et al*., 2002); IBV-like viruses have been isolated from turkeys, pheasants and guinea fowl. The disease is transmitted by the air-borne route, direct chicken-to-chicken contact, contact with infected faeces and indirectly through mechanical spread (contaminated poultry equipment or egg-packing materials, manure used as fertiliser, farm visits, etc.). IB occurs world-wide and assumes a variety of clinical forms, the principal one being respiratory disease that develops after infection of the respiratory tract tissues following inhalation or ingestion. Infection of the oviduct at a very young age can lead to permanent damage and, in hens, can lead to cessation of egg-laying or production of thin-walled and misshapen shells with loss of shell pigmentation. IB can be nephropathogenic causing acute nephritis, urolithiasis and mortality, especially in young birds. After apparent recovery, chronic nephritis can produce death at a later time. Vaccine and field strains of IBV may persist in the caecal tonsils of the intestinal tract and be excreted in faeces for weeks or longer in clinically normal chickens. For an in-depth review of IB, refer to Jackwood & de Wit, (~~2013~~ 2020). A detailed discussion of IBV antigen, genome and antibody detection assays prepared by de Wit (2000) is also available.

The clinical signs induced by IBV infections are not specific and could be caused by a number of other pathogens. There is therefore a need to adopt a broad diagnostic approach to understanding the role of other pathogens. The differential diagnosis for respiratory disease includes pathogens such as avian metapneumovirus, low pathogenic avian influenza, Newcastle disease virus, infectious laryngotracheitis virus, *Mycoplasma gallisepticum* and *synoviae*, and *Avibacterium paragallinarum*. For nephrosis and nephritis, the differential diagnosis includes, amongst others, astroviruses, infectious bursal disease virus, intoxications and dehydration. For drops in egg production, alternative causes include the respiratory pathogens mentioned but also avian encephalomyelitis or egg-drop syndrome virus.

There have been no reports of human infection with IBV.

B. DIAGNOSTIC TECHNIQUES

~~Confirmation of~~ Confirmatory diagnosis of IBV is based on detection of virus RNA, virus detection, or seroconversion. Extensive use is made of live and inactivated vaccinations, which may complicate diagnosis by serological methods as antibodies to vaccination and field infections cannot always be distinguished. Persistence of live vaccines may also confuse attempts at recovering or identifying the causative field strain of IBV.

Table 1. Test methods available for the diagnosis of infectious bronchitis virus and their purpose

Method	Purpose					
	Population freedom from infection[(a)]	Individual animal freedom from infection prior to movement[(b)]	Contribute to eradication policies[(c)]	Confirmation of clinical cases[(d)]	Prevalence of infection – surveillance[(e)]	Immune status in individual animals or populations (post-vaccination)[(f)]
Detection and identification of the agent						
Virus isolation (embryos or TOCs)	+[(g)]	++[(h)]	–	+++	+	+[(i)]
Staining by immunohistochemistry	–	–	–	++	+	+[(i)]
Gene sequencing (virus identification)	–	–	–	++	++[(j)]	–

Method	Purpose					
	Population freedom from infection[a]	Individual animal freedom from infection prior to movement[b]	Contribute to eradication policies[c]	Confirmation of clinical cases[d]	Prevalence of infection – surveillance[e]	Immune status in individual animals or populations (post-vaccination)[f]
Conventional RT-PCR (detection of virus-~~genome~~ derived RNA)	+[g, k]	++	++	±++	+++	+[i]
Real-time RT-PCR (conserved gene)	±+[g, k]	++	++	±++	+++	+[i]
Real-time RT-PCR (lineage or genotype specific)	++[k, l]	++	++	++	++	+[i]
VN (virus identification)	–	–	–	–	+	–
Detection of immune response						
VN (antibody detection)	–	–[l]	–	+[m]	+[n]	++
HIT (antibody detection)	–	–[l]	+	+[m]	+[n]	++
ELISA (antibody detection)	++[o]	+	++	++[m]	++[m, p]	++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
TOC = tracheal organ culture; RT-PCR = reverse-transcription polymerase chain reaction; VN = virus neutralisation;
HIT = haemagglutination inhibition test; ELISA = enzyme-linked immunosorbent assay.

[a] See Appendix 1 of this chapter for justification table for the scores given to the tests for this purpose.

[b] See Appendix 2 of this chapter for justification table for the scores given to the tests for this purpose.

[c] See Appendix 3 of this chapter for justification table for the scores given to the tests for this purpose.

[d] See Appendix 4 of this chapter for justification table for the scores given to the tests for this purpose.

[e] See Appendix 5 of this chapter for justification table for the scores given to the tests for this purpose.

[f] See Appendix 6 of this chapter for justification table for the scores given to the tests for this purpose.

[g] Suitable for ensuring lack of infection during the past 10 days;

[h] Suitable at the individual level only during excretion periods;

[i] Sometimes used in the evaluation of vaccines to assess protection against viral excretion, but can be positive even when good clinical protection is achieved.

[j] Especially suitable for surveillance of a given or an emerging genotype-lineage;

[k] Detection in cloacal samples can be several weeks longer than in tracheal samples, strain depending;

[l] Limited suitability for this purpose as it may be too specific due to the serotype used as an antigen or genotype, use of a panel of lineage-specific tests present in the region is recommended;

[m] Suitable provided paired samples collected a few weeks apart can be analysed;

[n] Serotype-specificity drops when chicken has been in contact to multiple types of IBV.

[o] Suitable for ensuring lack of infections dating back to more than 10 days;

[p] Especially suitable when IB surveillance is not focused on a given serotype;

1. Detection and identification of the agent

1.1. Sampling

Samples appropriate to the form of IB observed must be obtained as soon as signs of clinical disease are evident. For acute respiratory disease, swabs from the upper respiratory tract of live birds or tracheal and lung tissues from diseased birds should be harvested. For birds with nephritis or egg-production problems, samples from the kidneys, ~~or~~ oviduct or cloaca, respectively, should be collected in addition to respiratory specimens. Although virus isolation is a well-established method, IBV identification by reverse-transcriptase polymerase chain reaction (RT-PCR) and subsequent sequencing or the use of a panel of genotype-lineage-specific RT-PCRs will be the most used methods in the field. A positive RT-

PCR result is not proof that IBV is present in the kidney cells and causing damage; it could also be caused by viraemia or contamination of the swabs by organs other than the airsac. In situations where IB-induced nephritis is suspected, kidney samples should also be ~~selected~~ collected from fresh carcases for histochemistry or immunofluorescence to demonstrate local replication. A high degree ~~rate~~ of virus recovery has been reported from the caecal tonsil or faeces. However, isolates from the intestinal tract may have no relevance to the latest infection or clinical disease. IBV isolation may be facilitated using sentinel ~~specific~~ specified pathogen free (SPF) chickens placed at one or more times in contact with commercial poultry. Vaccinated chickens might be used to detect IBV strains for which the vaccination regimes used might not be sufficiently effective. Blood samples from acutely affected birds as well as convalescent chickens can also be submitted for serological testing.

1.2. Culture

Samples must be placed in cold transport media containing penicillin (10,000 International Units [IU]/ml) and streptomycin (10 mg/ml) and kept on ice and be frozen as soon as possible. Suspensions of tissues (10–20% w/v) are prepared in sterile phosphate buffered saline (PBS) or nutrient broth for egg inoculation, or in tissue culture medium for chicken tracheal organ culture (TOC) inoculation (Cook *et al.*, 1976). The suspensions are clarified by low-speed centrifugation and filtration through bacteriological filters (0.2 µ) before inoculation of SPF embryonated chicken eggs or TOCs.

SPF embryonated chicken eggs and/or TOCs are used for primary isolation of IBV. Cell cultures are not recommended for primary isolation as it is often necessary to adapt IBV isolates to growth in chicken embryos before cytopathic effect (CPE) is produced in chick embryo kidney cells.

Embryonated eggs used for virus isolation should originate preferably from SPF chickens or from breeder sources that have been neither infected nor vaccinated with IBV. Most commonly, 0.1–0.2 ml of sample supernatant is inoculated into the allantoic cavity of 9–11-day-old embryos. Eggs are candled daily for 7 days with mortality within the first 24 hours being considered nonspecific. The initial inoculation usually has limited macroscopic effects on the embryo unless the strain is derived from a vaccine and is already egg adapted. Normally, the allantoic fluids of all eggs are pooled after harvesting 3–6 days after infection; this pool is diluted 1/5 or 1/10 in antibiotic broth and used to infect another set of eggs for up to a total of three to four passages. Typically, a field strain will induce observable embryonic changes consisting of stunted and curled embryos with feather dystrophy (clubbing) and urate deposits in the mesonephros on the second to fourth passage. Embryo mortality in later passages may occur as the strain becomes more egg adapted. RT-PCR can be used to check whether the last passage is really negative for IBV. Other viruses, notably adenoviruses that are common to the respiratory tract, also produce embryo lesions indistinguishable from IBV. The IBV-laden allantoic fluid should not agglutinate red blood cells and isolation of IBV must be confirmed by serotyping or genotyping. Infective allantoic fluids are kept at –20°C or below for short-term storage, –60°C or below for long-term storage or at 4°C after lyophilisation.

TOCs prepared from 19- to 20-day-old embryos can be used to isolate IBV directly from field material (Cook *et al.*, 1976). An automatic tissue-chopper is desirable for the large-scale production of suitable transverse sections or rings of the trachea for this technique (Darbyshire *et al.*, 1978). The rings are about 0.5–1.0 mm thick, and are maintained in a medium consisting of Eagle's N-2-hydroxyethylpiperazine N'-2-ethanesulphonic acid (HEPES) in roller drums (15 rev/hour) at 37°C. Infection of tracheal organ cultures usually ~~produce~~ results in ciliostasis within 24–48 hours. Ciliostasis may be produced by other viruses and suspect IBV cases must be confirmed by serotyping or genotyping methods.

1.3. Methods for detection and identification ~~and detection~~

The initial tests performed on IBV isolates are directed at eliminating other viruses from diagnostic consideration. Chorioallantoic membranes from infected eggs are collected, homogenised, and tested for ~~avian~~ Aviadenovirus ~~group 1~~ by immunodiffusion or PCR. ~~Group 1 avian~~ Aviadenovirus infections of commercial chickens are common, and the virus often produces stunted embryos indistinguishable from IBV-infected embryos. Furthermore, harvested allantoic fluids do not hemagglutinate (HA) chick red blood cells. Genetic-based tests, RT-PCR, and sequence analysis are used commonly to identify an isolate as IBV. The presence of IBV in ~~infective~~ infected allantoic fluid or TOCs is usually ~~detected~~ confirmed by RT-PCR amplification. Other techniques may be used ~~as well~~, for example, cells present in the chorioallantoic membranes or allantoic fluid of infected eggs or TOCs may be tested for IBV antigen using fluorescent antibody tests, immunohistochemistry or an antigen- enzyme-linked immunosorbent assay~~s~~ (ELISA) using a group-specific monoclonal antibody (MAb) or polyclonal antiserum.

1.4. Serotype identification

Antigenic variation among IBV strains is common (Jackwood & de Wit, ~~2013~~ 2020), but at present there is no agreed definitive classification system for serotyping. Nevertheless, antigenic relationships and differences among strains are important, as vaccines based on one particular serotype may show little or no protection against viruses of a different antigenic group. As a result of the regular emergence of antigenic variants, the viruses, and hence the disease situation and vaccines used, may be quite different in different geographical locations. Ongoing assessment of the viruses present in the field is necessary to produce vaccines that will be efficacious in the face of antigenic variants that arise. Serotyping of IBV isolates and strains has been done using haemagglutination inhibition (HI) ~~(~~and virus neutralisation (VN) tests in chick embryos, TOCs and cell cultures. MAbs have been described and used in antigen-ELISAs or immunofluorescence tests for typing of IBV strains, but the availability ~~number~~ of suitable MAbs is very limited (de Wit, 2000). Owing to the number of variants, the requirements to raise a monospecific antiserum in SPF birds for each novel strain and the complexity of serotyping, means that serotyping is hardly used anymore.

1.5. Lineage or genotype identification

Development of improved techniques for nucleotide sequencing, the availability of IBV sequence data, especially relating to the S gene, in computer databases and the demonstration that even relatively short sequence lengths can allow meaningful results in phylogenetic analyses have led to an increase in such studies and have largely replaced HI and VN serotyping for determining the identity of a field strain. The molecular basis of antigenic variation has been investigated, usually by nucleotide sequencing of the gene coding for the spike (S) protein or, more specifically, nucleotide sequencing of the gene coding for the S1 subunit of the S protein where it is believed that the epitopes to which neutralising antibodies bind are found. A high correlation with HI or VN results has not been seen, while different serotypes generally have large differences (20–50%) in the deduced amino acid sequences of the S1 subunit, other viruses that are clearly distinguishable in neutralisation tests show only 2–3% differences in amino acid sequences.

The primary advantages of genotyping methods are a rapid turnaround time, and the ability to detect a variety of genotypes or lineages, depending on the primer sets used. Genetic diversity between IBV isolates has been confirmed, but viruses sharing temporal, geographical, antigenic or epidemiological parameters tend to fall into specific lineages and this has proven valuable in assessing both the global epidemiology and local spread of IBV (de Wit *et al.*, 2011; Jackwood, 2012; Valastro *et al.*, 2016). Sequence analysis of the S gene provides the required information for determining the level of genetic homology and the relationship of an IBV S gene to other IBV isolates including vaccine strains.

~~It has been~~ Work has shown that coronaviruses isolated from turkeys, pheasants and guinea fowl are genetically similar to IBV, having approximately 90% nucleotide identity in the highly conserved region II of the 3' untranslated region (UTR) of the IBV genome (Cavanagh *et al.*, 2001; 2002). The potential role of these coronaviruses in IBV infections has not been determined. This is the same for the gammacoronaviruses that have been detected in wild birds (Muradrasoli *et al.*, 2010)

The major uses of RT-PCR tests are virus identification at the genetic level and its application in the understanding of epidemiological investigations during IBV outbreaks. The RT-PCR tests, as they now exist however, do not provide information on viral pathogenicity.

1.5.1. Reverse-transcriptase polymerase chain reaction (RT-PCR)

The wider availability and increased speed of production of results obtained using commercially available kits for RT-PCR and automatic sequencers now means such studies are within the capabilities of many more diagnostic laboratories, providing more accurate and meaningful results. Tracheal or oropharyngeal swabs are ~~often~~ primarily used as the specimens of choice because they are easy to obtain and process. However, cloacal swabs are also taken in cases where the infection may be a bit older, especially in layer and breeder birds. Such samples usually contain little extraneous organic material that can interfere with RNA recovery and amplification by PCR. The system used for RNA extraction will also affect the success of RT-PCR on clinical specimens, and even with commercial kits care should be taken in selecting the most appropriate system validated for the samples to be analysed. The usual target region for IBV characterisation is the S1 subunit of the S glycoprotein gene.

Many one and two-step RT-PCR kits are commercially available from manufacturers claiming superior enzyme sensitivity and fidelity. Reverse transcription is performed according to the manufacturer's instructions. Most diagnostic laboratories determine the genotype or lineage of IBV isolates using partial sequence analysis, usually about 700 nt long, of the S1 region of the S

gene (Callison *et al.*, 2006). Given the diverse nature of the IBV S1 sequence, primer sets might need to be updated from time to time and the actual region sequenced may vary. Such analyses allow for rapid epidemiological assessment of the origins and spread of the viruses responsible for IBV outbreaks and the detection of vaccine strains within flocks. It should be noted that for more refined epidemiological studies and for more accurate studies on the evolution of IBV, the phylogenetic studies need to use complete S gene and even whole genome sequences.

1.5.2. Real-time RT-PCR analysis

Pathogen/ target gene	Primer/probe (5'–3')	Concentration	Cycling parameters[a]
Method 1: Roh *et al.*, 2014			
IBV 5'UTR	F: IBV 5' GU391: GCT-TTT-GAG-CCT-AGC-GTT R: IBV 5' GL533: GCC-ATG-TTG-TCA-CTG-TCT-ATT Probe: IBV 5' G: FAM-CAC-CAC-CAG-AAC-CTG-TCA-CCT-C-BHQ-1	10 µM 10 µM 4 µM	One cycle of 50°C/30 min and 95°C/15 min followed by 40 cycles of: 94°C/1 sec and 60°C/60 sec
IBV M41/S1	Mass-F': CGT-KTA-CTA-CTA-YCA-AAG-TGC Mass-R': CCA-TGA-ATA-RTA-CCA-ACA-RTA-CAC Mass-P: FAM-AGC-CTG-CAT-TAT-TAR-AT-MGBNFQ	10 µM 10 µM 4 µM	One cycle of 50°C/30 min and 95°C/15 min followed by 40 cycles of: 94°C/1 sec and 60°C/60 sec

[a] A denaturation step prior to cycling has not been included.

Rapid confirmation of IBV can be determined using a strategy that avoids post-amplification processing (sequence analysis). The most common method for IBV is real-time RT-PCR. The real-time RT-PCR assay uses specific probes against the target sequence, the use of fluorogenic hydrolysis probes or fluorescent dyes eliminate the requirement of any post-amplification processing step and results can be obtained in less than 3 hours.

A generic IBV real-time PCR that targets a conserved region in the IBV genome (e.g. the 5' UTR) can be used for the detection of IBV in a clinical sample (Callison *et al.*, 2006). Lineage-genotype-specific real-time PCRs targeting the S1 gene for genotypes lineages that are known to circulate in the region may be used in conjunction with this generic IBV PCR. Genotype-lineage specific primers and probes have been published for a number of genotypes such as Massachusetts (primer sets XCE3-(CAG-ATT-GCT-TAC-AAC-CAC-C) and MCE1+ (AAT-ACT-ACT-TTT-ACG-TTA-CAC), 793B primer sets XCE3-(CAG-ATT-GCT-TAC-AAC-CAC-C) and BCE1+ (AGT-AGT-TTT-GTG-TAT-AAA-CCA), Arkansas and others (Cavanagh *et al.,* 1999; Roh *et al.,* 2014). However, due to the high variability between and on-going evolution within genotypes (Valastro *et al.*, 2016), continual updating of the genotype-specific and genotype-lineage-specific primers and probes is needed (Molenaar *et al.*, 2020; 2024). When the genotype-lineage specific RT-PCRs are negative and the generic RT-PCR is positive, additional sequencing may be needed to identify the strain involved and to adapt the primers and probes used in the genotype specific PCRs accordingly. Using a genotype lineage-specific RT-PCR for every IBV vaccine strain used in the hatchery, each vaccine strain is expected to be detected around 5–7 days post-vaccination in a high percentage of the vaccinated birds confirming replication of the vaccine strain.

2. Serological tests

A number of tests have been described. Those considered here include VN, agar gel immunodiffusion (AGID), HI and ELISA. Each test has advantages and disadvantages in terms of practicality, specificity, sensitivity and cost. In general, ELISAs are most suitable for routine serological testing and can detect antibodies caused by vaccination and field exposure. ELISA can detect antibody responses to all serotypes, but a difference in sensitivity may exist for low antibody levels against strains of some serotypes. AGID also detects IBV antibodies against all types of IBV but lacks sensitivity and is liable to yield inconsistent results as the presence and duration of precipitating antibodies may vary with individual birds. VN and HI tests are more serotype specific, especially in young birds that have not been exposed to different types of IBV (de Wit, 2000). VN tests are too expensive and impractical for use in routine conditions. VN and HI when used on serial sera from young growing chickens such as pullets and broilers can give information on the serotype-specific antibody status of a flock. In layers and breeders, higher levels of HI or VN antibodies against the challenge virus are correlated with higher levels of protection against egg drop (Box *et al.*, 1988; De Wit *et al.*, 2019). Regular monitoring of sera from flocks for IB antibody titres may help to indicate the level of vaccine or field challenge responses. Because chicken sera from older birds that have been in contact with vaccines and the field strain of several serotypes contain antibodies that are highly cross-reactive against antigenically unrelated strains, serodiagnosis of suspected disease outbreaks of IB at the serotype level cannot be

used with a high degree of confidence as the detected antibodies against a certain serotype might be induced by infections of other types of IBV.

2.1. Virus neutralisation

In VN tests, all sera should first be heated to 56°C for 30 minutes. Virus is mixed with serum and incubated for 30–60 minutes at 37°C or room temperature. Chicken embryos are most often employed, but antibodies can be measured using TOC or cell culture systems. Two methods have been used to estimate neutralising antibodies. One employs a constant serum concentration reacted with varying dilutions of virus (the alpha method) and the other employs a constant amount of virus and varying dilutions of serum (the beta method).

In the alpha method, tenfold dilutions of egg-adapted virus are reacted with a fixed dilution (usually 1/5) of antiserum, and the mixtures are inoculated into groups of from five to ten eggs. The virus alone is titrated in parallel. End-points are calculated by the Kärber or the Reed and Muench methods. The results are expressed as a neutralisation index (NI) that represents the $\log_{10}$ difference in the titres of the virus alone and that of the virus/antiserum mixtures. The NI values may reach 4.5–7.0 in the case of homologous virus/serum mixtures; values of <1.5 are not specific, but a heterologous virus will give a value as low as 1.5.

The beta method is the more widely used neutralisation test for antibody assay with chicken embryos or cells. Two- or four-fold dilutions of antiserum are reacted in equal volumes with a dilution of virus, usually fixed at 100 or 200 EID_{50} (median embryo-infective doses) per 0.05 ml and 0.1 ml of each mixture inoculated into the allantoic cavity of each of from five to ten embryonated eggs. A control titration of the virus is performed simultaneously to confirm that the fixed virus dilution in the virus/serum mixtures was between $10^{1.5}$ and $10^{2.5}$ EID_{50}. End-points of the serum titres are determined by the Kärber or Reed and Muench method as before, but here are expressed as reciprocals of $\log_2$ dilutions. This fixed-virus/varying-serum method is also employed for neutralisation tests in tracheal organ cultures using five tubes per serum dilution, as is conventional with other viruses. The results are calculated according to Reed and Muench, and the virus titres are expressed as median ciliostatic doses per unit volume ($\log_{10}$ CD_{50}). Serum titres are again expressed as $\log_2$ dilution reciprocals. This test is more sensitive than others, but technical logistics hamper its more widespread adoption. For cell-adapted strains it is recommended to check the S1 sequence for mutations that might affect the antigenicity.

2.2. Haemagglutination inhibition

A standard protocol for a HI test for IBV has been described (Alexander *et al.*, 1983), and the test procedure detailed below is based on that standard. Most strains and isolates of IBV will agglutinate chicken red blood cells (RBCs) after neuraminidase treatment (Schultze *et al.*, 1992). The strain selected to produce antigen may be varied, depending on the requirements of diagnosis. The antigen for the HI test is prepared from IBV-laden allantoic fluids.

For HA and HI tests, procedures are carried out at 4°C or a validated higher temperature, such as 20°C.

2.2.1. Haemagglutination test

i) Dispense 0.025 ml of PBS, pH 7.0–7.4, into each well of a plastic ~~U or~~ V-bottom microtitre plate.

ii) Place 0.025 ml of virus antigen in the first well. For accurate determination of the HA content, this should be done from a close range of an initial series of dilutions, i.e. 1/2, 1/3, 1/5, 1/7 and 1/9.

iii) Make twofold dilutions of 0.025 ml volumes of the virus antigen across the plate.

iv) Dispense a further 0.025 ml of PBS into each well.

v) Dispense 0.025 ml of 1% (v/v) chicken RBCs to each well.

vi) Mix by tapping the plate gently and allow the RBCs to settle for 40–60 minutes at 4°C, when control RBCs should be settled to a distinct button.

vii) HA is ~~more easily~~ determined by tilting the plate and observing the presence or absence of tear-shaped streaming of the RBCs. The titration should be read to the highest dilution giving complete HA ~~in which there is~~ (no ~~sedimentation or~~ streaming); this ~~is 100% HA and~~ represents 1 HA unit (HAU) and can be calculated accurately from the initial range of dilutions.

2.2.2. Haemagglutination-inhibition test

The HI test is used in the diagnosis and routine flock monitoring of vaccine responses.

i) Dispense 0.025 ml of PBS into each well of a plastic ~~U or~~ V-bottom microtitre plate.

ii) Place 0.025 ml of serum into the first well of the plate.

iii) Make twofold dilutions of 0.025 ml volumes of the serum across the plate.

iv) Add 4 HAU of virus antigen in 0.025 ml to each well and leave for 30 minutes.

v) Add 0.025 ml of 1% (v/v) chicken RBCs to each well and, after gentle mixing, allow the RBCs to settle for 40–60 minutes when control RBCs should be settled to a distinct button.

vi) The HI titre is the highest dilution of serum causing complete inhibition of 4 HAU of antigen. The agglutination is assessed more exactly by tilting the plates. Only those wells in which the RBCs 'stream' at the same rate as the control wells (containing 0.025 ml RBC and 0.05 ml PBS only) should be considered to show inhibition.

vii) The validity of results should be assessed against a negative control serum, which should not give a titre $>2^2$, and a positive control serum, for which the titre should be within one dilution of the known titre.

viii) Sera are usually regarded as positive if they have a titre of 2^4 or more. However, it should be noted that even in SPF flocks, a very small proportion of birds may show a nonspecific titre of 2^4, but usually in birds over 1 year of age. Because chicken sera from older birds that have been in contact with several types of IBV contain antibodies that can be highly cross-reactive against antigenically unrelated strains, a cut-off of the HI test of 2^4 will be too low.

2.3. Enzyme-linked immunosorbent assay

Commercial kits for ELISAs are widely available and used. These ELISAs use different cut-offs and mathematical formulas to convert the ELISA result into a titre. This means that every ELISA has its own interpretation and the titre results of different ELISAs on the same serum will differ.

C. REQUIREMENTS FOR VACCINES

1. Background

Strains used in live virus vaccines generally require attenuation. At present, a minority of countries only permit~~s~~ the use of attenuated live vaccines of the Massachusetts type, such as the H120 strain. Most countries also have ~~licensed~~ approved vaccines against other serotypes depending on the local situation and needs. Commonly used administration routes for live vaccines are spray (course spray or aerosol) or in the drinking water (oral route). Administration by eye-drop, if carefully done, is a very good method but in many cases too expensive or not possible due to lack of vaccination crews.

The grouping of IBV strains that confer cross-protective immunity is the most important typing system from a practical point of view because it provides direct information about the efficacy of a vaccine (de Wit, 2000). The number of cross-protective groups that exists is unknown, but cross-challenge experiments in chicken tend to identify a smaller number of groups compared with serotypes and genotypes, presumably because they are measuring the complete immune response and not just a part of it. In general, there is a higher chance of a good level of cross-protection between strains with a high level of genetic homology in the S-gene than between strains with a low homology. However, the vaccination-challenge experiments have shown that the relationship is not very strong (de Wit *et al*., 2011). Therefore, a cross-immunisation study has to be performed to be able to determine the cross-protective immunity of a strain. Many factors can influence the outcome of such studies and should be accounted for (de Wit & Cook, 2014).

Live vaccines confer better local immunity in the respiratory tract and suitable combinations of vaccines of different serotypes also may protect against a wider antigenic spectrum of field strains (Cook *et al*., 1999; de Wit *et al*, 2013). Live vaccines carry a risk of residual pathogenicity associated with vaccine back-passage in flocks. However, proper application of vaccine can achieve uniform distribution of the vaccine in the flock and avoid back-passage. Furthermore, the use of vaccines at the manufacturer's recommended dosages will also help avoid back-passage reversion that may be caused by fractional dose application. For long-living birds, vaccination with live vaccines only during the rearing period will often not be sufficient to induce a long-lasting protection against challenge in the laying period. Boosting with inactivated vaccines of a good quality can be very efficient in increasing the level of protection against challenge in the laying period. The efficacy of inactivated vaccines depends heavily on proper priming with a live vaccine(s). Inactivated vaccines must be administered to birds individually, by intramuscular or

subcutaneous injection. Variant strains may be used to prepare inactivated autogenous vaccines for controlling IB in layers and breeders, subject to local legislative requirements.

There are prospects for genetically engineered vaccines (Armesto *et al*., 2011; Casais *et al*., 2003), and *in-ovo* vaccination (Tarpey *et al*., 2006; Wakenell *et al*., 1995), but the progress for live vaccines is slow compared with other poultry diseases.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature. National and international standards that apply in the country in which IB vaccines are manufactured must be complied with. The ~~licensing~~ regulatory authority should provide information and guidance on requirements. These are now often presented in general terms, as applying to all vaccines – avian and mammalian, live and inactivated, or viral and bacterial vaccines. There may also be specific requirements applying to IB vaccines, live and inactivated. As examples, references are given to the European and USA regulations (European Pharmacopoeia [~~2017a; 2017b~~ 11.1, 2023; 11.4, 2024] ; USDA, ~~2017~~ 2024).

For IB vaccines, important differences among countries may arise regarding the challenge virus to be used for potency tests, and its validation. Traditionally, the virulent M41 (Mass 41) strain of the Massachusetts (Mass) type has been used for challenge tests of both live and inactivated vaccines of the Mass serotype. Although this type is still common, it is not the only or the dominant type in any country and many countries allow the use of vaccines from other serotypes of IBV. Establishing criteria for validating the challenge virus may be more difficult for non-Massachusetts types, because of their varying virulence. Inactivated vaccines are often expected to protect against drops in egg production. The traditional M41 challenge should cause a drop of at least 67% in the unvaccinated controls, which was considered by many IB specialists as being excessive and also too dependent on the chicken genetic line and on particular challenge parameters. For other types of IBV, much lower drops in egg production may be regarded as satisfactory, depending on published evidence of the effects of these strains in the field. It therefore seems necessary to relax the criteria for Massachusetts type challenges, and the European Pharmacopoeia now defines a satisfactory drop in egg production for Massachusetts types to be at least 35%, and for non-Massachusetts types to be at least 15% in non-vaccinated birds, provided that the drop is 'commensurate with the documented evidence' (European Pharmacopoeia). However, under laboratory conditions, in many cases even a 35% drop in egg production by M41 or 15% for variant viruses is hard to achieve in the more modern genetic chicken lines than in the past. It can be recommended that an egg drop of at least 10–15% in non-vaccinated birds should be the minimal drop produced by any IB challenge virus. Appropriate statistical analyses should also be included in any vaccination-challenge study. Inactivated and live vaccines may also claim protection against other clinical signs such as respiratory signs, nephritis or loss of egg shell quality.

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production* and Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials for veterinary use*.

The seed-lot (master seed) system should be employed for whatever type of vaccine is produced. Each virus must be designated as to strain and origin and must be free from contamination with other strains of IBV and extraneous agents. The master seed virus is the backbone of the vaccine. A few samples of the master seed are used to produce a working seed. This working seed is used for production batches. The master seed should be of such a size that it is sufficient for the life span of a product.

For live virus vaccines, a minority of countries permit only strains of the Massachusetts type. Many countries allow other strains as well, usually on the basis that those strains are already present in their national flocks. The antigenic type incorporated in ~~both~~ live ~~and inactivated~~ vaccines requires justification if there is doubt as to its existence in a country.

2.1.1. Biological characteristics of the master seed

i) Live vaccines

Currently live IBV vaccines are normally attenuated by multiple repeat passage of a virulent virus in specific pathogen free (SPF) embryonated chicken eggs (Cavanagh, 2003). Spontaneous mutations may arise throughout the IBV genome some of which lead to attenuation of the virus, or minor populations present in the inoculum may be selected during passaging, leading to attenuation. However, as a consequence of this method the attenuated viruses produced by this approach have only a few mutations that are responsible for loss of virulence and these will differ between vaccine strains. Two major drawbacks of this method are that once the virus is used to inoculate chickens the mutations within the attenuated vaccine viruses may back-mutate or be re-selected resulting in virulent

virus, an undesirable consequence, or that as a consequence of multiple passage the immunogenicity of the attenuated virus will not result in adequate protection. Recent work by Keep *et al.* (2024) has shown that the Beaudette S protein expressed from a virulent M41 genomic background not only attenuates the recombinant virus but provides protection against challenge with virulent M41 indicating that multiple passage may not affect antigenicity with respect to protection. ~~Therefore~~ Nevertheless, it is recommended that the number of passages from the master seed to the working seeds is preferably not more than five. Results from efficacy studies using experimental batches at the highest passage claimed for production should be ~~taken into account~~ considered when setting the upper limit of passage allowed for the vaccine. ~~To test the worst-case scenario, it is recommended to test the~~ It is essential that the efficacy ~~on using~~ of the working seed that is used to produce the final product ~~that will be used in the field~~ must be determined. The master seed has to comply satisfactorily with the required tests for extraneous agents and safety. The working seeds must be grown in SPF chicken eggs to minimise the risk of introduction of potential pathogens.

ii) Inactivated vaccines

The IBV strains, for inactivated vaccines, are grown in SPF eggs, non-SPF eggs from healthy flocks (where allowed by the regulatory authority) or cell culture and are chemically inactivated usually by an agent that binds to and destroys the genomic RNA. Batches of inactivated virus suspension must be tested for residual infectivity using embryonated eggs or suitable cell cultures.

Every seed lot must be free from bacterial, fungal, mycoplasmal and viral contamination.

For the detection of extraneous viruses, the seed is first treated with a high-titred monospecific antiserum prepared against the strain under examination or against one of identical type. This mixture is cultured in a variety of ways, designed to confirm the absence of any viruses considered from past experience to be potential contaminants. The antiserum must not contain antibodies to adenovirus, avian encephalomyelitis virus, avian rotavirus, chicken anaemia virus, fowlpox virus, infectious laryngotracheitis virus, influenza A virus, Newcastle disease virus, infectious bursal disease virus, leukosis virus, reovirus, Marek's disease virus, turkey herpesvirus, adeno-associated virus, egg-drop syndrome 76 (EDS76) virus, avian nephritis virus, avian metapneumovirus or reticuloendotheliosis virus. The inoculum given to each unit of the culture system used should contain a quantity of the neutralised IBV component under test that had an initial infectivity of at least ten times the minimum field dose. These systems include:

1. SPF chicken embryos, incubated for 9–11 days, inoculated via both allantoic sac and chorioallantoic membrane (two passages);
2. Chicken embryo fibroblast cultures or other cells that are genetically susceptible for leukosis virus subgroups A, B, and J but not to endogenous avian leukosis virus. The COFAL test (test for avian leukosis using complement fixation), or double-antibody sandwich ELISA for group-specific leukosis antigen is performed on cell extracts harvested at 14 days. An immunofluorescence test for reticuloendotheliosis virus is done on cover-slip cultures after two passages.
3. SPF chicken kidney cultures that are examined for CPEs, cell inclusions and haemadsorbing agents passaged at intervals of no fewer than 5 days for up to 20 days' total incubation.
4. SPF chickens of minimum vaccination age inoculated intramuscularly with 100 field doses, and on to the conjunctiva with ten field doses; this is repeated 2–3 weeks later ~~when the chickens are also inoculated both into the foot pad and intranasally with ten field doses~~. Observations are made for 5–6 weeks overall, and serum is collected for tests for antibodies against the extraneous viruses listed above ~~avian encephalomyelitis, infectious bursal disease, Marek's disease, Newcastle disease and *Salmonella* Pullorum infection~~.

2.1.2. Validation as a vaccine strain

The vaccine virus shall be shown to be satisfactory with respect to safety and efficacy for the chickens for which it is intended. Tests on vaccine virus should include a test for reversion ~~any potential ability to revert~~ to virulence. Live and inactivated vaccine seed must be tested for safety as in Section C.2.2.4.

Efficacy should be demonstrated using a batch vaccine at the highest passage level intended to be ~~registered~~ approved.

For live vaccines, a minimum of ten SPF chickens that are not older than the minimum age to be recommended for vaccination are vaccinated by the route intended for field use (e.g. intranasally or by eyedrop) at the recommended dose. Ten unvaccinated control birds from the same age and source are retained separately. All birds of both groups are challenge inoculated either intranasally or by eyedrop 3–4 weeks later or other time interval in line with the desired claim for onset or duration of immunity, with $10^{3.0}$–$10^{5.0}$ EID_{50} of reference challenge virus, the optimal challenge dose may depend on the challenge strain. A swab of the trachea is taken from each bird 4–5 days after challenge and placed in 3 ml of antibiotic broth. Each fluid is tested for IBV by the inoculation (0.2 ml) of five embryonated eggs, 9–11 days of age. An alternative test to that of taking swabs is to kill birds at 4–6 days after challenge and examine microscopically the tracheal rings for ciliary activity. Failure to resist challenge is indicated by an extensive loss of ciliary motility. The live vaccine is suitable for use if at least 80% of the challenge vaccinated birds show no evidence of infectious IBV in their trachea, while 80% or more of the control birds should have evidence of the presence of the virus.

To assess an inactivated vaccine intended to protect laying birds, 30 or more SPF chickens are vaccinated as recommended at the earliest permitted age. If a primary vaccination with live vaccine is first undertaken, an additional group of birds is given only the primary vaccination. In both cases, these primary vaccinations should be done at no later than 3 weeks of age. The inactivated vaccine is given after the live priming vaccination according to the vaccination schedule to be recommended. A further group of 30 control birds are left unvaccinated. All groups are housed separately until 4 weeks before peak egg production, and then are housed together or in similar conditions. ~~Individual~~ Egg production is monitored and once it is regular, all birds are challenged, egg production being recorded for ~~a further 3–4 weeks~~ at least 4 weeks. The challenge should be sufficient to ensure a significant loss of production ~~during the 3 weeks~~ after challenge. The loss in the non-vaccinated control group should be at least 35% where challenge has been made with a Massachusetts-type strain unless justified. Where it is necessary to carry out a challenge with a strain of another serotype for which there is documented evidence that the strain will not cause a 35% drop in egg production, the challenge must produce a drop in egg production commensurate with the documented evidence and not less than 15% unless justified.

~~; the group that received primary live virus vaccine followed by inactivated vaccine should not significantly drop in production compared with the previous level, and the group given only a primary vaccination should show an intermediate drop in production. The vaccine complies with the test if egg production or quality is significantly better in the group having received the inactivated vaccine than in any control group.~~ The inactivated vaccine complies with the test if egg production or egg quality is significantly better in the group vaccinated with live and inactivated vaccine than in the group only vaccinated with live vaccine, and the egg production or egg quality of the group vaccinated with the inactivated vaccine is significantly better than in the non-vaccinated group.

Sera are collected from all birds at vaccination, 4 weeks later, and at challenge; there should be no response in the negative control birds.

To assess an inactivated vaccine intended to protect birds against respiratory disease, 20 SPF chickens aged 4 weeks are vaccinated as recommended. An additional 20 control birds of the same age and origin are housed with this first group. Antibody responses are determined 4 weeks later; there should be no response in the control birds. All birds are then challenged with 10^3 CID_{50} (50% chick infective dose) or other dose depending on the strain of virulent virus, killed 4–7 days later, and tracheal sections are examined for ciliary motility, or tracheal swabs evaluated for challenge virus recovery. At least 80% of the unvaccinated controls should display complete ciliostasis, whereas the tracheal cilia of a similar percentage of the vaccinated birds should remain unaffected. Tracheal swabs from at least 90% of the vaccinated birds should be negative for virus isolation, while tracheal swabs from at least 90% of the control birds should be positive for virus isolation.

Both live and inactivated multivalent vaccines containing other fractions such as Newcastle disease, infectious bursal disease, avian metapneumo-, reo- and EDS76 viruses are available. The efficacy of the different fractions of these vaccines should each be established; this should also be evaluated in the combination vaccine to assess possible interference between the different vaccine components.

2.2. Method of manufacture

2.2.1. Procedure

All virus strains destined for live attenuated vaccines are cultured in the allantoic sac of SPF chicken embryos or in suitable cell cultures. For inactivated vaccines, hens' eggs from healthy non-SPF flocks (where permitted by the regulatory authority) or suitable cell cultures may be used. The pooled fluid is clarified and then titrated for infectivity. For live vaccines this fluid is lyophilised or frozen in vials, tablets or other forms, and for inactivated vaccines, the inactivated virus ~~it~~ is blended with e.g. high-grade mineral oil or other suitable adjuvant to form an emulsion to which a preservative can be added.

2.2.2. Requirements for ingredients

See chapter 1.1.8 with special focus on products of biological origin (POBs) originating from a country with negligible risk for transmissible spongiform encephalopathies (TSEs).

2.2.3. In-process controls

The required antigen content is based on initial test batches of vaccine of proven efficacy in laboratory and field trials. Infectivity titrations are done in chicken embryos.

Live vaccine should have a titre not less than the minimum dose, which has been proven to be efficacious in the laboratory studies. For inactivated vaccines, which are produced on hens' eggs from healthy non-SPF flocks, validated methods must be in place to exclude the presence of potential contaminants in the eggs. Otherwise, the inactivating agent and inactivation procedure must be shown under manufacture not only to be effective on IBV, but also on other potential contaminants; with the use of beta-propiolactone or formalin, any live leukosis viruses and Salmonella species must be eliminated; and with other inactivating agents, the complete range of potential contaminants must be rendered ineffective. Before inactivation procedures, it is important to ensure homogeneity of suspensions, and a test of inactivation should be conducted on each batch of both bulk harvest after inactivation and the final product.

2.2.4. Final product batch tests

i) Sterility

Every batch of live vaccine should be tested for the absence of extraneous agents as for the seed virus (see chapter 1.1.9). For vaccines administered via drinking water, spray or ~~skin scarification~~ in ovo, one non-pathogenic micro-organism per dose is acceptable.

ii) Safety (target animal batch safety test)

a) For live attenuated vaccines

Use no fewer than ten chickens from an SPF flock that are of the minimum age stated on the label for vaccination. Administer by eyedrop to each chicken ten doses of the vaccine reconstituted so as to obtain a concentration suitable for the test. Observe the chickens for 21 days. For vaccines intended for chickens that are 2 weeks old or more, use the chickens inoculated in the 'test for extraneous agents using chickens' (see Section C.2.1.1 point 4). If during the period of observation, more than two chickens die from causes not attributable to the vaccine, repeat the test. The vaccine complies with the test if no chicken shows serious clinical signs, in particular respiratory signs, and no chicken dies from causes attributable to the vaccine.

b) For inactivated vaccines

Inject a double dose of vaccine by the recommended route into each of ten 14- to 28-day-old chickens from an SPF flock. Observe the chickens for 21 days. Ascertain that no abnormal local or systemic reaction occurs.

Safety tests in target animals are not required by many regulatory authorities for the release of each batch. Where required, standard procedures are generally conducted using fewer animals than are used in the safety tests required for the relevant regulatory approval.

iii) Batch potency

The potency test is developed from the results of efficacy tests on the furthest passage from the master seed virus. Live vaccines are tested for potency by titration of infectivity, and

inactivated vaccines by measuring antibody production or alternative methods. An example of a potency test for inactivated vaccine consists of vaccinating 10 SPF chickens, at least 2 weeks of age, and showing that their mean HI titre 4 weeks later is not significantly less than those obtained for a batch that has shown satisfactory efficacy.

iv) Stability

Vaccine must be shown to have the required potency to achieve the claimed duration of immunity at the end of the claimed shelf life.

At least three batches should be tested for stability and must give satisfactory results for 3 months beyond the claimed shelf life. The stability of a live vaccine must be measured by maintenance of an adequate infectivity titre. The stability of an inactivated vaccine is measured at intervals by batch potency tests. The concentration of preservative and persistence through the shelf life should be assessed. There should be no physical change in the vaccine and it should regain its former emulsion state after one quick shake.

There are maximum level requirements for the use of antibiotics, preservatives and residual inactivating agents.

2.3. Requirements for ~~authorisation/registration/licensing~~ regulatory approval

2.3.1. Manufacturing process

For ~~registration~~ approval of vaccine, all relevant details concerning manufacture of the vaccine and quality control testing (see Section C.2.1 and 2) should be submitted to the authorities. Information shall be provided from three consecutive vaccine batches to demonstrate consistency of production.

2.3.2. Safety requirements

Additional testing required for live IB vaccines and precautions:

i) The reversion-to-virulence test carried out should be consistent with VICH GL41 (Examination of live veterinary vaccines in target animals for absence of reversion to virulence, 2008[1]). Reversion-to-virulence for attenuated/live vaccines and environmental considerations (dissemination and spread of live vaccines and their potential to cause problems for non-vaccinated animals and non-target animals in case of genetically modified vaccines). The vaccine complies when there is no indication of an increase in virulence of virus recovered from the final bird passage compared with the original vaccine that was used for the first passage. It is recommended to use at least five sequential passages over birds by natural spreading or eye-drop application of a suspension of fresh mucosal tissue. Validated alternative methods may be used as well.

ii) Precautions (hazards)

IBV itself is not known to present any danger to staff employed in vaccine manufacture or testing. Extraneous agents may be harmful, however, and the initial stages of handling a new seed virus should be carried out in a safety cabinet. It is a wise precaution with all vaccine production to take steps to minimise exposure of staff to aerosols of foreign proteins. Persons allergic to egg materials must never be employed in this work. Manufacturers should provide adequate warnings that medical advice should be sought in the case of self-injection (including for adjuvants, oil-emulsion vaccine, preservatives, etc.) with warnings included on the product label/leaflet so that the vaccinator is aware of any danger.

2.3.3. Efficacy requirements

To ~~register~~ achieve regulatory approval, a commercial vaccine, a batch or batches produced according to the standard method and containing the minimum amount of antigen or potency value shall prove its efficacy (protection). Efficacy should be demonstrated using a batch vaccine at the highest passage level intended to be ~~registered~~ approved. Each batch of live vaccine should contain sufficient live virus per dose per bird to last until the expiry date, indicated as the minimum dose that has been proven to be efficacious in laboratory studies.

[1] https://www.ema.europa.eu/en/documents/scientific-guideline/vich-gl41-target-animal-safety-examination-live-veterinary-vaccines-target-animals-absence-reversion_en.pdf

Vaccine efficacy (protection) is estimated in vaccinated animals directly by evaluating their resistance to challenge. Vaccine efficacy should be established for each serotype of IBV against which protection is claimed.

The challenge models for determining efficacy are as outlined in Section C.~~2.2.2~~ 2.1.2.

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747 * *

748 **NB:** At the time of publication (~~2018~~ 2024) there were no WOAH Reference Laboratories
749 for avian infectious bronchitis ~~(adenomatosis)~~ (please consult the WOAH Web site:
750 https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).

751 **NB:** FIRST ADOPTED IN 1989. MOST RECENT UPDATES ADOPTED IN 2018.

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Appendix 1: Avian infectious bronchitis
Intended purpose of test: population freedom from infection

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
Virus isolation ± avian	Organ samples, swabs	High in acute phase of infection	Multiple studies	See reference	Detects infectious virus, basis for strains for experiments and antigens	Low availability	De Wit (2000)
Conventional RT-PCR ++ avian	RNA	High in acute phase of infection	Multiple studies	See reference	Basis for sequencing		De Wit (2000)
Real-time RT-PCR conserved gene ++ avian	RNA	High in acute and subacute phase of the infection	Multiple studies	See reference	Ct value provides information about quantity	No difference between vaccines and field strains of same genotype-lineage	De Wit (2000)
Real-time RT-PCR lineage or genotype specific ++ avian	RNA	High in acute and subacute phase of the infection	Multiple studies	See reference	Ct value provides information about quantity	No difference between vaccines and field strains of same genotype-lineage, panel must be appropriate for the region	De Wit (2000)
ELISA ++ avian	Serum antibodies	Positive in 2 weeks post-infection	Multiple studies	See reference	Group-specific, commercial ELISAs for chicken sera are widely available	Group-specific can remain negative in well protected young birds	De Wit (2000)

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Appendix 2: Avian infectious bronchitis
Intended purpose of test: individual animal freedom from infection prior to movement

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
Virus isolation ++ avian	Organ samples, swabs	High in acute phase of infection	Multiple studies	See reference	Detects infectious virus, basis for strains for experiments and antigens	Low availability	De Wit (2000)
Conventional RT-PCR ++ avian	RNA	High in acute phase of infection	Multiple studies	See reference	Basis for sequencing		De Wit (2000)
Real-time RT-PCR ++ avian	RNA	High in acute and subacute phase of the infection	Multiple studies	See reference	Ct value provides information about quantity	No difference between vaccines and field strains of same genotype-lineage	De Wit (2000)
ELISA ± avian	Serum antibodies	Positive in 2 weeks post-infection	Multiple studies	See reference	Group-specific, commercial ELISAs for chicken sera are widely available		De Wit (2000)

Appendix 3: Avian infectious bronchitis
Intended purpose of test: contribute to eradication policies

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
Conventional RT-PCR ++ avian	RNA	High in acute phase of infection	Multiple studies	See reference	Basis for sequencing		De Wit (2000)
Real-time RT-PCR conserved gene ++ avian	RNA	High in acute and subacute phase of the infection	Multiple studies	See reference	Ct value provides information about quantity	No difference between vaccines and field strains of same genotype-lineage	De Wit (2000)
Real-time RT-PCR lineage or genotype specific ++ avian	RNA	High in acute and subacute phase of the infection	Multiple studies	See reference	Ct value provides information about quantity	No difference between vaccines and field strains of same genotype-lineage, panel must be appropriate for the region	De Wit (2000)
HIT ± avian	Serum HI antibodies	Seroconversion usually within 2 weeks	Multiple studies	See reference	Serotype specificity, correlation with level of protection in layers and breeders	Serotype specificity drops in case of contact with multiple types of IBV, difference between laboratories can be significant	De Wit (2000)
ELISA ++ avian	Serum antibodies	Seroconversion usually within 2 weeks	Multiple studies	See reference	Group specific, commercial ELISAs for chicken sera are widely available	Group specific can remain negative in well protected young birds	De Wit (2000)

Appendix 4: Avian infectious bronchitis
Intended purpose of test: confirmation of clinical cases

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
Virus isolation +++ avian	Organ samples, swabs	High in acute phase of infection	Multiple studies	See reference	Detects infectious virus, basis for strains for experiments and antigens	Low availability	De Wit (2000)
Staining by immunohistochemistry ++ avian	Organs		Multiple studies	See reference	Shows infection of cells	Low availability	De Wit (2000)
Gene sequencing ++ avian	RNA	High	Multiple studies	See reference	Epidemiology, vaccines and field strains of same genotype lineage can be discriminated	Relevance of mutations hard to predict	De Wit (2000)
Conventional RT-PCR +++ avian	RNA	High in acute phase of infection	Multiple studies	See reference	Basis for sequencing		De Wit (2000)
Real-time RT-PCR conserved gene +++ avian	RNA	High in acute and subacute phase of the infection	Multiple studies	See reference	Ct value provides information about quantity	No difference between vaccines and field strains of same genotype lineage	De Wit (2000)
Real-time RT-PCR Lineage or genotype specific ++ avian	RNA	High in acute and subacute phase of the infection	Multiple studies	See reference	Ct value provides information about quantity	No difference between vaccines and field strains of same genotype lineage, panel must be appropriate for the region	De Wit (2000)
VNT ± avian	Serum neutralising antibodies	Seroconversion usually within 3 weeks	Multiple studies	See reference	Serotype specificity, correlation with level of protection in layers and breeders	Serotype specificity drops in case of contact with multiple types of IBV	De Wit (2000)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
HIT ± avian	Serum HI antibodies	Seroconversion usually within 2 weeks	Multiple studies	See reference	Serotype specificity, correlation with level of protection in layers and breeders	Serotype specificity drops in case of contact with multiple types of IBV, difference between laboratories can be significant	De Wit (2000)
ELISA ++ avian	Serum antibodies		Multiple studies	See reference	Group specific, commercial ELISAs for chicken sera are widely available	Group specific can remain negative in well protected young birds	De Wit (2000)

Appendix 5: Avian infectious bronchitis
Intended purpose of test: prevalence of infection – surveillance

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
Virus isolation ++ avian	Organ samples, swabs	High in acute phase of infection	Multiple studies	See reference	Detects infectious virus, basis for strains for experiments and antigens	Low availability	De Wit (2000)
Staining by immunohistochemistry ± avian	Organs		Multiple studies	See reference	Shows infection of cells	Low availability	De Wit (2000)
Gene sequencing ± avian	RNA	High	Multiple studies	See reference	Epidemiology, vaccines and field strains of same genotype-lineage can be discriminated	Relevance of mutations hard to predict	De Wit (2000)
Conventional RT-PCR +++ avian	RNA	High in acute phase of infection	Multiple studies	See reference	Basis for sequencing		De Wit (2000)
Real-time RT-PCR conserved gene +++ avian	RNA	High in acute and subacute phase of the infection	Multiple studies	See reference	Ct value provides information about quantity	No difference between vaccines and field strains of same genotype lineage	De Wit (2000)
Real-time RT-PCR Lineage or genotype specific ++ avian	RNA	High in acute and subacute phase of the infection	Multiple studies	See reference	Ct value provides information about quantity	No difference between vaccines and field strains of same genotype lineage, panel must be appropriate for the region	De Wit (2000)
VNT ± avian	Serum neutralising antibodies	Seroconversion usually within 3 weeks	Multiple studies	See reference	Serotype specificity, correlation with level of protection in layers and breeders	Serotype specificity drops in case of contact with multiple types of IBV	De Wit (2000)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
HIT ± avian	Serum HI antibodies	Seroconversion usually within 2 weeks	Multiple studies	See reference	Serotype specificity, correlation with level of protection in layers and breeders	Serotype specificity drops in case of contact with multiple types of IBV. Difference between laboratories can be significant	De Wit (2000)
ELISA ++ Avian	Serum antibodies		Multiple studies	See reference	Group specific, commercial ELISAs for chicken sera are widely available	Group specific can remain negative in well protected young birds	De Wit (2000)

Appendix 6: Avian infectious bronchitis

Intended purpose of test: Immune status in individual animals or populations post-vaccination

Test with score and species	Sample type and target analytes	Accuracy	Test population used to measure accuracy	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
VNT ++ avian	Serum neutralising antibodies	Seroconversion usually within 3 weeks	Multiple studies	See reference	Serotype specificity, correlation with level of protection in layers and breeders	Serotype specificity drops in case of contact with multiple types of IBV	De Wit (2000)
HIT ++ avian	Serum HI antibodies	Seroconversion usually within 2 weeks	Multiple studies	See reference	Serotype specificity, correlation with level of protection in layers and breeders	Serotype specificity drops in case of contact with multiple types of IBV. Difference between laboratories can be significant	De Wit (2000)
ELISA ++ avian	Serum antibodies		Multiple studies	See reference	Group specific, commercial ELISAs for chicken sera are widely available	Group specific can remain negative in well protected young birds	De Wit (2000)

Annexe 22. Chapitre 3.3.4. Influenza aviaire (y compris l'infection par les virus de l'influenza aviaire hautement pathogènes)

RÉUNION DE LA COMMISSION DES NORMES BIOLOGIQUES
Paris, 9–13 septembre 2024

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CHAPTER 3.3.4.

AVIAN INFLUENZA (INCLUDING INFECTION WITH HIGH PATHOGENICITY AVIAN INFLUENZA VIRUSES)

SUMMARY

~~*Influenza A*~~ *Avian influenza (AI) is a viral disease caused by* ~~*specified*~~ *viruses that* ~~*are members of*~~ *belong to the family* Orthomyxoviridae~~,~~ ~~*and placed in the*~~ *genus* Alphainfluenzavirus (Influenzavirus *A or influenza A virus*). *There are* ~~*seven influenza*~~ *four influenzavirus genera (Alphainfluenzavirus, Betainfluenzavirus, Gammainfluenzavirus, Deltainfluenzavirus), but only influenza A viruses are known to infect birds. Diagnosis is by detection and characterisation of fragments of their genome or by isolation of the virus* ~~*or by detection*~~ *and further characterisation* ~~*of fragments of its genome. This is because*~~ *Infections in birds can give rise to a wide variety of clinical signs that may vary according to the host (e.g. species, age, immune status, opportunistic co-infections), strain of virus*~~*, the host's immune status, presence of any secondary exacerbating organisms*~~ *and environmental conditions. Certain strains of avian influenza virus (AIV) can infect mammals and spillover events have sporadically been observed in humans.*

Detection and identification of the agent: ~~*Suspensions in antibiotic solution of oropharyngeal and cloacal swabs (or faeces) taken from live birds, or of faeces and pooled samples of organs from dead birds, are inoculated into the allantoic cavity of 9- to 11-day old embryonated chicken eggs. The eggs are incubated at 37°C (range 35–39°C) for 2–7 days. The allantoic fluid of any eggs containing dead or dying embryos during the incubation and all eggs at the end of the incubation period are tested for the presence of haemagglutinating activity.*~~ *The presence of influenza A virus can be determined by application of reverse-transcription polymerase chain reaction (RT-PCR) on oropharyngeal, tracheal or cloacal swabs taken from live or dead birds, fresh faeces and pooled samples of organs and tissues from dead birds. Swabs and organs can be taken also from species other than birds susceptible to influenza A infection. The approach to diagnosis using RT-PCR is based on initial generic detection of AIV RNA in clinical specimens, primarily by targeting fragments of one or more segments of the influenza A genome, which are highly conserved for all influenza A viruses. Virus isolation (VI) can be achieved using embryonated chicken eggs and allows the detection of infectious (replication-competent) virus and its characterisation. Compared with VI, molecular diagnostics are more rapid, sensitive and cost-effective laboratory tests that are adaptable to high throughputs.* ~~*The presence of influenza A virus can be confirmed by an immunodiffusion test between concentrated virus and an antiserum to the nucleoprotein and/or matrix antigens, both of which are common to all influenza A viruses, or by real-time reverse-transcription polymerase chain reaction (real-time RT-PCR) on the allantoic fluids.*~~ *For these reasons, isolation in embryos has largely been replaced with a rapid initial diagnosis of AIV by* ~~*direct detection in samples, of one or more segments of the influenza A genome using real-time RT-PCR or other*~~ *validated molecular techniques.*

For ~~*serological*~~ *virus subtyping* ~~*of the virus*~~*, the genome of specific H and N subtypes can be identified using molecular protocols with subtype-specific primers and probes (e.g. conventional or real-time RT-PCR) or sequencing analysis. For serological subtyping,* ~~*a Reference Laboratory*~~ *laboratories should conduct*

haemagglutination and neuraminidase inhibition tests against a battery of polyclonal or monospecific antisera to each of the 16 haemagglutinin (*H1–16*) and 9 neuraminidase (*N1–9*) subtypes of ~~*influenza A virus*~~ *avian influenza*. ~~*Alternatively, the genome of specific H and N subtypes is identified using RNA detection technologies with subtype specific primers and probes (e.g. real-time RT-PCR) or sequencing and phylogenetic analysis.*~~

As the general term 'high~~ly~~ pathogenicity avian influenza' and the historical term 'fowl plague' refer to infection with high pathogenicity (*HP*) strains of influenza A virus, it is necessary to assess the pathogenicity of influenza A virus isolates *at least for primary disease events* ~~*for domestic poultry*~~. All naturally occurring high pathogenicity avian influenza (*HPAI*) strains isolated to date have been either of the H5 or H7 subtype, with a subset of H5 or H7 isolates being of low pathogenicity (*LP*). *Verification of high or low pathogenicity of a virus isolate is achieved by obtaining an intravenous pathogenicity index (IVPI) following inoculation of a minimum of eight susceptible 4- to 8-week-old chickens with infectious virus; strains are considered to be of high pathogenicity if they cause more than 75% mortality within 10 days, or when the inoculation of 10 susceptible 4- to 8-week-old chickens* ~~*resulting*~~ *results in an IVPI greater than 1.2. Characterisation of suspected high pathogenicity strains of the virus should be conducted in a secure biocontainment laboratory facility capable of housing such pathogens according to local and international legislation.* The methods used for the determination of strain virulence for birds have evolved over recent years with a greater understanding of the molecular basis of pathogenicity. ~~*Regardless of their pathogenicity for chickens,*~~ H5 or H7 viruses with a *polybasic* HA0 cleavage site amino acid sequence similar to any of those that have been observed in ~~*high pathogenicity*~~*HP* viruses are considered to be influenza A viruses with high pathogenicity. H5 and H7 isolates that are not *of* high~~ly~~ pathogenic*ity* for chickens and do not have a HA0 cleavage site amino acid sequence similar to any of those that have been observed in highly pathogenic viruses are considered to ~~*have*~~ *be of* low pathogenicity. ~~*However, in some circumstances it is necessary to verify high or low pathogenicity of a virus isolate using the intravenous inoculation of a minimum of eight susceptible 4- to 8-week-old chickens with infectious virus; strains are considered to be of high pathogenicity if they cause more than 75% mortality within 10 days, or inoculation of 10 susceptible 4- to 8-week-old chickens resulting in an intravenous pathogenicity index (IVPI) of greater than 1.2. Characterisation of suspected highly pathogenic strains of the virus should be conducted in a virus-secure biocontainment laboratory.~~ *Defining pathogenicity by the molecular route aids in reducing animal experiments. Although classified as LPAI according to official definitions using IVPI or molecular criteria, certain* ~~*Low pathogenicity avian influenza (LPAI) in poultry*~~ *LPAI viruses* (*e.g H9 subtype*) may be accompanied by a sudden and unexpected increase in virulence (*emerging disease*) *in poultry or have proven* ~~*natural*~~ *transmission to humans* ~~*associated with severe consequences*~~. In these disease scenarios there should be formal monitoring in relevant poultry populations by national authorities. The occurrence of H5 and H7 ~~*low pathogenicity avian influenza viruses*~~ *LPAIVs* should be monitored as some have the potential to mutate into ~~*high pathogenicity avian influenza viruses*~~ *HPAIVs*.

Serological tests: *As all influenza A viruses have antigenically* ~~*similar*~~ *conserved nucleoprotein and matrix* ~~*antigens*~~ *proteins, these antigens are preferred targets of influenza A group serological screening methods. Enzyme-linked immunosorbent assays (ELISAs) are widely used to detect antibodies to these antigens in either host species-dependent (indirect) or species-independent (competitive) test formats. Haemagglutination inhibition (HI) tests* ~~*have*~~ *are also* ~~*been*~~ *employed in routine diagnostic serology, generally for subtyping activities, but* ~~*it is possible that this technique may miss some particular infections because*~~ *antibodies directed against the haemagglutinin (HA) are highly subtype- and even strain-specific within certain subtypes and thus HI can vary in specificity* ~~*is subtype specific*~~*. Therefore, depending on the testing purpose, the choice of HA antigen to be used in the HI test is critical to the performance of this test.*

Requirements for vaccines: ~~*The first use of*~~ *Traditionally, vaccination* ~~*in an avian influenza eradication programme was against LPAI. The*~~ *programmes used inactivated oil-emulsion vaccines with the same haemagglutinin and neuraminidase subtypes as the circulating field virus, and infected flocks were identified by detection of virus or antibodies against the virus in non-vaccinated sentinel birds. During the 1990s the prophylactic use of inactivated oil-emulsion vaccines was employed in Mexico and Pakistan to control widespread outbreaks of HPAI and H5/H7 LPAI. During the 1999–2001 outbreak of H7 LPAI in Italy, an inactivated vaccine was used with the same (i.e. homologous) haemagglutinin subtype to the field virus, but with a different (i.e. heterologous) neuraminidase. This allowed the serological differentiation of non-infected vaccinated birds from vaccinated birds infected with the field virus and ultimately* ~~*resulted*~~ *aided in eradication of the field virus. Prophylactic use of H5 and H7 vaccines has been practised in parts of Italy, aimed at preventing H5/H7 LPAI infections,* ~~*and several*~~ *Multiple countries in Asia, Africa,* ~~*and*~~ *the Middle East, Europe,*

Central and South America have already been using or have started to use vaccination as a supplementary aid in controlling or preventing H5/H7 HPAI ~~in China (People's Rep. of) forH7N9, and in Mexico for H7N3 HPAI virus infections~~. Native HPAI viruses ~~should~~ must not be used as the seed virus for production of vaccine.

If LPAI and HPAI viruses are used in challenge studies, an appropriate level of containment should be used as determined by risk assessment.

A. INTRODUCTION

Influenza in birds is caused by infection with viruses of the family *Orthomyxoviridae* placed in the genus *Alphainfluenzavirus* (*influenzavirus A* or influenza A virus) ~~(International Committee on Taxonomy of Viruses [ICTV], 2019). Influenza A viruses are the only orthomyxoviruses known to naturally affect birds (Swayne & Sims, 2020).~~ Among the four genera of influenzaviruses (Alphainfluenzavirus, Betainfluenzavirus, Gammainfluenzavirus, Deltainfluenzavirus) that infect vertebrates, only influenza A viruses are known to infect birds. Many species of birds have been shown to be susceptible to infection with influenza A viruses; aquatic birds form a major reservoir of these viruses, and the overwhelming majority of isolates have been of low pathogenicity (low virulence) for chickens and turkeys. Influenza A viruses have antigenically closely related nucleoprotein and matrix proteins, but are classified into subtypes on the basis of their haemagglutinin (H) and neuraminidase (N) antigens ~~(World Health Organization Expert Committee, 1980)~~. At present, 16 H subtypes (H1–H16) and 9 N subtypes (N1–N9) are recognised in birds, with a new H19 subtype recently proposed. In addition, further influenza A subtypes (H17N10, H18N11) ~~for influenza A viruses~~ have been identified in bats in Central and South America ~~from bats in Guatemala (ICTV 2019; Swayne *et al.*, 2020; Tong *et al.*, 2013)~~. Further to this, some avian influenza virus strains principally of H3, H5, H7, H9 and H10 subtypes, have caused sporadic zoonotic infections and some strains of these subtypes have been highlighted as potential pandemic risks should additional mutations occur that support sustained human-to-human transmission.

To date, naturally occurring high pathogenicity influenza A viruses (HPAIV) that produce acute clinical disease in chickens, turkeys and other birds of economic importance have been associated only with the H5 and H7 subtypes. There is the risk of a H5 or H7 virus of low pathogenicity (H5/H7 low pathogenicity avian influenza [LPAI]) becoming highly pathogenic by mutation at the H protein cleavage site. Low pathogenicity H5 and H7 occur widely in poultry and aquatic wild birds alongside a vast range of non-H5/H7 subtypes. ~~, although intercontinental spread of HPAI has received greater attention in recent years. There is the risk of a H5 or H7 virus of low pathogenicity (H5/H7 low pathogenicity avian influenza [LPAI]) becoming highly pathogenic by mutation. Some avian influenza virus strains have caused sporadic zoonotic infections principally of H5, H7 and H9 subtypes and these three subtypes have been highlighted as potential pandemic risks should additional mutations occur that support sustained human to human transmission (Cox et al., 2017).~~ Recently, HPAIV have caused a pandemic of unprecedented magnitude among avian species accompanied by severe losses to the poultry industry and wild bird species. This pandeminic event is due to HPAI H5 viruses of the goose/Guangdong lineage (Gs/GD), clade 2.3.4.4b (clade based upon H only), which have established enzootic status in several wild bird population worldwide. These HPAI H5 viruses have also caused infections in a remarkable number of terrestrial and marine mammals mainly following close contact with infected poultry, wild birds, captive birds or contaminated equipment, environment or feed.

Throughout this chapter of the *Terrestrial Manual,* the following terms will be used: 1) HPAI as an infection by an avian influenza virus that meets the definition of high pathogenicity, 2) LPAI as an infection with any H1–H16 avian influenza virus that is not of high pathogenicity, and 3) influenza A as an infection with any HPAI or LPAI virus.

Depending on the species, age and type of bird, specific characteristics of the viral strain involved~~,~~ and on environmental factors, the ~~highly pathogenic disease,~~ HPAI infection outcome in fully susceptible birds, may ~~vary from one of~~ range from sudden death with no preceding overt clinical signs, to a more characteristic disease with variable clinical presentations including respiratory and nervous signs~~, such as ocular and nasal discharges, coughing, snicking and dyspnoea, swelling of the sinuses and/or head, apathy, reduced vocalisation, marked reduction in feed and water intake, cyanosis of the unfeathered skin, wattles and comb, incoordination, and nervous signs and diarrhoea (Swayne *et al.*, 2020)~~. In laying birds, additional clinical features include a marked drop in egg production, usually accompanied by an increase in numbers of poor quality eggs. Typically, high morbidity is accompanied by high and rapidly escalating ~~unexplained~~ mortality (see Section B.1.4 *Assessment of pathogenicity* for details on clinical signs). However, none of these signs can be considered pathognomonic for infection with AIV. In certain host species such as Pekin ducks (*Anas platyrhynchos domesticus*) and its wildlife sister species, mallards and mandarin ducks, some HPAI viruses do not necessarily produce significant clinical disease. In addition, LPAI viruses which normally cause only a mild or no clinical disease, may in certain circumstances produce a spectrum of clinical signs, the severity of which may ~~approach that of~~ resemble that expected with HPAI infection, particularly ~~if exacerbating infections and/or adverse environmental conditions are present~~ where bird health may be impacted by other factors (e.g. other infectious agents or environmental factors that may affect disease severity). Confirmatory diagnosis of the disease, therefore, depends on the ~~isolation or~~ detection of the ~~causal virus~~ cause of infection and the demonstration that it fulfils one of the defined criteria described in Section B.1.4 *Assessment of pathogenicity*.

Testing sera from suspect birds using antibody detection methods may supplement diagnosis, but these methods are not suitable for ~~a definitive identification~~ either subtyping or pathotyping of infection. Diagnosis for official control purposes is established on the basis of agreed official criteria for pathogenicity according to *in-vivo* tests or ~~to~~ the haemagglutinin subtyping and characterisation of molecular determinants (i.e. the presence of a cleavage site of the haemagglutinin precursor protein HA0 consistent with HPAI virus) ~~and haemagglutinin subtyping~~. These definitions evolve as scientific knowledge of the disease increases.

Occasionally, where the infection pressure is high (e.g. following outbreaks in wild birds), spillover events into non-avian species have been described (Runstadler& Puryear, 2024). Infection of terrestrial and marine mammals with HPAI H5 viruses of clade 2.3.4.4b can result in a highly variable clinical picture. Signs described so far in the order Carnivora primarly include neurological signs such as circling, lack of coordination, ataxia, tremors and lethargy. Loss of appetite, apathy, hypersalivation, fever, dyspnea (shallow and accelerated breathing), nasal discharge, and abortion have been also reported. Both for domestic and wild mammals of the order Carnivora, subclinical or clinically mild infections have been also documented. Furthermore, HPAI H5N1 clade 2.3.4.4b virus has also been identified as the cause of udder infections leading to mastitis in dairy cattle in the USA. Clinical signs in infected cattle may include reduced feed intake with decreased rumination and rumen motility, respiratory signs such as clear nasal discharge, dehydration, fever and a sudden decrease in milk production. Affected cows may produce thicker, concentrated milk similar to colostrum or may stop producing milk. Other clinical signs may include abnormal faecal consistency, lethargy and abortion. Subclinical infection has been also reported[1].

Regardless of the species infected, HPAI should be subject to official control by national authorities. In addition, LPAI, particularly H5 and H7 subtypes, may be subject to national or state/provincial control. The viruses that cause ~~influenza A~~ avian influenza have the potential to spread from the laboratory if adequate levels of biosecurity and biosafety are not in place. Avian influenza viruses should be handled with appropriate measures as described in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*. Biocontainment measures should be determined by risk analysis as described in Chapter 1.1.4. The measures required may vary ~~among~~ depending on the genetics of the subtypes and pathotypes of influenza A viruses manipulated, with higher level containment being indicated for some LPAI and HPAI viruses~~,~~. Such viruses ~~and~~ may require additional procedural, equipment and facility enhancements under specific conditions such as high virus concentrations, housing infected animals or conducting procedures ~~with aerosol generating activities~~ that may result in the generation of aerosols. The occupational risk of operators working with zoonotic viruses should be carefully assessed to ensure safe working practises commensurate with the pathogens being handled. Countries lacking access to such a specialised national or regional laboratory facilities should send specimens to a WOAH Reference Laboratory depending on the purposes of handling the viruses.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of avian influenza and their purpose

Method	Purpose					
	Population freedom from infection[(a)]	Individual animal freedom from infection prior to movement[(b)]	Contribute to eradication policies[(c)]	Confirmation of clinical cases[(d)]	Prevalence of infection – surveillance (including vaccinated flocks)[(e)]	Immune status in individual animals or populations post-vaccination[(f)]
Detection and identification of the agent[(g)]						
Virus isolation	+	++~~+~~	+	+++	+	–
Antigen detection	+	+	+	+	+	–
Real-time RT-PCR	++±	+++	++±	+++	++±	–
Conventional RT-PCR	±	±	±	++	±	±

[1] https://www.aphis.usda.gov/sites/default/files/hpai-livestock-case-definition.pdf

Method	Purpose					
	Population freedom from infection[(a)]	Individual animal freedom from infection prior to movement[(b)]	Contribute to eradication policies[(c)]	Confirmation of clinical cases[(d)]	Prevalence of infection – surveillance (including vaccinated flocks)[(e)]	Immune status in individual animals or populations post-vaccination[(f)]
Detection of immune response						
AGID	+ (Influenza A)	+ (Influenza A)	++ (Influenza A)	+ (convalescent)	++ (Influenza A)	++ (Influenza A)
HI	+++ (H5 or H7)	++ (H5 or H7)	+++ (H5 or H7)	++ (convalescent)	+++ (H5 or H7)	+++ (H5 or H7)
ELISA	+++	+	++	+ (convalescent)	++	++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
RT-PCR = reverse-transcription polymerase chain reaction; AGID = agar gel immunodiffusion;
HI = haemagglutination inhibition test; ELISA = enzyme-linked immunosorbent assay.

[(a)] See Appendix 2 of this chapter for justification table for the scores given to the tests for this purpose.
[(b)] See Appendix 3 of this chapter for justification table for the scores given to the tests for this purpose.
[(c)] See Appendix 4 of this chapter for justification table for the scores given to the tests for this purpose.
[(d)] See Appendix 5 of this chapter for justification table for the scores given to the tests for this purpose.
[(e)] See Appendix 6 of this chapter for justification table for the scores given to the tests for this purpose.
[(f)] See Appendix 7 of this chapter for justification table for the scores given to the tests for this purpose.
[(g)] A combination of agent identification methods applied on the same clinical sample is recommended.

1. Detection and identification of the agent

Detection and identification of influenza A viruses as the cause of infections and disease in poultry and other birds requires a thorough diagnostic investigation to differentiate from similar diseases caused by other viral agents especially *Orthoavulavirus javaense*, commonly known as avian paramyxovirus type 1 virus ~~(APMV-1)~~ (Family *Paramyxoviridae*, subfamily *Avulavirinae*, genus *Orthoavulavirus* (APMV-1, used hereafter). Individual influenza A and APMV-1 virus isolates vary greatly in virulence, causing various disease outcomes ~~syndromes~~ evident as subclinical infections, drops in egg production, respiratory disease, and severe ~~and~~ disease with high mortality ~~disease~~. The latter clinical outcome ~~syndrome~~ can be caused by either HPAI or virulent APMV (Newcastle disease viruses). Therefore, as no disease manifestation is defined as being pathognomonic for influenza A or APMV-1 infection ~~it is judicious to have~~ a single sampling procedure is recommended. This sampling approach, coupled with ~~and~~ simultaneous~~ly conduct specific differentiating~~ differential diagnostic ~~tests~~ evaluation using assays specific for either ~~both~~ influenza A ~~and~~ or APMV-1 viruses on field samples, allows ~~to obtain~~ an accurate aetiological diagnosis of a single agent or, on occasion, confirmation of dual infection.

1.1. Samples for virus detection ~~isolation~~

When handling or sampling suspect cases of avian influenza, it is imperative to use appropriate biosafety measures as described in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*.

~~Virus isolation is the reference method but is laborious and time intensive, used primarily for diagnosis of a first clinical case in an outbreak and to obtain virus isolates for further laboratory analysis.~~

For both molecular and virological investigations of ~~severe~~ the disease ~~and high mortality~~ in poultry flocks, it is usual to attempt virus ~~isolation~~ detection from samples collected from recently dead birds or moribund birds that have been humanely killed ~~humanely~~. Samples taken from dead birds should include intestinal contents (faeces) or cloacal swabs and oropharyngeal or tracheal swabs. Samples from trachea, lungs, air sacs, brain, intestine, spleen, caecal tonsils, kidneys, ~~brain,~~ liver and heart should also be collected and processed either separately or as a pool. When pooling samples, the brain should be collected and processed first (to avoid cross contamination with other tissue types) and kept separate as presence of virus in the brain may be an indicator of HPAI or ~~NDV~~ virulent APMV-1. Further pools of tissues or organs should be made consistent with known virus tropisms between HPAI and LPAI, i.e. grouped at the level of respiratory or, ~~systemic and~~ gastrointestinal tracts.

Samples from live birds should include both oropharyngeal or tracheal and cloacal swabs, the latter should be visibly coated with faecal material. To avoid harming them, swabbing of small delicate birds should be done with the use of especially small swabs that are usually commercially available and intended for use in human paediatrics or the collection of fresh faeces may serve as an adequate alternative (caution that some influenza A viruses and APMV-1 ~~type 1 avian paramyxoviruses~~ in birds can have a strong respiratory tropism). ~~Similar~~ Swab samples can be pooled from the same anatomical site (i.e. cloacal swabs with cloacal swabs, oropharyngeal swabs with oropharyngeal swabs), from the same species and the same premises. Most commonly pooling of five is used or occasionally more, if validation of this approach has been undertaken to ensure no significant loss of sensitivity. ~~if appropriately validated not to reduce sensitivity of detection, but specific swab types should be used (Spackman *et al.*, 2013).~~ Furthermore, the type of swabs used may affect test sensitivity or validity with thin wire or plastic shafted swabs preferred. Swabs should be collected and tested individually from wild or captive waterfowl. These birds may carry multiple subtypes of AI virus, and pooling the swabs in a single tube may compromise the detection and the correct characterisation of one or more virus subtypes.

In addition to testing sick and dead birds, in active surveillance scenarios of populations appearing healthy, environmental samples collected from water, drinker biofilms, feeder troughs, floors, walls, nests, cages, or fans as well as air, have been proposed as a less invasive and more economical surveillance strategy (Harder *et al.*, 2023). Although progress has been made in exploiting environmental sample matrices for the detection of AIV, protocols harmonisation and proper validation of such techniques in comparison with routine methodology is mostly lacking and further investigations are needed to ascertain their suitability for surveillance of AIV.

As described above, the HPAI H5N1 clade 2.3.4.4b virus has demonstrated the capacity to infect a broad range of mammals, most likely as a consequence of exposure to infected poultry, wild birds, and captive birds or even to contaminated environment, materials or feed. In the majority of HPAI H5 clade 2.3.4.4b positive live domestic/farmed carnivores (e.g. cats, minks, artic foxes, raccoon dog) and wild mammals (e.g. foxes, sea lions), the virus has been detected in nasal or tracheal/oropharyngeal swabs and, to a lesser extent, in rectal swabs. In the post-mortem examination of dead or severely ill or deceased animals, the highest viral loads of HPAI H5 virus has mainly been found at the level of the central nervous system and in the lower respiratory tract; hence, both these anatomical sites should be examined. Of note, neurological disease induced by HPAI H5 viruses in mammals can manifest without clinical respiratory disease. In this case, respiratory samples can test negative despite efficient virus replication in the central nervous system. In infected lactating dairy cattle, milk samples have been shown to yield the highest virus concentration. Milk from each of the four quarters (3–10 ml per animal) are the preferred sample from lactating animals. Deep nasal swabs should be used for non-lactating animals (see OFFLU website[2]).

The clinical samples should be placed in virus transport media (isotonic phosphate-buffered saline (PBS) pH 7.0–7.4 or normal saline (0.9% (w/v) NaCl) or protein based media such as brain-heart-infusion or tris-buffered tryptose broth ~~isotonic phosphate-buffered saline (PBS), pH 7.0–7.4~~ with antibiotics ~~or a solution containing protein and antibiotics~~. The antibiotics to be used and their concentration can be varied according to local conditions, but could be, for example, penicillin (2000 units/ml), streptomycin (2 mg/ml), gentamycin (50 µg/ml) and mycostatin (1000 units/ml) for tissues and oropharyngeal or tracheal swabs, but at five-fold higher concentrations for faeces and cloacal swabs. It is important to re-adjust the pH of the solution to pH 7.0–7.4 following the addition of the antibiotics. It is ~~recommended~~ advisable that a solution for transport of the swabs should contain protein to stabilise the virus (e.g. brain–heart infusion, up to 5% [v/v] cattle serum, 0.5% [w/v] bovine albumen or similar commercially available transport media). Indeed, brain-heart-infusion (BHI) broth has been demonstrated to give added stability than PBS for virus recovery. If control of *Chlamydophila* is desired, 0.05–0.1 mg/ml oxytetracycline should be included. Faeces and finely minced tissues should be prepared as 10–20% (w/v) suspensions in the antibiotic solution. Suspensions should be processed as soon as possible after incubation for 1–2 hours at room temperature. In case the use of transport media is prevented by logistic or economical issues, swabs must be returned to their casing and submitted dry to the laboratory as quickly as possible (<24 hours), assuring they are immediately chilled on ice or with frozen gel packs. When immediate processing is impractical, samples may be stored at 4°C for up to 4 days. For a prolonged storage, diagnostic samples and isolates should be kept at –80°C but for transport, ~~on~~ dry ice (≤–50°C) is widely used. Repeated freezing and thawing should be avoided.

Preserving the quality and integrity of biological samples during transportation may be challenging in remote areas where laboratory facilities are limited and cold chain maintenance is difficult. For these reasons, the use of technologies and reagents (Rattanamas *et al.*, 2022) that increase the capacity to detect and diagnose viral infections in these scenarios are becoming increasingly common for the transport of samples to laboratories for the direct detection of AIV via nucleic acid assays. However, whilst these methods may enable molecular detection, the processes involved will inactivate virus infectivity, thus preventing virus characterisation by isolation.

[2] https://www.offlu.org/wp-content/uploads/2024/05/2024_05_10_HPAI_Dairy-cattle.pdf

As such, it is advisable to limit their use to contexts where proper conditions for sample preservation are difficult to achieve.

1.2. Virus isolation

The preferred method of growing influenza A viruses is by the inoculation of specific pathogen free (SPF) embryonated chicken eggs, or specific antibody negative (SAN) eggs. The supernatant fluids of faeces, swabs or tissue suspensions can be obtained through clarification by centrifugation at 1000 ***g*** for approximately 10 minutes at a temperature not exceeding 25°C. Clarified preparations can be inoculated into three to five embryonated SPF or SAN chicken eggs of 9–11 days' incubation using a number of routes including the amniotic sac, chorioallantoic sac or membrane (at least one of which should be used for primary isolation) and allantoic sacs. The inoculated eggs are typically incubated at 37°C (range 35–39°C) for 2–7 days. Recent studies have generated validation data to support a shortening of this time period to a 4-day time period by adopting a 'rapid-passage approach'. Eggs containing dead or dying embryos as ~~they arise~~ detected by candling, and all eggs remaining at the end of the incubation period, should first be chilled to 4°C for 4 hours or overnight (the latter is the preferred option). After checking that the embryos have died, the amnio-allantoic fluids should be recovered and tested with a screening test (such as haemagglutination [HA] test), influenza A type-specific test (such as agar gel immunodiffusion test [AGID] or solid-phase antigen-capture enzyme-linked immunosorbent assays [ELISA]~~)~~ or lateral-flow immunochromatography) or influenza A subtype-specific test (such as haemagglutination inhibition [HI] and neuraminidase [N] inhibition [NI] tests) or a molecular test to detect influenza A specific nucleic acid signatures (such as real-time reverse transcription polymerase chain reaction [RT-PCR]) as described later (see Section B.1.5.2 *Direct RNA detection* ~~1.2.2~~). Detection of HA activity, in bacteria-free amnio-allantoic fluids verified by microbiological assay, indicates a high probability of the presence of an influenza A virus or of an APMV-1 ~~avian orthoavulavirus (formerly avian paramyxovirus)~~. Fluids that give a negative reaction should be passaged into at least one further batch of eggs, and up to three passages.

Routine checks for bacterial contamination should be conducted by streaking samples in Luria ~~Broth~~ Bertani agar plates and reading these at 24 and 48 hours of incubation against a light source. BHI agar and blood agar plates may also be used. For larger numbers of samples, initial cultures could be in tryptose phosphate broth. Contaminated samples can be treated by incubation with increased antibiotic concentrations for 2–4 hours (gentamicin, penicillin g, and amphotericin b solutions at final concentrations to a maximum of 1 mg/ml, 10,000 U/ml, and 20 µg/ml, respectively). Samples heavily contaminated by bacteria that cannot be removed by centrifugation or controlled by antibiotics can be filtrated through 0.45 and 0.2 micron sterile filters. Filtration should be used only when other methods fail because ~~aggregation~~ it may significantly reduce virus titre.

1.3. Virus identification

The presence of influenza A virus can be confirmed in AGID tests by demonstrating the presence of the nucleoprotein or matrix antigens, both of which are common to all influenza A viruses (see Section B.2.2 *Agar gel immunodiffusion*). The antigens may be prepared by concentrating the virus from infective allantoic fluid or extracting the infected chorioallantoic membranes; these are tested against known positive antisera. Virus may be concentrated from infective allantoic fluid by ultracentrifugation, or by precipitation under acid conditions. The latter method consists of the addition of 1.0 M HCl to infective allantoic fluid until it is approximately pH 4.0. The mixture is placed in an ice bath for 1 hour and then clarified by centrifugation at 1000 ***g*** at 4°C. The supernatant fluid is discarded. The virus concentrates are resuspended in glycine/sarcosyl buffer consisting ~~of~~ of 1% (w/v) sodium lauroyl sarcosinate buffered to pH 9.0 ~~with~~ and 0.5 M glycine. These concentrates contain both nucleoprotein and matrix polypeptides and infectivity is destroyed.

Preparations of nucleoprotein-rich antigen can also be obtained from chorioallantoic membranes for use in the AGID test ~~(Beard, 1970)~~. This method involves removal of the chorioallantoic membranes from infected eggs that have allantoic fluids with HA activity. The membranes are then homogenised or ground to a paste. This is subjected to three freeze–thaw cycles, followed by centrifugation at 1000 ***g*** for 10 minutes. The pellet is discarded and the supernatant is used as an antigen following treatment with 0.1% formalin or 1% betapropiolactone.

Use of the AGID test to demonstrate nucleoprotein or matrix antigens is a satisfactory way to indicate the presence of influenza A virus in amnioallantoic fluid, but lacks sensitivity compared to other methods including molecular assays ~~(see Section 1.2.2), but~~ Various experimental and commercial rapid, solid-phase antigen-capture ELISAs (AC-ELISAs) are an effective and easy to use alternative ~~(Swayne *et al.*, 2020)~~. Most AC-ELISAs have been approved and marketed to detect human influenza A virus in clinical specimens. Some have demonstrated effectiveness for detection of influenza A, but many of these commercial tests have had low sensitivity (Slomka *et al.,* 2012). Those validated for veterinary use are preferred (see Section B.1.5.1 *Antigen detection*).

Any HA activity of sterile fluids harvested from the inoculated eggs is most likely to be caused by an influenza A virus or APMV-1 virus ~~an avian paramyxovirus, but~~ However, a few strains of avian reovirus, as well as nonsterile

fluid containing HA of bacterial origin (e.g. mycoplasms) can cause the agglutination of red blood cells (RBCs) so multiple approaches to define the cause of HA activity may be required. Most laboratories will have antiserum specific to Newcastle disease virus (~~avian paramyxovirus type 1~~ virulent~~,~~ APMV1), and in view of its widespread occurrence and almost universal use as a live vaccine in poultry, it is best to evaluate its presence by haemagglutination inhibition (HI) tests (see Chapter 3.3.14 *Newcastle disease*).

Alternatively, the presence of influenza virus can be confirmed by the use of conventional RT-PCR or real-time RT-PCR targeting conserved viral genome segments, e.g. the matrix gene ~~using nucleoprotein specific or matrix specific conserved primers~~ (Hassan *et al.*, 2022; Nagy *et al.*, ~~2020~~ 2021; Spackman *et al.*, 2002; 2020). The presence of subtype H5 or H7 influenza virus can also be confirmed by using H5- or H7-specific primers (Hassan *et al.*, 2022; Slomka *et al.*, 2007b; Spackman *et al.*, 2002). See Section B.1.5.2 *Direct RNA detection* for details on molecular procedures.

Antigenic subtyping can be accomplished by monospecific antisera prepared against purified or recombinant H and N subtype-specific proteins, used in HI and NI tests, or polyclonal antisera raised against a range of intact influenza viruses and used in HI and NI tests. For laboratories conducting the HI test to H subtype it is strongly recommended that two sera for each H subtype is used but with a heterologous N and should ideally use antisera to contemporary viruses relevant to the region in which the virus is detected. Subtyping can also be accomplished using H and N subtype specific primers in RT-PCR and real-time RT-PCR tests~~;~~ or using sequence analysis of H and N genes (Section B.1.5.2 *Direct RNA detection*). Subtype identification by these techniques is becoming increasingly common but ~~is beyond the scope of many~~ diagnostic laboratories not ~~specialising~~ specialised in influenza viruses~~. Assistance is available from the~~ can be assisted by WOAH Reference Laboratories and Collaborating Centres (see WOAH website for up-to-date list).

1.4. Assessment of pathogenicity

The term HPAI relates to the assessment of pathogenicity in chickens and implies the involvement of high pathogenicity strains of virus. It is used to describe a disease of fully susceptible chickens with clinical signs that may include one or more of the following: ocular and nasal discharges, coughing, snicking and dyspnoea, swelling of the sinuses and/or head, listlessness, reduced vocalisation, marked reduction in feed and water intake, cyanosis of the unfeathered skin, wattles and comb, incoordination, nervous signs and diarrhoea. In laying birds, additional clinical features include a marked drop in egg production usually accompanied by an increase in numbers of poor-quality eggs. Typically, high morbidity is accompanied by high and rapidly escalating unexplained mortality. However, none of these signs can be considered pathognomonic and high mortality may occur in their absence. In addition, LPAI viruses that normally cause only mild or no clinical disease, may cause a much more severe disease if predisposing infections or adverse environmental factors are present and, in certain circumstances, the spectrum of clinical signs may mimic HPAI.

The historical term 'fowl plague' has been abandoned in favour of the more accurate term HPAI. Because all naturally occurring HPAI viruses to date have been H5 and H7 subtypes and genomic studies have determined HPAI viruses arise by mutation of H5/H7 LPAI viruses, all H5/H7 LPAI viruses may potentially become HPAI but predicting which LPAI strains will mutate to HPAI is not possible. Pathogenicity shifts have been associated with changes to the proteolytic cleavage site of the haemagglutinin including: 1) substitutions of non-basic with basic amino acids (arginine or lysine); 2) insertions of multiple basic amino acids from codons duplicated from the haemagglutinin cleavage site; 3) short insertions of basic and non-basic amino acids from unknown source; 4) recombination with inserts from other influenza A virus gene segments or avian host cellular genome (e.g. 28S rRNA) that lengthen the proteolytic cleavage site; and 5) loss of the shielding glycosylation site at residue 13 in combination with multiple basic amino acids at the cleavage site[3]. Nucleotide sequencing and amino acid deduction ~~sequencing~~ of the cleavage sites of H5 and H7 subtype influenza A isolates of low pathogenicity for birds may identify viruses that have the capacity, following simple mutation, to have high pathogenicity for poultry.

The following methods and criteria have been adopted by the WOAH for determining pathogenicity of an influenza A virus:

a) *Molecular assessment:* For all H5 and H7 viruses in chickens, the amino acid sequence of the proteolytic cleavage site must be determined. The presence of multiple basic amino acids in the HA0 cleavage site is the genotypic standard for HPAI strains; therefore, if the isolate being tested has an HA0 cleavage site motif identical to previous HPAI viruses, it should be designated as HPAI irrespective of a low or high pathogenicity outcome following pathotyping in chickens (see the table that lists all the reported haemagglutinin proteolytic cleavage sites of HA0 protein for H5 and H7 LPAI and HPAI viruses based on deduced amino acid sequence, which can be found on the OFFLU site (see footnote ~~2~~ 1). Furthermore, any

[3] ~~http://www.offlu.org/wp-content/uploads/2021/01/Influenza_A_Cleavage_Sites.pdf~~ https://www.offlu.org/wp-content/uploads/2022/01/Influenza-A-Cleavage-Sites-Final-04-01-2022.pdf

isolate with a new motif must be tested *in vivo* by IVPI. In case of difficulties in the interpretation of the cleavage site motif, WOAH or FAO reference laboratories should be consulted.

b) *In-vivo* assessment: One of the two following *in-vivo* tests ~~methods~~ to determine pathogenicity in chickens is used. A high pathogenicity influenza A virus is:

i) any influenza A virus that is lethal[4] for six, seven or eight of eight 4- to 8-week-old susceptible chickens within 10 days following intravenous inoculation with 0.2 ml of a 1/10 dilution of a bacteria-free, infective allantoic fluid

or

ii) any influenza A virus that has an intravenous pathogenicity index (IVPI) greater than 1.2~~.~~ when using the following ~~is the IVPI~~ procedure:

- Fresh infective allantoic fluid, confirmed free at least from APMV-1 and ~~other extraneous agents~~ bacteria, with a HA titre >1/16 (>24 or >$\log_2$ 4 when expressed as the reciprocal) is diluted 1/10 in sterile isotonic saline.
- 0.1 ml of the diluted virus is injected intravenously into each of ten 4- to 8-week-old SAN susceptible chickens; if possible, SPF chickens should be used.
- Birds are examined at ~~24 hour~~ intervals agreed according to local ethical guidance for 10 days. At each observation, each bird is scored 0 if normal, 1 if sick, 2 if severely sick, 3 if dead. (The judgement of sick and severely sick birds is a subjective clinical assessment. Normally, 'sick' birds would show one of the following signs and 'severely sick' more than one of the following signs: respiratory involvement, depression, diarrhoea, cyanosis of the exposed skin or wattles, oedema of the face and/or head, nervous signs. Dead individuals must be scored as 3 at each of the remaining daily observations after death[5]).
- The IVPI is the mean score per bird per observation over the 10-day period. An index of 3.00 means that all birds died within 24 hours, and an index of 0.00 means that no bird showed any clinical signs ~~during the 10-day observation period~~.

~~b) For all H5 and H7 viruses of low pathogenicity in chickens, the amino acid sequence of the connecting peptide of the haemagglutinin molecule (HA0) (i.e. the cleavage site) must be determined. The presence of several multiple basic amino acids, inserts of cellular or viral nucleic acids or loss of specific glycosylation sites in the HA0 cleavage site is the genotypic standard for HPAI strains; therefore, if the isolate being tested has an HA0 cleavage site motif identical to previous HPAI viruses, it should be designated as HPAI irrespective of a low or high pathogenicity determined by pathotyping in chickens (see the table that lists all the reported haemagglutinin proteolytic cleavage sites of HA0 protein for H5 and H7 LPAI and HPAI viruses based on deduced amino acid sequence, which can be found on the OFFLU site (see footnote 2 1). Furthermore, any isolate with a new motif must be tested *in vivo* by IVPI. In case of difficulties in the interpretation of the cleavage site motif, WOAH and/or FAO reference laboratories should be consulted.~~

The WOAH classification system to identify influenza A viruses for which disease notification and control measures should be taken is defined in the *Terrestrial Code* in Chapter 10.4 *Infection with high pathogenicity avian influenza viruses* and Chapter 1.1 *Notification of diseases and provision of epidemiological information*.

A variety of strategies and techniques, including commercial kits and automated sequencers, have successfully been used ~~successfully~~ to define the nucleotide sequence of the ~~nucleotides at that portion of the~~ HA ~~gene coding for the~~ cleavage site ~~region of the haemagglutinin of H5 and H7 subtypes of avian influenza virus,~~ enabling the amino acids there to be deduced. ~~This can be done by RNA extraction from the sample and direct sequencing of the haemagglutinin proteolytic cleavage site. Various stages in the procedure can be facilitated using commercially available kits and automated sequencers.~~

Furthermore, real-time RT-PCR techniques have become critical in rapidly defining viral pathotype (James *et al.*, 2022) often significantly reducing turnaround times in diagnostic laboratories. These assays can be used to pathotype viruses in the following scenarios: in case of an overt and widespread circulation of a definite genotype in wild birds in a specific area; in the event of documented secondary outbreaks linked

[4] To minimise pain and distress, when birds ~~are too sick to eat or drink~~ reach well defined ethically accepted humane endpoints, they should be ~~killed humanely~~ euthanised.

[5] To minimise pain and distress, when birds reach humane endpoints ~~are too sick to eat or drink~~, they should be euthanised ~~killed humanely~~ and scored as dead at the next observation.

to H5/H7 confirmed cases for which the cleavage site sequence has been determined; to quickly confirm a strong clinical suspicion.

Determination of the cleavage site by sequencing or real-time RT-PCR ~~other methods~~ has become the method of choice for initial assessment of the pathogenicity of these viruses and has been incorporated into agreed definitions. This has reduced the number of *in-vivo* tests, although the initial ~~Sanger~~ sequencing result of a HA cleavage site for an H5 or H7 LPAI virus should be confirmed by either inoculation of birds or deep sequencing using high throughput sequencing technologies assuring a high depth of coverage ~~with a minimum of 1000 reads~~ to exclude the presence of any HPAI virus in the background of an LPAI virus infection of the same H5 or H7 subtype.

Although all the truly HPAI viruses isolated to date have been of H5 or H7 subtypes, at least three isolates, all of H10 subtype (H10N1, H10N4 and H10N5), have been reported that would have fulfilled ~~both~~ the WOAH ~~and EU~~ *in-vivo* definitions for HPAI viruses ~~(Bonfante *et al.*, 2014; Wood *et al.*, 1996)~~ as they killed 6/10, 7/10 and 8/10 chickens with IVPI values >1.2 when the birds were inoculated intravenously. However, these viruses did not induce death or signs of disease when inoculated intranasally and did not have a haemagglutinin cleavage site sequence compatible with HPAI virus. Similarly, other intravenously inoculated influenza A viruses are nephrotropic and birds that die have high titres of virus in their kidneys indicating a renal pathogenic mechanism ~~(Slemons & Swayne, 1990)~~, but such laboratory-induced pathobiology is not comparable to multi-organ infection and systemic disease caused by HPAI viruses. An H4N2 virus isolated from quail had a multibasic cleavage site sequence (PEKRRTR/GLF) but with an IVPI value of 0.0 ~~(Wong *et al.*, 2014)~~ suggesting the multibasic cleavage site in viruses other than H5 and H7 alone may not be sufficient for declaration of HPAI virus and the *in-vivo* test should be carried out. Conversely, four viruses (A/chicken/Pennsylvania/1/83 [H5N2] and A/goose/Guangdong/2/96 [H5N1], A/turkey/England/87-92BFC/91 [H5N1] or A/chicken/Texas/298313/04 [H5N2]) have been described that have HA0 cleavage sites containing multiple basic amino acids, but which show low pathogenicity (IVPI <1.2) when inoculated intravenously into 6-week-old chickens ~~(Londt *et al.*, 2007)~~. No single explanation including the presence of a glycosylation site masking the HA0 cleavage site was reported emphasising both intra-haemagglutinin and multigenic influences in rare circumstances upon phenotypic expression of high pathogenicity. The presence of high pathogenicity haemagglutinin cleavage site in H5 and H7 influenza A viruses necessitates declaration of high pathogenicity to facilitate immediate control of the disease, otherwise a delay to complete *in-vivo* testing may result in continued onward transmission and spread between premises with severe consequence for future eradication once confirmed as a HPAI virus.

~~A table is available on the OFFLU website that lists all the reported haemagglutinin proteolytic cleavage site of HA0 protein for H5 and H7 LPAI and HPAI viruses based on deduced amino acid sequence. This table will be updated as new viruses are characterised; it can be found on the OFFLU site (see footnote 2 1).~~

1.5. Antigen capture and molecular techniques

At present, conventional virus isolation and characterisation techniques for the diagnosis of influenza A viruses remain ~~a~~ key method~~s~~ for initial diagnosis of influenza A infection in a primary disease event and to provide virus for more detailed analyses including *in-vivo* testing and gene sequencing. Furthermore, they may be invaluable in confirming or disproving the presence of infectious virus when other test results including conventional and real-time RT-PCR are inconclusive ~~all weakly positive~~. However, ~~conventional~~ classical methods tend to be costly, labour intensive and slow and the delays associated with these approaches are often not compatible with the implementation of control measures. There have been enormous developments and improvements in molecular and other diagnostic techniques, many of which are now routinely applied as a first choice for the diagnosis of influenza A infections (Figure 1).

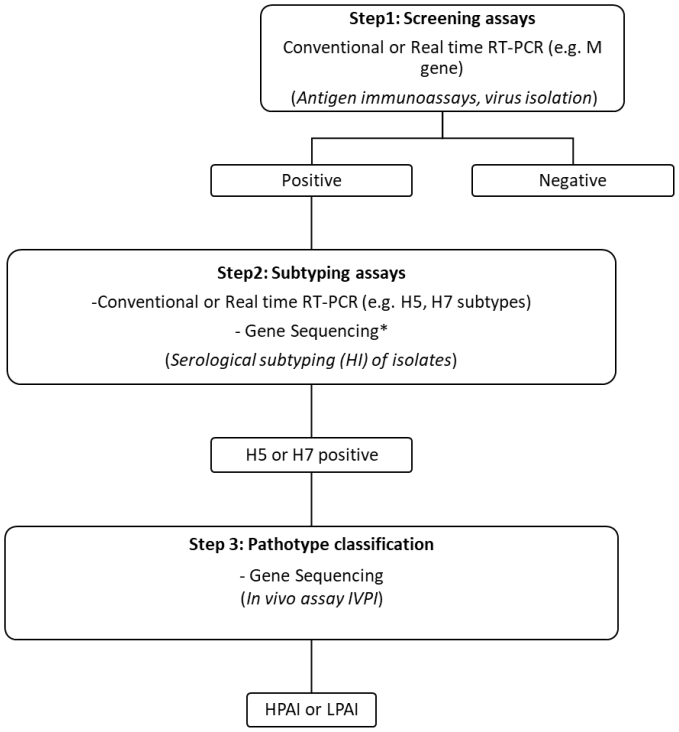

Fig. 1. Schematic overview of screening tests, subtyping assays, and characterisation methods for AIV. As rapid and sensitive diagnosis of AIV is of paramount importance in its early control and eradication, molecular-based tests are recommended as the first choice for the diagnosis of the pathogen. Tests in italics and brackets in the table may be used as alternative diagnostic methods, depending on the primary objective of the investigation and the virus being targeted.

1.5.1. Antigen detection

There are several commercially available AC-ELISA kits that can detect the presence of influenza A viruses in poultry ~~(Swayne *et al.*, 2020)~~. Most of the kits are enzyme immunoassays or are based on immunochromatography (lateral flow devices, LFD) and use a monoclonal antibody against the nucleoprotein; they should be able to detect any influenza A virus. The main advantages of LFDs are ~~these tests is~~ that they can demonstrate the presence of influenza A virus within 15 minutes, they are very easy to use and may be used "pen-side". The disadvantages are that they may lack sensitivity, they may not have been validated for different species of birds, H and N subtype identification is not achieved and the kits are expensive. The tests should only be interpreted on a flock basis and not as an individual bird test. Oropharyngeal or tracheal samples from clinically affected or dead birds provide the best sensitivity. Nevertheless, the lack of sensitivity is a major drawback to the use of available antigen detection tests. Test sensitivities may vary between cloacal and tracheal swabs, whilst the tests can perform less well with samples from waterfowl or wild birds compared with chickens. Improved but moderate sensitivity of LFDs ~~in so named lateral flow devices~~ was reported when using samples of feather follicles from birds infected with HPAI (Slomka *et al.,* 2012). Because of low sensitivity, antigen detection is mainly used for initial field screening of high mortality clinical cases for suspected influenza A virus infections followed by confirmation of results using a more sensitive laboratory-based test. Negative LFD test results do not necessarily rule out an influenza A infection and other laboratory-based testing pipelines should be used for confirmatory diagnosis where suspicion is raised.

1.5.2. Direct RNA detection

Table 2. Molecular assy as for the detection of type A avian influenza virus*

Primer/probe	Primer/probe sequence (5'–3')	Target
Heine *et al.* (2015) https://www.izsvenezie.com/reference-laboratories/avian-influenza-newcastle-disease/diagnostic-protocols/		
Forward	AGA-TGA-GYC-TTC-TAA-CCG-AGG-TCG	AI M gene
Reverse	TGC-AAA-AAC-ATC-YTC-AAG-TCT-CTG	
Reverse	TGC-AAA-CAC-ATC-YTC-AAG-TCT-CTG	
Reverse	TGC-AAA-GAC-ATC-YTC-AAG-TCT-CTG	
Reverse	TGC-AAA-TAC-ATC-YTC-AAG-TCT-CTG	
Probe	FAM-TCA-GGC-CCC-CTC-AAA-GCC-GA-TAMRA/BHQ1	
Nagy *et al.* (2021) https://science.vla.gov.uk/flu-lab-net/protocols.html	Nagy et al., 2021. https://www.izsvenezie.com/reference-laboratories/avian-influenza-newcastle-disease/diagnostic-protocols/	
Forward	GGC-CCC-CTC-AAA-GCC-GA	AI M gene
Reverse	CGT-CTA-CGY-TGC-AGT-CC	
Probe	FAM-TCA-CTK-GGC-ACG-GTG-AGC-GT-MGB	
Hassan *et al.* (2022)		
Forward	AGA-TGA-GYC-TTC-TAA-CCG-AGG-TCG	AI M gene
Reverse	TGC-AAA-AAC-ATC-TTC-AAG-TYT-CTG	
Reverse	TGC-AAA-GAC-ACT-TTC-CAG-TCT-CTG	
Reverse	TGC-AAA-I(Inosine)AC-ATC-YTC-AAG-TYT-CTG	
Probe	FAM-TCA-GGC-CCC-CTC-AAA-GCC-GA-BHQ1	

*Not all the molecular methods described in Section B.1.5.2 *Direct RNA detection* are included in this table and only some of the methods used for influenza A screening are reported as examples

As demonstrated by the current definitions of HPAI, molecular techniques are used preferentially for diagnosis for some time now including~~. Furthermore, there have recently been developments towards their application to the~~ detection and characterisation of influenza A viruses directly from clinical specimens of infected birds. It is imperative that when using highly sensitive molecular detection methods that allow rapid direct detection of viral RNA for confirmatory laboratory diagnosis of influenza A infections, stringent protocols are in place to prevent the risk of cross-contamination between clinical samples. In addition, RNA detection test methodologies should be validated to the WOAH standard (see Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals*) using clinical material and virus isolates to demonstrate the tests as being 'fit for purpose' for application in a field diagnostic setting, which ~~may~~ should include the use of internal test standards. The control reactions enable greater confidence in the integrity of the molecular reactions, clinical samples and results.

Furthermore, these evaluations enable the appropriate setting of test thresholds for interpretation between positive and negative samples. The increased sensitivity of real-time RT-PCR may leads to the detection of viral RNA in samples in the absence of actively infectious viruses ~~and care should be taken when interpreting outputs with small detection limits that may not be indicative of active infection~~. This problem can be overcome through ~~the~~ serial testing of multiple samples from the same cohort of ~~infected~~ birds, and is especially relevant when testing samples from domestic poultry for disease investigation.

In settings with more limited facilities, conventional RT-PCR techniques on clinical samples can, with the correctly defined primers, result in rapid detection and subtype identification (at least of H5 and H7 ~~and H9~~ subtypes~~, and more recently developed assays are also available for other subtypes~~), including a cDNA product that can be used for nucleotide sequencing (Slomka *et al.*, 2007a). However, these approaches have now been largely replaced by the preferred molecular detection tests for influenza A virus by real-time RT-PCR, a modification to the RT-PCR that reduces the time and cross contamination risks, and increases sensitivity and specificity for both virus identification ~~of virus subtype and sequencing~~ and subtyping. These are ideal qualities for influenza A outbreak management, where the period of time in which an unequivocal diagnosis can be obtained is crucial for decision making by the relevant Veterinary Authority. In addition, RT-PCR systems can be designed to operate in a 96-well format and combined with high-throughput robotic RNA extraction from specimens.

~~For example, Spackman et al. (2002) used a single step real-time RT-PCR primer/fluorogenic hydrolysis probe system to allow detection of influenza A viruses and determination of subtype H5 or H7. The test performed well relative to virus isolation and offered a cheaper and much more rapid alternative, with diagnosis on clinical samples in less than 3 hours. In additional studies, the real-time RT-PCR was shown to have sensitivity and specificity equivalent to virus isolation in numerous settings but updates to primer/probe design can be beneficial over time to accommodate genetic evolution in gene regions targeted by assays (Laconi et al., 2020). These~~ Properly designed molecular tests can provide high sensitivity and specificity similar to those of virus isolation when used on tracheal and oropharyngeal swabs of chickens and turkeys, but may lack sensitivity for detection of influenza A virus in faecal swabs, faeces and tissues in some bird species, because of the presence of PCR inhibitors resulting in false-negative results ~~(Das *et al.*, 2006)~~. Incorporation of a positive internal control during RNA extraction into the test will verify a proper test run. In addition, improvements in RNA extraction and RNA treatment kits have been made available in the market ~~developed~~ to eliminate most PCR inhibitors from test samples.

~~Real-time RT-PCR, usually based around the hydrolysis probe method for generation of the target-specific fluorescence signal, has become the method of choice in many laboratories for at least partial diagnosis directly from clinical specimens. The method offers rapid results, with sensitivity and specificity comparable to virus isolation. These are ideal qualities for influenza A outbreak management, where the period of time in which an unequivocal diagnosis can be obtained is crucial for decision making by the relevant Veterinary Authority. In addition, real time RT PCR systems can be designed to operate in a 96 well format and combined with high throughput robotic RNA extraction from specimens (Aguero *et al.,* 2007).~~

The approach to diagnosis using real-time RT-PCR adopted in most laboratories ~~has been~~is based on the initial ~~generic detection of~~ screening for influenza A virus in clinical samples, primarily by initially targeting the matrix (M) gene, which is highly conserved for all influenza A viruses (Table 2)~~, although further generic PCR targets have been identified in the NP and PB1 gene segments~~, ~~followed by~~ Specific real-time RT-PCR testing for H5 and H7 subtype viruses are then used to follow up on M gene positive results. Numerous assays have been reported for a highly sensitive detection of M ~~(or NP)~~ gene fulfilling the criteria for a suitable screening test. For example, Spackman *et al.* (2002) used a single-step real-time RT-PCR primer/fluorogenic hydrolysis probe system to allow detection of influenza A viruses. The test performed well relative to virus isolation and offered an inexpensive and much more rapid alternative, with diagnosis on clinical samples in less than 3 hours. *In-silico* monitoring of influenza A screening tools should be conducted and updates applied as available; this is the case of the Spackman *et al.* (2002) protocol, which has been updated via the introduction of degenerated bases in the primer sequences targeting the M gene (Hassan *et al.*, 2022; Laconi *et al.*, 2020; Spackman *et al.*, 2020). These updates also allow the inclusion of an internal process control to prevent false negative results arising from PCR inhibition or failure in nucleic acids extraction. With improvements in assay design and using novel biochemical approaches, screening assays relevant to all influenza A viruses from all hosts (animal and human) have been developed (Nagy *et al.*, 2021) with high relevance to an avian–'other' host interface.

In cases where laboratories do not have access to a real-time RT-PCR platform for screening samples for the M gene, an alternative solution could be to use the primer set described in the real-time RT-PCR protocols from Spackman *et al.* (2022) or Heine *et al.* (2015) or Hassan *et al.* (2022). However, it should be noted that validation data for this type of use is not currently available and, in addition, due to the small

size of the amplicon (less than 100 bp), the RT-PCR results should be visualised on silver-stained SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) gels or 2–3% agarose gel.

Subtyping by PCR represents a greater challenge due to the diversity within subtypes. Maintaining sensitive and specific tools for subtyping requires costant *in-silico* monitoring and routine updates to primer/probe design to accommodate genetic evolution in gene regions targeted by molecular assays. For subtype identification, oligonucleotides ~~primers~~ used in real-time RT-PCR are often targeted at the HA2 region, as this is relatively well conserved within the haemagglutinin genes of the H5 and H7 subtypes (Spackman *et al*., 2008; Spackman & Suarez, 2008). ~~It has therefore served as the target region for these subtypes.~~ Spackman *et al.* (2002) demonstrated specific detection of these subtypes, but cautioned that ~~their~~ these H5 and H7 primer/probe sequences ~~had been~~ were designed for the detection of North American H5 and H7 ~~isolates~~ viruses and might not be suitable for all H5 and H7 ~~isolates~~ viruses. This proved to be the case. Slomka *et al.* (2007b) described modification of the H5 oligonucleotide sequences used by Spackman *et al.* (2002) to enable the detection of the Eurasian 'Goose/Guangdong lineage' (Gs/GD) H5N1 subtype and other Eurasian H5 subtypes ~~that have been isolated within the past 15 years~~ in both poultry and wild birds. As the group of 'Gs/GD' viruses diversified and spread across several continents, it has become important that diagnostics in all settings have proven fit for purpose detection of this H5 lineage of viruses divided into multiple clades (World Health Organization/World Organisation for Animal Health/Food and Agriculture Organization [WHO/WOAH/FAO] & H5N1 Evolution Working Group, 2014). Eastern and western hemisphere H7 AIVs are known to be phylogenetically distinguishable from each other; hence, real-time RT-PCRs for the detection of Eurasian H7 AIVs in biological samples have been developed and tested on a variety of H7 viruses collected from geographically and temporally distinct H7 outbreaks (Hassan *et al.*, 2022; Liu *et al.*, 2018; Slomka *et al.*, 2009).

When the presence of H5/H7 subtypes is ruled out in clinical samples testing positive for influenza A, the diagnostic investigations should continue until subtype identification. Because of the economic implications to the poultry sector and the zoonotic risk in endemic areas, detection of H9 is generally prioritised over other subtypes, and new diagnostic tools have recently been made available to improve detection of any H9 lineage (Panzarin *et al.*, 2022). Due to challenges with maintaining sensitive and specific subtyping PCR tools, protocols that leverage influenza A detection followed by subtype attempts are best placed for highest success. Newer rapid methods for HA and NA subtyping have been developed ~~that~~ to enable the simultaneous detection and subtyping speeding the time to achieve rapid identification of an influenza A virus using arrays (Hassan *et al.*, 2022; ~~Hoffmann *et al.*, 2016;~~ James *et al.*, 2018) ~~or microchip (Kwon *et al.*, 2019) technologies~~. ~~The validated Eurasian real-time RT-PCR have proven valuable in the investigation of many H5Nx HPAI clinical samples and other subtypes submitted to International Reference Laboratories from Europe, Africa, Asia and North America since 2005 (Liu *et al.*, 2018; Slomka *et al.*, 2007).~~ Each set of primers and probes needs to be validated against a diverse set of viruses to make the test applicable in a diverse range of avian species, and in viruses from broad geographic areas and time periods. ~~In addition, real-time RT-PCR methods are now widely used for the rapid and accurate determination of the neuraminidase subtype (James *et al.*, 2018).~~

One of the problems with rapidly emerging new tests is that methods and protocols may be developed and reported without the test being properly validated. ~~This has been addressed for some of the real-time RT-PCR protocols. In the European Union, National Reference Laboratories have collaborated to define and validate protocols that can be recommended for use within Europe (Hoffmann *et al.*, 2016; Nagy *et al.*, 2020; Slomka *et al.* 2007).~~ Importantly this should include routine analysis of detected viruses (coordinated through WOAH Reference Laboratories) in standard assays to ensure reliable specific detection of contemporary viruses affecting poultry and other populations. In addition, given the high variability in the influenza A genome it is imperative that assays used in routine diagnosis and surveillance have ongoing demonstration of their fitness for detection of contemporary viruses validated for use in the region where they are applied. There should be an appropriate match for local strains taking account of significant regional and intercontinental variability amongst particular endemic viruses. ~~Laconi *et al.* (2020) in reviewing five validated well used real-time RT-PCR methods concluded that continuous monitoring of assay performance using both in silico and *in vitro* methodology was important as the emergence of new strains containing mutations within primer and probe binding areas might significantly affect the positive outcome of a test. Increasingly with improvements in assay design and using novel biochemical approaches screening assays relevant to all influenza A viruses from all hosts (animal and human) have been developed (Nagy *et al.*, 2020) with high relevance to an avian 'other' host interface.~~

As stated before (see Section B.1.4 *Assessment of pathogenicity*), the molecular pathotyping of H5/H7 is largely based on the determination of the cleavage site sequence of the HA0 precursor of these subtypes. This can be achieved by RT-PCR followed by Sanger sequencing of the amplification product. Real-time RT-PCR protocols that amplify regions across the cleavage site of the HA0 gene have also been ~~described~~ developed ~~that amplify regions across the cleavage site of the HA0 gene~~ to determine the viral pathotype. ~~This may result in useful tests for specific viruses. For example, Hoffman *et al.* (2007) have described a~~

~~real-time RT-PCR test specific to the Eurasian HPAI H5N1 Qinghai-like clade 2.2 viruses that represents a rapid means of determining the pathotype for this subgroup of H5N1 HPAI viruses without sequencing.~~ In situations where large numbers of positive samples/cases are detected during ~~disease events~~ the same outbreak, these specific targeted real-time RT-PCR assays ~~have been developed for~~ allow the simultaneous sensitive detection and pathotyping of viruses. This can prove to be very useful, particularly when applying to early warning systems such as surveillance of wild bird populations for local presence of HPAI (Graaf *et al.,* 2017; Hassan *et al.,* 2022; James *et al.,* 2022; Naguib *et al.,* 2017).

In low-resource setting laboratories, the detection of AIV can be hampered by the need to maintain a cold chain for wet reagents along with the adequate provision of reagents. In such circumstances, the use of lyophilised molecular reagents for increased thermal stability should be considered to maximise surveillance capacities. The interchangeability of the wet and lyophlised reagents may be different between distinct molecular protocols and thus validation is required before their routine use.

Modifications to the straightforward RT-PCR method of detection of viral RNA have been designed to reduce the effect of inhibitory substances in the sample taken, the possibility of contaminating nucleic acids and the time taken to produce a result. The loop-mediated isothermal amplification (LAMP) system for H5 and H7 detection appears to show high sensitivity and reliable specificity ~~(Ahn *et al.,* 2019; Bao *et al.,* 2014)~~, but may have limited application because of susceptibility to viral mutations affecting the target regions, reducing virus detection ~~(Postel *et al.,* 2010)~~.

~~Increasing innovation and technological improvements have made it possible that molecular based and improved antigen detection technologies have developed sufficiently to permit rapid flock side tests for the detection of presence of influenza A virus specific subtypes and pathogenicity markers (Inui *et al.,* 2019).~~ Furthermore, innovations in test design have enabled ~~for example~~ the development of point of care ~~chip based ultrafast PCR approaches~~ tests such as chip-based PCR and recombinase polymerase amplification (RPA) ~~(Kwon *et al.,* 2018)~~ with increasing application anticipated in the future.

1.5.3. Gene sequencing

Currently real-time RT-PCR is the preferred method of virus surveillance because the test provides rapid sensitive diagnostics for influenza A, as well as subtypes H5, H7 and H9 and is available in high throughputs. However, greater use of sequencing technologies, particularly as unit costs reduce with improvement in technology, offers powerful opportunities to simultaneously detect and sequence viruses directly from clinical samples in a laboratory or field setting, for example applying nanopore technology (Ip *et al.,* 2023 ~~King *et al.,* 2020~~).

Increasingly gene sequencing is being applied not only to detailed characterisation of viruses for use in molecular epidemiology but also in virus subtyping, ~~and~~ defining host range markers, ~~for host range~~ including the ones with zoonotic ~~risk~~ implications, and detecting the emergence of new genotpes through reassortment. Sanger sequencing methodology has been widely used for decades and enables the rapid determination of typically a single (H) target gene in 24~~-36~~ hours to define virus pathogenicity (see Section B.1.4.b ~~B.1.1.1~~) and still has widespread utility. However, as genomic data can be rapidly determined using next generation sequencing technology, ~~it enables a broader~~ a more resolved analysis using a range of bioinformatics tools is possible ~~(Zhang *et al.,* 2017)~~. For example, with the advent of greater access to sequencing methodology either through specialised laboratories or commercial providers it is now possible to determine the genomic sequences of influenza A viruses from birds to provide a level of characterisation important in rapid pathogen identification and outbreak intervention. Conventionally, nucleotide sequences have been used in outbreak epidemiology to infer virus origin and ~~precise~~ glean relationships between different viruses associated within the same event (by phylogeny) to support outbreak management. ~~Virus gene~~ Either whole genome or partial hemagglutinin and neuramindase sequences ~~of haemagglutinin and neuraminidase~~ can rapidly be compared to known sequences of all subtypes in gene databases and used to reveal closest match thereby identifying the virus subtype and phylogenetic relationships. This often avoids the need to culture the virus for rapid identification although reliability and quality of data reduces with increasing cycle threshold values in samples from real-time RT-PCR testing.

1.5.4. Molecular epidemiology and phylogenetics

Phylogenetic techniques ~~Increasingly such analyses~~ are now being applied at the whole genome level to reveal virus genotypes and provide greater ~~analytical specificity~~ resolution to ~~the analyses~~ outbreak investigations. Such approaches are especially valuable to track ~~since~~ virus evolution which can be more precisely mapped and also determine new genotypes that might arise as a result of ~~including change through~~ genetic reassortment, a key mechanism associated with virus diversity and fitness for birds. This approach is especially valuable for early or first incursions in a new event as it enables greater precision in determining virus origin and the mechanisms leading to the emergence of a virus. However, it also

represents a tool to track movements of the virus between infected premises (IPs) and follow its evolutionary trajectories. ~~This~~ Whole genome sequencing has become increasingly important in characterising the rapid evolution and wide diversity of Gs/GD lineage viruses associated with transcontinental spread. The WHO/WOAH/FAO H5N1 Evolution Working Group (2014) developed criteria to distinguish genetic groups specific to the H5 hemagglutinin (HA) gene of the Gs/GD lineage, including a dynamic nomenclature system to define subclades within that lineage. The continuing circulation of HPAI H5N1 viruses in poultry or wild birds has resulted in the ongoing evolution of the H5 HA with the emergence of multiple HA subclades. Since 2005, there have been five intercontinental movements of multiple clades of the H5Nx Gs/GD HPAI lineage viruses: in 2005 clade 2.2; in 2008–2010 clade 2.3.2.1; in 2014–2015 clades 2.3.4.4c and 2.3.2.1; in 2016–2017 clade 2.3.4.4b; and in 2020–2023 clade 2.3.4.4b.

Translation of nucleotide sequences of all genomic segments into amino acid sequences enables data mining for other virus characteristics or traits such as tropism, host range markers including zoonotic and predicted antiviral drug susceptibility which are invaluable for informing outbreak management.

Full access to comprehensive genetic sequences and related metadata from AI outbreaks is crucial not only to identify the determinants of its transmissibility and pathogenicity but also to reconstruct the dissemination dynamics of AIV and to follow the evolution of the virus. Multiple public databases for influenza virus sequence data exist and allow rapid and complete information sharing (e.g. GISAID, GenBank) with the international scientific community. As there is an urgent need to make genomic data publicly available during ongoing epidemics, in case of difficulties in generating and analysing whole genome sequence data, WOAH or FAO Reference Laboratories should be consulted.

2. Serological tests

2.1. Enzyme-linked immunosorbent assay (ELISA)

Commercial ELISA kits that detect antibodies against the influenza A nucleoprotein are available. Kits with an indirect and competitive/blocking format have been developed and validated and are now being used to detect influenza A virus-specific antibodies in different animal species. Several avian influenza competitive ELISA (AIV C-ELISA) or blocking ELISA (AIV B-ELISA) have been developed and validated as a more sensitive alternative to the AGID test (see Section B.2.2 *Agar gel immunodiffusion*) for the detection of influenza A group reactive antibodies in sera from chickens and other bird species ~~(SCAHLS, 2009)~~. This AIV ELISA platform, ~~as~~ in either a "competitive" or "blocking" format, detects antibodies to influenza A viruses by allowing these antibodies to compete for antigen binding sites with a monoclonal antibody against an epitope on the nucleoprotein that is conserved in all influenza A viruses.

The kits should be validated for the specific species of interest and for the specific purpose(s) for which they are to be used. Several different test and antigen preparation methods are used. Such tests have usually been evaluated and validated by the manufacturer, and it is therefore important to carefully follow ~~that~~ the instructions specified for their intended use ~~be followed carefully~~. Please see the WOAH Register for kits certified by the WOAH[6]. ELISA kits are of moderate cost and are amenable to high throughput screening for influenza A virus infections and have strong utility for application to large-scale serosurveillance programmes and compare favourably to HI ~~(Arnold *et al.*, 2018)~~. However, all positive results must ~~be followed by~~ undergo HI test for subtyping to H5 and H7. Some subtype-specific ELISA kits are available, e.g. for antibodies to H5, H7, H9 and some N subtypes (i.e. N1) but generally are of lower sensitivity than influenza A ELISA. Recently, employing bead-based immunoassay technology and recombinantly expressed influenza virus proteins, multiplex serological assays have been developed that offer options for improved diagnostic bandwidth (combining several HA and NA subtypes or further targets of notifiable poultry diseases) and an economising testing schedule. Further validation is required to prove them fit for purpose in routine laboratory settings.

To test serum samples obtained from mammals for exposure to avian influenza viruses, a preliminary screening can be performed with a commercially available multispecies competitive ELISA kit targeting antibodies specific for nucleoprotein (NP) of type A influenza viruses. Prior to routine testing, it is recommended to carefully evaluate the validity of ELISAs in the species under examination due to the unknown diagnostic performance of these commercial assays with mammalian sera other than the ones declared by the kit manufacturer. Additional confirmatory serological tests (e.g. HI) are required.

[6] https://www.woah.org/en/what-we-offer/veterinary-products/#ui-id-5/

2.2. Agar gel immunodiffusion

Whilst the AGID is relatively inexpensive and suitable for resource limited settings, it is advisable to use more sensitive platforms such as ELISA for flock level serological investigations including pre-export/import screening of birds for historical exposure to influenza A.

All influenza A viruses have ~~antigenically similar~~ a degree of conservation across protein structures for the nucleoprotein and ~~antigenically similar~~ matrix ~~antigens~~ proteins. As such, ~~Owing to this fact~~ AGID tests are able to detect the presence or absence of antibodies to any influenza A virus~~.~~ based on reactivity to these conserved proteins as concentrated virus preparations~~, as described above,~~ will contain an abundance of both matrix and nucleoprotein ~~antigens; the matrix antigen diffuses more rapidly than the nucleoprotein antigen~~ proteins. AGID tests have been widely and routinely used to detect specific antibodies in chicken and turkey flocks as an indication of infection, but AGID tests are less reliable at detecting antibodies following infection with influenza A viruses in other avian species. These have generally employed nucleoprotein-enriched preparations made from the chorioallantoic membranes of embryonated chicken eggs ~~(Beard, 1970)~~ that have been infected at 10 days of age, homogenised, freeze–thawed three times, and centrifuged at 1000 ***g***. The supernatant fluids are inactivated by the addition of 0.1% formalin or 1% betapropiolactone, recentrifuged and used as antigen. Not all avian species may produce precipitating antibodies following infection with influenza viruses, for example ducks. The AGID is a low-cost serological screening test of reduced sensitivity for detection of generic influenza A infections, but must be followed by HI tests for subtyping influenza A positive~~s as to H5 and H7~~ sera.

Tests are usually carried out using gels of 1% (w/v) agarose or purified type II agar and 8% (w/v) NaCl in 0.01 M phosphate buffer, pH 7.2, poured to a thickness of 2–3 mm in Petri dishes or on microscope slides, and incubated in a humidified chamber. Using a template and cutter, wells of approximately 5 mm in diameter are cut into the agar at a distance of about 3 mm from each other. A pattern of wells must place each suspect serum adjacent to a known positive serum and antigen. Each well should have reagent added to fill the well, corresponding to the top of the meniscus with the top of the gel, but do not over fill. Approximately 25–30 µl of each reagent should be required per well, but this depends on thickness of the gel, with thicker gels requiring an additional volume of reagent.

Wells should be examined for precipitin lines at 24 hours, and weak positive samples or samples for which specific lines have not formed should be incubated longer and examined again at 48 hours. The time to formation of visible precipitin line is dependent on the concentrations of the antibody and the antigen. The precipitin lines are best observed against a dark background that is illuminated from behind. A specific, positive result is recorded when the precipitin line between the known positive control wells is continuous with the line between the antigen and the test well. Crossed lines are interpreted to be caused by the test serum lacking identity with the antibodies in the positive control well.

~~Whilst the AGID is relatively inexpensive and suitable for resource limited settings it is being increasingly replaced by other platforms such as ELISA for flock level serological investigations including pre-export/import screening of birds for historical exposure to influenza A.~~

2.3. Haemagglutination and haemagglutination inhibition tests

Variations in the procedures for HA and HI tests are practised in different laboratories. The following recommended examples apply to the use of V-bottomed microwell plastic plates in which the final volume for both types of test is 0.075 ml. U-bottomed plates can be used but care in reading is required as the clarity is less defined. The reagents required for these tests are isotonic PBS (0.01 M), pH 7.0–7.4, and RBCs taken from a minimum of three SPF or SAN chickens and pooled into an equal volume of Alsever's solution. Cells should be washed three times in PBS before use as a 1% (packed cell v/v) suspension. Positive and negative control antigens and antisera should be run with each test, as appropriate.

2.3.1. Haemagglutination test

i) Dispense 0.025 ml of PBS into each well of a plastic V-bottomed microtitre plate.

ii) Place 0.025 ml of virus suspension (i.e. infective allantoic fluid) in the first well. For accurate determination of the HA content, this should be done from a close range of an initial series of dilutions, i.e. 1/3, 1/4, 1/5, 1/6, etc.

iii) Make twofold dilutions of 0.025 ml volumes of the virus suspension across the plate.

iv) Dispense a further 0.025 ml of PBS to each well.

v) Dispense 0.025 ml of 1% (v/v) chicken RBCs to each well.

vi) Mix by tapping the plate gently and then allow the RBCs to settle for about 40 minutes at room temperature, i.e. about 20°C, or for 60 minutes at 4°C, if ambient temperatures are high, by which time control RBCs should have formed a distinct button.

vii) HA is determined by tilting the plate and observing the presence or absence of tear-shaped streaming of the RBCs. The titration should be read to the highest dilution giving complete HA (no streaming); this represents 1 HA unit (HAU) and can be calculated accurately from the initial range of dilutions.

2.3.2. Haemagglutination inhibition test

i) Dispense 0.025 ml of PBS into each well of a plastic V-bottomed microtitre plate.

ii) Place 0.025 ml of serum into the first well of the plate.

iii) Make twofold dilutions of 0.025 ml volumes of the serum across the plate.

iv) Add 4 HAU of virus/antigen in 0.025 ml to each well and leave for a minimum of 30 minutes at room temperature (i.e. about 20°C) or 60 minutes at 4°C.

v) Add 0.025 ml of 1% (v/v) chicken RBCs to each well and mix gently, allow the RBCs to settle for about 40 minutes at room temperature, i.e. about 20°C, or for 60 minutes at 4°C if ambient temperatures are high, by which time control RBCs should have formed a distinct button.

vi) The HI titre is the highest dilution of serum causing complete inhibition of 4 HAU of antigen. The agglutination is assessed by tilting the plates. Only those wells in which the RBCs stream at the same rate as the control wells (containing 0.025 ml RBCs and 0.05 ml PBS only) should be considered to show inhibition.

vii) The validity of results should be assessed against a negative control serum, which should not give a titre >1/4 (>2^2 or >$\log_2$ 2 when expressed as the reciprocal), and a positive control serum for which the titre should be within one dilution of the known titre.

The HI test is primarily used to determine if antibodies indicating influenza A virus infections are subtyped as H5 and H7 or other H subtypes (H1-4, H6, H8-16). HI titres may be regarded as being positive if there is inhibition at a serum dilution of 1/16 (2^4 or $\log_2$ 4 when expressed as the reciprocal) or more against 4 HAU of antigen. Some laboratories prefer to use 8 HAU in HI tests. While this is permissible, it affects the interpretation of results so that a positive titre is 1/8 (2^3 or $\log_2$ 3) or more. The meaning of a minimum positive titre should not be misinterpreted; it does not imply, for example, that immunised birds with that titre will be protected against challenge or that birds with lower titres will be susceptible to challenge. Appropriate virus/antigen control, positive control serum and RBC control well should be included with each batch of HI tests.

Chicken sera rarely give nonspecific positive agglutination reactions in this test and any pretreatment of the sera is unnecessary. Sera from species other than chickens may sometimes cause agglutination of chicken RBCs resulting in nonspecific agglutination. Therefore, each serum should first be tested for this idiosyncrasy and, if present, it should be inhibited by adsorption of the serum with chicken RBCs. This is done by adding 0.025 ml of packed chicken RBCs to each 0.5 ml of antisera, mixing gently and leaving for at least 30 minutes; the RBCs are then pelleted by centrifugation at 800 ***g*** for 2–5 minutes and the adsorbed sera are decanted. Alternatively, RBCs of the avian species under investigation could be used. Nonspecific inhibition of agglutination can be caused by steric inhibition when the tested serum contains antibodies against the same N subtype as the H antigen used in the HI test. The steric inhibition reaction can result in RBC buttoning in the bottom of the plate or streaming at the same rate as the control. If using whole virus antigen in HI test for subtyping, it is important to ensure that two antigens for each haemagglutinin subtype are used with heterologous neuraminidase i.e. H5N1 and H5N6 to eliminate the possibility of interference in the assay with anti-N antibodies that can lead to false typing results. Alternatively, the H antigen used can be recombinant or purified H protein that lacks N protein. The HI test is based on antigenic binding between the H antigen and antisera and thus other factors may cause nonspecific binding of the H antigen and sera leading to a nonspecific inhibition reaction. At this time there are no documented cross reactions or nonspecific inhibition reactions between the different haemagglutinin subtypes of influenza A.

Where influenza A viruses of avian origin emerge in non-avian species, the detection of antibodies against avian influenza in mammals may be required. For this a pretreatment of the sera is necessary. Three volumes of appropriately diluted receptor-destroying enzyme (RDE) are added to one volume of serum. The mixture is incubated overnight at 37°C, and subsequently inactivated at 56°C for 30 minutes and brought to a final dilution of 1/10 by adding six volumes of PBS. To remove nonspecific haemagglutinating factors, if present, one volume of packed chicken erythrocytes can be added to 20 volumes of serum and incubated at 4°C under gentle shaking for 1 hour before removing erythrocytes by centrifugation at 300 ***g*** for 5 minutes. HI tests are performed using four haemagglutinating units of virus with 0.5% chicken erythrocytes according to standard procedures described above.

2.4. Neuraminidase antibody detection ~~inhibition~~ tests

The neuraminidase-inhibition test has been used to identify the influenza A neuraminidase type of isolates as well as to characterise the presence of anti-N antibodies ~~antibody~~ in infected birds. The procedure requires specialised expertise and reagents; consequently, this testing is usually done in a WOAH Reference Laboratory. ~~The~~ A DIVA (differentiating infected from vaccinated animals) strategy ~~used previously in Italy also relies on a serological test to detect specific anti-N antibodies; the test procedure~~ has also been described (Capua *et al*., 2003). Recent developments favoured an enzyme-linked lectin assay (ELLA) to measure NA-specific antibodies which allows for improved test harmonisation.

C. REQUIREMENTS FOR VACCINES

1. Background

The past two decades have been marked by a significant change in the epidemiological and ecological dynamics of HPAI viruses. An increasing number of HPAI lineages have emerged in poultry and one of them, the H5Nx Gs/GD Eurasian lineage with its descendant clades, has demonstrated the ability of HPAI viruses to expand their host range well beyond domestic birds. As detailed in Section A. *Introduction*, the Gs/GD clade 2.3.4.4b virus extensively circulates in wild bird populations and since 2022, it has reached a global spread. As such, and due to the impact on both wild bird and poultry populations, vaccination has been considered in areas that have previously relied on conventional control measures of biosecurity, early detection, stamping-out and movement restrictions. Vaccination using appropriately matched vaccines is an important complementary tool to be taken into account for the control of HPAI. When properly implemented, vaccination can reduce virus circulation, limit economic losses, improve animal welfare and minimise environmental impact, thus reducing the risk of spillover to wild animals and humans (Harder *et al.*, 2023).

However, for multiple reasons, vaccination alone is not a universal panacea for the ~~solution to the~~ control of HPAI if eradication is the desired result. Without the application of monitoring systems, strict biosecurity and depopulation in the face of infection, HPAI ~~will~~ might become endemic in vaccinated poultry populations. Furthermore, ~~Long term~~ circulation of ~~the~~ wildtype virus in ~~a~~ vaccinated populations may result in both antigenic and genetic changes as has occurred with H5Nx (Gs/GD lineage), H7N3, H7N9 and H9N2 influenza A viruses in Mexico, and various Middle Eastern and Asian countries ~~(Swayne & Sims, 2020)~~. The currently used vaccines and the use of vaccination have been reviewed and both challenges and opportunities of application considered (EFSA Panel on Animal Health and Animal Welfare [AHAW] *et al.*, 2023~~; FAO, 2016; Swayne & Sims, 2020~~).

The haemagglutinin is the primary influenza A viral protein that elicits a protective humoral immune response used in officially approved poultry vaccines and such immunity is haemagglutinin subtype specific. ~~To date~~ Historically, the majority of influenza A vaccines used in poultry have been inactivated whole virus vaccines prepared from infective allantoic fluid of embryonated chicken eggs, inactivated by beta-propiolactone or formalin and emulsified with mineral oil adjuvants. Because of the potential for reassortment leading to increased virulence, live-attenuated conventional influenza vaccines against any subtype are not recommended. However, biotechnology holds great potential to generate live avian influenza virus vaccines with altered gene segments which reduce the risk of reassortment, limit replication and abrogate negative aspects of live influenza A virus vaccines ~~(Song *et al.*, 2007)~~. The existence of a large number of haemagglutinin subtypes (i.e. H1–16), together with the known ~~variation~~ antigenic diversity seen across of different strains within a single subtype pose serious problems when selecting strains to produce inactivated influenza A vaccines. In addition, some isolates do not grow to a sufficiently high titre to produce adequately potent vaccines without costly pre-concentration. While some vaccination strategies use autogenous vaccines, i.e. vaccines prepared from isolates specifically involved in an epizootic, others rely on vaccines prepared from biologically characterised, fully approved seed strain viruses possessing the same haemagglutinin subtype as the field virus and capable of yielding high concentrations of antigen. Historically, inactivated vaccines for LPAI and HPAI control have used LPAI viruses with a matching haemagglutinin subtype from outbreaks as seed strains, but with development of resistance in the field associated with prolonged vaccine use, the majority of inactivated vaccine seed strains are now reverse genetic derived virus with antigenically close matching haemagglutinin, and sometimes neuraminidase, to circulating field viruses. Use of native HPAI viruses as inactivated vaccine seed strains is strongly discouraged because of biosafety concerns during production and when in use.

Since the 1970s in the USA, inactivated influenza A vaccines have been used primarily in turkeys against LPAI viruses under emergency vaccination programmes, but since the 2000s, most vaccines have been against H1 and H3 swine influenza A viruses used under a routine preventative vaccination programme in breeder turkeys ~~(Swayne *et al.*, 2020)~~. Since the early 1990s, vaccination of chickens against H9N2 LPAI virus has been used extensively in Asia and the Middle East using billions of inactivated vaccine doses ~~(Swayne & Sims, 2020)~~. Vaccination was used to control multiple incursions of avian influenza viruses of the H5 and H7 subtypes between 2000 and 2006 in Italy (EFSA Panel on AHAW *et al.*, 2023). Vaccination against HPAI was first used in Mexico during the H5N2 outbreaks of 1994–1995 ~~(Villarreal, 2007),~~ and in Pakistan ~~(Naeem, 1998)~~ during the H7N3 outbreaks of 1995. Beginning with H5N1 ~~goose/Guangdong~~ (Gs/GD~~)~~ lineage HPAI outbreaks in Hong Kong

in 2002 (Sims, 2003), a vaccination policy was adopted using H5N2 LPAI vaccine seed strains and subsequently replaced with H5Nx reverse genetic vaccine seed strains in China (People's Rep. of), as the field virus spread throughout and outside of China Between 2002 and 2010, 113 billion doses of vaccine was used to control HPAI with 95% being inactivated and 5% recombinant vaccines, and a similar usage rate continues (Swayne *et al.*, 2011; Swayne & Sims, 2020). As the H5Nx Gs/GD lineage HPAI spread across the globe, additional countries have implemented emergency and/or preventative vaccination programmes for HPAI control. Similarly, preventive and emergency vaccination against H5N1 HPAI has been permitted implemented for outdoor poultry and zoo birds in several European Union countries in the 2000s.

Live recombinant virus-vectored vaccines with H5 influenza A virus haemagglutinin gene inserts have been approved and used in a few countries increasingly since 1997, mostly in chickens, and include recombinant fowl poxvirus (rFPV), recombinant Newcastle disease virus (rNDV) and recombinant herpesvirus turkey vaccines (rHVT). Since 2015, non-replicating, haemagglutinin based H5 RNA particle, H5 expressed baculovirus and H5 DNA vaccines have also been approved for vaccination of poultry but have had limited use (EFSA Panel on AHAW *et al.*, 2023; Swayne & Sims, 2020).

1.1. Rationale and intended use of the ~~product~~ vaccines

Experimental work has shown, for HPAI and LPAI, that potent and properly administered vaccines increase resistance to , or prevent infection, protect against reduce clinical signs and mortality, prevent drops reduction in egg production, reduce virus shedding from respiratory and intestinal tracts, protect from diverse field viruses within the same haemagglutinin subtype, protect from low and high challenge exposure, and reduce excretion and thus prevent contact transmission of challenge virus (Capua *et al.*, 2004; EFSA Panel on AHAW *et al.*, 2023; Swayne & Sims, 2020). Although, in experimental vaccination studies, a challenge virus is still able to infect and replicate in clinically healthy vaccinated SPF birds when exposed to high doses, the vaccination should reduce virus shedding to quantities shed may be that are insufficient for onward transmission of the virus (Van der Goot *et al.*, 2005). Vaccinated herds in which the reproduction factor of infection is suppressed to below 1 (R0 < 1) are unlikely to pose a risk of further spread of field viruses (EFSA Panel on AHAW *et al.*, 2024). Most of the work evaluating vaccines has been done in chickens and to a lesser extent in turkeys and ducks, so turkeys and some care must be taken in extrapolating the results obtained to other species. It is reccomended to first evaluate each specific vaccine in the species in which it is to be used. Most national HPAI and LPAI control regulations reserve the right to use vaccines in emergencies as 1) preventive tool, based on the outcome of risk assessments for disease introduction or spread, when other preventive measures are considered insufficient, or 2) in emergency scenarios as an immediate response to an outbreak to create barriers to further spread of the disease. In all cases, an adequate surveillance plan to guarantee the absence of circulation of the virus in the face of vaccination must be developed (EFSA Panel on AHAW *et al.*, 2024; WOAH *Terrestrial Code*, Chapter 10.4 *Infection with high pathogenicity avian influenza viruses*).

The conditions that determine risk assessments on the implementation of vaccination against HPAI in poultry depend on multiple variables, such as i) the epidemiological situation (e.g. high number of poultry outbreaks, infected wild bird populations, high risk of virus introduction from neighbouring areas); ii) the characteristics of the susceptible poultry population (e.g. high density of poultry farms, presence of species with high genetic value, presence of species that may not show clinical signs of infection); iii) environmental considerations (e.g. farms near wetlands, areas with a high density of migratory wild birds), iv) social and trade considerations (i.e. traditions of live poultry marketing, transboundary trade) (EFSA Panel on AHAW *et al.*, 2023).

There may be regional specific rules on the use of vaccination as a further layer of protection against HPAI (e.g. European Commission [EC], 2023).

2. Outline of production and minimum requirements for conventional vaccines

The information below is based primarily on the experiences in the USA and the guidance and policy for regulatory approval of influenza A vaccines in that country (United States Department of Agriculture, 1995 [updated 2006]). The basic principles for producing vaccines, particularly inactivated vaccines, are common to several viruses e.g. Newcastle disease (chapter 3.3.14).

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

The vaccine production facility should operate under the appropriate biosecurity procedures and practices. If HPAI virus is to be used in challenge studies, the facility used for such studies should meet the competent veterinary authority within the country minimum requirements for Containment Group 3 pathogens as outlined in chapter 1.1.4.

2.1. Characteristics of the seed

2.1.1. Biological characteristics

For any subtype, only well characterised influenza A virus of proven low pathogenicity, preferably obtained from an international or national repository, should be used to establish a master seed for inactivated vaccines. HPAI viruses should not be used as seed virus for vaccine. For HPAI, reverse genetics produced vaccine seed strains based on haemagglutinin gene of the HPAI virus are preferred, but should have the cleavage site sequence ~~altered~~ de-engineered to contain that of a H5/H7 LPAI virus.

A master seed is established from which a working seed is obtained. The master seed and working seed are produced in SPF or SAN embryonated eggs. The establishment of a master culture may only involve producing a large volume of infective allantoic fluid (minimum 100 ml), which can be stored as lyophilised aliquots (0.5 ml).

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

The established master seed should be controlled/examined for sterility, safety, potency and absence of specified extraneous agents (Chapter 1.1.8).

2.2. Method of manufacture

2.2.1. Procedure

For vaccine production, a working seed, from which batches of vaccine are produced, is first established in SPF or SAN embryonated eggs by expansion of an aliquot of master seed to a sufficient volume to allow vaccine production for 12–18 months. It is best to store the working seed in liquid form at below –60°C as lyophilised virus does not always multiply to high titre on subsequent first passage.

The routine procedure is to dilute the working seed in sterile isotonic buffer (e.g. PBS, pH 7.2), so that about 10^3–10^4 EID_{50} in 0.1 ml are inoculated into each allantoic cavity of 9- to 11-day-old embryonated SPF or SAN chicken eggs. These are then incubated at 37°C. Eggs containing embryos that die within 24 hours should be discarded. The incubation time will depend on the virus strain being used and will be predetermined to ensure maximum yield with the minimum number of embryo deaths.

The infected eggs should be chilled at 4°C before being harvested. The tops of the eggs are disinfected, removed and the allantoic fluids collected by suction. The inclusion of any yolk material and albumin should be avoided. All fluids should be stored immediately at 4°C and tested for bacterial contamination.

In the manufacture of inactivated vaccines, the harvested allantoic fluid is treated with either formaldehyde (a typical final concentration is 1/1000, i.e. 0.1% formalin) or beta-propiolactone (BPL) (a typical final concentration is 1/1000–1/4000, i.e. 0.1–0.025% of 99% pure BPL) or other approved inactivation methods. The exposure time required must be sufficient to ensure freedom from ~~live virus~~ any infectious virus. Most inactivated vaccines are formulated with non-concentrated inactivated allantoic fluid (active ingredient). However, active ingredients may be concentrated for easier storage of antigen. The active ingredient is usually emulsified with mineral or vegetable oil and surfactants in the final product formulation. The exact content of formulations are generally commercially sensitive and are not released ~~secrets~~.

2.2.2. Requirements for substrates and media

The inactivated influenza A vaccines prepared from conventional or reverse genetics generated viruses are produced in 9- to 11-day-old embryonated SPF or SAN chicken eggs. The method of production is basically the same as for propagating the virus aseptically (see Section B.1.2 *Virus isolation*); all procedures are performed under ~~sterile~~ aseptic conditions.

2.2.3. In-process controls

For inactivated vaccines, completion of the inactivation process should be tested in embryonated eggs, taking at least 10 aliquots of 0.2 ml from each batch and passaging each aliquot at least twice through SPF or SAN embryos. Viral infectivity must not remain.

2.2.4. Final product batch tests

Most countries have published specifications for the control of production and testing of vaccines, which include the definition of the obligatory tests on vaccines during and after manufacture.

i) Sterility and purity

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9.

ii) Safety

For inactivated vaccines, a double dose is administered by the recommended route to ten 3-week-old birds, and these are observed for 2 weeks for absence of clinical signs of disease or local lesions.

iii) Batch potency

Potency of influenza A vaccine is generally evaluated by testing the ability of the vaccine to induce a significant HI titre in SPF or SAN birds. Indeed, because of their ability to block virus attachment and entry into host cells of influenza type A viruses, the antibodies against the HA protein measured by such a test are recognised to be the most powerful mediators of resistance to influenza infection and are considered the primary immune correlate of protection. Conventional potency testing involving the use of three diluted doses and challenge with HPAI virus may also be used for vaccines prepared to give protection against LPAI subtypes. For inactivated vaccines against HPAI or LPAI virus, potency tests may rely on the measurement of immune response or challenge and assessment of morbidity, mortality (HPAI only) and quantitative reduction in challenge virus replication in respiratory ~~(oropharyngeal or tracheal)~~ and intestinal ~~(cloaca)~~ tracts (the latter parameter assessed by collection of oropharyngeal/tracheal and cloacal swabs). Assessment of haemagglutinin antigen content could allow for *in-vitro* extrapolation to potency for subsequent vaccine batches.

iv) Preservatives

A preservative may be used for vaccine in multidose containers.

2.3. Requirements for regulatory approval

2.3.1. Safety requirements

i) Target and non-target animal safety

Most inactivated influenza A vaccines are approved for use in chickens and turkeys. Field trials in the target species should be conducted to determine tolerance and safety of the vaccine at full dose. Recently the use of inactivated influenza A vaccines has been expanded to ducks, geese, other poultry and zoo birds. Any ~~extra~~ off-label use of the vaccines should be done cautiously and under the supervision of a veterinarian experienced in disease control through vaccination in the test species. Care must be taken to avoid self-injection with oil emulsion vaccines.

ii) Reversion-to-virulence for attenuated/live vaccines

Only inactivated influenza A virus vaccines are recommended. Live conventional influenza vaccines against any subtype are not ~~recommended~~ acceptable because of the risk for reassortment of gene segments of vaccine virus with field virus, potentially creating more pathogenic field viruses.

iii) Environmental consideration

None

2.3.2. Efficacy requirements

i) For animal production

For regulatory purposes, influenza A vaccines should pass an efficacy challenge test using a statistically relevant number of SPF or SAN chickens per group. The challenge should occur at a minimum of three weeks post-vaccination, using a challenge HPAI virus dose that causes 90% or greater mortality in the sham population. A standardised challenge dose of 10^6 mean chicken embryo infectious doses is most widely used. Protection from mortality in the vaccine group should be a minimum of ~~80~~ 90%. For LPAI, mortality is not a feature of challenge models, therefore a statistically significant reduction in virus shedding titre and/or the number of birds shedding virus from oropharynx or cloaca should be observed between sham and test vaccine groups. Other metrics of protection can be used to determine efficacy such as prevention of drops in egg production.

In establishing minimum antigen requirements, 50 PD_{50} or 3 µg of haemagglutinin per dose have been recommended ~~(Swayne & Sims, 2020)~~. Minimum HI serological titres in field birds should be

1/32 to protect from mortality or greater than 1/128 to provide reduction in challenge virus replication and shedding for antigenically close related vaccine and challenge viruses.

Use of appropriate antigens (i.e. antigenically matched to circulating strains) is critical for the achievement of adequate vaccine efficacy (clinical protection and significant reduction of shedding) against field viruses and to minimise vaccine failures regardless of the vaccine platform. Antigen selection should be based on the molecular and antigenic relatedness of the candidate vaccine strain and of the field isolates in the HA1 protein. Several studies have demonstrated that HI titres correlate with protection when the vaccine and field strains are antigenically closely related, in particular for inactivated vaccines. For novel vaccine platforms (e.g. HVT-vectored or replicon particles) a less clear association between HI titres and protection has been demonstrated, putatively as the result of a major stimulation of cell-mediated immunity.

ii) For control and eradication

Efficacy should be the same as for animal production.

2.3.3. Stability

When stored under the recommended conditions, the final vaccine product should maintain its potency for at least 1 year. Inactivated vaccines must not be frozen.

3. Vaccines based on biotechnology

3.1. Vaccines available and their advantages

Recombinant live vaccines for influenza A viruses have been produced by inserting the gene coding for the influenza A virus haemagglutinin into a non-influenza live virus vector and using this recombinant virus to immunise poultry against influenza A ~~(Swayne & Sims, 2020)~~. Recombinant live vector vaccines have several advantages over inactivated influenza A vaccines: 1) they induce mucosal, humoral and cellular immunity; 2) they can be mass administered *in ovo* or to 1-day-old birds in the biosecure hatchery to induce early protection; and 3) they enable easy serological differentiation of infected from non-infected vaccinated birds because they do not induce the production of antibodies against the nucleoprotein or matrix antigens that are common to all influenza A viruses: i.e. differentiation of infected from vaccinated animals (DIVA). Therefore, only field-infected birds will exhibit antibodies in the AGID test or ELISAs directed towards the detection of influenza group A (nucleoprotein and/or matrix) antibodies. However, recombinant live vaccines have limitations in that they may have reduced replication and thus induce no or only partial protective immunity in birds that have had field exposure to or vaccine induced immunity against the vector virus or the H gene insert ~~(Bertran *et al.*, 2018; Swayne & Sims, 2020)~~. If used in day-old or young birds, the effect of maternal antibodies to the vector virus on vaccine efficacy may vary with the vector type; i.e. most severe inhibition in decreasing order for Newcastle disease virus, fowl poxvirus and HVT vectors. In addition, because the vectors are live viruses that may have a restricted host range, the use of such vaccines must be restricted to species in which efficacy has been demonstrated. These limitations may prevent the choice of these vaccine candidates under emergency vaccination campaigns, which require the simultaneous administration of birds of different ages and species (EFSA Panel on AHAW *et al.*, 2023).

A rFPV-H5 vaccine, with H gene insert for A/turkey/Ireland/1378/1983 (H5N8), was developed in the early 1980s and ~~authorised~~ approved beginning in 1998 for use against H5N2 LPAI of Mexico ~~(Swayne & Sims, 2020)~~. This vaccine has principally been used in Mexico with expansion into several other countries within Central America and Vietnam with over 9 billion doses used between 1998 and 2016. This rFPV-H5 has had the H gene insert updated to A/chicken/Mexico/P-14/2016 (H5N2) ~~(Bertran *et al.*, 2020)~~. An rFPV-H7 with haemagglutinin insert from A/chicken/Guanajuato/07437-15/2015 (H7N3) has been developed and approved with deployment to Mexico in 2018 against H7N3 HPAI, and a rFPV-H5 with H and N gene inserts from A/goose/Guangdong/1996 (H5N1, clade 0) was used in China against the H5N1 HPAI during 2005 ~~(Chen & Bu, 2009; Criado *et al.*, 2019; Swayne & Sims, 2020)~~. rFPV can be effective when given to 1-day-old chicks with varying levels of maternal immunity ~~(Arriola *et al.*, 1999)~~. However, when very high levels of inhibitory immunity is anticipated because of previous infection or vaccination, the efficacy of the recombinant live vaccine in such day-old chicks should be confirmed and may require a prime-boost application of recombinant vaccine followed at a minimum 10 days later by inactivated influenza A vaccine boost to give optimal immunity (EFSA Panel on AHAW *et al.*, 2023; ~~Richard-Mazet *et al.*, 2014; Swayne & Sims, 2020~~).

Newcastle disease virus can also be used as a vector for expressing influenza haemagglutinin genes. A recombinant Newcastle disease vaccine virus (rNDV) expressing a H5 HA gene (rNDV-H5) was shown to protect SPF chickens against challenge with both virulent Newcastle disease virus and a HPAI H5N2 virus ~~(Veits *et al.*, 2006)~~. A similar recombinant virus based on Newcastle disease virus vaccine strain La Sota and expressing H

gene of A/goose/Guangdong/1996 (clade 0) (H5N1) was produced in China (the People's Rep. of) ~~(Ge *et al.*, 2007)~~ and reported to be efficacious in protection studies with either virus. This rNDV-H5 (clade 0) vaccine has been used widely with subsequent updating of HA insert twice with clade 2.3.4 and 2.3.2 clade haemagglutinin inserts ~~(Swayne & Sims, 2020)~~. An rNDV-H5 with H gene insert from A/chicken/Mexico/435/2005 (H5N2) has been developed, approved and deployed in Mexico against H5N2 LPAI ~~(Swayne & Sims, 2020)~~. An rNDV-H5 vaccine with H gene insert from A/chicken/Iowa/04-20/2015 (H5N2) (Gs/GD lineage, clade 2.3.4.4) insert was effective in protecting chickens against challenge with homologous H5N2 HPAI virus in chickens lacking immunity to the Newcastle disease virus vector or the H gene insert, but rNDV-H5 vaccine was ineffective as a primary or booster vaccine in poultry with maternal immunity or well-immunised against Newcastle disease or the H5 haemagglutinin protein ~~(Bertran *et al.*, 2018)~~. rNDV-H5 vaccines are effective as a primary vaccine if used in Newcastle disease or H5 antibody negative chickens, or as a priming vaccine followed by a boost with an inactivated influenza A vaccine in Newcastle disease or H5 antibody positive chickens. The major advantage of rNDV-H5 is the ability for low cost mass application by spray in the hatchery or field ~~(Swayne & Sims, 2020)~~.

Since 2010, a rHVT-H5 with haemagglutinin insert of A/swan/Hungary/4999/2006 (Gs/GD lineage, clade 2.2) has been approved and used in several countries such as Bangladesh, Egypt, Iraq, Pakistan and Vietnam ~~Egypt and Bangladesh~~ against H5Nx Gs/GD lineage HPAI and in Mexico against H5N2 LPAI (EFSA Panel on AHAW *et al.*, 2023; ~~Rauw *et al.*, 2011; Swayne & Sims, 2020~~). This rHVT-H5 vaccine has produced broad protection across diverse H5 HPAI viruses ~~(Rauw *et al.*, 2011)~~. Vaccine trials using HVT-H5 vaccines in combination with a DNA or a subunit vaccine have also been conducted in Europe providing protection against clade 2.3.4.4b in layers in the Netherlands and in fattening turkeys in Italy reaching 100% survival after challenge at 50 days with HPAI 2.3.4.4b isolate (EFSA Panel on AHAW *et al.*, 2023). Furthermore, maternally derived antibodies to rHVT vector or H5 haemagglutinin protein have had minimal negative impact on the effectiveness of the vaccine in broiler chickens after a single vaccination at 1 day of age ~~(Bertran *et al.*, 2018)~~. The rHVT-H5 is limited to application only *in ovo* or at 1 day of age to chickens in the hatchery, as application later on the farm is not feasible because of the ubiquitous infection by Marek's disease viruses or use of Marek's disease vaccines. Furthermore, rHVT-H5 vaccines are in the later stages of development. Some of these products have been used in strategies for HA insert optimisation by computational models in an effort to increase the breadth of protection offered against viruses belonging to different H5 clades. In addition, rHVT-H9 vaccines have also been tested in some Asian countries.

Because of the induction of broader immunity across mucosal, humoral and cellular areas, recombinant live vectored vaccines have had a longer use life in the field before appearance of field viruses that are resistant to the vaccine strains as compared to inactivated whole virus vaccines which produce primarily a strong humoral immunity and require more frequent updates to maintain efficacy in the field. Several ~~A~~ recombinant duck enteritis virus~~es~~ ~~in domestic ducks~~ for the prevention of H5 and/or H7 HPAI ~~has~~ have been developed and shown efficacy ~~but is pending regulatory approval and deployment~~ in domestic ducks under experimental conditions in China (People's Rep. of) ~~(Liu *et al.*, 2011)~~.

Non-replicating haemagglutinin-based RNA particle and DNA vaccines with the H gene from A/Gyrfalcon/Washington/40188-6/2014 (H5N8) (Gs/GD lineage, clade 2.3.4.4) have been approved for poultry use in the USA ~~(Swayne & Sims, 2020)~~. The H5 RNA particle vaccine is part of the USA emergency vaccine bank, along with rHVT-H5 and an inactivated H5N2 vaccines. The H5 RNA particle vaccine has been demonstrated to be an effective booster vaccine to replace ~~rg~~ reverse genetics generated inactivated H5Nx vaccine ~~(Bertran *et al.*, 2017)~~. A baculovirus with the H gene insert from A/duck/China/E319-2/2003 (Gs/GD lineage, clade 2.3.~~3~~2) has been approved for poultry use in several countries such as Iran, Iraq, Egypt, Jordan ~~Bangladesh, Egypt~~ and Mexico (EFSA Panel on AHAW *et al.*, 2023; ~~Swayne & Sims, 2020~~). This vaccine technology induced a statistically significant reduction of HPAI clade 2.3.4.4b shedding after challenge at 7 weeks of age on conventional mule ducks and, since autumn 2023, it has been used in the preventive vaccination campaign in ducks in France (EFSA Panel on AHAW *et al.*, 2023). Since this ~~category~~ type of vaccine only contains the specific influenza A haemagglutinin protein, ~~they~~ it ~~are~~ is easily amenable to serological DIVA testing using assays designed for identifying antibodies to the nucleoprotein/matrix protein. However, field reports of protection with vectored and conventional influenza A vaccines suggest that protection by single dose of the vectored vaccines for long lived poultry is not feasible, with long-term field protection requiring a booster with inactivated influenza A vaccine or non-replicating, haemagglutinin-based vaccine ~~(Swayne & Sims, 2020)~~. Other baculovirus-related recombinant protein-based vaccine candidates have been designed also against other HA subtypes.

More recently, a vaccine based on RNA replicon technology has become available. Recombinant RNA replicons are able to provide single-cycle replication *in vivo*, which restricts the spread of the vaccine replicon between vaccinated birds, prevents the replicon from causing disease and, at the same time, allows the stimulation of both humoral and cell-mediated immunity. Vaccine trials have been conducted in France in mule ducks, which turned out to be effective in reducing virus shedding following the challenge with clade 2.3.4.4b. The vaccine has also undergone a field trial in geese in Hungary (EFSA Panel on AHAW *et al.*, 2023).

The use of engineered and packaged mRNA is being considered for future developments and applications in poultry. These approaches can overcome the obstacles of the species-specificity of some viral vectors and therefore allow vaccination of multiple poultry species. Pure mRNA vaccines can also prevent the induction of immunity against replicon components that could interfere with booster applications. Although veterinary applications of such technology are currently limited in particular for AIV, vaccines for use in humans are in later stages of development.

In addition ~~to these approved vaccines~~, various experimental haemagglutinin-based H5 and H7 influenza A vaccines have been described using *in-vivo* or *in-vitro* expression systems including recombinant adenoviruses, salmonella, lactobacillae, vaccinia, avian leucosis virus, various eukaryotic systems (plants or cell cultures) and infectious laryngotracheitis virus ~~(Swayne & Sims, 2020)~~. Even the use of virus-like particles (VLPs) for vaccine production in veterinary vaccinology has increased in the past few years thanks to the favourable characteristics intrinsic to VLPs, such as high immunogenicity, safety and versatility. VLPs are self-assembled viral protein complexes the structure of which closely mimic those of their parent viruses in conformation and organisation. Prototypes of AI vaccines based on VLPs have shown promising results in experimental trials.

3.2. Special requirements for biotechnological vaccines, if any

Live recombinant vectored vaccines with influenza A haemagglutinin gene inserts should have an environmental impact assessment completed to determine the risk of the vaccine to be virulent in non-target avian species and will not increase in virulence in the target avian species.

4. ~~Surveillance methods for detecting~~ Detection of infection in vaccinated flocks and vaccinated birds

A strategy that allows differentiation of infected from vaccinated animals (DIVA), has been put forward as a possible solution to the eventual eradication of HPAI and H5/H7 LPAI without involving mass culling of birds and the resulting economic damage, especially in developing countries ~~(FAO, 2004)~~. This strategy has the benefits of vaccination (less virus in the environment), but the ability to identify infected flocks would still allow the implementation of additional control measures, including stamping out of infected flocks. DIVA strategies use one of two broad detection schemes within the vaccinated population: 1) detection of influenza A virus ('virus DIVA'), or 2) detection of antibodies against influenza A field virus infection ('serological DIVA'). At the flock level, a simple method consists of regularly monitoring sentinel birds left unvaccinated in each vaccinated flock, but this approach does have some management problems, particularly with regards to identifying the sentinels in large flocks. As an alternative or adjunct system, testing for field exposure may be performed on the vaccinated birds either by detection of field virus or antibodies against the virus. To detect the field virus, oropharyngeal or cloacal swabs from baseline daily mortality ("bucket sampling") or sick birds can be tested, individually or as pools, by molecular methods, such as real-time RT-PCR ~~or AC-ELISA~~ of the vaccinated populations ~~(Swayne & Kapczynski, 2008)~~. Molecular methods are the most sensitive tools to be used to monitor vaccinated poultry flocks for circulating AI viruses. They enable a direct and rapid insight into virus circulation within the flock at the time point of sampling and can be used to provide information on the subtype and pathotype of the virus. The efficacy of this approach mainly depends on the number of samples taken and the frequency of sampling (Harder *et al.*, 2023). Surveillance strategies to be implemented following the vaccination of poultry holdings against HPAI were assessed in an EFSA Scientific Opinion (EFSA Panel on AHAW *et al.,* 2024). One of the most effective surveillance strategies, identified by simulations and models described in the Opinion, includes sampling all dead birds (up to a number of 5, 10, 15 and 20, according to the target species or the desired sensitivity) collected within 48 hours before sample submission at defined sampling intervals. Reducing the sampling intervals, but not increasing the number of sampled birds, optimizes the early detection surveillance sensitivity. To demonstrate disease freedom and for early detection of HPAI virus following preventive vaccination, monthly virological testing of all dead birds up to 15 per flock is recommended, combined with passive surveillance in both vaccinated and unvaccinated flocks. It remains to be proven in well-controlled validation studies whether environmental sampling, in the form of swabs or boot socks taken from floors, walls, feeder troughs, nests, cages, or fans as well as air, water and drinker biofilms, may represent a future option for less invasive and economical virological surveillance in scenarios of subclinical infection usually observed in vaccinated poultry (EFSA Panel on AHAW *et al.,* 2024; Harder *et al.*, 2023).

To use serological DIVA schemes, vaccination systems that enable the detection of field exposure in vaccinated populations should be used. Several systems have been used. First, use of a vaccine containing a virus of the same haemagglutinin subtype but a different neuraminidase (N) from the field virus. Antibodies to the N of the field virus act as natural markers of infection. This system was used in Italy following the re-emergence of a H7N1 LPAI virus in 2000, ~~and used~~ where an H7N3 inactivated vaccine was used with the detection of N3-specific antibodies indicating a vaccinated flock, N1 antibodies indicating infection, and both N1 and N3 antibodies indicating an infected, vaccinated flock (Capua *et al.*, 2003). However, this method is labour intensive and time consuming and these inherent problems have prevented its wider use. In addition, problems with this system or with recently developed NA subtype-specific ELISAs would arise if a field virus emerges that has a different N antigen to the existing field virus or if subtypes with different N antigens are already circulating in the field as is present in many low and middle income countries with H5Nx (Gs/GD lineage), H9N2 and other NA subtypes in live poultry markets ~~(Swayne & Sims, 2020)~~. A second serological DIVA option is the use of vaccines that contain only HA, e.g.

replicating or non-replicating recombinant vaccines or subunit vaccines, which allows validated, classical AGID and nucleoprotein (NP)- or matrix protein-based ELISAs to be used to detect antibodies indicative of infection in vaccinated birds. Finally, for inactivated vaccines, a test that detects antibodies to the nonstructural viral or M2e proteins have been described (~~Avellaneda *et al.*, 2010;~~ Lambrecht *et al.,* 2007). These systems are yet to be validated in the field.

DIVA serological surveillance in long-lived poultry is useful to retrospectively demonstrate freedom from infection in a region, zone or compartment as it demonstrates the absence of infection in the preceding period. However, one problem with DIVA serological testing is the challenge of interpreting results in a scenario of endemic co-circulation of LPAI viruses in poultry (e.g. H9N2 in Asia, Africa and the Middle East). This is because most of the DIVA serological tests available rely on the detection of antibody to NP, which is common to all influenza A viruses. For vaccinated flocks in areas with endemic LPAIV, therefore, routine screening by subtype-specific PCR for the subtype of concern is the most appropriate method.

Implementation of any vaccination plan should go hand in hand with a system of enhanced surveillance, which needs to be developed according to the epidemiological scenario, the socioeconomic context and the aim of the vaccination. Delegated regulation (EU) 2023/361[7] allows the possibility of vaccination against HPAI in the European Union and provides specific requirements for surveillance in the context of emergency and preventive vaccination programmes.

5. Continued evaluation and updating of vaccine seed strains to protect against emergent variant field virus strains

Historically, H5 LPAI inactivated vaccine seed strains and recombinant fowl poxviruses with H5 gene inserts have shown broad cross protection in chickens against challenge by diverse H5 HPAI viruses from Eurasia and North America ~~(Swayne & Kapczynski, 2008)~~. In 1995, Mexico implemented influenza A vaccine use for poultry as one tool in the HPAI control strategy, with eradication of HPAI strain by June 1995, but as H5N2 LPAI viruses continued to circulate, H5N2 vaccination was maintained ~~(Villarreal, 2007)~~. Within a few years, multiple lineages of antigenically ~~variant~~ divergent H5N2 LPAI field viruses emerged that escaped from immunity induced by the original 1994 inactivated vaccine seed strain ~~(Lee *et al.,* 2004)~~. Similarly, emergent H5Nx HPAI Gs/GD lineage field viruses have arisen in China (the People's Rep. of), Indonesia, Egypt and various other Asian and Middle Eastern countries since 2005 that escaped from immunity induced by classical H5 inactivated LPAI vaccine seed strains and even ~~rg~~ reverse genetics generated H5 vaccine seed strains used in commercial vaccines ~~(Grund *et al.*, 2011; Liu *et al.,* 2020; Swayne & Sims, 2020)~~. Similarly, H9N2 LPAI field viruses resistant to inactivated vaccine seed strains have arisen in multiple countries in Asian and Middle East after prolonged usage of a single inactivated vaccine seed strain. It is not clear whether the emergence of these antigenic variants is related to use of vaccines or improper use of vaccines, but the emergence of ~~resistance~~ escape mutants necessitated the change in vaccine seed strains to antigenically match the circulating field strains ~~(Cattoli *et al.,* 2011; Lee *et al.,* 2016)~~. China ~~as~~ is the largest user of avian influenza vaccines. To ensure a complete protection against the different clades of viruses, China has updated its inactivated H5Nx (Gs/GD lineage) and H7N9 seed strains ten ~~eight times~~ and four times ~~once~~, respectively, with the life span of a seed strain ranging from 3 to 7 years ~~(Liu *et al.,* 2020; Swayne & Sims, 2020)~~. Mexico has updated its H5N2 inactivated seed strains twice and its rFPV-H5 once over a 20-year period of H5 vaccine use ~~(Swayne & Sims, 2020)~~. Initially H9N2 inactivated vaccine usage in South Korea, was associated with decreased field virus diversity, as vaccinal immunity completely inhibited antigenically closely related field virus replication ~~(Lee *et al.*, 2016)~~. However, over time, field virus diversity increases as antigenic variants arise in the field and expand their populations. ~~The live recombinant vectored vaccines have been updated less frequently, suggesting a broader immunity, requiring less frequent insert updates as compared inactivated vaccine seed strains.~~

All influenza A vaccination programmes should have an epidemiologically relevant surveillance programme that includes all relevant geographical regions and production sectors (Harder *et al.*, 2023). The resulting isolates, along with viruses obtained from outbreaks, should be assessed for genetic and antigenic variation as part of an ongoing programme for assessing vaccine effectiveness in the field. Initially, the viruses should be sequenced and analysed for critical amino acid changes within the five major antigenic epitopes of the HA. A representative subset of antigenic variants should be tested for cross-reactivity in a HI test using a panel of standard antisera produced against diverse influenza A viruses from the same HA subtype and the data analysed for quantitative changes by antigenic cartography ~~(Fouchier & Smith, 2010)~~. Based on this cartographic data, a few of the predominant circulating influenza A viruses and selected antigenic variants should be used in challenge efficacy studies ~~(Swayne *et al.*, 2015)~~. Vaccines that are not protective should be discontinued and replaced with vaccines containing updated inactivated vaccine seed strains or HA inserts within other vaccine platforms. Based on the timeline for emergence of antigenic variants for H5N1 viruses in China (People's Rep. of), vaccines should be assessed at a minimum every 2–3 years for efficacy against predominant circulating field viruses of the country or region. Alternatively, vaccine seed strains should be updated when a vaccine-escape mutant accounts for more than 30% of the relevant AIV subtype ~~(Liu *et al.*, 2020)~~. Based on this scientific information, the competent Veterinary Authority within the country should establish, in consultation with leading veterinary vaccine scientists and international organisations, naturally isolated or reverse genetics derived LPAI vaccine seed strains for conventional inactivated vaccines ~~, and~~ or H5 and H7 haemagglutinin gene insert cassettes for recombinant vaccines optimised to ensure relevance to the emergent wildtype viral populations. In some situations, more than one seed strain or vaccines type may be necessary to cover all production sectors within a country. Only high quality and potent vaccines should be approved for use in control programmes. Proper administration of

[7] http://data.europa.eu/eli/reg_del/2023/361/oj

high quality, potent vaccines ~~is~~ and optimal management of the farms are critical in inducing protective immunity in poultry populations.

In 2022, an OFFLU initiative (OFFLU Avian Influenza Matching – AIM) was established to provide improved information on the antigenic characteristics of AIVs to support vaccination programmes particularly with vaccine strain selection. Global updates will be regularly posted on the OFFLU website to provide information on antigenic characteristics of circulating AIVs, including vaccine challenge trials, that can support decisions when designing or modifying vaccination programmes. As the efficacy of OFFLU AIM activity depends on information and samples from countries experiencing outbreaks of avian influenza especially outbreaks that occur in vaccinated flocks, laboratories/national authorities are encouraged to share these data with OFFLU (see OFFLU website for up-to-date information[8]).

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NB: There are WOAH Reference Laboratories for avian influenza (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3). Please contact the WOAH Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for avian influenza

NB: FIRST ADOPTED IN 1989 AS AVIAN INFLUENZA (FOWL PLAGUE). MOST RECENT UPDATES ADOPTED IN 2021.

APPENDIX ~~3.3.4.~~1.

BIOSAFETY GUIDELINES FOR HANDLING HIGH PATHOGENICITY AVIAN INFLUENZA VIRUSES IN VETERINARY DIAGNOSTIC LABORATORIES

INTRODUCTION

The spread of high pathogenicity H5Nx avian influenza throughout Asia, Africa and Europe has led to an increase in the number of laboratories performing diagnostics for this pathogen. High pathogenicity avian influenza (HPAI) viruses, in general, are a serious threat to birds and mortality is often 100% in susceptible chickens. In addition, the agents can also pose a serious zoonotic threat, with approximately 60% mortality reported in humans infected with H5N1 HPAI virus. In recognition of the need for guidance on how to handle these viruses safely, the WOAH has established the following biocontainment guidelines for handling specimens that may contain HPAI virus. They are based on Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities,* the World Health Organization[9], and Centers for Disease Control and Prevention[10].

BIOCONTAINMENT LEVELS

Samples for diagnostic testing for HPAI virus using the following techniques do not require high-level containment but should be carried out at an appropriate biosafety and containment level determined by risk analysis (see Chapter 1.1.4. *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*):

- Conventional and real-time reverse transcriptase polymerase chain reaction (RT-PCR)
- Antigen-capture assays
- Serology

Virus isolation and identification procedures for handling specimens that may contain high-titred replication-competent HPAI virus should as a minimum, include the following:

- Personnel protective equipment should be worn, including solid-front laboratory coats, gloves, safety glasses and respirators with greater than or equal to 95% efficiency.
- Specimens from potentially infected birds or animals should only be processed in type II or type III biological safety cabinets (BSC).
- Necropsies of birds should be performed in a Type II BSC while wearing respiratory protection, such as a N95 respirator, or in a Type III biological safety cabinet, or other primary containment devices with 95% efficient air filtration.
- Centrifugation should be performed in sealed centrifuge cups.
- Centrifugation rotors should be opened and unloaded in a BSC.
- Work surfaces and equipment should be decontaminated after specimen processing.
- Contaminated materials should be decontaminated by autoclaving or disinfection before disposal or should be incinerated.

If chickens or other birds or mammals are inoculated with HPAI viruses, inoculation should be done in appropriate containment including:

[9] WHO laboratory biosafety guidelines for handling specimens suspected of containing avian influenza A virus, 12 January 2005.

[10] Biosafety in Microbiological and Biomedical Laboratories, 5th edition. HHS Publication No. (CDC) 21-1112. https://www.cdc.gov/labs/pdf/CDC-BiosafetyMicrobiologicalBiomedicalLaboratories-2009-P.PDF 1 December 2009.

- Inoculated chickens should be held in animal isolation cabinets or other primary containment devices, or non-isolation cages/floor pens in specially designed containment rooms
- Animal isolation cabinets should be in a separate facility that is equipped to handle the appropriate biocontainment for HPAI.
- The room should be under negative pressure to the outside and the animal isolation cabinets should be under negative pressure to the room.
- Animal isolation cabinets should have HEPA-filtered inlet and exhaust air.
- Biosafety cabinet or other primary containment devices should be available in the animal facility to perform post-mortem examinations and to collect specimens.

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NOTE ON APPENDICES 2 TO 7

In 2023, a new section was added to WOAH Terrestrial Manual *chapters to describe the rationale behind the selection of tests for different purposes given in Table 1* Test methods available and their purpose *and an explanation of their score. It was felt that this information would help the reader to find relevant information for the selection of tests while making sure that the selection process is science-based and transparent. The information will also address queries received from Members and provide justification for different tests.*

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Appendix 2: Avian influenza
Intended purpose of test: population freedom from infection

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
Detection and identification of the agent							
Real-time RT-PCR M gene (Nagy *et al.* [2021]) +++	Extracted RNA from clinical (swabs and tissues) and environmental specimens	Originally validated as for Nagy *et al.* (2021). Stringently assessed further, in context of H5N1 clade 2.3.4.4b HPAIV outbreaks. Slomka *et al.* (2023) defined a highly stringent criterion adapted to this validation of 514 poultry swabs: For a given swab, if it registered a positive result (Ct<36) by any of the three AIV real-time RT-PCRs (M-gene Nagy *et al.* [2021], H5[HA2] [Slomka *et al.* (2007b)] and H5[HP] [James *et al.* (2022)]), then by consensus it is considered as an H5Nx positive swab. If all three tests are negative (Ct>36), then by consensus it is considered as AIV negative. Se and Sp for the three AIVRT-PCRs were calculated for this assay relative to consensus swab status (positive/negative) DSe: 90.6% DSp: 100% (affirmed 100% Sp originally demonstrated by Nagy *et al.* [2021])	Additional validation based on H5N1 (clade 2.3.4.4b) HPAIV testing at 12 UK poultry outbreak premises (514 swabs, equal numbers of oro-pharyngeal and cloacal) during autumn 2021, which featured in Slomka *et al.* (2023) Chickens: eight premises, 350 swabs Turkeys: three premises, 124 swabs Ducks: One premise: 40 swabs	See references Validated by WOAH Reference Laboratory in UK – report available on request^	-Use of 99,353 M-gene sequences to ensure that as far as possible, the primer and probe designs match a vast range of animal and human influenza A viruses -Assessed in several independent European National Reference Laboratories, featuring an extensive range of human and animal influenza A viruses (laboratory isolates and 1455 clinical specimens) - Sensitively detect many contemporary swine, equine and human influenza A virus strains -Can be used to detect infection in vaccinated birds (DIVA approach) for monitoring freedom from infection in vaccinated or unvaccinated poultry flocks. -Viable virus is not requested -Adaptable to high throughput -Rapid	-Despite the thorough bioinformatics input to the primer/probe design (see left), no influenza A virus real-time RT-PCR can guarantee sensitive detection of all strains that may be encountered, as accepted by Nagy *et al.* (2021). Therefore, in an outbreak/epizootic threat context, it is prudent to apply an appropriately informed AIV real-time RT-PCR testing strategy that may feature more than one assay (Slomka *et al.* [2023]). -Does not give information on the pathotype of the virus (HPAI or LPAI virus) -Does not give information on whether live or inactivated virus -Risk of cross contamination -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA -Needs specialised equipment	Nagy *et al.* (2021). Slomka *et al.* (2023)
Real-time RT-PCR M gene (Heine *et al.* [2015]; Laconi *et al.* [2020], protocol 3) +++	Extracted RNA from clinical specimens (swabs and tissues)	Reference test was genetic sequencing DSe = 100% DSp = 100%	92 avian samples and 117 mammalian samples of known infectious status (121 true positive and 88 true negative samples)	See references Validated by WOAH Reference Laboratory in Italy – report available on request^	-*In silico* evaluation of primers/probe match using 4088 AIV M-gene sequences (Laconi *et al.* [2020]) -Assessed on extensive range of avian influenza A viruses belonging to different subtypes and genotypes -Tested on multiple sample matrixes from mammalian and avian species -Assessed in several independent European National Reference Laboratories -Can be used to detect infection in vaccinated birds (DIVA approach)	-Despite being tested on a variety of AIV subtypes, no influenza A virus real-time RT-PCR can guarantee sensitive detection of all strains that may be encountered. Therefore, in an outbreak/epizootic threat context, it is prudent to apply an appropriately informed AIV real-time RT-PCR testing strategy that may feature more than one assay (Slomka *et al.* [2023]).	Heine *et al.* (2015). Laconi *et al.* (2020)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
					for monitoring freedom from infection in vaccinated or unvaccinated poultry flocks -Viable virus is not requested -Adaptable to high throughput -Rapid	-*In silico* evaluation demonstrated that the test may have decreased sensitivity in the detection of a cluster of H5N1 viruses related to the A/chicken/East_Java/Av1955/2022 (EPI_ISL_13690275), belonging to the 2.3.2.1c clade, due to primers mismatches -Does not give information on the pathotype of the virus (HPAI or LPAI virus) -Does not give information on whether live or inactivated virus -Risk of cross contamination -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA -Needs specialised equipment	
Real-time RT-PCR M gene (Hassan *et al*. [2022]) +++	Extracted RNA from clinical specimens (swabs and tissues) and egg-grown isolates	Reference test was genome sequencing DSe = 100% DSp=100%	122 clinical samples 428 virus isolates	See reference	-*In silico* evaluation of primers/probe match using >3000 AIV M-gene sequences -Assessed on extensive range of avian and porcine influenza A viruses belonging to different subtypes and genotypes -Assessed also on a minor selection of human IAV isolates (<20) -Can be used to detect infection in vaccinated birds (DIVA approach) for monitoring freedom from infection in vaccinated or unvaccinated poultry flocks. -Viable virus is not requested -Adaptable to high throughput -Rapid	-Despite tested on a variety of AIV subtypes, no influenza A virus real-time RT-PCR can guarantee sensitive detection of all strains that may be encountered. Therefore, in an outbreak/epizootic threat context, it is prudent to apply an appropriately informed AIV real-time RT-PCR testing strategy that may feature more than one assay (Slomka *et al.* [2023]) -Does not give information on the pathotype of the virus (HPAI or LPAI virus) -Does not give information on whether live or inactivated virus -Risk of cross contamination -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA -Needs specialised equipment	Hassan *et al.* (2022)
Real-time RT-PCR H5 (Hassan *et al*. [2022])	Extracted RNA from clinical specimens	Reference test was genome sequencing DSe = 100%	122 clinical samples 428 virus isolates	See reference	-*In silico* evaluation of primers/probe match using >700 AIV H5-gene sequences	-Despite tested on a variety of AIVs, no influenza A virus real-time RT-PCR can guarantee	Hassan *et al.* (2022)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
+++	(swabs and tissues) and egg-grown isolates	DSp=100%			-Specificity assessed on extensive range of avian, porcine and human influenza A viruses belonging to different subtypes and genotypes -Assessed also on a minor selection of human IAV isolates (<20) - Combines two distinct target regions along the H5 HA gene -Viable virus is not requested -Adaptable to high throughput -Rapid	sensitive detection of all strains that may be encountered. Therefore, in an outbreak/epizootic threat context, it is prudent to apply an appropriately informed AIV real-time RT-PCR testing strategy that may feature more than one assay (Slomka *et al.* [2023]) -Failed to detect an individual Eurasian wild bird LP H5 isolate (EU-RL ring trial) -Does not give information on the pathotype of the virus (HPAI or LPAI virus) -Does not give information on whether live or inactivated virus -Risk of cross contamination -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA -Needs specialised equipment	
Real-time RT-PCR H5 (Slomka *et al.* [2007b]) +++	Extracted RNA from clinical (swabs and tissues) and environmental specimens	Originally validated as for Slomka *et al.* (2007b). Stringently assessed further, in context of H5N1 clade 2.3.4.4b HPAIV outbreaks. Slomka *et al.* (2023) defined a highly stringent criterion adapted to this validation of 514 poultry swabs: For a given swab, if it registered a positive result (Ct<36) by any of the three AIV real-time RT-PCRs (M-gene Nagy *et al.* [2021], H5[HA2] and H5[HP], then by consensus it is considered as an H5N1 positive swab. If all three tests are negative (Ct>36), then by consensus it is considered as AIV negative. Se and Sp for the three AIVRT-PCRs were calculated for this assay relative to consensus swab status (positive/negative): DSe: 96.2% DSp: 100%	514 poultry swabs, see above entry for Nagy *et al.* (2021) M-gene real-time RT-PCR: Chickens: Eight premises, 350 swabs Turkeys: Three premises, 124 swabs Ducks: One premises: 40 swabs	See references Validated by WOAH Reference Laboratory in UK – report available on request	-The real-time RT-PCR H5(HA2) has been proven to successfully and sensitively detect Eurasian-origin H5 AIVs, through careful targeting of the relatively conserved HA2 region of the H5 gene. Detected H5 AIVs include not only the H5Nx clade 2.3.4.4b HPAIVs, but earlier clades of the "goose/Guangdong" lineage (GsGd), as well as non-GsGd H5 LPAIVs (references cited by Slomka *et al.* (2023). -Viable virus is not requested -Adaptable to high throughput -Rapid	-The real-time RT-PCR H5(HA2) is optimised for detection of Eurasian H5 AIVs and may be notably less sensitive for detection of American lineage H5 AIVs (acknowledged by Slomka *et al.* [2007b]), due to divergence in the HA2 portion of the H5 gene -Despite tested on a variety of AIVs, no influenza A virus real-time RT-PCR can guarantee sensitive detection of all strains that may be encountered. Therefore, in an outbreak/epizootic threat context, it is prudent to apply an appropriately informed AIV real-time RT-PCR testing strategy that may feature more than one assay (Slomka *et al.* [2023]) -Does not give information on the pathotype of the virus (HPAI or LPAI virus)	Slomka *et al.* (2007b) Slomka *et al.* (2023)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
						-Does not give information on whether live or inactivated virus -Risk of cross contamination -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA -Needs specialised equipment	
Real-time RT-PCR H5 (Spackman *et al.* [2002]) +++	Extracted RNA from clinical specimens (swabs)	Reference test was HI Results for 100% of the samples were in agreement between real-time RT-PCR H5 and VI	Eight swab samples from live-bird markets (test population composed by 202 samples but only eight samples were tested by real-time RT-PCR H5 as the remaining 194 samples were typed as H7 positive samples by real-time RT-PCR H7 and VI)	See Reference	-The real-time RT-PCR H5(HA2) has been proven to successfully and sensitively detect American-origin H5 AIVs, through careful targeting of the relatively conserved HA2 region of the H5 gene -Viable virus is not requested -Adaptable to high throughput -Rapid	-The real-time RT-PCR H5(HA2) is optimised for detection of American H5 AIVs due to divergence in the HA2 portion of the H5 gene -Despite tested on a variety of AIVs, no AIV real-time RT-PCR can guarantee sensitive detection of all strains that may be encountered. Therefore, in an outbreak/epizootic threat context, it is prudent to apply an appropriately informed AIV testing strategy that may feature more than one assay (Slomka *et al.* [2023]) -Does not give information on the pathotype of the virus (HPAI or LPAI virus) -Does not give information on whether live or inactivated virus -Risk of cross contamination -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA -Needs specialised equipment	Spackman *et al.* (2002)
Real-time RT-PCR H5(HP) (James *et al.* [2022]) +++	Extracted RNA from clinical (swabs and tissues) and environmental specimens	Originally validated as from James *et al.* (2022). Stringently assessed further, in context of H5N1 clade 2.3.4.4b HPAIV outbreaks, relative to consensus (see above entry for Nagy *et al.* [2021] M-gene real-time RT-PCR and Slomka *et al.* [2007b] H5 real-time RT-PCR): DSe: 98.3% DSp: 100%	514 poultry swabs, see above entry for Nagy *et al.* (2021) M-gene real-time RT-PCR: Chickens: eight premises, 350 swabs	Validated by WOAH Reference Laboratory in UK – report available on request	-In addition to its description by James *et al.* (2022), the real-time RT-PCR H5(HP) has been shown to be highly sensitive in the detection of H5N1 HPAIV in swabs obtained from UK poultry outbreaks caused by the H5N1 clade 2.3.4.4b HPAIV during 2021–2022 (Slomka *et al.* [2023])	-There remains the possibility that future H5 HPAIV incursions (due to GsGd or non-GsGd lineage viruses) may include H5 gene sequence variants that may not be detected as sensitively as the current H5N1 clade 2.3.4.4b HPAIV strains circulating since autumn 2021. Clearly this concern applies to any AIV real-time RT-	James *et al.* (2022). Slomka *et al.* (2023)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
			Turkeys: three premises, 124 swabs Ducks: One premises: 40 swabs		-This assay can be used once a sample has been confirmed as positive by the generic influenza A virus screening or H5 HA2 RRT-PCR assays to detect the presence of a multi-basic cleavage site (MBCS) sequence indicative of H5 HPAIV -Viable virus is not requested -Adaptable to high throughput -Rapid	PCR, underlining the importance of ongoing validation, to ensure fitness-for-purpose for the sensitive detection of newly emerging viral strains -Does not give information on the pathotype of the virus (HPAI or LPAI virus) -Risk of cross contamination -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA -Needs specialised equipment	
Real-time RT-PCR H7 (Hassan *et al*. [2022]) +++	Extracted RNA from clinical specimens (swabs and tissues) and egg-grown isolates	Reference test was genome sequencing DSe = 100% DSp=100%	122 clinical samples 428 virus isolates	See Reference	-*In silico* evaluation of primers/probe match using >300 AIV H7-gene sequences -Specificity assessed on extensive range of avian influenza A viruses belonging to different subtypes and genotypes -Combines two distinct target regions along the H7 HA gene -Viable virus is not requested -Adaptable to high throughput -Rapid	Despite tested on a variety of AIVs, no influenza A virus-time RT-PCR can guarantee sensitive detection of all strains that may be encountered. Therefore, in an outbreak/epizootic threat context, it is prudent to apply an appropriately informed AIV real-time RT-PCR testing strategy that may feature more than one assay -Does not give information on the pathotype of the virus (HPAI or LPAI virus) -Does not give information on whether live or inactivated virus -Risk of cross contamination -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA -Needs specialised equipment	Hassan *et al.* (2022)
Real-time RT-PCR H7(HA2) (Slomka *et al.* [2009]) +++	Extracted RNA from clinical (swabs and tissues) and environmental specimens	Slomka *et al.* (2009) described and validated the H7(HA2) real-time RT-PCR relative to M-gene real-time RT-PCR of Spackman *et al.* (2002); see separate entry) DSe: 95.4% DSp: 97.9%	AIV laboratory isolates included: H7 AIVs: n=65; non-H7 AIVs: n=57. Clinical specimens from H7N7 (LP and HPAIV inoculations): n=117 swabs; plus 180 AIV negative swabs	See references	-The real-time RT-PCR H7(HA2) has been proven to successfully and sensitively detect Eurasian-origin H7 AIVs, through careful targeting of the relatively conserved HA2 region of the H7 gene. The real-time RT-PCR H7(HA2) demonstrated its value during H7 chicken outbreaks, both LP and HPAIV (Seekings *et al.* [2018], Reid *et al*. [2019]).	-The real-time RT-PCR H7(HA2) is optimised for detection of Eurasian H7 AIVs is less sensitive for detection of American lineage H7 AIVs (noted by Slomka *et al.* [2009]), due to divergence in the HA2 portion of the H7 gene. -Despite tested on a variety of AIVs, no influenza A virus real-time RT-PCR can guarantee	Slomka *et al.* (2009). Seekings *et al.* (2018). Reid *et al.* (2019)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
					-Viable virus is not requested -Adaptable to high throughput -Rapid	sensitive detection of all strains that may be encountered. Therefore, in an outbreak/epizootic threat context, it is prudent to apply an appropriately informed AIV real-time RT-PCR testing strategy that may feature more than one assay; -Does not give information on the pathotype of the virus (HPAI or LPAI virus) -Does not give information on whether live or inactivated virus; -Risk of cross contamination; -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA; -Needs specialised equipment	
Real-time RT-PCR H7 (Spackman *et al.* [2002]) +++		Reference test was HI Results for 98% of the samples were in agreement between real-time RT-PCR H7 and VI	202 swab samples from live-bird markets	See Reference	-The real-time RT-PCR H7(HA2) has been proven to successfully and sensitively detect American-origin H7 AIVs, through careful targeting of the relatively conserved HA2 region of the H7 gene -Viable virus is not requested -Adaptable to high throughput -Rapid	-The real-time RT-PCR H7(HA2) is optimised for detection of American H7 AIVs due to divergence in the HA2 portion of the H7 gene. -Despite tested on a variety of AIV subtypes, no H7 real-time RT-PCR can guarantee sensitive detection of all strains that may be encountered. Therefore, in an outbreak/epizootic threat context, it is prudent to apply an appropriately informed H7 testing strategy that may feature more than one assay. -Does not give information on the pathotype of the virus (HPAI or LPAI virus) -Does not give information on whether live or inactivated virus; -Risk of cross contamination; -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA; -Needs specialised equipment	Spackman *et al.* (2002)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
H5 conventional RT-PCR (Slomka *et al.* [2007a]) +++		Reference test was genome sequencing DSe = 99.36% DSp=100%	426 avian and 43 mammalian samples of known infectious status (472 know positive samples and 63 known negative samples)	Validated by WOAH Reference Laboratory in Italy – report available on request^	-The RT-PCR H5(HA2) has been proven to successfully and sensitively detect Eurasian-origin H5 AIVs -Can be used once a sample has been confirmed as positive by the generic influenza A virus screening or H5 HA2 RRT-PCR assays to detect the presence of a multi-basic cleavage site (MBCS) sequence indicative of H5 HPAIV by Sanger sequencing -Viable virus is not requested	-The RT-PCR H5(HA2) is optimised for detection of Eurasian H5 AIVs. Despite tested on a variety of AIVs, no H5RT-PCR can guarantee sensitive detection of all strains that may be encountered. Therefore, in an outbreak/epizootic threat context, it is prudent to apply an appropriately informed AIV testing strategy that may feature more than one assay. -Does not give information on the pathotype of the virus (HPAI or LPAI virus); -Risk of cross contamination; -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA; -Needs specialised equipment	Slomka *et al.* (2007a)
H7 conventional RT-PCR (Slomka *et al.* [2007a]) +++		Reference test was genome sequencing DSe = 100% DSp=100%	89 avian samples of known infectious status (59 know positive samples and 30 known negative samples)	Validated by WOAH Reference Laboratory in Italy – report available on request^	-The RT-PCR H7(HA2) has been proven to successfully and sensitively detect Eurasian-origin H7 AIVs. -Can be used once a sample has been confirmed as positive by the generic influenza A virus screening or H7 HA2 RRT-PCR assays to detect the presence of a multi-basic cleavage site (MBCS) sequence indicative of H7 HPAIV by Sanger sequencing -Viable virus is not requested	The RT-PCR H7(HA2) is optimised for detection of Eurasian H7 AIVs. Despite tested on a variety of AIVs, no H7RT-PCR can guarantee sensitive detection of all strains that may be encountered. Therefore, in an outbreak/epizootic threat context, it is prudent to apply an appropriately informed AIV testing strategy that may feature more than one assay	Slomka *et al.* (2007a)
Virus isolation +	Tracheal swabs, cloacal swabs, and organ samples Target: Infectious virus	Reference tests were real-time RT-PCR for H5 subtype and sequencing DSe=76% Negative results by VI for H5 could be explained by considering the condition of the clinical samples at the time of their arrival (cold chain was not constantly	725 avian samples	See reference	-Demonstrates the presence of infectious (replication-competent) virus (essential to assess the efficacy of inactivation processes and for AIV phenotypic characterisation) -High specificity -Detects all AIV subtypes	-Time consuming -Relies on proper care to protect the virus in the samples from environmental damage, and use of an adequate transport system to maintain infectivity and replication competence -Difficult to manage for high throughput -Require high-level containment	Monne *et al.* (2008)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
		maintained during shipping, compromising the viability of the viruses) DSp=100%					
	Swabs Target: Infectious virus	Reference test was real-time RT-PCR for type A influenza Overall the results of the two assays agreed on 1385 samples (89%) and disagreed on 165 samples (11%)	1550 clinical swab samples from live-bird markets	See reference	As in the above line	As in the above line	Spackman *et al.* (2002)
Antigen-capture immunoassays +	Tracheal and cloacal swabs Feather pulp Target: influenza virus internal proteins	DSe and DSp differ based on the antigen-capture immunoassay used. The accuracy data presented here are the ones from Slomka *et al.* (2012). Reference test was real-time RT-PCR DSe = between 31·6% and 36·8% for tracheal and cloacal swabs and between 33.3% and 84·0% for feathers depending on which device was assessed	282 avian samples	See reference	-Fast (about 15 minutes after addition of the sample) -Specific -May be used "pen-side"	-Poor sensitivity	Slomka *et al.* (2012)
Detection of immune response							
Haemagglutination inhibition test +++	Serum samples Antibodies against the hemagglutinin (HA) antigen	A Bayesian version of latent class analysis was used to compare the results of multiple serological tests from different study settings reported in the literature DSe = 98·8% DSp= 99·5%	114 serum sample	See reference	-Identify AIV subtype-specific antibodies such as H5 and H7 -Relatively inexpensive on a per sample basis -No special equipment is needed -Results can be obtained in about 2 hours -Not species-specific	-Serum and antigen must be antigenically matched -The HI assay as an antibody screening assay is not practical unless one is targeting specific subtypes or lineages -The accuracy can be affected by steric inhibition from the NA	Comin *et al.* (2013)
ELISA +++	Serum samples Antibodies against the Influenza virus internal proteins	DSe and DSp differ based on the ELISA used and the antibodies tested The values reported here are estimated applying AGID and blocking ELISA on sera from AI virus experimental-infection trials (Brown *et al.* [2009]) DSe = 82% DSp= 100%	281 serum sample (178 positive and 103 negative)	See reference	-Rapid results -Ability to screen large numbers of samples in a semiautomated manner -NP-ELISA assays can be used to demonstrate freedom from infection in vaccinated birds (DIVA approach) if compatible vaccines are applied (i.e. vaccines that do not contain the AI-NP in the formulation)	-Indirect ELISA is species-specific -Requires some specialised equipment -Sensitivity and specificity may vary between ELISA assays used	Brown *et al.* (2009)
AGID +	Serum samples	Estimated applying AGID and blocking ELISA on sera from AI virus experimental-infection trials.	281 serum sample (178 positive and 103 negative)	See reference	-Simple and economical test -Does not require specialised laboratory equipment	-Electrolyte concentration, buffer, pH, and incubation temperatures can affect precipitate formation	Brown *et al.* (2009)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
	Antibodies against the Influenza virus internal proteins	DSe = 67,4% DSp= 100%				-Sera from waterfowl should not be tested by this method (lack of sensitivity as these birds do not produce precipitating antibodies) -Time consuming	

1619 **Appendix 3: Avian Influenza***
1620 **Intended purpose of test: individual animal freedom from infection prior to movement**

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
Detection and identification of the agent							
Real-time RT-PCR M gene (Nagy *et al.*, [2021]) +++	Extracted RNA from clinical (swabs and tissues) and environmental specimens	Originally validated as for Nagy *et al.* (2021). Stringently assessed further, in context of H5N1 clade 2.3.4.4b HPAIV outbreaks. Slomka *et al.* (2023) defined a highly stringent criterion adapted to this validation of 514 poultry swabs: For a given swab, if it registered a positive result (Ct<36) by any of the three AIV real-time RT-PCRs (M-gene Nagy *et al.* [2021], H5[HA2] [Slomka *et al.* (2007b)] and H5[HP] [James *et al.* (2022)]), then by consensus it is considered as an H5Nx positive swab. If all three tests are negative (Ct>36), then by consensus it is considered as AIV negative. Se and Sp for the three AIVRT-PCRs were calculated for this assay relative to consensus swab status (positive/negative): DSe: 90.6% DSp: 100% (affirmed 100% Sp originally demonstrated by Nagy *et al.* [2021])	Additional validation based on H5N1 (clade 2.3.4.4b) HPAIV testing at 12 UK poultry outbreak premises (514 swabs, equal numbers of oro-pharyngeal and cloacal) during autumn 2021, which featured in Slomka *et al.* (2023) Chickens: eight premises, 350 swabs Turkeys: three premises, 124 swabs Ducks: One premise: 40 swabs	See references Validated by WOAH Reference Laboratory in UK – report available on request	-Use of 99,353 M-gene sequences to ensure that as far as possible, the primer and probe designs match a vast range of animal and human influenza A viruses -Assessed in several independent European National Reference Laboratories, featuring an extensive range of human and animal influenza A viruses (laboratory isolates and 1455 clinical specimens) - Sensitively detect many contemporary swine, equine and human influenza A virus strains -Viable virus is not requested -Adaptable to high throughput -Rapid -Can be used to detect infection in vaccinated birds (DIVA approach) for monitoring freedom from infection in vaccinated or unvaccinated poultry flocks	-Despite the thorough bioinformatics input to the primer/probe design (see left), no influenza A virus real-time RT-PCR can guarantee sensitive detection of all strains that may be encountered, as accepted by Nagy *et al.* (2021). Therefore, in an outbreak / epizootic threat context, it is prudent to apply an appropriately informed AIV real-time RT-PCR testing strategy that may feature more than one assay (Slomka *et al.* [2023]) -Does not give information on the pathotype of the virus (HPAI or LPAI virus) -Does not give information on whether live or inactivated virus -Risk of cross contamination -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA -Needs specialised equipment	Nagy *et al.* (2021) Slomka *et al.* (2023)
Real-time RT-PCR M gene (Heine *et al.* [2015]; Laconi *et al.* [2020], protocol 3) +++	Extracted RNA from clinical specimens (swabs and tissues)	Reference test was genetic sequencing DSe = 100% DSp = 100%	92 avian samples and 117 mammalian samples of known infectious status (121 true positive and 88 true negative samples)	See references Validated by WOAH Reference Laboratory in Italy – report available on request^	-*In silico* evaluation of primers/probe match using 4088 AIV M-gene sequences (Laconi *et al.* [2020]) -Assessed on extensive range of avian influenza A viruses belonging to different subtypes and genotypes -Tested on multiple sample matrixes from mammalian and avian species -Assessed in several independent European National Reference Laboratories	-Despite tested on a variety of AIV subtypes, no influenza A virus real-time RT-PCR can guarantee sensitive detection of all strains that may be encountered. Therefore, in an outbreak/epizootic threat context, it is prudent to apply an appropriately informed AIV real-time RT-PCR testing strategy that may feature more than one assay (Slomka *et al.* [2023]).	Heine *et al.* (2015) Laconi *et al.* (2020)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
					-Can be used to detect infection in vaccinated birds (DIVA approach) for monitoring freedom from infection in vaccinated or unvaccinated poultry flocks. -Viable virus is not requested -Adaptable to high throughput -Rapid	-*In silico* evaluation demonstrated that the test may have decreased sensitivity in the detection of a cluster of H5N1 viruses related to the A/chicken/East_Java/Av1955/2022 (EPI_ISL_13690275), belonging to the 2.3.2.1c clade, due to primers mismatches. -Does not give information on the pathotype of the virus (HPAI or LPAI virus) -Does not give information on whether live or inactivated virus -Risk of cross contamination -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA -Needs specialised equipment	
Real-time RT-PCR M gene (Hassan *et al*. [2022]) +++	Extracted RNA from clinical specimens (swabs and tissues) and egg-grown isolates	Reference test was genome sequencing DSe = 100% DSp=100%	122 clinical samples 428 virus isolates	See reference	- In silico evaluation of primers/probe match using >3000 AIV M-gene sequences -Assessed on extensive range of avian and porcine influenza A viruses belonging to different subtypes and genotypes - Assessed also on a minor selection of human IAV isolates (<20) -Can be used to detect infection in vaccinated birds (DIVA approach) for monitoring freedom from infection in vaccinated or unvaccinated poultry flocks. -Viable virus is not requested -Adaptable to high throughput -Rapid	-Despite tested on a variety of AIV subtypes, no influenza A virus real-time RT-PCR can guarantee sensitive detection of all strains that may be encountered. Therefore, in an outbreak/epizootic threat context, it is prudent to apply an appropriately informed AIV real-time RT-PCR testing strategy that may feature more than one assay (Slomka *et al.* [2023]) -Does not give information on the pathotype of the virus (HPAI or LPAI virus) -Does not give information on whether live or inactivated virus -Risk of cross contamination -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA -Needs specialised equipment	Hassan *et al.* (2022)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
Real-time RT-PCR H5 (Hassan *et al*. [2022]) +++	Extracted RNA from clinical specimens (swabs and tissues) and egg-grown isolates	Reference test was genome sequencing DSe = 100% DSp=100%	122 clinical samples 428 virus isolates	See reference	- *In silico* evaluation of primers/probe match using >700 AIV H5-gene sequences -Specificity assessed on extensive range of avian, porcine and human influenza A viruses belonging to different subtypes and genotypes - Assessed also on a minor selection of human IAV isolates (<20) - Combines two distinct target regions along the H5 HA gene -Viable virus is not requested -Adaptable to high throughput -Rapid	-Despite tested on a variety of AIVs, no influenza A virus real-time RT-PCR can guarantee sensitive detection of all strains that may be encountered. Therefore, in an outbreak / epizootic threat context, it is prudent to apply an appropriately informed AIV real-time RT-PCR testing strategy that may feature more than one assay (Slomka *et al.* [2023]) -Failed to detect an individual Eurasian wild bird LP H5 isolate (EU-RL ring trial) -Does not give information on the pathotype of the virus (HPAI or LPAI virus) -Does not give information on whether live or inactivated virus -Risk of cross contamination -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA -Needs specialised equipment	Hassan *et al.* (2022)
Real-time RT-PCR H5 (Slomka *et al.* [2007b]) +++	Extracted RNA from clinical (swabs and tissues) and environmental specimens	Originally validated as for Slomka *et al.* (2007b). Stringently assessed further, in context of H5N1 clade 2.3.4.4b HPAIV outbreaks. Slomka *et al.* (2023) defined a highly stringent criterion adapted to this validation of 514 poultry swabs: For a given swab, if it registered a positive result (Ct<36) by any of the three AIV real-time RT-PCRs (M-gene Nagy *et al.* [2021], H5[HA2] and H5[HP]), then by consensus it is considered as an H5N1 positive swab. If all three tests are negative (Ct>36), then by consensus it is considered as AIV negative. Se and Sp for the three AIVRT-PCRs	514 poultry swabs, see above entry for Nagy *et al. (*2021) M-gene real-time RT-PCR: Chickens: eight premises, 350 swabs Turkeys: three premises, 124 swabs Ducks: one premises: 40 swabs	See references Validated by WOAH Reference Laboratory in UK – report available on request°.	-The real-time RT-PCR H5(HA2) has been proven to successfully and sensitively detect Eurasian-origin H5 AIVs, through careful targeting of the relatively conserved HA2 region of the H5 gene. Detected H5 AIVs include not only the H5Nx clade 2.3.4.4b HPAIVs, but earlier clades of the "goose/Guangdong" lineage (GsGd), as well as non-GsGd H5 LPAIVs (references cited by Slomka *et al.* (2023) -Viable virus is not requested -Adaptable to high throughput -Rapid	-The real-time RT-PCR H5(HA2) is optimised for detection of Eurasian H5 AIVs and may be notably less sensitive for detection of American lineage H5 AIVs (acknowledged by Slomka *et al.* [2007b]), due to divergence in the HA2 portion of the H5 gene -Despite tested on a variety of AIVs, no influenza A virus real-time RT-PCR can guarantee sensitive detection of all strains that may be encountered. Therefore, in an outbreak/epizootic threat context, it is prudent to apply an appropriately informed AIV real-time RT-PCR testing strategy that may feature more	Slomka *et al.* (2007b). Slomka *et al.* (2023)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
		were calculated for this assay relative to consensus swab status (positive/negative) DSe: 96.2% DSp: 100%				than one assay (Slomka *et al.* [2023]) -Does not give information on the pathotype of the virus (HPAI or LPAI virus) -Does not give information on whether live or inactivated virus -Risk of cross contamination -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA -Needs specialised equipment	
Real-time RT-PCR H5 (Spackman *et al.* [2002]) +++	Extracted RNA from clinical specimens (swabs)	Reference test was HI Results for 100% of the samples were in agreement between real-time RT-PCR H5 and VI	Eight swab samples from live-bird markets (test population composed by 202 samples but only eight samples were tested by real-time RT-PCR H5 as the remaining 194 samples were typed as H7 positive samples by real-time RT-PCR H7 and VI)	See Reference	-The real-time RT-PCR H5(HA2) has been proven to successfully and sensitively detect American-origin H5 AIVs, through careful targeting of the relatively conserved HA2 region of the H5 gene. -Viable virus is not requested -Adaptable to high throughput -Rapid	-The real-time RT-PCR H5(HA2) is optimised for detection of American H5 AIVs due to divergence in the HA2 portion of the H5 gene. -Despite tested on a variety of AIVs, no AIV real-time RT-PCR can guarantee sensitive detection of all strains that may be encountered. Therefore, in an outbreak/epizootic threat context, it is prudent to apply an appropriately informed AIV testing strategy that may feature more than one assay (Slomka *et al.* [2023]). -Does not give information on the pathotype of the virus (HPAI or LPAI virus) -Does not give information on whether live or inactivated virus -Risk of cross contamination -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA -Needs specialised equipment	Spackman *et al.* (2002)
Real-time RT-PCR H5(HP) (James *et al.* [2022]) +++	Extracted RNA from clinical (swabs and tissues) and	Originally validated as from James *et al.* (2022). Stringently assessed further, in context of H5N1 clade 2.3.4.4b HPAIV outbreaks, relative	514 poultry swabs, see above entry for Nagy *et al.* (2021)	Validated by WOAH Reference Laboratory in	-In addition to its description by James *et al.* (2022), the real-time RT-PCR H5(HP) has been shown to be highly sensitive in the detection of	-There remains the possibility that future H5 HPAIV incursions (due to GsGd or non-GsGd lineage viruses) may include H5 gene	James *et al.* (2022) Slomka *et al.* (2023)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
	environmental specimens	to consensus (see above entry for Nagy *et al.* (2021) M-gene real-time RT-PCR and Slomka *et al.* (2007b) H5 real-time RT-PCR: DSe: 98.3% DSp: 100%	M-gene real-time RT-PCR: Chickens: eight premises, 350 swabs Turkeys: three premises, 124 swabs Ducks: one premises: 40 swabs	UK – report available on request	H5N1 HPAIV in swabs obtained from UK poultry outbreaks caused by the H5N1 clade 2.3.4.4b HPAIV during 2021–2022 (Slomka *et al.* [2023]). -This assay can be used once a sample has been confirmed as positive by the generic influenza A virus screening or H5 HA2 RRT-PCR assays to detect the presence of a multi-basic cleavage site (MBCS) sequence indicative of H5 HPAIV -Viable virus is not requested -Adaptable to high throughput -Rapid	sequence variants which may not be detected as sensitively as the current H5N1 clade 2.3.4.4b HPAIV strains circulating since autumn 2021. Clearly this concern applies to any AIV real-time RT-PCR, underlining the importance of ongoing validation, to ensure fitness-for-purpose for the sensitive detection of newly emerging viral strains. -Does not give information on whether live or inactivated virus -Risk of cross contamination -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA -Needs specialised equipment	
Real-time RT-PCR H7 (Hassan *et al.* [2022]) +++	Extracted RNA from clinical specimens (swabs and tissues) and egg-grown isolates	Reference test was genome sequencing DSe = 100% DSp=100%	122 clinical samples 428 virus isolates	See Reference	- *In silico* evaluation of primers/probe match using >300 AIV H7-gene sequences -Specificity assessed on extensive range of avian influenza A viruses belonging to different subtypes and genotypes - Combines two distinct target regions along the H7 HA gene -Viable virus is not requested -Adaptable to high throughput -Rapid	-Despite tested on a variety of AIVs, no influenza A virus real-time RT-PCR can guarantee sensitive detection of all strains that may be encountered. Therefore, in an outbreak / epizootic threat context, it is prudent to apply an appropriately informed AIV real-time RT-PCR testing strategy that may feature more than one assay-Does not give information on the pathotype of the virus (HPAI or LPAI virus) -Does not give information on whether live or inactivated virus -Risk of cross contamination -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA -Needs specialised equipment	Hassan *et al.* (2022)
Real-time RT-PCR H7(HA2) (Slomka *et al.* [2009]) +++	Extracted RNA from clinical (swabs and tissues) and	Slomka *et al.* (2009) described and validated the H7(HA2) real-time RT-PCR relative to M-gene	AIV laboratory isolates included: H7 AIVs: n=65; non-H7 AIVs: n=57.	See references	-The real-time RT-PCR H7(HA2) has been proven to successfully and sensitively detect Eurasian-origin H7 AIVs, through careful targeting of the	-The real-time RT-PCR H7(HA2) is optimised for detection of Eurasian H7 AIVs is less sensitive for detection of American lineage H7	Slomka *et al.* (2009) Seekings *et al.* (2018)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
	environmental specimens	real-time RT-PCR of Spackman *et al. (*2002; see separate entry): DSe: 95.4% DSp: 97.9%	Clinical specimens from H7N7 (LP and HPAIV inoculations): n=117 swabs; plus 180 AIV negative swabs		relatively conserved HA2 region of the H7 gene. The real-time RT-PCR H7(HA2) demonstrated its value during H7 chicken outbreaks, both LP and HPAIV (Seekings *et al.* [2018], Reid *et al.* [2019]). -Viable virus is not requested -Adaptable to high throughput -Rapid	AIVs (noted by Slomka *et al.* [2009]), due to divergence in the HA2 portion of the H7 gene. -Despite tested on a variety of AIVs, no influenza A virus real-time RT-PCR can guarantee sensitive detection of all strains that may be encountered. Therefore, in an outbreak/epizootic threat context, it is prudent to apply an appropriately informed AIV real-time RT-PCR testing strategy that may feature more than one assay; -Does not give information on the pathotype of the virus (HPAI or LPAI virus) -Does not give information on whether live or inactivated virus; -Risk of cross contamination; -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA; -Needs specialised equipment	Reid *et al.* (2019)
Real-time RT-PCR H7 (Spackman *et al.* [2002]) +++		Reference test was HI Results for 98% of the samples were in agreement between real-time RT-PCR H7 and VI	202 swab samples from live-bird markets	See Reference	-The real-time RT-PCR H7(HA2) has been proven to successfully and sensitively detect American-origin H7 AIVs, through careful targeting of the relatively conserved HA2 region of the H7 gene -Viable virus is not requested -Adaptable to high throughput -Rapid	-The real-time RT-PCR H7(HA2) is optimised for detection of American H7 AIVs due to divergence in the HA2 portion of the H7 gene. -Despite tested on a variety of AIV subtypes, no H7 real-time RT-PCR can guarantee sensitive detection of all strains that may be encountered. Therefore, in an outbreak/epizootic threat context, it is prudent to apply an appropriately informed H7 testing strategy that may feature more than one assay. -Does not give information on the pathotype of the virus (HPAI or LPAI virus)	Spackman *et al.* (2002)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
						-Does not give information on whether live or inactivated virus; -Risk of cross contamination; -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA; -Needs specialised equipment	
H5 conventional RT-PCRs (Slomka *et al.* [2007a]) **+++**		Reference test was genome sequencing DSe = 99.36% DSp=100%	426 avian and 43 mammalian samples of known infectious status (472 know positive samples and 63 known negative samples)	Validated by WOAH Reference Laboratory in Italy – report available on request^	-The RT-PCR H5(HA2) has been proven to successfully and sensitively detect Eurasian-origin H5 AIVs -Can be used once a sample has been confirmed as positive by the generic influenza A virus screening or H5 HA2 RRT-PCR assays to detect the presence of a multi-basic cleavage site (MBCS) sequence indicative of H5 HPAIV by Sanger sequencing -Viable virus is not requested	-The RT-PCR H5(HA2) is optimised for detection of Eurasian H5 AIVs Despite tested on a variety of AIVs, no H5RT-PCR can guarantee sensitive detection of all strains that may be encountered. Therefore, in an outbreak/epizootic threat context, it is prudent to apply an appropriately informed AIV testing strategy that may feature more than one assay -Does not give information on whether live or inactivated virus; -Risk of cross contamination; -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA; -Needs specialised equipment	Slomka *et al.* (2007)
H7 conventional RT-PCRs (Slomka *et al.* [2007a]) **+++**		Reference test was genome sequencing DSe = 100% DSp=100%	89 avian samples of known infectious status (59 know positive samples and 30 known negative samples)	Validated by WOAH Reference Laboratory in Italy – report available on request^	The RT-PCR H7(HA2) has been proven to successfully and sensitively detect Eurasian-origin H7 AIVs -Can be used once a sample has been confirmed as positive by the generic influenza A virus screening or H7 HA2 RRT-PCR assays to detect the presence of a multi-basic cleavage site (MBCS) sequence indicative of H7 HPAIV by Sanger sequencing -Viable virus is not requested	The RT-PCR H7(HA2) is optimised for detection of Eurasian H7 AIVs. Despite tested on a variety of AIVs, no H7RT-PCR can guarantee sensitive detection of all strains that may be encountered. Therefore, in an outbreak / epizootic threat context, it is prudent to apply an appropriately informed AIV testing strategy that may feature more than one assay	Slomka *et al.* (2007)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
Virus isolation +++	Tracheal swabs, cloacal swabs, and organ samples Target: infectious virus	Reference tests were real-time RT-PCR for H5 subtype and sequencing DSe=76% Negative results by VI for H5 could be explained by considering the condition of the clinical samples at the time of their arrival (cold chain was not constantly maintained during shipping, compromising the viability of the viruses) DSp=100%	725 avian samples	See reference	-Demonstrates the presence of infectious (replication-competent) virus (essential to assess the efficacy of inactivation processes and for AIV phenotypic characterisation) -High specificity -Detects all AIV subtypes	-Time consuming -Relies on proper care to protect the virus in the samples from environmental damage, and use of an adequate transport system to maintain infectivity and replication competence. -Difficult to manage for high throughput -Require high-level containment	Monne *et al.* (2008)
	Swabs Target: Infectious virus	Reference test was real-time RT-PCR for type A influenza Overall the results of the two assays agreed on 1,385 samples (89%) and disagreed on 165 samples (11%)	1550 clinical swab samples from live-bird markets	See reference	As in the above line	As in the above line	Spackman *et al.* (2002)
Antigen-capture immunoassays +	Tracheal and cloacal swabs Feather pulp Target: Influenza virus internal proteins	DSe and DSp differ based on the antigen-capture immunoassay used The accuracy data presented here are the ones from Slomka *et al.* [2012] Reference test was real-time RT-PCR DSe = between 31·6% and 36·8% for tracheal and cloacal swabs and between 33.3% and 84·0% for feathers depending on which device was assessed	282 avian samples	See reference	-Fast (about 15 minutes after addition of the sample) -Specific -May be used “pen-side”	-Poor sensitivity	Slomka *et al.* (2012)
Detection of immune response							
Haemagglutination inhibition test ++	Serum samples Antibodies against the hemagglutinin (HA) antigen	A Bayesian version of latent class analysis was used to compare the results of multiple serological tests from different study settings reported in the literature DSe = 98·8% DSp= 99·5%	114 serum sample	See reference	-Identify AIV subtype-specific antibodies such as H5 and H7 -Relatively inexpensive on a per sample basis -No special equipment is needed -Results can be obtained in about 2 hours -Not species-specific	-Serum and antigen must be antigenically matched -The HI assay as an antibody screening assay is not practical unless one is targeting specific subtypes or lineages -The accuracy can be affected by steric inhibition from the NA	Comin *et al.* (2013)
ELISA +	Serum samples	DSe and DSp differ based on the ELISA used and the antibodies	281 serum sample	See reference	-Rapid results	-Indirect ELISA is species-specific	Brown *et al.* (2009)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
	Antibodies against the Influenza virus internal proteins	tested The values reported here are estimated applying AGID and blocking ELISA on sera from AI virus experimental-infection trials (Brown *et al.* [2009]) DSe = 82% DSp= 100%	(178 positive and 103 negative)		-Ability to screen large numbers of samples in a semiautomated manner	-Requires some specialised equipment -Sensitivity and specificity may vary between ELISA assays used	
AGID +	Serum samples Antibodies against the Influenza virus internal proteins	Estimated applying AGID and blocking ELISA on sera from AI virus experimental-infection trials. DSe = 67,4% DSp= 100%	281 serum sample (178 positive and 103 negative)	See reference	-Simple and economical test -Does not require specialised laboratory equipment	-Electrolyte concentration, buffer, pH, and incubation temperatures can affect precipitate formation -Sera from waterfowl should not be tested by this method (lack of sensitivity as these birds do not produce precipitating antibodies) -Time consuming	Brown *et al.* (2009)

1621
1622

Appendix 4: Avian Influenza*
Intended purpose of test: contribute to eradication policies

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
Detection and identification of the agent							
Real-time RT-PCR M gene (Nagy *et al.* [2021]) **+++**	Extracted RNA from clinical (swabs and tissues) and environmental specimens	Originally validated as for Nagy *et al.* (2021). Stringently assessed further, in context of H5N1 clade 2.3.4.4b HPAIV outbreaks. Slomka *et al.* (2023) defined a highly stringent criterion adapted to this validation of 514 poultry swabs: For a given swab, if it registered a positive result (Ct<36) by any of the three AIV real-time RT-PCRs (M-gene Nagy *et al.* [2021], H5[HA2] [Slomka *et al.* (2007b)] and H5[HP] [James *et al.* [2022]), then by consensus it is considered as an H5Nx positive swab. If all three tests are negative (Ct>36), then by consensus it is considered as AIV negative. Se and Sp for the three AIVRT-PCRs were calculated for this assay relative to consensus swab status (positive/negative): DSe: 90.6% DSp: 100% (affirmed 100% Sp originally demonstrated by Nagy *et al.* [2021])	Additional validation based on H5N1 (clade 2.3.4.4b) HPAIV testing at 12 UK poultry outbreak premises (514 swabs, equal numbers of oro-pharyngeal and cloacal) during autumn 2021, which featured in Slomka *et al.* (2023) Chickens: eight premises, 350 swabs Turkeys: three premises, 124 swabs Ducks: one premise: 40 swabs	See references Validated by WOAH Reference Laboratory in UK – report available on request°.	-Use of 99,353 M-gene sequences to ensure that as far as possible, the primer and probe designs match a vast range of animal and human influenza A viruses -Assessed in several independent European National Reference Laboratories, featuring an extensive range of human and animal influenza A viruses (laboratory isolates and 1455 clinical specimens) - Sensitively detect many contemporary swine, equine and human influenza A virus strains -Can be used to detect infection in vaccinated birds (DIVA approach) for monitoring freedom from infection in vaccinated or unvaccinated poultry flocks. -Viable virus is not requested -Adaptable to high throughput -Rapid	-Despite the thorough bioinformatics input to the primer/probe design (see left), no influenza A virus real-time RT-PCR can guarantee sensitive detection of all strains that may be encountered, as accepted by Nagy *et al.* (2021). Therefore, in an outbreak / epizootic threat context, it is prudent to apply an appropriately informed AIV real-time RT-PCR testing strategy that may feature more than one assay (Slomka *et al.* [2023]) -Does not give information on the pathotype of the virus (HPAI or LPAI virus) -Does not give information on whether live or inactivated virus -Risk of cross contamination -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA -Needs specialised equipment	Nagy *et al.* (2021). Slomka *et al.* (2023)
Real-time RT-PCR M gene (Heine *et al.* [2015]; Laconi *et al.* [2020], protocol 3) **+++**	Extracted RNA from clinical specimens (swabs and tissues)	Reference test was genetic sequencing DSe = 100% DSp = 100%	92 avian samples and 117 mammalian samples of known infectious status (121 true positive and 88 true negative samples)	See references Validated by WOAH Reference Laboratory in Italy – report available on request^	-*In silico* evaluation of primers/probe match using 4088 AIV M-gene sequences (Laconi *et al.* [2020]) -Assessed on extensive range of avian influenza A viruses belonging to different subtypes and genotypes -Tested on multiple sample matrixes from mammalian and avian species -Assessed in several independent European National Reference Laboratories	-Despite tested on a variety of AIV subtypes, no influenza A virus real-time RT-PCR can guarantee sensitive detection of all strains that may be encountered. Therefore, in an outbreak/epizootic threat context, it is prudent to apply an appropriately informed AIV real-time RT-PCR testing strategy that may feature more than one assay (Slomka *et al.* [2023]).	Heine *et al.* (2015). Laconi *et al.* (2020)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
					-Can be used to detect infection in vaccinated birds (DIVA approach) for monitoring freedom from infection in vaccinated or unvaccinated poultry flocks. -Viable virus is not requested -Adaptable to high throughput -Rapid	-*In silico* evaluation demonstrated that the test may have decreased sensitivity in the detection of a cluster of H5N1 viruses related to the A/chicken/East_Java/Av1955/2022 (EPI_ISL_13690275), belonging to the 2.3.2.1c clade, due to primers mismatches. -Does not give information on the pathotype of the virus (HPAI or LPAI virus) -Does not give information on whether live or inactivated virus -Risk of cross contamination -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA -Needs specialised equipment	
Real-time RT-PCR M gene (Hassan *et al*. [2022]) +++	Extracted RNA from clinical specimens (swabs and tissues) and egg-grown isolates	Reference test was genome sequencing DSe = 100% DSp=100%	122 clinical samples 428 virus isolates	See reference	- In silico evaluation of primers/probe match using >3000 AIV M-gene sequences -Assessed on extensive range of avian and porcine influenza A viruses belonging to different subtypes and genotypes - Assessed also on a minor selection of human IAV isolates (<20) -Can be used to detect infection in vaccinated birds (DIVA approach) for monitoring freedom from infection in vaccinated or unvaccinated poultry flocks. -Viable virus is not requested -Adaptable to high throughput -Rapid	-Despite tested on a variety of AIV subtypes, no influenza A virus real-time RT-PCR can guarantee sensitive detection of all strains that may be encountered. Therefore, in an outbreak/epizootic threat context, it is prudent to apply an appropriately informed AIV real-time RT-PCR testing strategy that may feature more than one assay (Slomka *et al*. [2023]) -Does not give information on the pathotype of the virus (HPAI or LPAI virus) -Does not give information on whether live or inactivated virus -Risk of cross contamination -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA -Needs specialised equipment	Hassan *et al*. (2022)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
Real-time RT-PCR H5 (Hassan *et al*. [2022]) **+++**	Extracted RNA from clinical specimens (swabs and tissues) and egg-grown isolates	Reference test was genome sequencing DSe = 100% DSp=100%	122 clinical samples 428 virus isolates	See reference	- *In silico* evaluation of primers/probe match using >700 AIV H5-gene sequences -Specificity assessed on extensive range of avian, porcine and human influenza A viruses belonging to different subtypes and genotypes - Assessed also on a minor selection of human IAV isolates (<20) - Combines two distinct target regions along the H5 HA gene -Viable virus is not requested -Adaptable to high throughput -Rapid	-Despite tested on a variety of AIVs, no influenza A virus real-time RT-PCR can guarantee sensitive detection of all strains that may be encountered. Therefore, in an outbreak / epizootic threat context, it is prudent to apply an appropriately informed AIV real-time RT-PCR testing strategy that may feature more than one assay (Slomka *et al.* [2023]) -Failed to detect an individual Eurasian wild bird LP H5 isolate (EU-RL ring trial) -Does not give information on the pathotype of the virus (HPAI or LPAI virus) -Does not give information on whether live or inactivated virus -Risk of cross contamination -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA -Needs specialised equipment	Hassan *et al.* (2022)
Real-time RT-PCR H5 (Slomka *et al*. [2007b]) **+++**	Extracted RNA from clinical (swabs and tissues) and environmental specimens	Originally validated as for Slomka *et al.* (2007b) Stringently assessed further, in context of H5N1 clade 2.3.4.4b HPAIV outbreaks. Slomka *et al.* (2023) defined a highly stringent criterion adapted to this validation of 514 poultry swabs: For a given swab, if it registered a positive result (Ct<36) by any of the three AIV real-time RT-PCRs (M-gene Nagy *et al.* [2021], H5[HA2] and H5[HP]), then by consensus it is considered as an H5N1 positive swab. If all three tests are negative (Ct>36), then by consensus it is considered as AIV negative. Se and Sp for the three AIVRT-PCRs were calculated for	514 poultry swabs, see above entry for Nagy *et al.* (2021) M-gene real-time RT-PCR: Chickens: eight premises, 350 swabs Turkeys: three premises, 124 swabs Ducks: one premises: 40 swabs	See references Validated by WOAH Reference Laboratory in UK – report available on request	-The real-time RT-PCR H5(HA2) has been proven to successfully and sensitively detect Eurasian-origin H5 AIVs, through careful targeting of the relatively conserved HA2 region of the H5 gene. Detected H5 AIVs include not only the H5Nx clade 2.3.4.4b HPAIVs, but earlier clades of the "goose/Guangdong" lineage (GsGd), as well as non-GsGd H5 LPAIVs (references cited by Slomka *et al.* [2023]). -Viable virus is not requested -Adaptable to high throughput -Rapid	-The real-time RT-PCR H5(HA2) is optimised for detection of Eurasian H5 AIVs and may be notably less sensitive for detection of American lineage H5 AIVs (acknowledged by Slomka *et al.* [2007b]), due to divergence in the HA2 portion of the H5 gene -Despite tested on a variety of AIVs, no influenza A virus real-time RT-PCR can guarantee sensitive detection of all strains that may be encountered. Therefore, in an outbreak/epizootic threat context, it is prudent to apply an appropriately informed AIV real-time RT-PCR testing strategy that may feature more	Slomka *et al.* (2007b) Slomka *et al.* (2023)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
		this assay relative to consensus swab status (positive/negative) DSe: 96.2% DSp: 100%				than one assay (Slomka *et al.* [2023]) -Does not give information on the pathotype of the virus (HPAI or LPAI virus) -Does not give information on whether live or inactivated virus -Risk of cross contamination -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA -Needs specialised equipment	
Real-time RT-PCR H5 (Spackman *et al.* [2002]) **+++**	Extracted RNA from clinical specimens (swabs)	Reference test was HI Results for 100% of the samples were in agreement between real-time RT-PCR H5 and VI	Eight swab samples from live-bird markets (test population composed by 202 samples but only eight samples were tested by real-time RT-PCR H5 as the remaining 194 samples were typed as H7 positive samples by real-time RT-PCR H7 and VI)	See Reference	-The real-time RT-PCR H5(HA2) has been proven to successfully and sensitively detect American-origin H5 AIVs, through careful targeting of the relatively conserved HA2 region of the H5 gene. -Viable virus is not requested -Adaptable to high throughput -Rapid	-The real-time RT-PCR H5(HA2) is optimised for detection of American H5 AIVs due to divergence in the HA2 portion of the H5 gene. -Despite tested on a variety of AIVs, no AIV real-time RT-PCR can guarantee sensitive detection of all strains that may be encountered. Therefore, in an outbreak/epizootic threat context, it is prudent to apply an appropriately informed AIV testing strategy that may feature more than one assay (Slomka *et al.* [2023]). -Does not give information on the pathotype of the virus (HPAI or LPAI virus) -Does not give information on whether live or inactivated virus -Risk of cross contamination -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA -Needs specialised equipment	Spackman *et al.* (2002)
Real-time RT-PCR H5(HP) (James *et al.* [2022]) **+++**	Extracted RNA from clinical (swabs and tissues) and	Originally validated as from James *et al. (*2022) Stringently assessed further, in context of H5N1 clade 2.3.4.4b HPAIV outbreaks, relative	514 poultry swabs, see above entry for Nagy *et al. (*2021)	Validated by WOAH Reference Laboratory in	-In addition to its description by James *et al.* (2022), the real-time RT-PCR H5(HP) has been shown to be highly sensitive in the detection of	-There remains the possibility that future H5 HPAIV incursions (due to GsGd or non-GsGd lineage viruses) may include H5 gene	James *et al.* (2022) Slomka *et al.* (2023)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
	environmental specimens	to consensus (see above entry for Nagy *et al.* (2021) M-gene real-time RT-PCR and Slomka *et al.* (2007b) H5 real-time RT-PCR: DSe: 98.3% DSp: 100%	M-gene real-time RT-PCR: Chickens: eight premises, 350 swabs Turkeys: three premises, 124 swabs Ducks: one premises: 40 swabs	UK – report available on request	H5N1 HPAIV in swabs obtained from UK poultry outbreaks caused by the H5N1 clade 2.3.4.4b HPAIV during 2021-2022 (Slomka *et al.* [2023]). -This assay can be used once a sample has been confirmed as positive by the generic influenza A virus screening or H5 HA2 RRT-PCR assays to detect the presence of a multi-basic cleavage site (MBCS) sequence indicative of H5 HPAIV -Viable virus is not requested -Adaptable to high throughput -Rapid	sequence variants which may not be detected as sensitively as the current H5N1 clade 2.3.4.4b HPAIV strains circulating since autumn 2021. Clearly this concern applies to any AIV real-time RT-PCR, underlining the importance of ongoing validation, to ensure fitness-for-purpose for the sensitive detection of newly emerging viral strains. -Does not give information on whether live or inactivated virus -Risk of cross contamination -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA -Needs specialised equipment	
Real-time RT-PCR H7 (Hassan *et al*. [2022]) +++	Extracted RNA from clinical specimens (swabs and tissues) and egg-grown isolates	Reference test was genome sequencing DSe = 100% DSp=100%	122 clinical samples 428 virus isolates	See Reference	- In silico evaluation of primers/probe match using >300 AIV H7-gene sequences -Specificity assessed on extensive range of avian influenza A viruses belonging to different subtypes and genotypes - Combines two distinct target regions along the H7 HA gene -Viable virus is not requested -Adaptable to high throughput -Rapid	Despite tested on a variety of AIVs, no influenza A virus real-time RT-PCR can guarantee sensitive detection of all strains that may be encountered. Therefore, in an outbreak/epizootic threat context, it is prudent to apply an appropriately informed AIV real-time RT-PCR testing strategy that may feature more than one assay -Does not give information on the pathotype of the virus (HPAI or LPAI virus) -Does not give information on whether live or inactivated virus -Risk of cross contamination -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA -Needs specialised equipment	Hassan *et al.* (2022)
Real-time RT-PCR H7(HA2) (Slomka *et al*. [2009])	Extracted RNA from clinical (swabs and	Slomka *et al.* (2009) described and validated the H7(HA2) real-time RT-PCR relative to M-gene	AIV laboratory isolates included:	See references	-The real-time RT-PCR H7(HA2) has been proven to successfully and sensitively detect Eurasian-origin H7	-The real-time RT-PCR H7(HA2) is optimised for detection of Eurasian H7 AIVs is less sensitive for	Slomka *et al.* (2009). Seekings *et al.*

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
+++	tissues) and environmental specimens	real-time RT-PCR of Spackman *et al.* (2002; see separate entry): DSe: 95.4% DSp: 97.9%	H7 AIVs: n=65; non-H7 AIVs: n=57. Clinical specimens from H7N7 (LP and HPAIV inoculations): n=117 swabs; plus 180 AIV negative swabs.		AIVs, through careful targeting of the relatively conserved HA2 region of the H7 gene. The real-time RT-PCR H7(HA2) demonstrated its value during H7 chicken outbreaks, both LP and HPAIV (Seekings *et al.,* 2018, Reid *et al.,* 2019). -Viable virus is not requested -Adaptable to high throughput -Rapid	detection of American lineage H7 AIVs (noted by Slomka *et al.* [2009]), due to divergence in the HA2 portion of the H7 gene. -Despite tested on a variety of AIVs, no influenza A virus real-time RT-PCR can guarantee sensitive detection of all strains that may be encountered. Therefore, in an outbreak / epizootic threat context, it is prudent to apply an appropriately informed AIV real-time RT-PCR testing strategy that may feature more than one assay; -Does not give information on the pathotype of the virus (HPAI or LPAI virus) -Does not give information on whether live or inactivated virus; -Risk of cross contamination; -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA; -Needs specialised equipment	(2018). Reid *et al.* (2019)
Real-time RT-PCR H7 (Spackman *et al.* [2002]) +++		Reference test was HI Results for 98% of the samples were in agreement between real-time RT-PCR H7 and VI	202 swab samples from live-bird markets	See Reference	-The real-time RT-PCR H7(HA2) has been proven to successfully and sensitively detect American-origin H7 AIVs, through careful targeting of the relatively conserved HA2 region of the H7 gene. -Viable virus is not requested -Adaptable to high throughput -Rapid	-The real-time RT-PCR H7(HA2) is optimised for detection of American H7 AIVs due to divergence in the HA2 portion of the H7 gene. -Despite tested on a variety of AIV subtypes, no H7 real-time RT-PCR can guarantee sensitive detection of all strains that may be encountered. Therefore, in an outbreak / epizootic threat context, it is prudent to apply an appropriately informed H7 testing strategy that may feature more than one assay. -Does not give information on the pathotype of the virus (HPAI or LPAI virus)	Spackman *et al.* (2002)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
						-Does not give information on whether live or inactivated virus; -Risk of cross contamination; -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA; -Needs specialised equipment	
H5 conventional RT-PCRs (Slomka *et al.* [2007a]) **+++**		Reference test was genome sequencing DSe = 99.36% DSp=100%	426 avian and 43 mammalian samples of known infectious status (472 know positive samples and 63 known negative samples)	Validated by WOAH Reference Laboratory in Italy – report available on request^.	-The RT-PCR H5(HA2) has been proven to successfully and sensitively detect Eurasian-origin H5 AIVs. -Can be used once a sample has been confirmed as positive by the generic influenza A virus screening or H5 HA2 RRT-PCR assays to detect the presence of a multi-basic cleavage site (MBCS) sequence indicative of H5 HPAIV by Sanger sequencing -Viable virus is not requested	The RT-PCR H5(HA2) is optimised for detection of Eurasian H5 AIVs. Despite tested on a variety of AIVs, no H5RT-PCR can guarantee sensitive detection of all strains that may be encountered. Therefore, in an outbreak / epizootic threat context, it is prudent to apply an appropriately informed AIV testing strategy that may feature more than one assay. -Does not give information on whether live or inactivated virus; -Risk of cross contamination; -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA; -Needs specialised equipment	Slomka *et al.* (2007a)
H7 conventional RT-PCRs (Slomka *et al.* [2007a]) **+++**		Reference test was genome sequencing DSe = 100% DSp=100%	89 avian samples of known infectious status (59 know positive samples and 30 known negative samples)	Validated by WOAH Reference Laboratory in Italy – report available on request^.	The RT-PCR H7(HA2) has been proven to successfully and sensitively detect Eurasian-origin H7 AIVs. -Can be used once a sample has been confirmed as positive by the generic influenza A virus screening or H7 HA2 RRT-PCR assays to detect the presence of a multi-basic cleavage site (MBCS) sequence indicative of H7 HPAIV by Sanger sequencing -Viable virus is not requested	The RT-PCR H7(HA2) is optimised for detection of Eurasian H7 AIVs. Despite tested on a variety of AIVs, no H7RT-PCR can guarantee sensitive detection of all strains that may be encountered. Therefore, in an outbreak / epizootic threat context, it is prudent to apply an appropriately informed AIV testing strategy that may feature more than one assay	Slomka *et al.* (2007a)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
Virus isolation +	Tracheal swabs, cloacal swabs, and organ samples Target: Infectious virus	Reference tests were real-time RT-PCR for H5 subtype and sequencing DSe=76% Negative results by VI for H5 could be explained by considering the condition of the clinical samples at the time of their arrival (cold chain was not constantly maintained during shipping, compromising the viability of the viruses) DSp=100%	725 avian samples	See reference	-Demonstrates the presence of infectious (replication-competent) virus (essential to assess the efficacy of inactivation processes and for AIV phenotypic characterisation) -High specificity -Detects all AIV subtypes	-Time consuming -Relies on proper care to protect the virus in the samples from environmental damage, and use of an adequate transport system to maintain infectivity and replication competence. -Difficult to manage for high throughput -Require high-level containment	Monne *et al.* (2008)
	Swabs Target: Infectious virus	Reference test was real-time RT-PCR for type A influenza Overall the results of the two assays agreed on 1,385 samples (89%) and disagreed on 165 samples (11%)	1,550 clinical swab samples from live-bird markets	See reference	As in the above line	As in the above line	Spackman *et al.* (2002)
Antigen-capture immunoassays +	Tracheal and cloacal swabs Feather pulp Target: Influenza virus internal proteins	DSe and DSp differ based on the antigen-capture immunoassay used. The accuracy data presented here are the ones from Slomka *et al.* (2012). Reference test was real-time RT-PCR DSe = between 31·6% and 36·8% for tracheal and cloacal swabs and between 33.3% and 84·0% for feathers depending on which device was assessed	282 avian samples	See reference	-Fast (about 15 min after addition of the sample) -Specific -May be used “pen-side”	-Poor sensitivity	Slomka *et al.* (2012)
Detection of immune response							
Haemagglutination inhibition test +++	Serum samples Antibodies against the hemagglutinin (HA) antigen	A Bayesian version of latent class analysis was used to compare the results of multiple serological tests from different study settings reported in the literature DSe = 98·8% DSp= 99·5%	114 serum sample	See reference	-Identify AIV subtype-specific antibodies such as H5 and H7 -Relatively inexpensive on a per sample basis -No special equipment is needed -Results can be obtained in about 2 hours -Not species-specific	-Serum and antigen must be antigenically matched -The HI assay as an antibody screening assay is not practical unless one is targeting specific subtypes or lineages -The accuracy can be affected by steric inhibition from the NA	Comin *et al.* (2013)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
ELISA ++	Serum samples Antibodies against the Influenza virus internal proteins	DSe and DSp differ based on the ELISA used and the antibodies tested. The values reported here are estimated applying AGID and blocking ELISA on sera from AI virus experimental-infection trials (Brown *et al.* [2009]) DSe = 82% DSp= 100%	281 serum sample (178 positive and 103 negative)	See reference	-Rapid results -Ability to screen large numbers of samples in a semiautomated manner	-Indirect ELISA is species-specific -Requires some specialised equipment -Sensitivity and specificity may vary between ELISA assays used.	Brown *et al.* (2009)
AGID ++	Serum samples Antibodies against the Influenza virus internal proteins	Estimated applying AGID and blocking ELISA on sera from AI virus experimental-infection trials. DSe = 67,4% DSp= 100%	281 serum sample (178 positive and 103 negative)	See reference	-Simple and economical test -Does not require specialised laboratory equipment	-Electrolyte concentration, buffer, pH, and incubation temperatures can affect precipitate formation -Sera from waterfowl should not be tested by this method (lack of sensitivity as these birds do not produce precipitating antibodies) -Time consuming	Brown *et al.* (2009)

1623
1624

Appendix 5: Avian Influenza*
Intended purpose of test: confirmation of clinical cases

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
Detection and identification of the agent							
Real-time RT-PCR M gene (Nagy *et al.* [2021]) +++	Extracted RNA from clinical (swabs and tissues) and environmental specimens	Originally validated as for Nagy *et al.* (2021) Stringently assessed further, in context of H5N1 clade 2.3.4.4b HPAIV outbreaks. Slomka *et al.* (2023) defined a highly stringent criterion adapted to this validation of 514 poultry swabs: For a given swab, if it registered a positive result (Ct<36) by any of the three AIV real-time RT-PCRs (M-gene Nagy *et al.* [2021], H5[HA2] [Slomka *et al.* (2007b)] and H5[HP] [James *et al.* (2022)]), then by consensus it is considered as an H5Nx positive swab. If all three tests are negative (Ct>36), then by consensus it is considered as AIV negative. Se and Sp for the three AIVRT-PCRs were calculated for this assay relative to consensus swab status (positive/negative): DSe: 90.6% DSp: 100% (affirmed 100% Sp originally demonstrated by Nagy *et al.* (2021)	Additional validation based on H5N1 (clade 2.3.4.4b) HPAIV testing at 12 UK poultry outbreak premises (514 swabs, equal numbers of oro-pharyngeal and cloacal) during autumn 2021, which featured in Slomka *et al.* (2023) Chickens: eight premises, 350 swabs Turkeys: three premises, 124 swabs Ducks: one premise: 40 swabs	See references Validated by WOAH Reference Laboratory in UK – report available on request	-Use of 99,353 M-gene sequences to ensure that as far as possible, the primer and probe designs match a vast range of animal and human influenza A viruses -Assessed in several independent European National Reference Laboratories, featuring an extensive range of human and animal influenza A viruses (laboratory isolates and 1455 clinical specimens) - Sensitively detect many contemporary swine, equine and human influenza A virus strains -Can be used to detect infection in vaccinated birds (DIVA approach) for monitoring freedom from infection in vaccinated or unvaccinated poultry flocks. -Viable virus is not requested -Adaptable to high throughput -Rapid	-Despite the thorough bioinformatics input to the primer / probe design (see left), no influenza A virus real-time RT-PCR can guarantee sensitive detection of all strains that may be encountered, as accepted by Nagy *et al* (2021). Therefore, in an outbreak / epizootic threat context, it is prudent to apply an appropriately informed AIV real-time RT-PCR testing strategy that may feature more than one assay (Slomka *et al.* [2023]) -Does not give information on the pathotype of the virus (HPAI or LPAI virus) -Does not give information on whether live or inactivated virus -Risk of cross contamination -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA -Needs specialised equipment	Nagy *et al.* (2021) Slomka *et al.* (2023)
Real-time RT-PCR M gene (Heine *et al.* [2015]; Laconi *et al.* [2020], protocol 3) +++	Extracted RNA from clinical specimens (swabs and tissues)	Reference test was genetic sequencing DSe = 100% DSp = 100%	92 avian samples and 117 mammalian samples of known infectious status (121 true positive and 88 true negative samples)	See references Validated by WOAH Reference Laboratory in Italy – report available on request^	-Is silico evaluation of primers/probe match using 4088 AIV M-gene sequences (Laconi *et al.* [2020]) -Assessed on extensive range of avian influenza A viruses belonging to different subtypes and genotypes -Tested on multiple sample matrixes from mammalian and avian species -Assessed in several independent European National Reference Laboratories	-Despite tested on a variety of AIV subtypes, no influenza A virus real-time RT-PCR can guarantee sensitive detection of all strains that may be encountered. Therefore, in an outbreak / epizootic threat context, it is prudent to apply an appropriately informed AIV real-time RT-PCR testing strategy that may feature more than one assay (Slomka *et al.* [2023])	Heine *et al.* (2015) Laconi *et al.* (2020)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
					-Can be used to detect infection in vaccinated birds (DIVA approach) for monitoring freedom from infection in vaccinated or unvaccinated poultry flocks. -Viable virus is not requested -Adaptable to high throughput -Rapid	-In silico evaluation demonstrated that the test may have decreased sensitivity in the detection of a cluster of H5N1 viruses related to the A/chicken/East_Java/Av1955/2022 (EPI_ISL_13690275), belonging to the 2.3.2.1c clade, due to primers mismatches. -Does not give information on the pathotype of the virus (HPAI or LPAI virus) -Does not give information on whether live or inactivated virus -Risk of cross contamination -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA -Needs specialised equipment	
Real-time RT-PCR M gene (Hassan *et al*. [2022]) +++	Extracted RNA from clinical specimens (swabs and tissues) and egg-grown isolates	Reference test was genome sequencing DSe = 100% DSp=100%	122 clinical samples 428 virus isolates	See reference	- In silico evaluation of primers/probe match using >3000 AIV M-gene sequences -Assessed on extensive range of avian and porcine influenza A viruses belonging to different subtypes and genotypes - Assessed also on a minor selection of human IAV isolates (<20) -Can be used to detect infection in vaccinated birds (DIVA approach) for monitoring freedom from infection in vaccinated or unvaccinated poultry flocks. -Viable virus is not requested -Adaptable to high throughput -Rapid	-Despite tested on a variety of AIV subtypes, no influenza A virus real-time RT-PCR can guarantee sensitive detection of all strains that may be encountered. Therefore, in an outbreak/epizootic threat context, it is prudent to apply an appropriately informed AIV real-time RT-PCR testing strategy that may feature more than one assay (Slomka *et al.* [2023]) -Does not give information on the pathotype of the virus (HPAI or LPAI virus) -Does not give information on whether live or inactivated virus -Risk of cross contamination -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA -Needs specialised equipment	Hassan *et al.* (2022)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
Real-time RT-PCR H5 (Hassan *et al.* [2022]) +++	Extracted RNA from clinical specimens (swabs and tissues) and egg-grown isolates	Reference test was genome sequencing DSe = 100% DSp=100%	122 clinical samples 428 virus isolates	See reference	- In silico evaluation of primers/probe match using >700 AIV H5-gene sequences -Specificity assessed on extensive range of avian, porcine and human influenza A viruses belonging to different subtypes and genotypes - Assessed also on a minor selection of human IAV isolates (<20) - Combines two distinct target regions along the H5 HA gene -Viable virus is not requested -Adaptable to high throughput -Rapid	-Despite tested on a variety of AIVs, no influenza A virus real-time RT-PCR can guarantee sensitive detection of all strains that may be encountered. Therefore, in an outbreak / epizootic threat context, it is prudent to apply an appropriately informed AIV real-time RT-PCR testing strategy that may feature more than one assay (Slomka *et al.* [2023]) -Failed to detect an individual Eurasian wild bird LP H5 isolate (EU-RL ringtrial) -Does not give information on the pathotype of the virus (HPAI or LPAI virus) -Does not give information on whether live or inactivated virus -Risk of cross contamination -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA -Needs specialised equipment	Hassan *et al.* (2022)
Real-time RT-PCR H5 (Slomka *et al.* [2007b]) +++	Extracted RNA from clinical (swabs and tissues) and environmental specimens	Originally validated as for Slomka *et al.,* (2007b). Stringently assessed further, in context of H5N1 clade 2.3.4.4b HPAIV outbreaks. Slomka *et al.* (2023) defined a highly stringent criterion adapted to this validation of 514 poultry swabs: For a given swab, if it registered a positive result (Ct<36) by any of the three AIV real-time RT-PCRs (M-gene Nagy *et al.* [2021], H5[HA2] and H5[HP]), then by consensus it is considered as an H5N1 positive swab. If all three tests are negative (Ct>36), then by consensus it is considered as AIV negative. Se and Sp for the three AIVRT-PCRs	514 poultry swabs, see above entry for Nagy *et al. (*2021) M-gene real-time RT-PCR: Chickens: eight premises, 350 swabs Turkeys: three premises, 124 swabs Ducks: one premises: 40 swabs	See references Validated by WOAH Reference Laboratory in UK – report available on request	-The real-time RT-PCR H5(HA2) has been proven to successfully and sensitively detect Eurasian-origin H5 AIVs, through careful targeting of the relatively conserved HA2 region of the H5 gene. Detected H5 AIVs include not only the H5Nx clade 2.3.4.4b HPAIVs, but earlier clades of the "goose/Guangdong" lineage (GsGd), as well as non-GsGd H5 LPAIVs (references cited by Slomka *et al.* (2023). -Viable virus is not requested -Adaptable to high throughput -Rapid	-The real-time RT-PCR H5(HA2) is optimised for detection of Eurasian H5 AIVs and may be notably less sensitive for detection of American lineage H5 AIVs (acknowledged by Slomka *et al.* 2007b), due to divergence in the HA2 portion of the H5 gene -Despite tested on a variety of AIVs, no influenza A virus real-time RT-PCR can guarantee sensitive detection of all strains that may be encountered. Therefore, in an outbreak/epizootic threat context, it is prudent to apply an appropriately informed AIV real-time RT-PCR testing strategy that may feature more	Slomka *et al. (*2007b) Slomka *et al. (*2023)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
		were calculated for this assay relative to consensus swab status (positive/negative): DSe: 96.2% DSp: 100%				than one assay (Slomka *et al.* [2023]) -Does not give information on the pathotype of the virus (HPAI or LPAI virus) -Does not give information on whether live or inactivated virus -Risk of cross contamination -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA -Needs specialised equipment	
Real-time RT-PCR H5 (Spackman *et al.* [2002]) +++	Extracted RNA from clinical specimens (swabs)	Reference test was HI Results for 100% of the samples were in agreement between real-time RT-PCR H5 and VI	Eight swab samples from live-bird markets (test population composed by 202 samples but only eight samples were tested by real-time RT-PCR H5 as the remaining 194 samples were typed as H7 positive samples by real-time RT-PCR H7 and VI)	See Reference	-The real-time RT-PCR H5(HA2) has been proven to successfully and sensitively detect American-origin H5 AIVs, through careful targeting of the relatively conserved HA2 region of the H5 gene -Viable virus is not requested -Adaptable to high throughput -Rapid	-The real-time RT-PCR H5(HA2) is optimised for detection of American H5 AIVs due to divergence in the HA2 portion of the H5 gene. -Despite tested on a variety of AIVs, no AIV real-time RT-PCR can guarantee sensitive detection of all strains that may be encountered. Therefore, in an outbreak / epizootic threat context, it is prudent to apply an appropriately informed AIV testing strategy that may feature more than one assay (Slomka *et al.* [2023]) -Does not give information on the pathotype of the virus (HPAI or LPAI virus) -Does not give information on whether live or inactivated virus -Risk of cross contamination -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA -Needs specialised equipment	Spackman *et al.* (2002)
Real-time RT-PCR H5(HP) (James *et al.* [2022]) +++	Extracted RNA from clinical (swabs and tissues) and	Originally validated as from James *et al. (*2022). Stringently assessed further, in context of H5N1 clade 2.3.4.4b HPAIV outbreaks, relative	514 poultry swabs, see above entry for Nagy *et al. (*2021)	Validated by WOAH Reference Laboratory in	-In addition to its description by James *et al. (*2022), the real-time RT-PCR H5(HP) has been shown to be highly sensitive in the detection of	-There remains the possibility that future H5 HPAIV incursions (due to GsGd or non-GsGd lineage viruses) may include H5 gene	James *et al. (*2022) Slomka *et al. (*2023)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
	environmental specimens	to consensus (see above entry for Nagy *et al. (*2021) M-gene real-time RT-PCR and Slomka *et al. (*2007b) H5 real-time RT-PCR: DSe: 98.3% DSp: 100%	M-gene real-time RT-PCR: Chickens: eight premises, 350 swabs Turkeys: three premises, 124 swabs Ducks: one premises: 40 swabs	UK – report available on request	H5N1 HPAIV in swabs obtained from UK poultry outbreaks caused by the H5N1 clade 2.3.4.4b HPAIV during 2021-2022 (Slomka *et al.,* 2023). -This assay can be used once a sample has been confirmed as positive by the generic influenza A virus screening or H5 HA2 RRT-PCR assays to detect the presence of a multi-basic cleavage site (MBCS) sequence indicative of H5 HPAIV -Viable virus is not requested -Adaptable to high throughput -Rapid	sequence variants which may not be detected as sensitively as the current H5N1 clade 2.3.4.4b HPAIV strains circulating since autumn 2021. Clearly this concern applies to any AIV real-time RT-PCR, underlining the importance of ongoing validation, to ensure fitness-for-purpose for the sensitive detection of newly emerging viral strains. -Does not give information on whether live or inactivated virus -Risk of cross contamination -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA -Needs specialised equipment	
Real-time RT-PCR H7 (Hassan *et al.* [2022]) +++	Extracted RNA from clinical specimens (swabs and tissues) and egg-grown isolates	Reference test was genome sequencing DSe = 100% DSp=100%	122 clinical samples 428 virus isolates	See Reference	- *In silico* evaluation of primers/probe match using >300 AIV H7-gene sequences -Specificity assessed on extensive range of avian influenza A viruses belonging to different subtypes and genotypes - Combines two distinct target regions along the H7 HA gene -Viable virus is not requested -Adaptable to high throughput -Rapid	Despite tested on a variety of AIVs, no influenza A virus real-time RT-PCR can guarantee sensitive detection of all strains that may be encountered. Therefore, in an outbreak / epizootic threat context, it is prudent to apply an appropriately informed AIV real-time RT-PCR testing strategy that may feature more than one assay-Does not give information on the pathotype of the virus (HPAI or LPAI virus) -Does not give information on whether live or inactivated virus -Risk of cross contamination -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA -Needs specialised equipment	Hassan *et al.* (2022)
Real-time RT-PCR H7(HA2) (Slomka *et al.* [2009]) +++	Extracted RNA from clinical (swabs and tissues) and	Slomka *et al. (*2009) described and validated the H7(HA2) real-time RT-PCR relative to M-gene	AIV laboratory isolates included: H7 AIVs: n=65; non-H7 AIVs: n=57.	See references	-The real-time RT-PCR H7(HA2) has been proven to successfully and sensitively detect Eurasian-origin H7 AIVs, through careful targeting of the	-The real-time RT-PCR H7(HA2) is optimised for detection of Eurasian H7 AIVs is less sensitive for detection of American lineage H7	Slomka *et al. (*2009) Seekings *et al. (*2018)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
	environmental specimens	real-time RT-PCR of Spackman *et al. (*2002; see separate entry): DSe: 95.4% DSp: 97.9%	Clinical specimens from H7N7 (LP and HPAIV inoculations): n=117 swabs; plus 180 AIV negative swabs		relatively conserved HA2 region of the H7 gene. The real-time RT-PCR H7(HA2) demonstrated its value during H7 chicken outbreaks, both LP and HPAIV (Seekings *et al.* [2018], Reid *et al.* [2019]) -Viable virus is not requested -Adaptable to high throughput -Rapid	AIVs (noted by Slomka *et al.* [2009]), due to divergence in the HA2 portion of the H7 gene. -Despite tested on a variety of AIVs, no influenza A virus real-time RT-PCR can guarantee sensitive detection of all strains that may be encountered. Therefore, in an outbreak / epizootic threat context, it is prudent to apply an appropriately informed AIV real-time RT-PCR testing strategy that may feature more than one assay; -Does not give information on the pathotype of the virus (HPAI or LPAI virus) -Does not give information on whether live or inactivated virus; -Risk of cross contamination; -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA; -Needs specialised equipment	Reid *et al. (*2019)
Real-time RT-PCR H7 (Spackman *et al.* [2002]) **+++**		Reference test was HI Results for 98% of the samples were in agreement between real-time RT-PCR H7 and VI	202 swab samples from live-bird markets	See Reference	-The real-time RT-PCR H7(HA2) has been proven to successfully and sensitively detect American-origin H7 AIVs, through careful targeting of the relatively conserved HA2 region of the H7 gene -Viable virus is not requested -Adaptable to high throughput -Rapid	-The real-time RT-PCR H7(HA2) is optimised for detection of American H7 AIVs due to divergence in the HA2 portion of the H7 gene. -Despite tested on a variety of AIV subtypes, no H7 real-time RT-PCR can guarantee sensitive detection of all strains that may be encountered. Therefore, in an outbreak / epizootic threat context, it is prudent to apply an appropriately informed H7 testing strategy that may feature more than one assay. -Does not give information on the pathotype of the virus (HPAI or LPAI virus)	Spackman *et al.* (2002)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
						-Does not give information on whether live or inactivated virus; -Risk of cross contamination; -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA; -Needs specialised equipment	
H5 conventional RT-PCR (Slomka *et al.* [2007a]) **+++**		Reference test was genome sequencing DSe = 99.36% DSp=100%	426 avian and 43 mammalian samples of known infectious status (472 know positive samples and 63 known negative samples)	Validated by WOAH Reference Laboratory in Italy – report available on request^	-The RT-PCR H5(HA2) has been proven to successfully and sensitively detect Eurasian-origin H5 AIVs. -Can be used once a sample has been confirmed as positive by the generic influenza A virus screening or H5 HA2 RRT-PCR assays to detect the presence of a multi-basic cleavage site (MBCS) sequence indicative of H5 HPAIV by Sanger sequencing -Viable virus is not requested	The RT-PCR H5(HA2) is optimised for detection of Eurasian H5 AIVs. Despite tested on a variety of AIVs, no H5RT-PCR can guarantee sensitive detection of all strains that may be encountered. Therefore, in an outbreak/epizootic threat context, it is prudent to apply an appropriately informed AIV testing strategy that may feature more than one assay -Does not give information on whether live or inactivated virus; -Risk of cross contamination; -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA; -Needs specialised equipment	Slomka *et al.* (2007a)
H7 conventional RT-PCR (Slomka *et al.* [2007a]) **+++**		Reference test was genome sequencing DSe = 100% DSp=100%	89 avian samples of known infectious status (59 know positive samples and 30 known negative samples)	Validated by WOAH Reference Laboratory in Italy – report available on request^	The RT-PCR H7(HA2) has been proven to successfully and sensitively detect Eurasian-origin H7 AIVs. -Can be used once a sample has been confirmed as positive by the generic influenza A virus screening or H7 HA2 RRT-PCR assays to detect the presence of a multi-basic cleavage site (MBCS) sequence indicative of H7 HPAIV by Sanger sequencing -Viable virus is not requested	The RT-PCR H7(HA2) is optimised for detection of Eurasian H7 AIVs. Despite tested on a variety of AIVs, no H7RT-PCR can guarantee sensitive detection of all strains that may be encountered. Therefore, in an outbreak / epizootic threat context, it is prudent to apply an appropriately informed AIV testing strategy that may feature more than one assay	Slomka *et al.* (2007a)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
Virus isolation +++	Tracheal swabs, cloacal swabs, and organ samples Target: Infectious virus	Reference tests were real-time RT-PCR for H5 subtype and sequencing DSe=76% Negative results by VI for H5 could be explained by considering the condition of the clinical samples at the time of their arrival (cold chain was not constantly maintained during shipping, compromising the viability of the viruses) DSp=100%	725 avian samples	See reference	-Demonstrates the presence of infectious (replication-competent) virus (essential to assess the efficacy of inactivation processes and for AIV phenotypic characterisation) -High specificity -Detects all AIV subtypes	-Time consuming -Relies on proper care to protect the virus in the samples from environmental damage, and use of an adequate transport system to maintain infectivity and replication competence. -Difficult to manage for high throughput -Require high-level containment	Monne *et al.* (2008)
	Swabs Target: Infectious virus	Reference test was real-time RT-PCR for type A influenza Overall, the results of the two assays agreed on 1,385 samples (89%) and disagreed on 165 samples (11%)	1550 clinical swab samples from live-bird markets	See reference	As in the above line	As in the above line	Spackman *et al.* (2002)
Antigen-capture immunoassays +	Tracheal and cloacal swabs Feather pulp Target: Influenza virus internal proteins	DSe and DSp differ based on the antigen-capture immunoassay used. The accuracy data presented here are the ones from Slomka *et al.* (2012) Reference test was real-time RT-PCR DSe = between 31·6% and 36·8% for tracheal and cloacal swabs and between 33.3% and 84·0% for feathers depending on which device was assessed	282 avian samples	See reference	-Fast (about 15 minutes after addition of the sample) -Specific -May be used “pen-side”	-Poor sensitivity	Slomka *et al.* (2012)
Detection of immune response							
Haemagglutination inhibition test ++	Serum samples Antibodies against the hemagglutinin (HA) antigen	A Bayesian version of latent class analysis was used to compare the results of multiple serological tests from different study settings reported in the literature DSe = 98·8% DSp= 99·5%	114 serum sample	See reference	-Identify AIV subtype-specific antibodies such as H5 and H7 -Relatively inexpensive on a per sample basis -No special equipment is needed, -Results can be obtained in about 2 hours -Not species-specific	-Provide information on historical exposure to IAV -No genetic data or phenotypic data -Cross-reactions can occur between different lineages within one subtype, or even among different subtypes -Serum and antigen must be antigenically matched	Comin *et al.* (2013)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
						-The HI assay as an antibody screening assay is not practical unless one is targeting specific subtypes or lineages -The accuracy can be affected by steric inhibition from the NA	
ELISA +	Serum samples Antibodies against the Influenza virus internal proteins	DSe and DSp differ based on the ELISA used and the antibodies tested The values reported here are estimated applying AGID and blocking ELISA on sera from AI virus experimental-infection trials (Brown *et al.* [2009]) DSe = 82% DSp= 100%	281 serum sample (178 positive and 103 negative)	See reference	-Rapid results -Ability to screen large numbers of samples in a semiautomated manner	-Provide information on historical exposure to IAV -Indirect ELISA is species-specific -Requires some specialised equipment -Sensitivity and specificity may vary between ELISA assays used	Brown *et al.* (2009)
AGID +	Serum samples Antibodies against the Influenza virus internal proteins	Estimated applying AGID and blocking ELISA on sera from AI virus experimental-infection trials. DSe = 67,4% DSp= 100%	281 serum sample (178 positive and 103 negative)	See reference	-Simple and economical test -Does not require specialised laboratory equipment	-Provide information on historical exposure to IAV -Electrolyte concentration, buffer, pH, and incubation temperatures can affect precipitate formation -Sera from waterfowl should not be tested by this method (lack of sensitivity as these birds do not produce precipitating antibodies) -Time consuming	Brown *et al.* (2009)

1625 **Appendix 6: Avian Influenza***

1626 **Intended purpose of test: prevalence of infection – surveillance (including vaccinated flocks)**

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
Detection and identification of the agent							
Real-time RT-PCR M gene (Nagy *et al.* [2021]) +++	Extracted RNA from clinical (swabs and tissues) and environmental specimens	Originally validated as for Nagy *et al.* (2021). Stringently assessed further, in context of H5N1 clade 2.3.4.4b HPAIV outbreaks. Slomka *et al.* (2023) defined a highly stringent criterion adapted to this validation of 514 poultry swabs: For a given swab, if it registered a positive result (Ct<36) by any of the three AIV real-time RT-PCRs (M-gene Nagy *et al.* [2021], H5[HA2] [Slomka *et al.* (2007b)] and H5[HP] [James *et al.* (2022)]), then by consensus it is considered as an H5Nx positive swab. If all three tests are negative (Ct>36), then by consensus it is considered as AIV negative. Se and Sp for the three AIVRT-PCRs were calculated for this assay relative to consensus swab status (positive/negative)): DSe: 90.6% DSp: 100% (affirmed 100% Sp originally demonstrated by Nagy *et al.* [2021])	Additional validation based on H5N1 (clade 2.3.4.4b) HPAIV testing at 12 UK poultry outbreak premises (514 swabs, equal numbers of oro-pharyngeal and cloacal) during autumn 2021, which featured in Slomka *et al.* (2023) Chickens: eight premises, 350 swabs Turkeys: three premises, 124 swabs Ducks: one premise: 40 swabs	See references Validated by WOAH Reference Laboratory in UK – report available on request	-Use of 99,353 M-gene sequences to ensure that as far as possible, the primer and probe designs match a vast range of animal and human influenza A viruses -Assessed in several independent European National Reference Laboratories, featuring an extensive range of human and animal influenza A viruses (laboratory isolates and 1455 clinical specimens) - Sensitively detect many contemporary swine, equine and human influenza A virus strains -Can be used to detect infection in vaccinated birds (DIVA approach) for early detection or monitoring freedom from infection in vaccinated or unvaccinated poultry flocks. -Viable virus is not requested -Adaptable to high throughput -Rapid	-Despite the thorough bioinformatics input to the primer/probe design (see left), no influenza A virus real-time RT-PCR can guarantee sensitive detection of all strains that may be encountered, as accepted by Nagy *et al.* (2021). Therefore, in an outbreak/epizootic threat context, it is prudent to apply an appropriately informed AIV real-time RT-PCR testing strategy that may feature more than one assay (Slomka *et al.* [2023]) -Does not give information on the pathotype of the virus (HPAI or LPAI virus) -Does not give information on whether live or inactivated virus -Risk of cross contamination -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA -Needs specialised equipment	Nagy *et al.* (2021) Slomka *et al.* (2023)
Real-time RT-PCR M gene (Heine *et al.* [2015]; Laconi *et al.* [2020], protocol 3) +++	Extracted RNA from clinical specimens (swabs and tissues)	Reference test was genetic sequencing DSe = 100% DSp = 100%	92 avian samples and 117 mammalian samples of known infectious status (121 true positive and 88 true negative samples)	See references Validated by WOAH Reference Laboratory in Italy – report available on request^.	-Is silico evaluation of primers/probe match using 4088 AIV M-gene sequences (Laconi *et al.* [2020]) -Assessed on extensive range of avian influenza A viruses belonging to different subtypes and genotypes -Tested on multiple sample matrixes from mammalian and avian species	-Despite tested on a variety of AIV subtypes, no influenza A virus real-time RT-PCR can guarantee sensitive detection of all strains that may be encountered. Therefore, in an outbreak/epizootic threat context, it is prudent to apply an appropriately informed AIV real-time RT-PCR testing strategy that may feature more	Heine *et al.* (2015) Laconi *et al.* (2020)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
					-Assessed in several independent European National Reference Laboratories -Can be used to detect infection in vaccinated birds (DIVA approach) for early detection or monitoring freedom from infection in vaccinated or unvaccinated poultry flocks. -Viable virus is not requested -Adaptable to high throughput -Rapid	than one assay (Slomka *et al.* [2023]) -*In silico* evaluation demonstrated that the test may have decreased sensitivity in the detection of a cluster of H5N1 viruses related to the A/chicken/East_Java/Av1955/2022 (EPI_ISL_13690275), belonging to the 2.3.2.1c clade, due to primers mismatches. -Does not give information on the pathotype of the virus (HPAI or LPAI virus) -Does not give information on whether live or inactivated virus -Risk of cross contamination -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA -Needs specialised equipment	
Real-time RT-PCR M gene (Hassan *et al*. [2022]) +++	Extracted RNA from clinical specimens (swabs and tissues) and egg-grown isolates	Reference test was genome sequencing DSe = 100% DSp=100%	122 clinical samples 428 virus isolates	See reference	- *In silico* evaluation of primers/probe match using >3000 AIV M-gene sequences -Assessed on extensive range of avian and porcine influenza A viruses belonging to different subtypes and genotypes - Assessed also on a minor selection of human IAV isolates (<20) -Can be used to detect infection in vaccinated birds (DIVA approach) for early detection or monitoring freedom from infection in vaccinated or unvaccinated poultry flocks. -Viable virus is not requested -Adaptable to high throughput -Rapid	-Despite tested on a variety of AIV subtypes, no influenza A virus real-time RT-PCR can guarantee sensitive detection of all strains that may be encountered. Therefore, in an outbreak / epizootic threat context, it is prudent to apply an appropriately informed AIV real-time RT-PCR testing strategy that may feature more than one assay (Slomka *et al.* [2023]) -Does not give information on the pathotype of the virus (HPAI or LPAI virus) -Does not give information on whether live or inactivated virus -Risk of cross contamination -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA	Hassan *et al.*(2022)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
						-Needs specialised equipment	
Real-time RT-PCR H5 (Hassan *et al.* [2022]) +++	Extracted RNA from clinical specimens (swabs and tissues) and egg-grown isolates	Reference test was genome sequencing DSe = 100% DSp=100%	122 clinical samples 428 virus isolates	See reference	- *In silico* evaluation of primers/probe match using >700 AIV H5-gene sequences -Specificity assessed on extensive range of avian, porcine and human influenza A viruses belonging to different subtypes and genotypes - Assessed also on a minor selection of human IAV isolates (<20) - Combines two distinct target regions along the H5 HA gene -Viable virus is not requested -Adaptable to high throughput -Rapid	-Despite tested on a variety of AIVs, no influenza A virus real-time RT-PCR can guarantee sensitive detection of all strains that may be encountered. Therefore, in an outbreak / epizootic threat context, it is prudent to apply an appropriately informed AIV real-time RT-PCR testing strategy that may feature more than one assay (Slomka *et al.* [2023]) -Failed to detect an individual Eurasian wild bird LP H5 isolate (EU-RL ring trial) -The interpretation of subtype-specific RT-qPCRs in flocks vaccinated with live vectored H5 vaccines could be affected (i.e. Organs where active replication of the vaccine is taking place would yield positive results to such assay even in the absence of field virus) -Does not give information on the pathotype of the virus (HPAI or LPAI virus) -Does not give information on whether live or inactivated virus -Risk of cross contamination -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA -Needs specialised equipment	Hassan *et al.* (2022)
Real-time RT-PCR H5 (Slomka *et al.* [2007b]) +++	Extracted RNA from clinical (swabs and tissues) and environmental specimens	Originally validated as for Slomka *et al.* (2007b). Stringently assessed further, in context of H5N1 clade 2.3.4.4b HPAIV outbreaks. Slomka *et al.* (2023) defined a highly stringent criterion adapted to this validation of 514 poultry swabs: For a given swab, if it registered a positive	514 poultry swabs, see above entry for Nagy *et al. (*2021) M-gene real-time RT-PCR: Chickens: eight premises, 350 swabs	See references Validated by WOAH Reference Laboratory in UK – report available on request	-The real-time RT-PCR H5(HA2) has been proven to successfully and sensitively detect Eurasian-origin H5 AIVs, through careful targeting of the relatively conserved HA2 region of the H5 gene. Detected H5 AIVs include not only the H5Nx clade 2.3.4.4b HPAIVs, but earlier clades of the	-The real-time RT-PCR H5(HA2) is optimised for detection of Eurasian H5 AIVs and may be notably less sensitive for detection of American lineage H5 AIVs (acknowledged by Slomka *et al.* [2007b]), due to divergence in the HA2 portion of the H5 gene	Slomka *et al. (*2007b). Slomka *et al. (*2023)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
		result (Ct<36) by any of the three AIV real-time RT-PCRs (M-gene Nagy *et al.* [2021], H5[HA2] H5[HP]), then by consensus it is considered as an H5N1 positive swab. If all three tests are negative (Ct>36), then by consensus it is considered as AIV negative. Se and Sp for the three AIVRT-PCRs were calculated for this assay relative to consensus swab status (positive/negative)): DSe: 96.2% DSp: 100%	Turkeys: three premises, 124 swabs Ducks: one premises: 40 swabs		"goose/Guangdong" lineage (GsGd), as well as non-GsGd H5 LPAIVs (references cited by Slomka *et al.,* 2023). -Viable virus is not requested -Adaptable to high throughput -Rapid	-Despite tested on a variety of AIVs, no influenza A virus real-time RT-PCR can guarantee sensitive detection of all strains that may be encountered. Therefore, in an outbreak / epizootic threat context, it is prudent to apply an appropriately informed AIV real-time RT-PCR testing strategy that may feature more than one assay (Slomka *et al*. [2023)] -The interpretation of subtype-specific RT-qPCRs in flocks vaccinated with live vectored H5 vaccines could be affected (i.e. Organs where active replication of the vaccine is taking place would yield positive results to such assay even in the absence of field virus) -Does not give information on the pathotype of the virus (HPAI or LPAI virus) -Does not give information on whether live or inactivated virus -Risk of cross contamination -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA -Needs specialised equipment	
Real-time RT-PCR H5 (Spackman *et al.* [2002]) +++	Extracted RNA from clinical specimens (swabs)	Reference test was HI Results for 100% of the samples were in agreement between real-time RT-PCR H5 and VI	Eight swab samples from live-bird markets (test population composed by 202 samples but only eight samples were tested by real-time RT-PCR H5 as the remaining 194 samples were typed as H7 positive	See Reference	-The real-time RT-PCR H5(HA2) has been proven to successfully and sensitively detect American-origin H5 AIVs, through careful targeting of the relatively conserved HA2 region of the H5 gene -Viable virus is not requested -Adaptable to high throughput -Rapid	-The real-time RT-PCR H5(HA2) is optimised for detection of American H5 AIVs due to divergence in the HA2 portion of the H5 gene -Despite tested on a variety of AIVs, no AIV real-time RT-PCR can guarantee sensitive detection of all strains that may be encountered. Therefore, in an outbreak/epizootic threat context, it is prudent to apply an appropriately informed AIV testing	Spackman *et al.* (2002)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
			samples by real-time RT-PCR H7 and VI)			strategy that may feature more than one assay (Slomka *et al.* [2023]) -The interpretation of subtype-specific RT-qPCRs in flocks vaccinated with live vectored H5 vaccines could be affected (i.e. Organs where active replication of the vaccine is taking place would yield positive results to such assay even in the absence of field virus) -Does not give information on the pathotype of the virus (HPAI or LPAI virus) -Does not give information on whether live or inactivated virus -Risk of cross contamination -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA -Needs specialised equipment	
Real-time RT-PCR H5(HP) (James *et al.* [2022]) +++	Extracted RNA from clinical (swabs and tissues) and environmental specimens	Originally validated as for James *et al.* (2022). Stringently assessed further, in context of H5N1 clade 2.3.4.4b HPAIV outbreaks, relative to consensus (see above entry for Nagy *et al. (*2021) M-gene real-time RT-PCR and Slomka *et al. (*2007b) H5 real-time RT-PCR: DSe: 98.3% DSp: 100%	514 poultry swabs, see above entry for Nagy *et al. (*2021) M-gene real-time RT-PCR: Chickens: eight premises, 350 swabs Turkeys: three premises, 124 swabs Ducks: one premises: 40 swabs	Validated by WOAH Reference Laboratory in UK – report available on request	-In addition to its description by James *et al. (*2022), the real-time RT-PCR H5(HP) has been shown to be highly sensitive in the detection of H5N1 HPAIV in swabs obtained from UK poultry outbreaks caused by the H5N1 clade 2.3.4.4b HPAIV during 2021-2022 (Slomka *et al.* [2023]) -Can be used once a sample has been confirmed as positive by the generic influenza A virus screening or H5 HA2 RRT-PCR assays to detect the presence of a multi-basic cleavage site (MBCS) sequence indicative of H5 HPAIV -Viable virus is not requested -Adaptable to high throughput -Rapid	-There remains the possibility that future H5 HPAIV incursions (due to GsGd or non-GsGd lineage viruses) may include H5 gene sequence variants which may not be detected as sensitively as the current H5N1 clade 2.3.4.4b HPAIV strains circulating since autumn 2021. Clearly this concern applies to any AIV real-time RT-PCR, underlining the importance of ongoing validation, to ensure fitness-for-purpose for the sensitive detection of newly emerging viral strains. -Does not give information on the pathotype of the virus (HPAI or LPAI virus) -Risk of cross contamination -Risk of false negative results in case of PCR inhibitors, extraction	James *et al. (*2022)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
						inefficiency, genetic diversity of viruses, instability of RNA -Needs specialised equipment	
Real-time RT-PCR H7 (Hassan *et al.* [2022]) +++	Extracted RNA from clinical specimens (swabs and tissues) and egg-grown isolates	Reference test was genome sequencing DSe = 100% DSp=100%	122 clinical samples 428 virus isolates	See Reference	- In silico evaluation of primers/probe match using >300 AIV H7-gene sequences -Specificity assessed on extensive range of avian influenza A viruses belonging to different subtypes and genotypes - Combines two distinct target regions along the H7 HA gene -Viable virus is not requested -Adaptable to high throughput -Rapid	Despite tested on a variety of AIVs, no influenza A virus real-time RT-PCR can guarantee sensitive detection of all strains that may be encountered. Therefore, in an outbreak/epizootic threat context, it is prudent to apply an appropriately informed AIV real-time RT-PCR testing strategy that may feature more than one assay. -The interpretation of subtype-specific RT-qPCRs in flocks vaccinated with live vectored H7 vaccines could be affected (i.e. Organs where active replication of the vaccine is taking place would yield positive results to such assay even in the absence of field virus) -Does not give information on the pathotype of the virus (HPAI or LPAI virus) -Does not give information on whether live or inactivated virus -Risk of cross contamination -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA -Needs specialised equipment	Hassan *et al.* (2022)
Real-time RT-PCR H7(HA2) (Slomka *et al.* [2009]) +++	Extracted RNA from clinical (swabs and tissues) and environmental specimens	Slomka *et al. (*2009) described and validated the H7(HA2) real-time RT-PCR relative to M-gene real-time RT-PCR of Spackman *et al. (*2002; see separate entry): DSe: 95.4% DSp: 97.9%	AIV laboratory isolates included: H7 AIVs: n=65; non-H7 AIVs: n=57. Clinical specimens from H7N7 (LP and HPAIV inoculations): n=117 swabs; plus 180 AIV negative swabs.	See references	-The real-time RT-PCR H7(HA2) has been proven to successfully and sensitively detect Eurasian-origin H7 AIVs, through careful targeting of the relatively conserved HA2 region of the H7 gene. The real-time RT-PCR H7(HA2) demonstrated its value during H7 chicken outbreaks, both LP and HPAIV (Seekings *et al.,* 2018, Reid *et al.,* 2019).	-The real-time RT-PCR H7(HA2) is optimised for detection of Eurasian H7 AIVs is less sensitive for detection of American lineage H7 AIVs (noted by Slomka *et al.* [2009]), due to divergence in the HA2 portion of the H7 gene -Despite tested on a variety of AIVs, no influenza A virus real-time RT-PCR can guarantee sensitive detection of all strains	Slomka *et al. (*2009). Seekings *et al. (*2018) Reid *et al. (*2019)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
					-Viable virus is not requested -Adaptable to high throughput -Rapid	that may be encountered. Therefore, in an outbreak/epizootic threat context, it is prudent to apply an appropriately informed AIV real-time RT-PCR testing strategy that may feature more than one assay; -The interpretation of subtype-specific RT-qPCRs in flocks vaccinated with live vectored H7 vaccines could be affected (i.e. Organs where active replication of the vaccine is taking place would yield positive results to such assay even in the absence of field virus) -Does not give information on the pathotype of the virus (HPAI or LPAI virus) -Does not give information on the pathotype of the virus (HPAI or LPAI virus); -Risk of cross contamination; -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA -Needs specialised equipment	
real-time RT-PCR H7 (Spackman *et al.* [2002]) +++		Reference test was HI Results for 98% of the samples were in agreement between real-time RT-PCR H7 and VI	202 swab samples from live-bird markets	See Reference	-The real-time RT-PCR H5(HA2) has been proven to successfully and sensitively detect American-origin H7 AIVs, through careful targeting of the relatively conserved HA2 region of the H7 gene. -Viable virus is not requested -Adaptable to high throughput -Rapid	-The real-time RT-PCR H7(HA2) is optimised for detection of American H7 AIVs due to divergence in the HA2 portion of the H7 gene. -Despite tested on a variety of AIV subtypes, no H7 real-time RT-PCR can guarantee sensitive detection of all strains that may be encountered. Therefore, in an outbreak/epizootic threat context, it is prudent to apply an appropriately informed H7 testing strategy that may feature more than one assay. -The interpretation of subtype-specific RT-qPCRs in flocks	Spackman *et al.* (2002)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
						vaccinated with live vectored H7 vaccines could be affected (i.e. Organs where active replication of the vaccine is taking place would yield positive results to such assay even in the absence of field virus) -Does not give information on the pathotype of the virus (HPAI or LPAI virus) -Does not give information on whether live or inactivated virus; -Risk of cross contamination; -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA; -Needs specialised equipment	
H5 conventional RT-PCR (Slomka *et al.* [2007a]) +++		Reference test was genome sequencing DSe = 99.36% DSp=100%	426 avian and 43 mammalian samples of known infectious status (472 know positive samples and 63 known negative samples)	Validated by WOAH Reference Laboratory in Italy – report available on request^	-The RT-PCR H5(HA2) has been proven to successfully and sensitively detect Eurasian-origin H5 AIVs. -Can be used once a sample has been confirmed as positive by the generic influenza A virus screening or H5 HA2 RRT-PCR assays to detect the presence of a multi-basic cleavage site (MBCS) sequence indicative of H5 HPAIV by Sanger sequencing -Viable virus is not requested	The RT-PCR H5(HA2) is optimised for detection of Eurasian H5 AIVs. Despite tested on a variety of AIVs, no H5RT-PCR can guarantee sensitive detection of all strains that may be encountered. Therefore, in an outbreak / epizootic threat context, it is prudent to apply an appropriately informed AIV testing strategy that may feature more than one assay. -Does not give information on whether live or inactivated virus; -Risk of cross contamination; -Risk of false negative results in case of PCR inhibitors, extraction inefficiency, genetic diversity of viruses, instability of RNA; -Needs specialised equipment	Slomka *et al.* (2007a)
H7 conventional RT-PCR (Slomka *et al.* [2007a]) +++		Reference test was genome sequencing DSe = 100% DSp=100%	89 avian samples of known infectious status (59 know positive samples and 30 known negative samples)	Validated by WOAH Reference Laboratory in Italy – report available on request^.	The RT-PCR H7(HA2) has been proven to successfully and sensitively detect Eurasian-origin H7 AIVs. -Can be used once a sample has been confirmed as positive	The RT-PCR H7(HA2) is optimised for detection of Eurasian H7 AIVs. Despite tested on a variety of AIVs, no H7RT-PCR can guarantee sensitive detection of all strains that may be encountered.	Slomka *et al.* (2007a)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
					by the generic influenza A virus screening or H7 HA2 RRT-PCR assays to detect the presence of a multi-basic cleavage site (MBCS) sequence indicative of H7 HPAIV by Sanger sequencing -Viable virus is not requested	Therefore, in an outbreak / epizootic threat context, it is prudent to apply an appropriately informed AIV testing strategy that may feature more than one assay	
Virus isolation +	Tracheal swabs, cloacal swabs, and organ samples Target: Infectious virus	Reference tests were real-time RT-PCR for H5 subtype and sequencing DSe=76% Negative results by VI for H5 could be explained by considering the condition of the clinical samples at the time of their arrival (cold chain was not constantly maintained during shipping, compromising the viability of the viruses) DSp=100%	725 avian samples	See reference	-Demonstrates the presence of infectious (replication-competent) virus (essential to assess the efficacy of inactivation processes and for AIV phenotypic characterisation) -High specificity -Detects all AIV subtypes	-Time consuming -Relies on proper care to protect the virus in the samples from environmental damage, and use of an adequate transport system to maintain infectivity and replication competence. -Difficult to manage for high throughput -Require high-level containment	Monne *et al.* (2008)
	Swabs Target: Infectious virus	Reference test was real-time RT-PCR for type A influenza Overall, the results of the two assays agreed on 1,385 samples (89%) and disagreed on 165 samples (11%)	1550 clinical swab samples from live-bird markets	See reference	As in the above line	As in the above line	Spackman *et al.* (2002)
Antigen-capture immunoassays +	Tracheal and cloacal swabs Feather pulp Target: Influenza virus internal proteins	DSe and DSp differ based on the antigen-capture immunoassay used. The accuracy data presented here are the ones from assays described in Slomka *et al.* (2012). Reference test was real-time RT-PCR DSe = between 31·6% and 36·8% for tracheal and cloacal swabs and between 33.3% and 84·0% for feathers depending on which device was assessed	282 avian samples	See reference	-Fast (about 15 minutes after addition of the sample) -Specific -May be used “pen-side”	-Poor sensitivity	Slomka *et al.* (2012)
Detection of immune response							
Haemagglutination inhibition test	Serum samples	A Bayesian version of latent class analysis was used to compare the	114 serum sample	See reference	-Identify AIV subtype-specific antibodies such as H5 and H7	-Serum and antigen must be antigenically matched	Comin *et al.* (2013)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
+++	Antibodies against the hemagglutinin (HA) antigen	results of multiple serological tests from different study settings reported in the literature DSe = 98·8% DSp= 99·5%			-Relatively inexpensive on a per sample basis -No special equipment is needed, -Results can be obtained in about 2 hours -Not species-specific	-The HI assay as an antibody screening assay is not practical unless one is targeting specific subtypes or lineages -The accuracy can be affected by steric inhibition from the NA	
ELISA +++	Serum samples Antibodies against the Influenza virus internal proteins	DSe and DSp differ based on the ELISA used The values reported here are estimated applying AGID and blocking ELISA on sera from AI virus experimental-infection trials (Brown *et al.* [2009]) DSe = 82% DSp= 100%	281 serum sample (178 positive and 103 negative)	See reference	-Rapid results, -Ability to screen large numbers of samples in a semiautomated manner -NP-ELISA assays can be used to demonstrate freedom from infection in vaccinated birds (DIVA approach) if compatible vaccines are applied (i.e. vaccines that do not contain the AI-NP in the formulation)	-Indirect ELISA is species-specific -Requires some specialised equipment -Sensitivity and specificity may vary between ELISA assays used	Brown *et al.* (2009)
AGID ++	Serum samples Antibodies against the Influenza virus internal proteins	Estimated applying AGID and blocking ELISA on sera from AI virus experimental-infection trials. DSe = 67,4% DSp= 100%	281 serum sample (178 positive and 103 negative)	See reference	-Simple and economical test -Does not require specialised laboratory equipment -Can be used for surveillance in flocks vaccinated with DIVA vaccines with NP as a negative marker (i.e. vaccines that do not contain the AI-NP in the formulation)	-Electrolyte concentration, buffer, pH, and incubation temperatures can affect precipitate formation -Sera from waterfowl should not be tested by this method (lack of sensitivity as these birds do not produce precipitating antibodies) -Qualitative only -Time consuming	Brown *et al.* (2009)

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Appendix 7: Avian Influenza*
Immune status in individual animals or populations post-vaccination

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
Detection of immune response							
Haemagglutination inhibition test +++	Serum samples Antibodies against the hemagglutinin (HA) antigen	A Bayesian version of latent class analysis was used to compare the results of multiple serological tests from different study settings reported in the literature DSe = 98·8% DSp= 99·5%	114 serum sample	See reference	-Allow to assess the immune status in individual animals or populations post-vaccination (vaccine coverage) using antigens antigenically close to the vaccine; -Identify AIV subtype-specific antibodies such as H5 and H7 -Relatively inexpensive on a per sample basis -No special equipment is needed -Results can be obtained in about 2 hours -Not species-specific	-Serum and antigen must be antigenically matched -The HI assay as an antibody screening assay is not practical unless one is targeting specific subtypes or lineages -The accuracy can be affected by steric inhibition from the NA -To assess vaccine coverage, it should be considered that it can perform poorly in flocks that have received only replicating-vectored vaccines as these predominantly induce T-cell over humoral immune responses	Comin *et al.* (2013)
AGID ++	Serum samples Antibodies against the Influenza virus internal proteins	Estimated applying AGID and blocking ELISA on sera from AI virus experimental-infection trials DSe = 67,4% DSp= 100%	281 serum sample (178 positive and 103 negative)	See reference	-Simple and economical test -Does not require specialised laboratory equipment -Can be used to detect past infection in vaccinated birds (DIVA approach) if compatible vaccines are applied (i.e. vaccines that do not contain the AI-NP in the formulation)	-Electrolyte concentration, buffer, pH, and incubation temperatures can affect precipitate formation -Sera from waterfowl should not be tested by this method (lack of sensitivity as these birds do not produce precipitating antibodies) -Qualitative only -Targets virus proteins not involved in mounting protective immunity (not suitable to assess vaccine coverage) -Time consuming	Brown *et al.* (2009)
ELISA ++	Serum samples Antibodies against the Influenza virus internal proteins	DSe and DSp differ based on the ELISA used and the antibodies tested. The values reported here are estimated applying AGID and blocking ELISA on sera from AI virus experimental-infection trials (Brown *et al.* [2009]) DSe = 82% DSp= 100%	281 serum sample (178 positive and 103 negative)	See reference	-Rapid results -Ability to screen large numbers of samples in a semiautomated manner -NP-ELISA assays can be used to detect past infection in vaccinated birds (DIVA approach) if compatible vaccines are applied (i.e. vaccines that do not contain the AI-NP in the formulation)	-Indirect ELISA is species-specific -Requires some specialised equipment -Sensitivity and specificity may vary between ELISA assays used. -NP-ELISA targets virus internal proteins not involved in mounting protective immunity (not suitable to assess vaccine coverage)	Brown *et al.* (2009)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
					- NP-ELISA assays can be used to monitor the vaccination uptake if inactivated whole virus vaccines are used and endemic LPAIV infections are excluded from flocks		

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°contact: AIWRL@APHA.gov.uk

*IMPORTANT NOTES:

It is important to clarify that the accuracy data reported here refer to the results of specific investigations carried out according to the criteria described in the scientific literature or in the validation dossiers as shown in the table. The results of DSe and DSp must therefore be interpreted with caution considering the characteristics of the virus used and the conditions under which the test was applied (e.g. sample type, reference test used, purpose of the investigation, commercial kit used, etc.). The choice of one test rather than another must not be attributed solely to the accuracy of the specific test. The most appropriate diagnostic approach to be used will be dependent on the primary objective of the investigation and the virus that is being targeted. The table provides an explanation of the utility of different assays for different purposes and can be used by laboratory scientists to guide them in deciding how to approach a specific diagnostic need.

Not all the molecular methods described in Chapter 3.3.4 are included in this table and only some of the methods used for influenza A screening and H5 and H7 subtyping are reported as examples. Importantly, AIV exhibits rapid evolutionary dynamics and therefore, there is the possibility that future AIV incursions may be caused by viral strains which may not be detected as sensitively as expected by the molecular tests described here. This underlines the importance of ongoing validation, to ensure fitness-for-purpose for the sensitive detection of newly emerging viral strains.

Annexe 23. Chapitre 3.4.9. Leucose bovine enzootique

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CHAPTER 3.4.9.

ENZOOTIC BOVINE LEUKOSIS

SUMMARY

Description and importance of the disease: *Enzootic bovine leukosis* (*EBL*) *is a disease of cattle caused by the bovine leukaemia virus* (*BLV*), *a member of the family* Retroviridae. *Cattle may be infected at any age, including the embryonic stage. Infection is lifelong and results in a persistent antibody response. Most infections are subclinical, but a proportion of cattle* (*~30%*) *over 3 years old develop persistent lymphocytosis, and a smaller proportion develop lymphosarcomas* (*tumours*) *in various internal organs. Natural infection has also been recorded in water buffaloes and capybaras. Clinical signs, if present, depend on the organs affected. Cattle with lymphosarcomas may die suddenly, or weeks or months after the onset of clinical signs dependent on the location and number of tumours and the tumour's growth characteristics.*

Detection and identification of the agent: *Virus can be detected in the culture supernatant following* in-vitro *culture of peripheral blood mononuclear cells* (*PBMC*) *from infected animals, by BLV antigen detection, by polymerase chain reaction* (*PCR*) *or by electron microscopy. Proviral DNA can also be detected in PBMC or tumours of infected animals by PCR.*

Serological tests: *The antibody detection methods widely used are the agar gel immunodiffusion* (*AGID*) *assay using serum and the enzyme-linked immunosorbent assay* (*ELISA*) *using serum or milk. These tests have formed the basis for successful eradication policies in many countries. Other tests, such as radio-immunoassay, can also be used. There are a number of AGID and ELISA kits are commercially available AGID and ELISA kits.*

Requirements for vaccines: *No vaccine against BLV is available.*

A. INTRODUCTION

There may be several causes of lymposarcomas in cattle, but the only definitely known cause is the retrovirus, bovine leukaemia virus (BLV), which causes enzootic bovine leukosis (EBL).

1. Description and impact of the disease

Enzootic bovine leukosis (EBL) is a disease of cattle caused by the bovine leukaemia virus (BLV), a member of the family Retroviridae. The term sporadic bovine leukosis (SBL) is usually reserved for young animals (calves) as well as cutaneous and thymic types of lymphoma, which is defined by the age of the animal affected and the distribution of the tumours. The cause of SBL is not known. There may also be lymphosarcomatous conditions that do not fall into either the SBL or EBL categories, i.e. adult multicentric lymphoma with sporadic occurrence of unknown aetiology. Only lymphomas caused by BLV infection should be termed leukosis or enzootic bovine leukosis (Gillet *et al.*, 2007).

Although animals can become infected with BLV at any age, tumours (lymphosarcomas) are seen typically in animals over 3 years of age. Infections are usually subclinical; only 30–70% of infected cattle develop persistent

lymphocytosis, and 0.1–10% of the infected animals develop tumours (lymphosarcomas). Although animals can become infected at any age, tumours are seen typically in animals over 4 years of age. Clinical signs will depend on the site of the tumours and may include digestive disturbances, inappetence, weight loss, weakness or general debility and sometimes neurological manifestations. Superficial lymph nodes may be obviously enlarged and may be palpable under the skin and by rectal examination. At necropsy, lymph nodes and a wide range of tissues are found to be infiltrated by neoplastic cells. Organs most frequently involved are the abomasum, right auricle of the heart, spleen, intestine, liver, kidney, omasum, lung, and uterus. The susceptibility of cattle to persistent lymphocytosis, and perhaps also to tumour development, is genetically determined.

There is ~~growing~~ evidence of the role of the virus in immunological dysfunctions and in increased culling rates. Two large-scale investigations estimated the mean decline in milk production per cow among test-positive BLV herds compared with test-negative herds as very similar at 2.5% and 2.7%, respectively (Emanuelsson *et al.*, 1992; Ott *et al.*, 2003). Such findings have been again confirmed in recent studies (Nekouei *et al.,* 2016; Norby *et al.,* 2016). In addition, a 7% lower conception rate in BLV test-positive cows compared with test-negative cows has been reported. Increased culling rates and a greater susceptibility to other diseases with infectious aetiology, e.g. mastitis, diarrhoea and pneumonia were also demonstrated among test-positive BLV herds (Emanuelsson *et al.*, 1992). Reduced protective immunity following vaccination in BLV infected cattle has also been reported (Frie *et al.,* 2016; Puentes *et al.,* 2016). Therefore, despite no obvious clinical signs during the long subclinical infection period, economic losses caused by persistent BLV infections are relevant.

~~Virus can be detected by *in vitro* cultivation of peripheral blood mononuclear cells (PBMC). The virus is present in PBMC and in tumour cells as provirus integrates into the DNA of infected cells. Virus is also found in the cellular fraction of various body fluids (nasal and bronchial fluids, saliva, milk). Natural transmission depends on the transfer of infected cells, for example during parturition. Artificial transmission occurs, e.g. by blood-contaminated needles, surgical equipment, gloves used for rectal examinations. Lateral transmission in the absence of these contributory factors is usually slow (Monti *et al.*, 2005). In regions where blood-sucking insects occur in large numbers, especially tabanids, these may transmit the virus mechanically. Viral antigens and proviral DNA can be identified in semen, milk and colostrum of infected animals (Dus Santos *et al.*, 2007; **Romero *et al.*, 1983**). Natural transmission through these secretions, however, has not clearly been demonstrated.~~

~~Although several species can be infected by inoculation of the virus, natural infection occurs only in cattle (*Bos taurus* and *Bos indicus*), water buffaloes, and capybaras. Sheep are very susceptible to experimental inoculation and develop tumours more often at a younger age than cattle. A persistent antibody response can also be detected after experimental infection in deer, rabbits, rats, guinea-pigs, cats, dogs, sheep, rhesus monkeys, chimpanzees, antelopes, pigs, goats and buffaloes.~~

BLV was probably present in Europe during the 19th century, from where it spread to the American continent in the first half of the 20th century. It may then have spread back into Europe and been introduced into other countries for the first time by the import of cattle from North America (Johnson & Kaneene, 1992). Despite the global BLV presence, a number of countries, particularly in Western Europe, are recognised as officially free from BLV infection due to continued surveillance programmes.

2. Nature and classification of the pathogen

BLV, a delta retrovirus, is the causative agent of EBL. As with all retroviruses, BLV integrates into the host's cell genome (provirus) thereby leading to a lifelong infection. Although several species can be infected by inoculation of the virus, natural infection occurs only in cattle (*Bos taurus* and *Bos indicus*), water buffaloes, and capybaras. Sheep are very susceptible to experimental inoculation and develop tumours more often at a younger age than cattle. A persistent antibody response can also be detected after experimental infection in deer, rabbits, rats, guinea-pigs, cats, dogs, sheep, rhesus monkeys, chimpanzees, antelopes, pigs, goats and buffaloes.

3. Zoonotic potential and biosafety and biosecurity requirements

Several studies have been carried out in an attempt to determine whether BLV causes disease in humans, especially through the consumption of milk from infected cows (Burmeister *et al*., 2007; Perzova *et al*., 2000). There was speculation about the involvement in BLV in human breast cancers (Buehring *et al.,* 2015), however such findings were not confirmed by other researchers (Gillet & Willems, 2016; Zhang *et al.,* 2016). Hence with no conclusive evidence of zoonotic transmission, it is ~~now~~ generally thought that BLV is not a hazard to humans. BLV is present in peripheral blood mononuclear cells (PBMC) and in tumour cells. Virus is also found in the cellular fraction of various body fluids (nasal and bronchial fluids, saliva, milk). Natural transmission depends on the transfer of infected cells, for example during parturition. Artificial transmission occurs, e.g. by blood-contaminated needles, surgical equipment, gloves used for rectal examinations. Lateral transmission in the absence of these contributory factors is usually slow (Monti *et al.*, 2005). In regions where blood-sucking insects occur in large numbers, especially tabanids, these may transmit the virus mechanically. Viral antigens and proviral DNA can be identified in semen,

milk and colostrum of infected animals (Dus Santos *et al.*, 2007; Romero *et al.*, 1983). Natural transmission through these secretions, however, has not clearly been demonstrated.

4. Differential diagnosis

There may be several causes of lymphosarcomas in cattle, but the only definitely known cause is BLV. The term sporadic bovine leukosis (SBL) is usually reserved for young animals (calves) as well as cutaneous and thymic types of lymphoma, which is defined by the age of the animal affected and the distribution of the tumours. The cause of SBL is not known. There may also be lymphosarcomatous conditions that do not fall into either the SBL or EBL categories, i.e. adult multicentric lymphoma with sporadic occurrence of unknown aetiology. Only lymphomas caused by BLV infection should be termed leukosis or enzootic bovine leukosis (Gillet *et al.*, 2007).

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of enzootic bovine leukosis and their purpose

Method	Purpose					
	Population freedom from infection[(b)]	Individual animal freedom from infection prior to movement[(a) (b)]	Contribute to eradication policies[(b)]	Confirmation of clinical cases[(a) (b)]	Prevalence of infection – surveillance[(b)]	Immune status in individual animals or populations post-vaccination
Detection and identification of the agent[(c)]						
Virus isolation[(c)]	–	+	–	+	–	–
PCR	+	++	+	++	+	–
Detection of immune response						
AGID	++~~+~~	++~~+~~	++~~+~~	++~~+~~	++~~+~~	–
ELISA	+++	+++	+++	+++	+++	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; AGID = agar gel immunodiffusion; ELISA = enzyme-linked immunosorbent assay.
[(a)] See Appendix 1 of this chapter for justification table for the scores given to the tests for this purpose.
[(b)] See Appendix 2 of this chapter for justification table for the scores given to the tests for this purpose.
[(c)] A combination of agent identification methods applied on the same clinical sample is recommended.

1. Detection and identification of the agent

BLV is an exogenous retrovirus and belongs to the genus *Deltaretrovirus* within the subfamily *Orthoretrovirinae* and the family *Retroviridae.* It is structurally and functionally related to the primate T-lymphotropic viruses 1, 2 and 3 (STLV-1, -2, -3) and human T-lymphotropic virus[es] 1 and 2 (HTLV-1 and -2). The major target cells of BLV are B lymphocytes (Beyer *ET AL.*, 2002; Gillet *et al.*, 2007). The virus particle consists of two positive sense single-stranded RNA that encode for the nucleoprotein p12, capsid (core) protein p24, transmembrane glycoprotein gp30, envelope glycoprotein gp51, and several enzymes, including the reverse transcriptase. Proviral DNA, which is generated after reverse transcription of the viral genome, integrates randomly into the DNA of the host cell where it persists without constant production of viral progeny. When infected cells are cultured *in-vitro*, usually by co-cultivation of PBMC with indicator cells, infectious virus is produced, most readily through stimulation with mitogens.

1.1. Virus isolation

PBMC from 1.5 ml of peripheral blood in ethylene diamine tetra-acetic acid (EDTA) are separated on a ficoll/sodium metrizoate density gradient, cultured with 2×10^6 fetal bovine lung (FBL) cells, and subsequently grown for 3–4 days in 40 ml of minimal essential medium (MEM) containing 20% fetal calf serum. Virus causes syncytia to develop in the cell monolayer of the FBL cells. Short-term cultures can be prepared by culturing PBMC in the absence of FBL cells in 24-well plates for 3 days (Miller *et al.*, 1985). The p24 and gp51 antigens can subsequently be detected in the supernatant of the cultures by

~~radio-immunoassay (RIA),~~ enzyme-linked immunosorbent assay (ELISA), immunoblot or agar gel immunodiffusion (AGID), and the presence of the BLV particles or BLV-provirus can be demonstrated by electron microscopy and by PCR, respectively.

1.2. Nucleic acid detection by polymerase chain reaction (PCR)

The use of the polymerase chain reaction (PCR) to detect BLV provirus has been described by various workers (Fechner *et al.*, 1996; Rola-Łuszczak *et al.,* 2013). Primers constructed to match the *gag, pol* and *env* regions of the genome have all been used with variable success. So far, real-time PCR is the most rapid and sensitive method. The methods described are conventional PCRs based on primer sequences from the *env* gene, coding for gp51 and a real-time method based on detection of the pol gene. The ~~technique is restricted to those laboratories that have the facilities for molecular virology, and the~~ usual precautions and control procedures must be in place to ensure validity of the test results (see Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals*).

PCR is mainly used as an adjunct to serology for confirmatory testing. The detection of BLV infection in individual animals by PCR can be useful in the following circumstances:

i) Young calves with colostral antibodies;

ii) Tumour cases, for differentiation between sporadic and infectious lymphoma;

iii) Tumour tissue from suspected cases collected at slaughterhouses;

iv) New infections, before development of antibodies to BLV;

v) Cases of weak positive or uncertain results in ELISA;

vi) The systematic screening of cattle in progeny-testing stations (before introduction into artificial insemination centres);

vii) Cattle used for production of vaccines, ensuring that they are BLV free.

1.2.1. Sensitivity and reliability of the method

i) *Analytical sensitivity*

Although the PCR assay has a theoretical sensitivity of one target molecule, in practice the analytical sensitivity may be approximately five to ten target molecules of proviral DNA.

ii) *False-positive results*

The high sensitivity of the nested PCR may cause problems of false-positive results due to contamination between samples (Belak & Ballagi-Pordany, 1993). To minimise this, several special procedures are adopted throughout the protocol, such as the use of laminar air-flow hoods, separate rooms for different steps of the procedure, new gloves or the use of special tube openers for each individual assay and negative controls (e.g. water blanks).

iii) *False-negative results*

It should be noted that only a small proportion of the PBMC can be infected, thus limiting the sensitivity of the assay. The presence of inhibitory substances in some samples may cause false-negative results. To ~~detect~~ confirm this, at least one ~~positive~~ internal amplification control is used on each sample ~~every test run~~. In addition, assays can use internal controls (mimics) that are added to each sample. ~~The mimic is a modified target molecule that is amplified with the same primers as the real target, but that generates a PCR product with different size, which can be visualised by agarose gel electrophoresis. The mimic is added at a low concentration that favours the amplification of the real target (& Belak, 1996). However, it is possible for the mimic to compete with the true target. It may therefore be necessary to analyse each sample with or without the mimic.~~

1.2.2. Sample preparation

PBMC are separated from EDTA blood samples by using a density separation centrifugation method. Alternatively buffy coat may be used, or even whole blood, e.g. where samples have been frozen.

Tumours or other tissues should be homogenised to a 10% suspension.

1.2.3. DNA extraction

Purification of total DNA is a prerequisite for achieving optimal sensitivity. Various purification methods are commercially available and suitable for the assay.

Special precautions should be taken during all steps to minimise the risk of contamination (Belak & Ballagi-Pordany, 1993).

1.2.4. Nested PCR procedure

Several PCR protocols for the detection of BLV provirus sequences have been published; as an example, an assay developed by Fechner *et al.* (1996) is described in detail. The BLV region used as target is the env gene, encoding for gp51 protein. The sequence used for designing the primers is available from GenBank, accession No. K02120.

1.2.4.1. Method developed by Fechner *et al.* (1996)

i) Primer design and sequences

Oligo	Env-Sequence (5'–3')	Position
BLV-env-1	TCT-GTG-CCA-AGT-CTC-CCA-GAT-A	5032–5053
BLV-env-2	AAC-AAC-AAC-CTC-TGG-GAA-GGG	5629–5608
BLV-env-3	CCC-ACA-AGG-GCG-GCG-CCG-GTT-T	5099–5121
BLV-env-4	GCG-AGG-CCG-GGT-CCA-GAG-CTG-G	5542–5521

The BLV-env-1/BLV-env-2 PCR-product size is 598 bp. The BLV-env-3/BLV-env-4 PCR-product size is 444 bp

ii) Reaction mixtures

Reaction solutions are mixed (except DNA sample) before adding to the separate reaction tubes. One negative control (double distilled H_2O) per five samples, and one positive control should be added. Total volumes of mixtures are calculated by multiplying the indicated volumes by the total number of samples, including controls, plus one.

The first PCR can be performed using a 50 µl reaction volume. For one reaction, the assay is optimised to 5 µl (10×) PCR buffer, 20 µl DNA (~1 µg of DNA), 1.25 µl each of the env-specific primers BLV-env-1 and BLV-env-2 (20 pmol/µl), 0.15 dNTP (each 25 mM), 3 µl $MgCl_2$ (25 mM), 0.25 µl Taq polymerase (1.25U), and 19.1 µl of distilled H_2O. The reaction follows the temperature profile: 2 minutes denaturation at 94°C; 30 cycles of 30 seconds at 95°C, 30 seconds at 58°C and 60 seconds at 72°C; followed by 4 minutes at 72°C.

The nested PCR can be performed using a 50 µl reaction volume. For one reaction, the assay is optimised to 3 µl PCR product of the first PCR, 5 µl (10×) PCR buffer, 1.25 µl each of the env-specific primers BLV-env-3 and BLV-env-4 (20 pmol/µl), 0.15 dNTP (each 25 mM), 0.25 µl Taq polymerase (1.25U), and 36.1 µl of distilled H_2O. The reaction follows the temperature profile: 2 minutes denaturation at 94°C; 30 cycles of 30 seconds at 95°C, 30 seconds at 58°C and 60 seconds at 72°C; followed by 4 minutes at 72°C.

iii) Laboratory procedure

Mix PCR-reagents for the first or nested PCR and use separate gloves or tube openers for each individual tube when adding the DNA samples. Put the samples on ice. Heat the thermoblock to 94°C. Place samples in the thermoblock and start the PCR-programmes accordingly.

iv) Agarose gel electrophoresis

Load approximately 10 µl of the nested PCR products with 20 µl loading buffer on a 2% agarose gel containing 0.01% ethidiumbromide (or alternative, safer stains for visualising PCR products). ~~Using 0.5 × Tris/borate/EDTA (TBE) buffer, electrophoresis is performed with 90 mA for 2 hours.~~ To control the size of the amplification products, a 100 bp ladder is recommended. ~~Analysis of PCR products is done by UV illumination.~~

v) Interpretation of the results

a) Positive samples should have PCR products of the expected size (444 bp), similar to the positive control.

b) Negative samples should have no PCR products of the expected size (444 bp).

c) The assay must be repeated if the positive control remained negative, or if the negative water controls are positive.

vi) Confirmatory testing

For confirmatory identification, the PCR products can be sequenced, hybridised to a probe, or analysed by restriction fragment length polymorphism (RFLP) analysis (Fechner *et al.,* 1997).

1.2.5. Real-time PCR procedure

Several real-time PCR protocols for the detection of BLV provirus sequences have been published (Jaworski *et al.*, 2018; Pluta *et al.*, 2024). The Rola-Łuszczak *et al.* (2013) method is described in detail here as an example. The BLV region used as target is the pol gene. The sequence used for designing the primers is available from GenBank, accession No. K02120.

1.2.5.1. Method developed by Rola-Łuszczak *et al.* (2013)

i) Primer design and sequences

Oligo	Pol-Sequence (5'–3')	Position
MRBLVL	CCT-CAA-TTC-CCT-TTA-AAC-TA	2321-2340
MRBLVR	GTA-CCG-GGA-AGA-CTG-GAT-TA	2421-2440
MRBLV probe	6FAM GAA-CGC-CTC-CAG-GCC-CTT-CA BHQ1	2341-2360

PCR-product size: 120 bp

ii) Reaction mixtures

Reaction solutions are mixed (except DNA sample) before adding to the separate reaction tubes. One negative control (double-distilled H_2O) per five samples and one positive control should be added. Total volumes of mixtures are calculated by multiplying the indicated volumes by the total number of samples, including controls, plus one.

The reaction mixture for each PCR test contains 12.5 µl of 2 × PCR master mix, 0.4 µM of each of the primers and 0.2 µM of the specific BLV probe and 500 ng of extracted genomic DNA, using a final reaction volume of 25 µl. Amplification was performed according to the following conditions: initial incubation and polymerase activation at 95°C for 15 minutes, denaturation at 94°C for 60 seconds, annealing at 60°C for 60 seconds through 50 cycles.

iii) Laboratory procedure

Mix PCR-reagents and use separate gloves or tube openers for each individual tube when adding the DNA samples. Put the samples on ice. Place samples in the thermoblock and run at appropriate parameters.

iv) Interpretation of the results

a) Test controls: all controls should give the expected results with positive control falling within the designated range and both the negative control (NC) and no template control (NTC) should have no Ct (threshold cycle) values.

b) Test samples

1) Positive ~~samples are those with Ct~~ result: any sample that has a ~~cycle threshold (~~Ct~~)~~ value less than or equal to 40~~.95~~ is regarded as positive.

~~b~~2) Negative~~ samples are those without a~~ result: any sample that shows no Ct value or those with a value greater than 40~~.96~~ is regarded as negative.

c) The assay must be repeated if the positive control ~~remained~~ displays as negative, or if the negative ~~water~~ no template controls ~~are positive~~ have Ct values. Samples on the borderline of cut-off (i.e. Ct of 40) should be retested and confirmed.

~~v~~d) Confirmatory testing ~~For confirmatory identification~~; if required, the PCR products can be sequenced.

2. Serological tests

Infection with the virus in cattle is lifelong and gives rise to a persistent antibody response. Antibodies can first be detected 3–16 weeks after infection. Maternally derived antibodies may take up to 6 or 7 months to disappear. There is no way of distinguishing passively transferred antibodies from those resulting from active infection. Active infection, however, can be confirmed by the detection of BLV provirus by the PCR. Passive antibody tends to protect calves against infection. During the periparturient period, cows may have serum antibody that is undetectable by AGID because of an antibody shift from the dam's circulation to her colostrum. Therefore, when using the AGID test, a negative test result on serum taken at this time (2–6 weeks pre- and 1–2 weeks post-partum) is not conclusive and the test should be repeated. However, the AGID can be performed at this stage with first-phase colostrum.

The antibodies most readily detected are those directed towards the gp51 and p24 of the virus. Most AGID tests and ELISAs in routine use detect antibodies to the glycoprotein gp51, as these appear earlier. Methods of performing these tests have been published (Dimmock *et al.,* 1987; European Commission, 2009). ELISAs are usually more sensitive than the AGID tests.

~~Weak positive and negative~~ WOAH reference sera for use in ELISA ~~are~~ is available in freeze-dried irradiated form, from the ~~WOAH~~ national reference laboratory in Germany (FLI Reims)[1]. The ~~calibration of these sera is based on the accredited~~ WOAH reference serum, named E05, ~~which~~ has been validated against the former reference serum E4 by different AGID and ELISAs.

2.1. Enzyme-linked immunosorbent assay

Either an indirect or blocking ELISA may be used. Assays based on both of these are available commercially; different kits may be required for serum or milk samples. Some ELISAs are sufficiently sensitive to be used with pooled samples. ELISAs are carried out in solid-phase microplates. BLV antigen is used to coat the plates either directly or by the use of a capture polyclonal or monoclonal antibody (MAb). The antigen is prepared from the cell culture supernatant of persistently BLV-infected cell lines. Fetal lamb kidney (FLK) cells are most commonly used for commercial tests (Van der Maaten & Miller, 1976). Since 2004, a new BLV-producing cell line, PO714, which is free from other viral infections and contains a provirus of the Belgian subgroup, has been made available (Beier *et al.*, 2004). The antigen is used at a predetermined dilution (e.g. 1/10) in phosphate buffered saline. In kit form, the plates are sometimes purchased precoated. Some preservatives may be added to milk samples to prevent souring. Preserved samples will not usually deteriorate significantly if stored for up to 6 weeks at 4°C.

2.1.1. Blocking enzyme-linked immunosorbent assay – serum ELISA

The following method is suitable for antibody detection in single or pooled serum samples.

2.1.1.1. Test procedure

i) Coating the plate

All wells are coated with BLV antibody, prediluted in coating buffer (100 µl/well), the plate is sealed and incubated for 18 hours at 4°C. A wash cycle (standard wash) is performed, which is three washes filling wells to the top, with a 3-minute soak in between each wash, and then the plate is blotted. BLV antigen is added, prediluted in wash buffer (100 µl/well), the plate is sealed and incubated for 2 hours at 37°C. A standard wash cycle is performed.

ii) Preparation and addition of samples and controls

The positive and negative control sera are prediluted (1/2) in wash buffer and the solution is added to four wells per control (100 µl/well). For testing pooled samples, 80 sera may be bulked then diluted (1/2) using wash buffer and the solution is added to two wells (100 µl/well) per sample. Single samples should be diluted 1/100 using wash buffer and the solution added to two wells (100 µl/well) per sample. After plating out the samples, the plate

[1] https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3

is sealed and incubated for 18 hours at 4°C. A brief wash is performed by filling the wells and immediately emptying them.

iii) Preparation and addition of conjugates and substrate

Prediluted biotinylated antibody is added (100 µl/well) to all wells – predilute using wash buffer + 10% fetal calf serum – the plate is sealed and incubated on a rocking table for 1 hour at 37°C. A standard wash is performed as described earlier. The peroxidase-conjugated avidin is prediluted in wash buffer and the solution is added to all wells (100 µl/well). The plate is sealed and incubated on a rocking table for 30 minutes at 37°C. A standard wash is performed. 100 µl orthophenylamine diamine substrate is added to all wells, the plate is covered and left in the dark for 9 minutes. The reaction is stopped with 100 µl of 0.5 M sulphuric acid per well.

2.1.1.2. Reading and interpretation of results

The plate reader is blanked on air and the absorbance is read at 490 nm. For dual wave-length readers a reference filter between 620 nm and 650 nm is used. Results are read within 60 minutes after the addition of stop solution.

The absorbance of the negative control should be about 1.1 ± 0.4; if the absorbance is below 0.7, the colour development time in step iii above (preparation and addition of conjugates and substrate) should be increased. Conversely, the time should be shortened if the absorbance is above 1.5. The absorbance of the positive control should be less than the absorbance of the negative control × 0.25.

A sample is positive when the absorbance of each of the two test wells is identical with or less than the mean absorbance of the four negative wells × 0.5.

A sample is negative when the absorbance of each of the two test wells is identical with or higher than the mean absorbance of the four negative control wells × 0.65.

For samples giving values between the absorbance of the negative control × 0.5 and × 0.65, it is recommended to retest the animal, using a sample taken 1 month later.

2.1.1.3. Sensitivity of the enzyme-linked immunosorbent assay

The sensitivity of pooled milk ELISAs can be evaluated using the WOAH ~~weak positive and~~ reference sera, E05. Assays should give a positive result on ~~WOAH reference sera~~ E05 diluted in negative milk 250 times more than the number of individual milks in the pool (EU Directive 88/406). For example, for pools of 60 milks, E05 should be diluted 1/250 × 60 = 1/15,000. For individual milk samples the positive WOAH reference sera E05 diluted 1/250 in negative milk must be positive.

Where pooled serum samples are tested, the WOAH reference serum E05 must test positive at a dilution 10 times higher than the number of individual animals in the pool. For example, for a pool of 50 individual samples, the WOAH reference serum diluted 1/500 in negative serum should give a positive result. In assays where serum samples are tested individually, WOAH reference serum E05 diluted 1/10 must be positive.

For some ELISA kits, a positive result is not recommended as the sole determinant of individual animal disease status; verification by a secondary method is recommended.

2.1.2. Indirect enzyme-linked immunosorbent assay – Milk ELISA

The following method is suitable for antibody detection in pooled milk samples.

2.1.2.1. Controls

Strong positive, weak positive, negative milk and diluent controls should be included in each assay. A strong positive control should be prepared by diluting the WOAH reference serum E05 1/25 in negative milk. A weak positive control should be prepared by diluting, in negative milk, the WOAH reference serum E05 25 times the number of individual milk samples in the pool under test. The milk used for diluting the WOAH reference serum controls should be unpasteurised, cream free and preserved.

2.1.2.2. Example test procedure

i) Milk samples must be stored, undisturbed in a refrigerator until a definite cream layer has formed (24–48 hours), or alternatively, centrifuged at 2000 rpm for 10 minutes, the cream layer should be removed prior to testing.

ii) A BLV antigen and a control negative antigen are precoated in alternate columns in the plate. 100 µl of test sample is added to 100 µl wash buffer in the plate to make a 1/2 dilution, adding to two control antigen wells and two BLV antigen wells.

iii) The plate is sealed and mixed on a shaker.

iv) The plate is incubated between 14 and 18 hours at 2–8°C.

v) 300 µl per well of wash diluent is added and discarded, and then 200 µl per well wash diluent is added, shaken for 10 seconds and discarded. Finally, 300 µl of wash diluent is added and soaked for 3 minutes and discarded.

vi) 200 µl per well of anti-bovine IgG-horseradish peroxidase affinity-purified conjugate diluted in wash diluent is added and the plate is incubated for 90 minutes at room temperature.

vii) The plate is washed by adding 300 µl of wash diluent per well; this is then discarded and a further 300 µl of wash diluent is added. This is left to soak for 3 minutes and discarded. Steps ~~vi and~~ vii ~~are~~ is repeated.

viii) 200 µl of ABTS (2,2'-azino-bis-[3-ethylbenzothiazoline-6-sulphonic acid]) substrate (prewarmed to 25°C) is added and the plate is incubated for 20 minutes at room temperature in the dark. The reaction may be stopped by adding 50 µl of stopping solution.

2.1.2.3. Reading and interpreting the results

The plate reader is blanked on air and the absorbance is read at 405 nm. All microplate wells must be read within 2 hours of addition of stopper. The absorbance readings of the wells containing negative antigen are subtracted from the readings of wells containing the positive antigen. The two net absorbance values for each test sample should be averaged. The same applies for the replicate weak positive controls. Replicates should be within 0.1 absorbance units of each other.

For the test to be considered valid, the averaged net absorbance of the weak positive (WP) controls should be 0.2–0.6 absorbance units. The net absorbance of the strong positive control should be >1.0 absorbance units. The net absorbance of the negative and diluent controls should be less than the lower limit of the inconclusive range.

Assuming that the above criteria are met:

i) Test samples are positive if their net absorbance value is greater than or equal to that of the WP control.

ii) Test samples are inconclusive if their net absorbance value is 75% or less of the net absorbance value of the WP control:

i.e. if the WP control net absorbance = 0.40

then the lower limit of the inconclusive range = 0.40 × 0.750 = 0.30

the inconclusive range in this example would be 0.30–0.39

and samples of ≥0.40 are considered positive.

iii) Test samples are negative if their net absorbance value is less than the lower limit of the 'inconclusive' range (<0.30 in the example).

2.2. Agar gel immunodiffusion

The AGID test is a specific, but not very sensitive, test for detecting antibody in serum samples from individual animals. It is, however, unsuitable for milk samples (except first colostrums) because of lack of specificity and sensitivity. The AGID is simple and easy to perform and has proven to be highly useful and efficient as a basis for eradication schemes. Reference sera are included with commercial AGID test kits.

2.2.1. Agar gel

A 0.8–1.2% solution of agar or agarose is prepared in 0.2 M Tris buffer, pH 7.2, with 8.5% NaCl. One method of preparing the agar is to dissolve 24.23 g of Tris methylamine in 1 litre of distilled water and adjust to pH 7.2 with 2.5 M HCl. Sodium chloride (85 g) is dissolved in 250 ml Tris/HCl and made up to 1 litre. Agarose (8 g) is added and the mixture is heated in a pressure cooker or autoclave at 4.55 kg/sq. cm for 10 minutes. The mixture is dispensed in 15 ml aliquots, which can be stored at 4°C for up to approximately 6 weeks.

2.2.2. Antigen

The antigen must contain specific glycoprotein gp51 of BLV. Antigen is prepared in a suitable cell culture system, such as permanently infected FLK cell monolayers. The cells used to produce the BLV antigen should be free from noncytopathic bovine viral diarrhoea virus and of bovine retroviruses, bovine immunodeficiency-like virus (lentivirus), and bovine syncytial virus (spumavirus). After 3–4 days' culture at 37°C, the growth medium is replaced with maintenance medium. The cells are harvested after 7 days using standard trypsin/versene solution. The cell suspension is centrifuged at 500 ***g*** for 10 minutes. Cells are resuspended in growth medium; 30% of the cells are returned to the culture vessel and the remainder is discarded. All culture supernatants are collected. The supernatants are concentrated 50–100-fold by available methods. This can be done by concentration in Visking tubing immersed in polyethylene glycol, or by precipitation with ammonium sulphate followed by ultrafiltration, or by precipitation in polyethylene glycol followed by desalting and size separation on a polyacrylamide bead column. The antigen contains gp51 predominantly, but may also contain p24.

The antigen may be standardised for glycoprotein gp51 by titration against the WOAH reference serum E05 as follows: a twofold dilution of the antigen preparation is made. The highest dilution that, when tested against undiluted WOAH reference serum E05, gives a precipitation line equidistant between the antigen and the serum will contain one unit. Two units of antigen are used in the test.

2.2.3. Known positive control serum

The positive control serum comes from a naturally or experimentally infected animal (cattle or sheep). The precipitation line formed should be a sharp distinct line midway between the antigen and the control serum wells. A dilution of the control positive serum that gives a weak positive result should be included in the test as an indicator of the test's sensitivity.

2.2.4. Known negative control serum

Serum from uninfected animals (cattle, sheep) is used.

2.2.5. Test sera

Sera from any species of animal are suitable.

2.2.6. Test procedure

i) The agar is melted by heating in a water bath and poured into Petri dishes (15 ml per Petri dish of diameter 8.5 cm). The poured plates are allowed to cool at 4°C for about 1 hour before holes are cut in the agar. A punch is used that cuts a hexagonal arrangement of six wells round a central well. Various dimensions of wells can be used; one satisfactory pattern has been produced using wells of 6.5 mm in diameter with 3 mm between wells. For best results, agar plates are used the same day that they are poured and cut.

ii) Antigen is placed in the central wells of the hexagonally arranged patterns. Test sera are placed alternately with positive control serum in the outer wells. There should be one control pattern per plate with positive control serum, weak positive control serum and negative control serum in the place of test sera.

iii) The test plates are kept at room temperature (20–27°C) in a closed humid chamber, and read at 24, 48 and 72 hours.

iv) *Interpretation of the results:* A test serum is positive if it forms a specific precipitation line with the antigen and forms a line of identity with the control serum. A test serum is negative if it does not form a specific line with the antigen and if it does not bend the line of the control serum. Nonspecific lines may occur; these do not merge with or deflect the lines formed by the positive control. A test serum is a weak positive if it bends the line of the control serum towards the antigen well without forming a visible precipitation line with the antigen; the reaction is inconclusive if it cannot be read either as negative or positive. A test is invalid if

the controls do not give the expected results. Sera giving inconclusive or weak positive results can be concentrated and retested.

C. REQUIREMENTS FOR VACCINES

Despite advances in research on experimental vaccines there is, as yet, no commercially available vaccine for the control of EBL.

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NB: There are WOAH Reference Laboratories for enzootic bovine leukosis (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3). Please contact the WOAH Reference Laboratory for any further information on diagnostic tests and reagents for enzootic bovine leukosis

NB: FIRST ADOPTED IN 1991. MOST RECENT UPDATES ADOPTED IN 2018.

559 **Appendix 1: Enzootic bovine leukosis**

560 **Intended purpose of test: individual animal freedom from infection prior to movement; confirmation of clinical cases**

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
Virus isolation + Bovine	PBMC Cell cultures inoculated with PBMC	Reference tests were nested PCR and ELISA Two out of six cell cultures were positive by CPE (DSe=33%)	N/A: this test is not recommended for routine diagnosis but in instances where the virus isolate is desirable for downstream applications e.g. *in vivo*	See reference	The isolated virus may be used for additional *in-vitro* or *in-vivo* characterisation or applications	1) Slow, time-consuming, can be difficult and expensive 2) Selection of different susceptible cell type may be critical 3) May only be suitable for certain cases e.g. animals with persistent lymphocytosis	Khudhair *et al.* (2021)

Appendix 2: Enzootic bovine leukosis

Intended purpose of test: population freedom from infection; individual animal freedom from infection prior to movement; contribute to eradication policies; confirmation of clinical cases and prevalence of infection – surveillance

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
PCR Nested PCR Real time PCR + Bovine	Genomic DNA from PBMC, whole blood, tumour or tissue sample as lymph node and spleen	Nested PCR: reference tests were ELISA and AGID. Nested PCR showed 100% DSe, while ELISA and AGID showed DSe 82.7% and 71.2%, respectively	52 BLV infected, nested PCR positive cattle	See references	1. Direct, sensitive and rapid detection of infection before development of antibodies to BLV 2.Detects BLV during early phase of infection or in the presence of colostrum antibodies	1. In most cases requires DNA preparation 2. False-negative in case of low proviral load and the presence of PCR inhibitors 3. Possibilities of carry-over cross contamination	Fechner *et al.* (1996)
		Real-time PCR: reference test was ELISA. DSe varied from 56% to 98%, depending on the technique used	56 serologically positive cattle, selected from infected herds from seven countries		1) As with nested PCR 2) Possibility to quantify the proviral load 3) Suitable for high through-put testing 4) Less risk of cross contamination	1) As with points 1 and 2 for nested PCR 2) Expensive equipment and reagents e.g. probes may be difficult to source in LMICs	Jaworski *et al.* (2018)
		DSe varied from 98.4% to 99.4%, depending on the technique used	317 serologically positive cattle				Yoneyama *et al.* (2021)
AGID ++ Bovine	Serum, antibodies, p24, gp51	Reference test was western blot. DSe for AGID was 95.9% while DSp was 95.7%	399 cattle from 19 herds tested by tested by AGID and western blot	See reference	1) Excellent specificity 2) Technically simple. 3) Not expensive	1) Low sensitivity (Ab detected after 4–8 weeks a.i.) 2) Time consuming i.e. results typically read after 3 days 3) Interpretation may be subjective – two readers suggested	Reichel *et al.* (1998)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
ELISA +++ Bovine	Serum, milk, antibodies p24, gp51	When reference test was PCR relative DSe was 91.0 (CI: 87.8–94.3) and DSp was 83.9 (CI: 77.3–90.5) when Bayesian model was employed	524 cattle from six herds including: 1) herd with no serological evidence of BLV infection 2) herd with more than 50% of cattle seropositive 3) herd with less than 5% of adult cattle seropositive 4) three farms were of unknown BLV status	See references	1) High sensitivity (Abs detected after 2–4 weeks a.i.), good specificity 2) Quantitative readout 3) Test is performed in a few hours 4) Technically simple 5) Suitable for high-throughput screening	1) Lower specficity in comparison to the AGID, false positive can occur	Monti *et al.* (2005)
		Reference test was PCR. Relative DSe was 97.2% and DSp was 97.5%	339 dairy cattle tested by PCR. 178 were positive and 161 were negative				Trono *et al.* (2001)
		Relative DSe was 100% (CI: 87–100) and DSp varied from 95% (CI :83–99) to 100% (CI :87–100)	116 dairy cattle from five herds including 54 BLV positive and 62 negative. Serological status was estimated based on five ELISAs				Kuczewski *et al.* (2018)

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CHAPTER 3.4.13.

THEILERIOSIS IN CATTLE (INFECTION WITH *THEILERIA ANNULATA*, *T. ORIENTALIS* AND *T. PARVA*)

SUMMARY

***Description and importance of the disease**: Tick-transmitted* Theileria *parasites of cattle are a major constraint to the improvement of the livestock industry in large parts of Europe, Africa and Asia.* Theileria annulata, T. orientalis *and* T. parva*, the most economically important species, are responsible for mortality and losses in production. Bovine theileriosis is generally controlled by the use of acaricides to kill ticks, but this method is not sustainable. Acaricides are expensive, they cause environmental damage, and over time ticks develop resistance to them requiring newer acaricides to be developed. More sustainable and reliable methods for the control of theileriosis that deploy a combination of strategic tick control and vaccination are desirable. However, these are yet to be successfully applied on a large scale in endemic areas.*

***Detection and identification of the agent:** Diagnosis of a variety of disease syndromes caused by the parasites is principally based on clinical signs, knowledge of disease and vector distribution, and identification of parasites in Giemsa-stained blood and lymph node smears. The presence of multinucleate intra-cytoplasmic and free schizonts, in lymph node biopsy smears, is a characteristic diagnostic feature of acute infections with* T. parva *and* T. annulata*. Animals infected with* T. parva *show enlarged lymph nodes, starting with the parotid lymph node, fever, a gradually increasing respiratory rate, dyspnoea and occasional diarrhoea. Post-mortem lesions observed are pulmonary oedema with froth in the trachea, enlargement of lymph nodes and spleen, haemorrhages in internal organs, abomasal erosions, the presence of parasitised lymphocytes and lympho-proliferative infiltrations in visceral tissues. The gross pathology caused by schizonts of* T. annulata *resembles that of* T. parva*, while the piroplasm stages may also be pathogenic, causing anaemia and jaundice. For* T. annulata*, the first lymph nodes involved are the pre-crural ones as a result of the predilection sites of the vector ticks. Conversely,* T. orientalis *is not a lymphoproliferative disease but affects animals mainly via the intra-erythrocytic phase. This results in clinical signs of abortion, anaemia, fever, jaundice and lethargy.*

***Molecular-based tests:** In addition, molecular diagnostic tests, particularly those based on the polymerase chain reaction and reverse line blot hybridisation are proving to be powerful tools for detecting theileria parasites in the vertebrate and invertebrate hosts, characterising species and parasite polymorphisms, defining population genetics and generating epidemiological data.*

***Serological tests:** The most widely used diagnostic test for* Theileria *species has been the indirect fluorescent antibody (IFA) test. For the IFA test, both schizont and piroplasm antigens may be prepared on slides or in suspension and preserved by freezing at ≤ –20°C, except in the case of the piroplasm suspension, which is stored at 4°C. Test sera are diluted with phosphate-buffered saline and incubated with the antigen in suspension, and anti-bovine immunoglobulin conjugate is then added. Using the test as described, the fluorescence is specific to the causative agent. The IFA test*

is sensitive, fairly specific, and usually easy to perform. However, because of the problems of cross-reactivity among some Theileria *species, the test has limitations for large-scale surveys in areas where species distribution overlaps. The IFA test for* T. parva *does not distinguish among the different immunogenic stocks. An indirect enzyme-linked immunosorbent assay for* T. parva *and* T. mutans*, based on recombinant parasite-specific antigens, has demonstrated higher sensitivity and specificity. The IFA test for* T. orientalis *does not differentiate between pathogenic and non-pathogenic strains.*

Requirements for vaccines: *Reliable vaccines of known efficacy have been developed for* T. parva *and* T. annulata*. For* T. annulata*, the vaccine is prepared from schizont-infected cell lines that have been isolated from cattle and attenuated during* in vitro *culture. The vaccine must remain frozen until shortly before administration. Vaccination against* T. parva *is based on a method of infection and treatment in which cattle are given a subcutaneous dose of tick-derived sporozoites and a simultaneous treatment with a long-acting tetracycline formulation. This treatment results in a mild or inapparent East Coast fever reaction followed by recovery. Recovered animals demonstrate a robust immunity to homologous and, to varying degrees, heterologous challenge, which usually lasts for the lifetime of an animal. In endemic areas characterised by high transmission intensities, immunisation of animals with a stock(s) engendering a broad-spectrum immunity is desirable to cover a range of immunologically distinct* T. parva *strains that exist in the field. Immunised animals may become carriers of the immunising parasite stock. Safety precautions must be taken in the preparation and handling of* T. parva *vaccines to protect the workers and to avoid contamination of the stabilates. Consideration should also be given to the risk of introducing new isolates into an area where they may then become established through a carrier state.*

A. INTRODUCTION

Theileriae are obligate intracellular protozoan parasites that infect both wild and domestic *Bovidae* throughout much of the world. They are transmitted by ixodid ticks, and have complex life cycles in both vertebrate and invertebrate hosts. There are a number of species of *Theileria* spp. that infect cattle; the ~~two~~ three most pathogenic and economically important are *T. parva*, ~~and~~ *T. annulata* and pathogenic strains of *Theileria orientalis*. *Theileria parva* occurs in 13 countries in sub-Saharan Africa causing three disease syndromes namely East Coast fever (ECF), Corridor disease (CD) and Zimbabwean theileriosis (January disease, JD). These syndromes differ in the origin of the parasite, ECF and JD being tick transmitted from cattle to cattle (cattle-derived) and CD from buffalo to cattle (buffalo-derived). ~~whilst~~ *Theileria annulata* (tropical/Mediterranean theileriosis) occurs in southern Europe as well as North Africa and Asia. Endemic regions of *T. annulata* and *T. parva* do not overlap extensively. *Theileria annulata* can occur in cattle, yaks, water buffalo and camels and is transmitted by ticks of the genus *Hyalomma*. Tropical theileriosis is more severe in European breeds, with a mortality rate of 40–90%, while the mortality rate in indigenous breeds of cattle from endemic areas can be as low as 3%. In Spain, *T. annulata* infections are mainly restricted to the southern and Mediterranean areas such as Menorca island, where the tick vector (*Hyalomma* sp.) is present. In northern Spain, reports of the presence of *Hyalomma* ticks are sporadic, as are associated *T. annulata* infections. However, tick distribution might change because of changes in climatic conditions.

Theileria orientalis/buffeli complex is now thought to consist of two species has at least two strains of economic importance, namely Chitose and Ikeda ~~— *T. orientalis*, occurring in the far east, and *T. buffeli* having a global~~ distribution ~~(Gubbels *et al.*, 2000; Jeong *et al.*, 2010)~~. Infection is generally subclinical; however, disease can occur in cattle depending on a number of epidemiological factors (including previous exposure to theileriae, stress or health status, and variations in ~~the species~~ strain pathogenicity, as reported recently in ~~Australia and New Zealand~~ Australasia ~~(Gebrekidan *et al.*, 2015; McFadden *et al.*, 2011)~~. The main tick vector, *Haemaphysalis longicornis* accompanied by the Ikeda strain has spread from to North the America (Hutcheson *et al.*, 2019).

Theileria taurotragi and *T. mutans* generally cause no disease or mild disease, and *T. velifera* is nonpathogenic. These last three parasites are found mainly in Africa, and overlap in their distributions, complicating the epidemiology of theileriosis in cattle. This has been further complicated by the finding of multiple related genotypes in cattle and buffalo, suggesting a genetically diverse population of *Theileria* circulating in bovids.

~~*Theileria lestoquardi*, also transmitted by *Hyalomma* ticks, is the only species of economic significance infecting small ruminants and it also occurs in north Africa, the Mediterranean basin and Asia. In sheep and goats, the morbidity rate from *T. lestoquardi* can approach 100% with a mortality rate of 46–100% in the most susceptible breeds. *Theileria uilenbergi* and *T. luwenshuni* are pathogenic ovine piroplasms described in north-western China (People's Rep. of), though *Theileria* parasites with similar sequences have been found in sheep in northern Spain and Turkey, but apparently with a low pathogenicity. *Theileria luwenshuni* has also been detected in sheep in the United Kingdom associated with clinical signs (Phipps *et al.*, 2016).~~

Some *T. parva* stocks produce a carrier state in recovered cattle, and studies using DNA markers for parasite strains have shown that *T. parva* carrier animals are a source of infection that can be transmitted naturally by ticks in the field ~~(Bishop *et al.*, 1992; Kariuki *et al.*, 1995; Marcotty *et al.*, 2002; Maritim *et al.*, 1989)~~. The severity of ECF may vary depending on factors such as the virulence of the parasite strain, sporozoite infection rates in ticks and genetic background of infected animals. Indigenous cattle in ECF-endemic areas are often observed to experience mild disease or subclinical infection, while introduced indigenous or exotic cattle usually develop severe disease.

The most practical and widely used method for the control of theileriosis is the chemical control of ticks with acaricides. However, tick control practices are not always fully effective for a number of reasons, including development of acaricide resistance, the high cost of acaricides, poor management of tick control, and illegal cattle movement in many countries. Vaccination using attenuated schizont-infected cell lines has been widely used for *T. annulata*, while for *T. parva* control, infection and treatment using tick-derived sporozoites and tetracycline is being implemented in a number of countries in eastern, central and southern Africa.

Chemotherapeutic agents such as parvaquone, buparvaquone and halofuginone are available to treat *T. parva* and *T. annulata* infections. Treatments with these agents rely on early detection of clinically affected animals and do not completely bring about eradication of theilerial infections, leading to the development of carrier states in their hosts.

~~The immune response to theileriae parasites is complicated. Cell-mediated immunity is thought to be the most important protective response in *T. parva* and *T. annulata*. In *T. parva,* the principal protective responses are mediated through killing of infected cells by bovine major histocompatibility complex (MHC) class I-restricted cytotoxic T lymphocytes. *Theileria annulata* schizonts inhabit macrophages and B cells. Innate and adaptive immune responses cooperate to protect cattle against *T. annulata* theileriosis. Intracellular parasites are mostly affected by cell mediated immunity. Infection of leukocytes with *T. annulata* activates the release of cytokines, initiating an immune response and helping to present parasite antigen to CD4+ T cells. These cells produce interferon γ (IFN γ), which activates non infected macrophages to synthesise tumour necrosis factor α (TNF α) and nitric oxide (NO), which destroy schizont- and piroplasm-infected cells. CD8+ T cells have recently been shown to recognise parasite antigens presented by the MHC and to kill infected leukocytes. B cells produce antibody that along with NO kill extracellular merozoites and intracellular piroplasms. On the other hand, overproduction of cytokines, in particular TNF-α, by macrophages generates many of the clinical signs and pathological lesions that characterise *T. annulata* theileriosis and the outcome of the infection depends upon the fine balance between protective and pathological properties of the immune system.~~

B. DIAGNOSTIC TECHNIQUES

Diagnosis of acute theileriosis is based on clinical signs, knowledge of disease, and vector distribution as well as examination of Giemsa-stained blood, lymph node and tissue impression smears. *Theileria parva* and *T. annulata* are diagnosed by the detection of schizonts in white blood cells or piroplasms in erythrocytes. The piroplasmic stage follows the schizont stage and, in both *T. parva* and *T. annulata*, it is usually less pathogenic and is thus often found in recovering or less acute cases. Infection with other *Theileria* parasites that also produce schizonts or piroplasms complicate the use of microscopy as diagnostic technique. ~~It is hoped that~~ A combination of serological ~~enzyme-linked immunosorbent assay (ELISA)~~ and polymerase chain reaction (PCR) ~~will~~ greatly enhance our present capacity to identify infected animals, thus making possible accurate surveys of *Theileria* species. ~~Eventually, the aim would be to develop these technologies for the diagnosis of all the vector-borne diseases.~~

Table 1. Test methods available for the diagnosis of theileriosis and their purpose

Method	Purpose					
	Population freedom from infection[a]	Individual animal freedom from infection prior to movement[a]	Contribute to eradication policies	Confirmation of clinical cases[a]	Prevalence of infection – surveillance[a]	Immune status in individual animals or populations post-vaccination
Detection and identification of the agent[b]						
Microscopic examination	–	++ ±	–	+++	–	–
PCR	± +	± ++	++	+++	± +	–
Detection of immune response						
IFAT	+	+++	++	–	+++	–

Method	Purpose					
	Population freedom from infection[(a)]	Individual animal freedom from infection prior to movement[(a)]	Contribute to eradication policies	Confirmation of clinical cases[(a)]	Prevalence of infection – surveillance[(a)]	Immune status in individual animals or populations post-vaccination
ELISA	+	+	++	–	+++	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; IFAT = indirect fluorescent antibody test; ELISA = enzyme-linked immunosorbent assay.
[(a)]See Appendix 1 of this chapter for justification table for the scores giving to the tests for this purpose.
[(b)]A combination of agent identification methods applied on the same clinical sample is recommended. This is because parasitaemia may fluctuate below agent detection limits in carrier animals, while antibodies may still be detected using serology. Conversely, in early infection, or in cases where a carrier is not exposed to vector and parasite challenge, the antibody titre may fall below the detection limit, while still testing positive for the agent.

1. Detection and identification of the agent

1.1. Microscopic examination

Multinucleate intralymphocytic and extracellular schizonts can be found in Giemsa-stained biopsy smears of lymph nodes, and are a characteristic diagnostic feature of acute infections with *T. parva* and *T. annulata.* Both intracellular and free-lying schizonts may be detected, the latter having been released from parasitised cells during preparation of the smears. Schizonts are transitory in *T. mutans* and the *T. orientalis/buffeli* group, in which the piroplasm stage may be pathogenic. *Theileria taurotragi* schizonts are not readily detected in Giemsa-stained blood smears. A veil to the side of the piroplasm may ~~distinguish~~ indicate *T. velifera*. The schizonts of *T. mutans*, if detected, are distinct from *T. parva,* having larger, flattened, and irregular nuclear particles. The piroplasms (intra-erythrocytic stage) of *T. parva, T. annulata* and *T. mutans* are similar, but those of *T. annulata* and *T. mutans* are generally larger and may be seen to divide. However, for practical purposes schizonts and piroplasms of different theilerias are very difficult to discriminate in Giemsa-stained smears.

The schizont is the pathogenic stage of *T. parva* and *T. annulata.* It initially causes a lymphoproliferative, and later a lymphodestructive disease. The infected animal shows enlargement of the lymph nodes, fever, a gradually increasing respiratory rate, dyspnoea and/or diarrhoea. The most common post-mortem lesions are enlarged lymph nodes, a markedly enlarged spleen, pulmonary oedema, froth in the trachea, erosions and ulceration of the abomasum, and enteritis with necrosis of Peyer's patches. Lymphoid tissues become enlarged in the initial stages of the disease, but then atrophy if the animal survives into the chronic stages of the disease. When examined histologically, infiltrations of immature lymphocytes are present in lung, kidney, brain, liver, spleen, and lymph nodes. Schizont-parasitised cells may be found in impression smears from all tissues: lung, spleen, kidney and lymph node smears are particularly useful for demonstrating schizonts. In longer standing cases, foci of lymphocytic infiltrations in kidneys appear as white infarcts. In animals that recover, occasional relapses may occur. A nervous syndrome called 'turning sickness' is sometimes observed in *T. parva*-endemic areas, and is considered to be associated with the presence of intravascular and extravascular aggregations of schizont-infected lymphocytes, causing thrombosis and ischaemic necrosis throughout the brain.

In *T. annulata,* both the schizont and piroplasm stages may be pathogenic. Schizonts are scarce in the peripheral blood of acutely sick animals and their presence in blood smears indicates a poor prognosis. However, schizonts can be easily detected in smears from lymph nodes, spleen and liver tissues obtained by needle biopsy of these organs. The gross pathology caused by schizonts of *T. annulata* resembles that of *T. parva*, while anaemia and jaundice are features of both schizont and piroplasm pathology. Pathogenic strains of *T. mutans* also cause anaemia, as can strains from Japan and Korea referred to as *T. sergenti*, as well as the pathogenic *T. orientalis* strains.

Piroplasms of most species of *Theileria* may persist for months or years in recovered animals, and may be detected intermittently in subsequent examinations. However, negative results of microscopic examination of blood films do not exclude latent infection. Relapse parasitaemia can be induced with some *Theileria* species by splenectomy. Piroplasms are also seen in impression smears prepared at post-mortem, but the parasites appear shrunken and their cytoplasm is barely visible.

1.2. Molecular methods

The early DNA-based molecular methods to detect *Theileria* species were based on Southern blotting using a range of probes, derived from ribosomal RNA gene sequences, to detect all the *Theileria* species that are known to infect cattle (~~Allsopp *et al.*, 1993;~~ Bishop *et al.,* 1995). DNA probes specific for *T. parva* ~~(Allsopp & Allsopp, 1988; Conrad *et al.*, 1987; Morzaria *et al.*, 1999a)~~ and *T. mutans* ~~(Morzaria *et al.,* 1989)~~ were also developed. The Southern blotting methods have largely been supplanted by species-specific conventional PCR protocols designed to detect *T. annulata* or *T. parva* carrier cattle using PCR followed by agarose electrophoresis-based detection (~~Bishop *et al.,* 1992;~~ D'Oliveira *et al.,* 1995; Odongo *et al.,* 2010~~; Skilton *et al.,* 2002~~). Several PCR assays have also been developed using specific genes or satellite sequences for characterisation of different isolates/strains/clones of *T. parva* ~~(Geysen *et al.,* 1999; Oura *et al.,* 2003; Patel *et al.,* 2011)~~. Whole genome sequencing may become a method to differentiate between disease syndrome strains.

A reverse line blot (RLB) assay based on hybridisation of PCR products to specific oligonucleotide probes immobilised on a membrane for simultaneous detection of different *Theileria* species has been introduced (Gubbels *et al.,* 1999), and ~~fluorescence resonance energy transfer (FRET) based real-time assays have been~~ real-time PCR assays using species-specific or hydrolysis probes have also been developed and largely replace conventional PCR assays in diagnostic laboratories because of their ease of use and to prevent contamination ~~developed for specific diagnosis of *T. parva*~~ (Pienaar *et al.*, 2011; Ros-Garcia *et al.*, 2012; Sibeko *et al.,* 2008).

The primer and probe sequences for several of these assays are presented in Table 2, together with the cycling conditions for the commonly used p104 nested PCR for *T. parva*.

PCR amplification of the p33/34 genes of the *T. orientalis/buffeli* complex followed by restriction enzyme analysis can be used to characterise the various types (~~Kawazu *et al.,* 1992;~~ Kubota *et al.,* 1995). A multiplex real-time PCR has been developed that can detect and differentiate the buffeli, Ikeda, chitose and type 5 strains (Perera *et al.*, 2015). The multiplexed tandem PCR amplifies the 23-kDa piroplasm membrane protein, nuclear internal transcribed spacer I and the major piroplasm surface protein. The test is sold as a kit and details on the primers are proprietary. A quantitative real-time PCR able to detect *T. orientalis* Ikeda and Chitose strains has also been developed based on the major piroplasm surface protein (Bogema *et al.*, 2015).

Table 2. PCR primers and probes for the detection of Theileria annulata or T. parva

Target gene	Primer sequences (5'–3')	Probe sequences/cycling conditions	Reference
Theileria annulata			
30 kDa protein	GTA-ACC-TTT-AAA-AAC-GT GTT-ACG-AAC-ATG-GGT-TT	n/a	D'Oliviera *et al.* (1995)
18S rRNA (hydrolysis probe)	AGA-CCT-TAA-CCT-GCT-AAA-TAG-G CAT-CAC-AGA-CCT-GTT-ATT-GC	FAM-AAG- [+T]TT- [+C]TA-[+C] TG-[+T] CC-CGT-T-BHQ1 ([+N] indicate a LNA base)	Ros-Garcia *et al.* (2012)
Theileria parva			
~~p104~~	~~ATT-TAA-GGA-ACC-TGA-CGT-GAC-TGC TAA-GAT-GCC-GAC-TAT-TAA-TGA-CAC-C~~	~~n/a~~	~~Skilton *et al.* (2002)~~
p104 (nested)	Primary PCR: ATT-TAA-GGA-ACC-TGA-CGT-GAC-TGC TAA-GAT-GCC-GAC-TAT-TAA-TGA-CAC-C Secondary PCR: GGC-CAA-GGT-CTC-CTT-CAG-ATT-ACG TGG-GTG-TGT-TTC-CTC-GTC-ATC-TGC ~~Cycling conditions:~~ ~~Primary:~~ ~~• 94°C for 1 minute~~ ~~• 40 cycles of 94°C/1 minute, 60°C/1 minute, 72°C/1 minute~~	Cycling conditions: Primary: • 94°C/1 minute • 40 cycles of 94°C/1 minute, 60°C/1 minute, 72°C/1 minute • 72°C for 9 minutes after the last cycle. Secondary: use 1 µl primary product • 94°C/1 minute • 30 cycles of 94°C/1 minute, 55°C/1 minute, 72°C/1 minute	Odongo *et al.* (2010)

Target gene	Primer sequences (5'–3')	Probe sequences/cycling conditions	Reference
	~~• 72°C for 9 minutes after the last cycle.~~ ~~Secondary:~~ ~~• 94°C for 1 minute~~ ~~• 30 cycles of 94°C/1 minute, 55°C/1 minute, 72°C/1 minute~~ ~~• 72°C for 9 minute after the last cycle~~	• 72°C for 9 minutes after the last cycle ~~n/a~~	
18S RNA (RLB)	GAG-GTA-GTG-ACA-AGA-AAT-AAC-AAT-A TCT-TCG-ATC-CCC-TAA-CTT-TC	TTC-GGG-GTC-TCT-GCA-TGT	Gubbels *et al.*, (1999)
18S RNA (FRET)	CTG-CAT-CGC-TGT-GTC-CCT-T ACC-AAC-AAA-ATA-GAA-CCA-AAG-TC	GGG-TCT-CTG-CAT-GTG-GCT TAT–FL LCRed640-TCG-GAC-GGA-G TTC-GCT-PH	Sibeko *et al.* (2008)
18S rRNA (FRET)	GGT-AAT-TCC-AGC-TCC-AAT-AG AAA-GTA-AAC-ATC-CAG-ACA-AAG-CG	GGG-TCT-CTG-CAT-GTG-GCT-TAT–FL LCRed640-TCG-GAC-GGA-GTT-CGC-T—PH	Pienaar *et al.* (2011)
Theileria orientalis			
Major piroplasm surface protein	GCA-AAC-AAG-GAT-TTG-CAC-GC TGT-GAG-ACT-CAA-TGC-GCC-TAG-A	VIC-CAT-GAA-CAG-TGC-TTG-GC-MGB (Ikeda) NED-TCC-TCA-GCG-CTG-TTC-T-MGB (Chitose)	Bogema *et al.* (2015)

2. Serological tests

2.1. The indirect fluorescent antibody test

The indirect fluorescent antibody (IFA) test is the most widely used diagnostic test for *Theileria* spp. The IFA test is robust, easy to perform and provides adequate sensitivity and specificity for use in the field for detection of prior infection with *T. parva* and *T. annulata* under experimental situations and in a defined epidemiological environment where only one theilerial species is present. The IFA test has limitations for large-scale serological surveys due to its reduced specificity in field situations where several *Theileria* species co-exist. There is a need for tests that are more specific, easy to interpret, and robust enough to be used in field conditions. No serological tests exist yet for *T. orientalis* Chitose or Ikeda strains.

2.1.1. Preparation of schizont antigen

i) Schizont antigen slides

The antigens used for the IFA test are intracytoplasmic schizonts derived from infected lymphoblastoid cell lines for *T. parva* and from infected macrophage cell lines for *T. annulata.*

Cultures of 200 ml to 1 litre of either *T. parva* or *T. annulata* schizont-infected cells containing 10^6 cells/ml, of which at least 90% of the cells are infected, are centrifuged at 200 ***g*** for 20 minutes at 4°C. The supernatant fluid is removed and the cell pellet is resuspended in 100 ml of cold (4°C) phosphate-buffered saline (PBS), pH 7.2–7.4, and centrifuged as before. This washing procedure is repeated three times, and after the final wash the cell pellet is resuspended in PBS (approximately 20–100 ml) to give a final concentration of 10^7 cells/ml.

Using a template or pipette tip, thin layers of the cell suspension are placed on Teflon-coated multispot slides, or on ordinary slides using nail varnish for separation. The smears should give between 50 and 80 intact cells per field view when examined under a ×40 objective lens. The antigens are distributed on to the slides using multichannel or a 100 µl pipette. By dispensing and immediately sucking up the schizont suspension, a monolayer of schizonts remains in each well. This is performed for each enclosure until the volume is exhausted. With this method, approximately 600 good quality slides containing a total of 6000 individual antigen spots can be obtained. The slides are air-dried, fixed in acetone for 10 minutes, individually wrapped in tissue paper and then in groups of five in aluminium foil, and

stored in airtight, waterproof plastic containers at either –20°C or –70°C. The antigens keep for at least 1 year at –20°C and longer at –70°C.

ii) Schizont antigen in suspension

First, 500 ml of *T. parva*- or *T. annulata*-infected cells containing 10^6 cells/ml are centrifuged at 200 ***g*** for 10 minutes at 4°C, and the cell pellet obtained is washed twice in 100 ml of cold PBS. The viability of the cells is determined by eosin or trypan blue exclusion (it should be greater than 90%). The cells are resuspended at 10^7/ml in cold PBS. To this volume, two volumes of a cold fixative solution containing 80% acetone and 0.1% formaldehyde (0.25% formalin) in PBS are added drop by drop while the cell suspension is stirred gently and continuously in an ice bath. The cell suspension is kept at –20°C and allowed to fix for 24 hours. Siphon about 2/3 of the volume off, centrifuge and decant. The fixed cells are then washed three times in cold saline and centrifuged at 200–400 ***g*** for 20 minutes at 4°C. After the last wash, the cells are resuspended into 5 ml PBS + 0.2% BSA (bovine serum albumin) at 10^7/ml. The fixed cells are distributed in aliquots of 0.5 ml. The antigen is stable at 4°C with 0.2% sodium azide as preservative for 2 weeks, and keeps indefinitely at –20°C.This method can also be used to prepare schizont antigen for *T. taurotragi*.

2.1.2. Preparation of piroplasm antigen

The piroplasm stage of *Theileria* spp. cannot be maintained in culture, therefore the piroplasm antigen must be prepared from the blood of infected animals. Due regard should be paid to the principle of 'The Three Rs' as set out in the WOAH *Terrestrial Code*, Chapter 7.8 *Use of animals in research and education.*

i) Piroplasm antigen slides

Experimental infections are induced by infecting cattle subcutaneously with sporozoites, or applying ticks infected with *T. parva*, *T. annulata* or *T. taurotragi*. Infection with *T. annulata* is invariably produced by inoculation of blood drawn from cattle with acute theileriosis. Splenectomy of the recipient cattle prior to the infection considerably increases the piroplasm parasitaemia in red blood cells (RBC). Peak parasitaemias are of short duration and if animals survive the disease the percentage of infected RBC decreases considerably in a few days. Infections with the parasite group referred to as *T. orientalis/buffeli, T. mutans* or *T. velifera* are usually induced by inoculating splenectomised cattle intravenously with blood from a carrier animal, or with a blood stabilate, or by application of infected ticks. When the piroplasm parasitaemia is 10% or higher, 100 ml of the infected blood is collected from the jugular vein in a heparinised or ethylene diamine tetra-acetic acid (EDTA) vacutainer, and gently mixed with 2 litres of PBS. The mixture is centrifuged at 500 ***g*** for 10 minutes at 4°C; the plasma and buffy coat are removed, the RBC are again resuspended in 2 litres of PBS, and the centrifugation step is repeated. It is important to remove the buffy coat after each wash. This washing procedure is repeated four times. After the final wash, an aliquot of the packed RBC is used to make doubling dilutions in PBS, and a 5 µl drop of each dilution is placed on slides. The dried spots are fixed in methanol and stained with Giemsa's stain, and the concentration of RBC is examined using a light microscope. The dilution that gives a single layer of RBC spread uniformly on the spot is then selected for large-scale preparation of piroplasm antigen slides. Approximately 10,000 antigen slides (100,000 antigen spots) can be prepared from 100 ml of infected blood. The antigen smears are allowed to dry at room temperature before fixing in cold (4°C) acetone for 10 minutes. The fixed smears can be stored as for the schizont antigen slides, and kept for similar periods.

ii) Piroplasm antigen suspension

An alternative method of preparing antigens to that described above is available, and has been tested for *T. parva*. In this procedure, 100 ml of blood are taken from an animal with a high piroplasm parasitaemia and prepared as described previously, and the packed cell volume is adjusted to 5% in PBS.

One volume of the RBC suspension is added to two volumes of the fixative (see Section B.2.1.1.ii above) while stirring. The cells are allowed to fix at –20°C for 24 hours. The fixed cells are then washed three times with PBS and centrifuged at 1000 ***g*** for 30 minutes. The deposit is resuspended to the original volume of blood with PBS containing 0.2% sodium azide, and distributed in aliquots of 0.5 ml.

The piroplasm antigen is stable at 4°C when preserved with 0.2% sodium azide for a period of at least 3 years.

2.1.3. Standardisation of antigen

Schizont or piroplasm antigen suspensions are mixed on a rotor mixer and titrated in PBS by doubling dilution starting from undiluted through to 1/16. The dilution giving a cell distribution of approximately 50–80 schizont-infected cells or 150–200 infected RBC per field view when examined under a ×40 objective lens is recommended for use for that batch of antigen. Using this dilution, test antigen smears are prepared on slides. These antigen smears plus the antigen slides previously frozen (and thawed before use) are tested against a range of dilutions of a panel of known strong, intermediate and weak positive and negative control sera. If the positive control sera titrate to their known titres and the negative control sera give no fluorescence, the antigen is used in the routine IFA test.

Both types of antigen preparations, acetone-fixed smears stored at either –20°C or –70°C, and antigens fixed in suspension and stored at either 4°C or –20°C, are used routinely in many laboratories. The sensitivity of both types of antigen is comparable. In laboratories where adequate low temperature storage facilities and a reliable supply of electricity are available, the antigen slides can be used. However, such antigens can only be transported on dry ice or in liquid nitrogen. Antigens fixed in suspension have the advantage over antigen slides in that the initial method of preparation is simpler and quicker. A large batch of this antigen can be stored in one container, and aliquots may be taken out as necessary from which fresh smears are prepared for the IFA test. The need for a large storage facility is thereby avoided. The antigens fixed in suspension can also be stored at 4°C and can be safely transported at room temperature without loss of antigenicity.

2.1.3.1. Preparation of bovine lymphocyte lysate

A lymphocyte lysate is prepared ~~according to the method described by Goddeeris *et al.* (1982),~~ for use in tests with antigens of *T. parva* in suspension. Briefly, a 3-month-old calf is splenectomised and maintained in a tick-free environment. To exclude the possibility of latent theilerial infections, Giemsa-stained blood smears are examined daily for a period of 4 weeks for parasites. The parasite-free animal is killed and the thymus and all the accessible lymph nodes are removed. These tissues are sliced into small pieces in cold PBS containing 0.45% EDTA as anticoagulant. Cells are teased out of the tissue, separated from the debris by passing through a muslin cloth, and washed three times with PBS/EDTA by centrifugation at 200 ***g*** for 20 minutes at 4°C. The washed lymphocytes are resuspended in PBS without EDTA, to give a final concentration of 5×10^7 cells/ml. The cells are disrupted by sonication in 100-ml aliquots on ice for 5 minutes using the 3/8 probe. The sonicated material is centrifuged at 1000 ***g*** for 30 minutes at 4°C, and the supernatant, adjusted to 10 mg protein/ml, is stored at –20°C in 4-ml aliquots.

2.1.3.2. Test procedure

i) With schizont or piroplasm slide antigen

a) Remove antigen slides from freezer and allow to thaw for 30 minutes at 4°C and then for 30 minutes at room temperature.

b) Inactivate the sera to be tested for 30 minutes in a water bath at 56°C.

c) Unpack the slides and label the numbers of the sera tested.

d) Prepare 1/ 40 and 1/80 dilutions of sera to be tested. Validated positive and negative sera are included with each test as controls. Further doubling dilutions can be made if end-point antibody titres are desired.

e) Transfer 25 µl of each serum dilution to a spot of antigen.

f) Incubate in a humid chamber for 30 minutes at room temperature.

g) Remove the serum samples from the antigen wells by washing with PBS and rinse by immersing in two consecutive staining jars containing PBS for 10 minutes each time.

h) Distribute to each well 20 µl of diluted anti-bovine immunoglobulin fluorescein isothiocyanate conjugate at appropriate dilution (generally, dilutions recommended by manufacturers are suitable; however, minor adjustments may be necessary for optimal results). Incorporate Evans blue into the conjugate at a final dilution of 1/10,000 as a counterstain and incubate in a humid chamber for 30 minutes at room temperature.

i) Repeat step g and mount with a cover-slip in a drop of PBS/glycerol (50% volumes of each).

j) Read the slides under a fluorescent microscope equipped with epi-Koem illumination (100 W mercury lamp), UV filter block, ×6.3 eyepieces and Phaco FL 40/1.3 oil objective lens.

ii) With schizont antigen stored in suspension

a) Thaw frozen antigen at room temperature.

b) Distribute the antigen suspension on the spots of multispot slides, using multichannel or a 100-µl pipette. By dispensing and immediately sucking up the suspension a monolayer of schizont-infected cells remains on each well.

c) Allow slides to dry at room temperature or 37°C.

d) Dilute test and control sera 1/40 in lymphocyte lysate (195 µl lymphocyte lysate + 5 µl serum).

e) Proceed as described in steps e to j (Section B.2.1.3.2.i).

iii) With piroplasm antigen stored in suspension

a) Resuspend piroplasm antigen (stored at 4°C) by agitation and disperse RBC by passing the suspension through a 25-gauge needle to break the clumps.

b) Dilute the antigen to previously standardised dilutions (see preparation of piroplasm antigen).

c) Allow slides to dry at room temperature or 37°C.

d) Proceed as described in steps d and e (Section B.2.1.3.2.ii).

2.1.4. Characteristics of the indirect fluorescent test

The incorporation of Evans blue provides a good contrast, enabling good differentiation of non-infected cells from the infected ones under the fluorescent microscope. Mounting the slides in 50% glycerol, at pH 8.0, reduces the rapid fading of fluorescein isothiocyanate and makes photography of the preparation possible. Once prepared, slides are stable and can be read for up to 72 hours after preparation when kept at 4°C in the dark.

The sensitivity of the IFA test depends on the period of time that has elapsed since the onset of infection. Following infection with sporozoites, antibodies to *T. parva* and *T. annulata* are first detected between days 10 and 14 using the schizont antigen. Using the piroplasma antigen, antibodies are first detected between days 15 and 21. Antibodies last for a variable period of time after recovery, depending on such factors as the establishment of a carrier state, chemotherapeutic intervention, and presence or absence of a rechallenge. Following recovery from infection with *T. parva* or *T. annulata*, high levels of antibody are generally detected for 30–60 days. The antibody levels gradually decline and low antibody titres are still detectable 4–6 months after recovery. Later, antibody may become undetectable at a serum dilution of 1/40, but may persist for more than 1 year following a single challenge. In ECF endemic regions, the seroprevalence in cattle population fluctuates considerably depending on the level and regularity of challenge. In an endemic area where a seasonal transmission cycle of ECF occurs, IFA has been shown to lack sensitivity. The overall diagnostic sensitivity of the IFA test has been evaluated as 55% at a cut off titre 1/40 and 28% at cut off 1/160. The specificity of the test for the two cut off points was 86% and 95% respectively (Billiouw *et al.*, 2005).

The IFA test is useful for identifying herds that contain carriers of *T. annulata*, but is not always sufficiently sensitive to detect all infected individuals. Both schizont and merozoite (piroplasm) IFA antigens have failed to detect antibody in some animals despite carrying patent infection with piroplasms (Darghouth *et al.*, 1996).

In *T. mutans* infections induced by sporozoite inoculation, antibodies are first detected between days 10 and 15 after the appearance of piroplasms. Low titres are detectable for at least 12–24 months.

The *T. parva* IFA test is highly sensitive for detection of antibodies in an epidemiological situation where only one species of *Theileria* exists. However, if the test is used to detect antibodies where mixed infections of *Theileria* occur, the specificity of the test needs to be carefully evaluated. For example, *T. annulata* and *T. parva* cross-react, although these cross-reactions are four- to six-fold lower than with the homologous sera. The cross-reactivity between the two species has little practical significance as the geographical distribution of these two parasites does not overlap. In the IFA test such cross-reactivity does not occur between *T. parva* and *T. mutans* or between *T. annulata* and *T. mutans*. There is a low level of cross-reactivity between *T. parva* and *T. taurotragi*, reducing the specificity of these two tests in many parts of sub-Saharan Africa where

their distribution overlaps. In areas where African buffalo and cattle come into frequent contact, cross-reactivity between *T. parva* and *Theileria* sp. (buffalo) may occur in cattle.

A panel of monoclonal antibodies (MAbs) detecting various epitopes on the polymorphic immunodominant antigen of the *T. parva* schizont stage has been generated. This panel can be used in the IFA test using the schizont-infected lymphoblastoid cells to detect differences between certain stocks of *T. parva* and between *T. parva* and other theilerial species. This test has been deployed as one of the several characterisation tools to differentiate various stocks of *T. parva*, and for quality control during sporozoite stabilate preparation (Bishop *et al.*, 1994).

2.2. Enzyme-linked immunosorbent assays

Serological tests based on the enzyme-linked immunosorbent assay (ELISA) have been developed for the detection of antibodies to *T. annulata* (Gray *et al.*, 1980). Tests used for *T. parva* and *T. mutans* are indirect ELISAs based on parasite-specific antigens, PIM and p32, respectively (Katende *et al.*, 1998~~; Morzaria *et al.*, 1999a~~). These ELISAs provide higher (over 95%) sensitivity than the IFA tests. The ELISA reagents are available from the International Livestock Research Institute, Nairobi, Kenya. The PIM antigen shows cross-reactivity between *T. parva* and *T.* sp. (buffalo) and should be considered as a confounding factor where African buffalo and cattle come into contact.

C. REQUIREMENTS FOR VACCINES

C1. Cell culture live vaccines for *Theileria annulata*

1. Background

Vaccination against *T. parva* and *T. annulata* has been attempted since the causal organisms were first recognised early in the last century. However, reliable live vaccines of known potency are a more recent development. The most widely used are attenuated schizont cell culture vaccines against *T. annulata*. The procedures for production and safety testing have been described (Food and Agriculture Organization of the United Nations [FAO], 1984~~; Hashemi-Fesharki, 1988; Pipano, 1989b~~), and the vaccine is used in Israel, Iran, Turkey, Spain, India, northern Africa, central Asia and the People's Republic of China.

Despite the fact that vaccination with the cell culture vaccine against *T. annulata* has been available for more than three decades and has shown to be effective under field conditions, the use of this vaccine has been limited. The concern about the introduction of vaccine-derived parasites into the field tick population has led to individual countries developing vaccines from local isolates (Morisson & Mc Keever, 2006). Some attenuated cell lines have lost the ability to differentiate to erythrocytic merozoites (piroplasms) when inoculated to cattle and in one instance, *Hyalomma* nymphs fed on vaccinated cattle did not become infected ~~(Kachani *et al.*, 2004a)~~. However, in most cases the loss of differentiation is based on macroscopic examination of blood films from vaccine inoculated cattle. This drawback, the difficulties in standardisation of the antigenic composition of the cultured parasites and the need of a cold chain for distribution of the vaccine to the field are limiting factors in commercialisation of this vaccine (Morisson & Mc Keever, 2006).

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics of the master seed

Primary cultures of *T. annulata*-infected cells may be established from trypsinised lymph nodes, liver, or spleen taken aseptically from an infected animal after death, or from the buffy coat of heparinised peripheral blood separated on a density gradient (Ficoll Hypaque), or by lymphocytes harvested from lymph node biopsy material using a plastic syringe method (Brown, 1979; FAO, 1984).

Seed cultures are prepared from cryopreserved cell lines that have been isolated from cattle and attenuated as described below. Vaccines should be produced from a seed culture (master seed) that has been passed less than 30 times, because there is some uncertainty about the immunogenic stability of these cultures in long-term passage.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9. As cell culture material is derived from field animals, their cells might be potential sources of contamination of the vaccine with extraneous pathogens. Potential contaminants include bovine leukosis, mycoplasma, bovine viral diarrhoea virus, bovine spongiform encephalopathy (BSE) and other bacteria and viruses.

Cell cross-contamination in cell cultures is a common problem during cell culturing and use. The problem can be solved by increasing the awareness and by introducing regular quality control of cell cross-contamination.

2.1.3. Validation as a vaccine strain

Attenuation of *T. annulata* schizonts is achieved by prolonged growth and passage in culture ~~(Pipano, 1989b)~~. The loss of parasite virulence appears to be due to a change in parasite gene expression. Attenuation is assessed by the inoculation of the culture into susceptible calves every 20–30 passages. A sample of culture should be cryopreserved every ten passages in case of accidental loss or contamination. Complete attenuation is achieved when cultures do not cause fever or detectable schizonts and piroplasms in susceptible cattle but could take up to 300 passages. An attenuated culture will reliably infect cattle at 10^5 cells and induce a serological reaction, and will not produce disease at 10^9 cells. Cultures may be cryopreserved using either dimethyl sulphoxide (DMSO) or glycerol. Two methods of storing and delivering the vaccine are described below.

2.2. Method of manufacture

2.2.1. Procedure

The infected cells are cultured initially in Eagle's minimal essential medium (MEM) or Leibovitz L15 medium supplemented with 20% calf serum and containing penicillin (100 units/ml), streptomycin (50 µg/ml), and mycostatin (75 units/ml) in 25~~ ml~~ cm^2 plastic screw-cap tissue-culture flasks. An alternative medium is RPMI 1640 with 10% fetal calf serum, 2 mM glutamine, penicillin and streptomycin, and is usually used with established cultures. Medium is replenished every 3–4 days. The presence of bright refractile cells free in the medium (on examination using a phase-contrast or inverted microscope) is indicative of infected cell growth. The cultures may establish as a monolayer or in suspension. Passage is ~~effected~~ performed by decanting the medium, adding 0.025% EDTA (versene) for 15 minutes to monolayer cultures, dispersing the cells, then counting and dispensing according to flask size. Approximately 10^6 cells are introduced into a 25 cm^2 flask, and the same seed rate in 100–200 ml is used in larger flasks. The general culture technique is as described by Brown (1979).

Serum is essential for maintenance of these cultures, and is obtained either from calves up to the age of 6 months, or from commercial sources, and is tested for toxicity through three passages in an established cell line before use.

2.2.2. Requirements for ingredients

Before starting to produce vaccine, seed material with known characteristics is required ~~(Pipano, 1997)~~. Three types of seed material are distinguished:

i) Master seed

Schizont-infected cells from a specific passage that have been selected and permanently stored and from which all other passages are derived. The master seed should consist of a single uniform batch of seed that has been mixed and filled into containers as one batch. As *T. annulata* schizont-infected cells are used for the manufacturing process, the master seed also represents the master cell stock (see Chapter 1.1.8 *Principles of veterinary vaccine production*). To prepare a master seed, schizont-infected cells that have proved to be safe for cattle are propagated to obtain in a single culture passage approximately 5×10^8 cells. The cells are cryopreserved in about 100 cryotubes each containing 5×10^6 cells. A viability check of the master seed should be performed once the master seed has been cryopreserved for at least 24 hours by reviving one of the cryotubes.

ii) Working seed

Schizont-infected cells at a passage level between the master seed and the production seed. To prepare a working seed, the contents of a single cryotube of master seed are

transferred to a 10 ml centrifuge tube containing 8 ml complete medium. The tube is centrifuged at 600 ***g*** for 15 minutes at 4°C and the pellet is transferred into a 75 cm^2 culture flask containing 15–20 ml medium. The medium is replaced the next day, and 4 days later the cells are dispersed and subcultured in larger vessels. After 5–6 subcultivations, a sufficient number of infected cells is available to start the production run.

iii) Production seed

Schizont-infected cells from a specific passage level are used without further propagation for the preparation of a batch of vaccine. The production seed is obtained by propagating large numbers of cells in monolayer or suspension cultures. Monolayer cultures are grown in flasks, 150 cm^2 to 175 cm^2, which usually provide an average of from 7 × 10^7 to 8 × 10^7 cells per vessel. About 80 ml of complete medium per flask is required. In a roller bottle culture system, 1.2–1.5 × 10^8 cells can be obtained in a conventional roller bottle (700 cm^2) containing 100–120 ml of medium. To obtain optimal yield of cells, stationary cultures or roller bottles cultures are incubated for 6–7 days with culture media as described previously, see Section C1.2.2.1.

The schizont-infected cells from all vessels are harvested and pooled together and the total number is computed. Alternatively, about 20% of the cells may be seeded again to prepare another batch of vaccine. Several batches of vaccine can be produced using a portion of the production seed as working seed. As prolonged cultivation may generate alteration in the futures of the schizonts, such as immunogenic capacity, after several batches, subsequent vaccine is produced by making a fresh production seed from the master seed.

Schizont-infected cells are mixed with DMSO at a final concentration of 7% or glycerol at a final concentration of 10%, and dispensed in 1.8-ml aliquots into 2-ml plastic vials, each vial containing ten doses of concentrated vaccine. As DMSO immediately penetrates the cell membranes, the time spent in dispensing the vaccine into the vials should be as short as possible. When glycerol is used, an equilibration time of 30–40 minutes is required before freezing the vaccine. There is no consensus on how many schizont-infected cells should constitute one dose of the vaccine. A recommended practical approach is to prepare doses of 10^6–10^7 infected cells in order to counteract variable environmental conditions in the field. However, considerable protection against sporozoite-induced infection has been achieved by vaccination with 10^5 infected cells ~~(Kachani *et al.*, 2004b)~~.

The vaccine is frozen by introducing the vials in an ultracold deep freezer (–70°C) and transferring them 24 hours later to liquid nitrogen containers. Alternatively, vials can be introduced in gas phase liquid nitrogen for 3 hours and then immersed in the liquid nitrogen for storage ~~(Pipano, 1989b)~~. Vaccine is transported to the field in liquid nitrogen, and diluted 1/10 in isotonic buffered saline in a screw-cap bottle with a rubber or silicone septum for aseptic withdrawal. For dilution of vaccine frozen with glycerol, isotonic buffered saline should also contain 10% glycerol in order to avoid osmotic damage to the schizonts. The vaccine is administered subcutaneously within 30 minutes of thawing ~~(Pipano, 1977)~~.

The vaccination regimen in Iran up to 1990 was to inoculate two different mild strains 1 month apart. But, in order to reduce cost and save time, a new method was implemented involving only a single dose of a local live attenuated vaccine strain ~~(Hashemi-Fesharki, 1998)~~. A fresh culture vaccine is used in Morocco, usually at a tenfold lower dose (10^4 schizont-infected cells) ~~(Kachani *et al.*, 2004b)~~. However, there are problems with quality control of vaccines with short shelf life.

2.2.3. In-process controls

Records of the source and passages of the working seed material should be maintained. Seeds should be free of infective agents such as enzootic bovine leukosis, bovine immunodeficiency virus, bovine pestivirus, bovine syncytial virus, Rift Valley fever, etc. Test procedures will depend on availability preferably using DNA analysis.

pH, temperature and coloration of the solutions should be checked during the process and must have been shown to be free from contaminants. Numbers and contamination in growing cell cultures should be checked on a daily basis by examination using an inverted microscope.

2.2.4. Final product batch tests

i) Sterility

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9.

ii) Safety precautions

Safety tests in target animals are not required by many regulatory authorities for the release of each batch. Where required, standard procedures are generally conducted using fewer animals than are used in the safety tests required for the relevant regulatory approval.

Theileria annulata schizonts are not hazardous for humans or contagious for animals, therefore the main purpose in designing a vaccine production facility is to prevent contamination of the product by extraneous organisms.

a) Freedom from properties causing undue local or systemic reactions

For testing the safety of the master seed, two to four susceptible calves, of the most sensitive stock available, are inoculated with a tenfold greater dose than is recommended for immunisation. This dose should not produce clinical signs beyond a transient rise in temperature. With completely attenuated master seed, no schizonts or piroplasms will be seen in lymph node and liver smears or in blood films. However, different breeds of cattle may show different sensitivities to the vaccine. This should be borne in mind when vaccine from a partially attenuated master seed is to be administered to high-grade cattle stocks.

Following a successful test for safety of a sample, all subsequent batches produced from the same master seed can be released without further testing for safety. However, if parasites are detected in the blood or tissues of vaccinated field animals, or if clinical signs develop following the inoculation of the vaccine, the batch or a parallel batch, from the same master seed, should be retested for safety.

iii) Batch potency

In Israel the schizont vaccines are tested using a documented procedure ~~(Pipano, 1989a)~~ before release.

Usually, the schizont vaccine is produced in small individual batches (3–5 thousand doses), which makes the full testing of each batch impractical for economic reasons. It is recommended therefore that the first batch of vaccine produced from a master seed be tested for safety, efficacy, potency and sterility, while each subsequent batch be tested for sterility and potency only. This recommendation is based on the fact that once the cultured schizonts become attenuated, no reversion to virulence has ever been observed during further cultivation. As far as efficacy is concerned, no obvious alteration of the immunogenic properties has been observed during the limited number (20–30) of passages involved in producing the actual vaccine.

a) Viability of schizont-infected cells

The potency test is conducted by quantitative *in-vitro* methods. Frozen vaccine remains stable during the storage period, even for long periods, but some loss of viability occurs during the freezing and thawing processes. Viability should be tested under conditions as similar as possible to those obtained when the vaccine is used in the field. For this reason, vaccine should be thawed and the diluted suspension of schizont-infected cells should be left at ambient temperature for 60 minutes before performing the viability tests. A simple test for evaluating viability of the infected cells is nigrosin dye exclusion counting ~~(Wathanga *et al.*, 1986)~~. Vaccine that, after being thawed and diluted and left at room temperature for 1 hour, still contains 50% or more live cells can be released for use although in most cases 80–90% of live cells are found.

Viability of the schizonts is also reflected by the plating efficiency of the schizont-infected cells ~~(Wathanga *et al.*, 1986)~~, as only cells containing viable schizonts multiply in culture. For this purpose, the thawed, diluted vaccine is transferred from the bottle to a centrifuge tube. A sample for counting is taken and the suspension is centrifuged for 15 minutes at 600 ***g***. Meanwhile, the total number of cells (live and dead) is determined in order to ascertain that the frozen vaccine had the necessary initial concentration of cells. After centrifugation, the supernatant is discarded and the cells are resuspended to the original volume using complete culture medium. Serial tenfold dilutions of cells in complete medium are performed in sterile 10 ml tubes so that the last two dilutions contain 50, and 5 cells per ml, respectively. Twelve replicates of 200 µl from each of the last two dilutions are introduced into a 96-well culture plate. The plates are incubated at 37°C in a 5% CO_2 atmosphere and

cultures are checked with an inverted microscope 6 and 9 days after seeding. The number of wells theoretically containing 1 cell each in which growth is observed is counted. Vaccine showing a plating efficiency <2 (cells) are adequate for field use.

2.3. Requirements for ~~authorisation/registration/licensing~~ regulatory approval

2.3.1. Manufacturing process

For ~~registration~~ regulatory approval of vaccine, all relevant details concerning manufacture of the vaccine and quality control testing (see Sections C2.2.1 and C2.2.2) should be submitted to the regulatory authorities. This information shall be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume.

2.3.2. Safety requirements

i) Target and non-target animal safety

Theileria annulata shizonts are not contagious for animals. These vaccines produce no adverse effects in healthy cattle. However, animals with existing infections, particularly viral infections, may not tolerate vaccination well. The administration of a viral vaccine, such as for foot and mouth disease, during the immunisation period (reaction period) is not recommended as the immune response may be compromised ~~(Hashemi-Fesharki, 1988)~~. In Iran, it is not recommended to vaccinate cows that are over 5 months pregnant, although studies in pregnant cattle with the vaccine stocks used in Israel found no effect on pregnancy ~~(Pipano, 1989a)~~. The immunity engendered is long lasting.

In general, cattle should be immunised in the first few months of life, and tick challenge under natural conditions reinforces the immunity. Although antigenically different strains of *T. annulata* have been identified ~~(Pipano, 1977)~~, it is generally considered that there is sufficient cross-protection among strains to provide adequate protection against field challenge as observed in Israel. In the vast infected areas of central Asia, a single stock has proved immunologically effective in 1.5 million cattle (Dolan, 1989~~; Wathanga *et al.*, 1986~~). However, as described previously, two stocks are used routinely in Iran ~~(Hashemi-Fesharki, 1988)~~.

ii) Reversion-to-virulence for attenuated/live vaccines and environmental considerations

Once the cultured schizonts become attenuated, no reversion-to-virulence has ever been observed during further cultivation.

iii) Precautions (hazards)

Different breeds of cattle may show different sensitivities to the vaccine. This should be borne in mind when vaccine from a partially attenuated master seed is to be administered to high-grade cattle stocks.

2.3.3. Efficacy requirements

i) Capacity to protect against naturally transmitted theileriosis

The batch of experimental vaccine used for the safety test can also be used for testing efficacy of the culture-derived anti-theilerial vaccine. Three or four calves are vaccinated with a conventional dose of vaccine and 6 weeks later, the vaccinated calves and the same number of unvaccinated calves are infected with sporozoites of *T. annulata*. Infection can be induced by live adult ticks issued from *T. annulata*-infected pre-imaginal stages or by inoculation of stabilate prepared from macerated infected ticks (for techniques see Section C2.2) Experience shows that inoculation of stabilate (macerated ticks) generally induces a more severe response than an equivalent number of live, infected ticks allowed to feed on the cattle. However in the long run, the results obtained by challenge with stabilate appear to be more reproducible than those obtained with different batches of live ticks.

There are no internationally agreed standards for the size of a challenge dose used in testing the efficacy of *T. annulata* culture-derived vaccine. Five to ten female and the same number of infected, unfed male *Hyalomma* ticks have been used for infection of cattle. Alternatively, stabilate equivalent to 2–4 macerated ticks inoculated subcutaneusly in the neck area will invariably produce acute theileriosis. The responses to the challenge infection of the vaccinated and unvaccinated control calves are monitored using the following parameters: duration and severity of pyrexia, rate of schizont-infected cells in lymph node or liver biopsy smears, rate of piroplasm infected erythrocytes in the blood films, decrease in white and red

blood cell counts, and severity of clinical manifestations such us anorexia, depression and recumbency.

The results of the efficacy test depend on factors such as immunological characteristics of the *T. annulata* isolate grown and attenuated in culture, the virulence and dose of the field isolate used for challenge, the species of infected ticks used to produce sporozoites. Research ~~studies (Pipano, 1989b)~~ shows that calves vaccinated with schizont vaccine may exhibit an apparently near total protection or show a low level parasitaemia, accompanied by mild fever and insignificant alteration of the remaining parameters from their pre-vaccination values following a potentially lethal homologous challenge. A lesser degree of protection has been exhibited when cattle vaccinated with schizont vaccine were challenged with tick-derived parasites from a geographically remote area. In contrast, in most of the trials the non-vaccinated control calves have exhibited a high level of parasitaemia and pancytopenia accompanied by severe clinical manifestations. In the absence of specific medication, the majority of the control animals have succumbed to the infection ~~(Pipano, 1989b)~~.

Field observations have also been used for evaluation of the efficacy of anti-theilerial vaccines ~~(Pipano, 1989a; Stepanova & Zablotskii, 1989)~~. Susceptible indigenous cattle as well as high-grade exotic breeds were protected against clinical theileriosis and death in pastures on which nonvaccinated cattle succumbed to theileriosis. As completely attenuated schizont vaccine does not yield piroplasms, the presence of this theilerial stage in vaccinated cattle showing no clinical signs is considered to be the result of unapparent tick-induced infection.

The frozen vaccine is viably preserved in large liquid nitrogen refrigerators at production facility and transported to farms in smaller liquid nitrogen containers. Field centres for storage and supply of vaccine can be set up in theileriosis-enzootic areas. The basic equipment required for field application of frozen vaccine includes a wide mouthed jar for preparing a 40°C water bath, a thermometer for measuring the temperature of water, long forceps, face shield and temperature-resistant gloves. Application of the frozen vaccine to field cattle begins by donning the face shield and temperature-resistant gloves. The required numbers of vials are withdrawn with the forceps from the canister of the liquid nitrogen refrigerator. When withdrawing the vials, the canister should be kept as deep as possible in the neck of the refrigerator to avoid quick warming of the remaining vials. Each withdrawn vial should be checked in order to ascertain that liquid nitrogen has not leaked inside. The liquid nitrogen does not alter the vaccine, but may cause the vial to explode when introduced in the water bath. Such a vial should be held at ambient temperature for 1–2minutes to allow the nitrogen to escape and then processed in the usual way. Leaking of liquid nitrogen into a vial containing frozen vaccine has raised questions to about the sterility of the frozen vaccine. However the system has been used for decades with no significant problem observed. The vaccine is administered subcutaneously within 30 minutes of thawing ~~(Pipano, 1977)~~.

2.3.4. Duration of immunity

Controversial results about the length of immunity engendered by vaccination with the cell culture vaccine have been obtained. Periods of from more than 48 months ~~(Stepanova & Zablotskii, 1989)~~ to less than 13 months ~~(Ouelli *et al.*, 2004)~~ have been reported.

2.3.5. Stability

The frozen vaccine has a practically unlimited shelf life.

C2. Immunisation of cattle against *Theileria parva* by the infection and treatment method (live vaccine)

1. Background

Vaccination against *T. parva* is based on a method of infection and treatment in which an aliquot of viable sporozoites is inoculated subcutaneously, and the animals are simultaneously treated with a formulation of a long-acting tetracycline, the so-called infection and treatment method (ITM) (Radley, 1981). Tetracyclines reduce the severity of the infection, and the resulting mild infection is usually controlled by the host's immune response, so that a carrier state is achieved. There are always risks associated with the use of live parasites for immunisation, however, with appropriate quality control and careful determination of a safe and effective immunising dose, the

method can and is being used successfully in the field. Some *T. parva* stocks have been shown to infect cattle reliably without inducing disease, and these can be used without tetracycline treatment. One such stabilate is being applied in the field and offers considerable advantages over potentially lethal stabilate infections and savings in the cost of vaccination. However, different stabilates of these stocks can produce severe disease in cattle, emphasising the importance of a carefully controlled immunising dose.

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics of the master seed

Theileria parva infected ticks can be produced by feeding nymphal *Rhipicephalus appendiculatus* ticks on the ears of an animal undergoing an active ECF infection. After moulting, these ticks, when prefed for 4 days on rabbits will have infective sporozoites in their salivary glands. By grinding these prefed ticks in a specific medium, sporozoites will be released in the supernatants and a stabilate can be produced (FAO, 1984) that can be cryopreserved and when in sufficient quantity be earmarked as a master seed.

If needed, working seed stabilates are prepared by injecting cryopreserved sporozoites from a master seed into experimental cattle and producing a working seed stabilate as described below. Vaccines should be produced from a seed (working seed) that has not undergone further tick passages after its immunological characterisation because this might change after passage.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

i) Field collection of ticks

It is important that well characterised laboratory strains of *R. appendiculatus* be used during preparation of immunising stabilates.

If field ticks are collected for experimental purposes, then consideration should be given to the possible hazard to humans from pathogens present in these ticks. The most important pathogen that has been recognised is Crimean–Congo haemorrhagic fever virus, usually associated with ticks of the genus *Hyalomma* and widely prevalent within the geographical distribution of *R. appendiculatus*. Those handling field tick collections should, therefore, be made aware of potential hazards. Ticks of *Hyalomma* species generally should not be removed from hosts; engorged or partially engorged ticks should not be crushed between the fingers. If removed, ticks should be handled with a forceps.

ii) Tick-handling facilities

The handling of field-collected ticks in the laboratory must be controlled so as to avoid accidental attachment to personnel. Field-collected ticks should be fed on rabbits and cattle in isolation facilities. Animals on which laboratory-infected or field-collected ticks have fed should be destroyed. Following engorgement of field-collected ticks on laboratory animals, aliquots should be homogenised and tested for extraneous human pathogens by inoculation in baby hamster kidney (BHK) and Vero cells. The effects of these inoculations should be studied through three passages. Any unused ticks should be destroyed by chemical means or by incineration.

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9. As stabilate material is derived from field animals and rabbits, their cells might be potential sources of contamination of the vaccine with extraneous pathogens. Potential contaminants include bovine leukosis, mycoplasma, bovine viral diarrhoea virus, BSE or other *Theileria* species transmitted by *R. appendiculatus* and alongside other bacteria and viruses.

In the case of different stocks being used on the same premises, problems with labels could be minimised by using appropriate pencils and clear codes. Preparation of different stabilates should be done sequentially to avoid cross-contamination and mislabelling. Regular quality control should be introduced to ensure the right stock(s) are used.

2.1.3. Validation as a vaccine strain

A vaccine strain should be identified in cross-immunity trials. These are set up between a vaccine strain and stabilates from *T. parva* field isolates from the area where the protection of the vaccine

strain is required. Ideally, five animals and two controls should be used per test, taking into account that these should be set up in two ways. First using the vaccine as a challenge in animals immunised with the local isolates and confirmed by subsequent homologous challenge. Secondly, using the local strain(s) as challenge in animals immunised with the vaccine and confirmed by subsequent homologous challenge. This will give information as to what extent a vaccine strain will give protection. On the other hand, results will indicate if a breakthrough might occur from the local *T. parva* population, present in carrier animals in the region where the vaccine will be deployed. A second test consists in testing whether infection with the vaccine strain can be controlled by the intended tetracycline treatment during the vaccination process.

2.2. Method of manufacture

2.2.1. Procedure

Vaccine batches are produced in several institutes in eastern Africa, using different strains, possibly requiring different parameters than the ones described here. For consistency in immunisation in field, it is essential that tick-derived sporozoite stabilates of an immunising stock are prepared from a fully characterised 'working seed stabilate'. The 'working seed stabilate' should be derived directly from the reference 'master seed stabilate', which is available in suitable quantity for future preparation of immunising stabilates. Immunising stabilates can be prepared according to a proposed set of standards (Morzaria *et al*., 1999~~b~~).

2.2.2. Requirements for ingredients

Before starting to produce vaccine, seed material with known characteristics is required. Three types of seed material are distinguished:

i) Master seed

The master seed is a cryopreserved sporozoite stabilate from a specific stock that has been selected and permanently stored and from which all other seeds are derived. The master seed should consist of a single uniform batch of seed that has been mixed and filled into containers as one batch. As *T. parva*-infective sporozoites are used for the manufacturing process, the master seed also represents the master stock (see Chapter 1.1.8). To prepare a master seed, *T. parva*-infected adult *R. appendiculatus* ticks are used that have fed as nymphs on the ears of an animal going through an active ECF infection. After moulting, these ticks, when prefed during 4 days on rabbits will have infective sporozoites in their salivary glands. These can be quantified by dissecting prefed ticks and determining infection rates in the dissected salivary glands by coloration ~~(Walker *et al*., 1981)~~. By grinding these prefed ticks in a specific medium, sporozoites will be released in the supernatants and a stabilate can be produced (FAO, 1984) that can be cryopreserved in minimum 100 cryotubes each containing preferably a set of infected acinus equivalent per vial or in case of a new stock an equivalent of 10 infected ticks per vial. A viability check of the master seed should be performed once the master seed has been cryopreserved for at least 24 hours by reviving one of the cryotubes.

ii) Working seed

The working seed is derived from infective sporozoites at a passage level between the master seed and the production seed. To prepare a working seed, the contents of a single cryotube of master seed are injected into a naive healthy experimental animal to produce an acute ECF infection.

iii) Production seed

To prepare a vaccine batch, the contents of sufficient cryotubes of working seed is mixed and the appropriate dose is injected into the required number of naive experimental cattle to produce an acute ECF infection.

Infection is established, with the working seed stabilate of *T. parva,* by inoculation of healthy cattle serologically and, ideally, PCR-negative for tick-borne diseases. During the parasitaemic phase of the ensuing disease reaction, clean laboratory-raised nymphs of *R. appendiculatus* are fed on the animals, and the engorged infected ticks are collected. The resultant adult ticks, within 3 weeks to 4 months after moulting, are applied in ear-bags to healthy rabbits. About 600 ticks are applied to each ear and unattached ticks are removed after 24 hours. After 4 days, the ticks are removed and samples (usually 60 ticks) taken to determine infection rates in dissected salivary glands. The remaining ticks are counted into batches of approximately 1000. An estimate of the total number of ticks can be obtained by counting and weighing a given number of ticks

and then weighing the total number of ticks. The ticks are washed in a sieve under fast flowing tap water and may be surface disinfected in 1% benzalkonium chloride, or in 70% alcohol, and then rinsed again in distilled water.

The ticks are placed (~1000) in heavy glass specimen jars or plastic beakers, and 50 ml MEM with Hank's or Earle's salts and 3.5% bovine plasma albumin (BPA) is added. The jars are kept on ice, and the ticks are ground using a tissue homogeniser (for instance Silverson LR2) for 2 minutes using a large aperture disintegrating head, and for 3 minutes using a small aperture head (emulsor screen). For smaller batches, an alternative method may consist of grinding the ticks, in batches of 1000 ticks using a mortar and pestle. Ticks are then crushed continuously by teams of two people for 15–30 minutes in a mortar; 30–35 ml cooled MEM/3.5% BSA medium, without glycerine with 50–100 g crushed glass is initially used. The difference (from 50 ml) MEM/BSA without glycerine is used to rinse the mortar and pestle and glass material used in crushing the ticks. Note that most of the crushing is done at the sidewalks of the mortar. Check for good crushing under a stereoscopic microscope and otherwise add glass. ALWAYS KEEP MEDIA AT 4°C.

The ground-up tick material is made up to 50 ml for every 1000 ticks, then centrifuged at 50 ***g*** for 5 minutes, and the supernatant is harvested. An equal volume of cold 15% glycerol in MEM/BSA is added dropwise while the tick material is maintained chilled on ice and stirred by a magnetic stirrer. The final volume will contain sporozoites from the equivalent of ten ticks/ml. The number of tick-equivalents/ml can be adjusted if parasite infection rates in a particular tick batch were either very high or very low. The final concentration of glycerol in the sporozoite stabilate is 7.5%.

The bottle containing the ground up tick material (gut) is fitted with a dispenser. Cryotubes of 1 ml are filled with the stabilate (1 ml per vial) while constantly stirring on an ice bath. Aliquots are kept at 4°C. Alternatively, artificial insemination equipment, as used to dispense semen, has been used with pre-labelled plastic straws. This latter system is ideal for large volume stabilates, and colour coding and labelling provide additional check on the identity of the immunising stabilate. An equilibration time of 30–45 minutes should be allowed.

The aliquots are then stored in insulation trays and moved to a –80°C deep-freezer as soon as possible. They are kept there for 24 hours, to allow a gradual cooling down (step-freezing) of the stabilate. On the second day, the aliquots are transferred into liquid nitrogen until use. Alternatively vials can be introduced in gas-phase liquid nitrogen for 3 hours and then immersed in the liquid nitrogen for storage ~~(Pipano, 1989b)~~. Vaccine is transported to the field in liquid nitrogen. Vials are taken out at the place of vaccination, by immersing the liquid nitrogen vials in lukewarm water (38°C) for 30 minutes to allow for good regeneration. It should be administered within 60 minutes after withdrawal from the liquid nitrogen container. Once unfrozen it can be kept alive on ice (+4°C) for another 6 hours ~~(Marcotty *et al.*, 2001; Mbao *et al.*, 2007)~~. The infection and treatment method is usually applied using long-acting tetracycline intramuscularly, and it is recommended that the tetracyline be administered first, in case an animal escapes having received stabilate only. Thereafter the stabilate is inoculated subcutaneoulsy over the parotid lymph node at the base of the ear.

The procedure for the preparation and testing of a multi-valent ITM vaccine (the Muguga cocktail) has been described in detail (Patel *et al.,* 2016). It is important to note that each of the component stabilates is produced before combining the infected and fed ticks immediately before homogenisation. The number of ticks from each component is calculated to produce a final vaccine stabilate containing equal numbers of infected acini from each component.

2.2.3. In-process controls

Records of the source and passages of the working seed material should be maintained. Seeds should be free of infective agents like enzootic bovine leukosis, bovine immunodeficiency virus, bovine pestivirus, bovine syncytial virus, Rift Valley fever, etc. Test procedures will depend on availability preferably using DNA analysis.

2.2.4. Final product batch tests

i) Sterility

Tests for sterility and freedom from contamination of biological materials for veterinary use may be found in chapter 1.1.9.

ii) Safety

Both ticks and experimental mammals are potential sources of contamination of stabilates with extraneous pathogens. In both cases, potential contaminants include *Ehrlichia bovis*, bovine *Borrelia* sp., orbiviruses, bunyaviruses, and others. Field-collected ticks should therefore not be used for the preparation of stabilates to be used for immunisation. Well characterised and pathogen-free laboratory colonies of ticks should be used for this purpose. Only healthy cattle and rabbits, free from tick-borne parasites, should be used for tick feeding. Stabilates should be prepared under aseptic conditions. In some circumstances, the use of antibiotics at concentrations appropriate for tissue culture may be indicated. Prepared stabilates should be subjected to routine tests for any viral infections in BHK and Vero cells (as above). Stabilates should be subjected to routine characterisation *in vivo*, which should involve infectivity testing in intact susceptible cattle, sensitivity to tetracyclines and other anti-theilerial drugs, and cross-immunity studies. A characterised 'working seed stabilate' should be prepared to ensure the purity of the *T. parva* stocks in the daughter immunising stabilate.

During stabilate preparation care must also be taken to avoid extraneous contamination of the stock being used with other *T. parva* stocks. Quality assurance procedures must be enforced, for example for the handling of infected ticks, and the rules should be adhered to rigidly. Tick unit facilities should allow for strict separation of infected and uninfected ticks. Tick unit personnel should use separate overalls for each batch of ticks used in stabilate preparation, and the overalls should be sterilised daily. Simultaneous work on many different stocks should be avoided. Stabilate storage systems should incorporate clear labelling of each stabilate tube or straw also mentioning the preferred number of doses per vial or straw. This will vary according to use in small holder dairy or pastoral herds etc.

Quality control checks on the stabilate should determine the similarity to the parent seed stock and also detect any extraneous *T. parva* contamination.

iii) Batch potency

The evaluation of the number of acini infected with *T. parva* in dissected tick salivary glands, before grinding, is a useful indicator of the level of infection but does not take into account the variable loss of viability during stabilate preparation caused by the intensity of grinding and the freeze–thaw processes. Furthermore, the state of maturation of the sporozoites is difficult to estimate by histological examination of the tick salivary glands. Therefore, the infectivity of the stabilate is determined by inoculation of a standard dose of 1.0 ml into susceptible cattle. The contents of 2–4 randomly selected tubes are mixed and then titrated in cattle, and its infectivity and lethality at different dilutions are established for use in immunisation. As the response of cattle to the infection and treatment method is dependent upon their susceptibility to the infection, it is important to titrate stabilates in cattle of the same type as those to be immunised. Titration of vaccine stabilates remains a highly controversial matter. Ideally, a median infectious dose (ID_{50}) and median lethal dose (LD_{50}) should be determined by titration of the stabilate using a tenfold dilution range (~~Duchateau *et al.*, 1998; 1999~~ Speybroeck *et al.*, 2008). The ID_{99+} (corresponding to close to 100% infectivity and having minimal lethality) should then be quantified by means of a finer titration, using dilutions around the LD_{50}. With respect to compound vaccines, quantification of the vaccine dose is complex as different strains need to be put together, changing the total lethality of the vaccine (Speybroeck *et al.,* 2008). The sensitivity to tetracyclines is also determined, essentially to provide a dose of stabilate that is controlled, preferably by a single dose of long-acting tetracycline administered at the same time as inoculation. The immunising dose should induce a very mild or inapparent infection, and the animal should develop a serological titre and be immune to lethal homologous challenge. Should a single treatment with tetracycline fail to suppress the infection in all cattle, then either a lower dose of the immunising stabilate or two treatments of tetracycline (on days 0 and 4) may be used. A single dose of 30 mg/kg long-acting oxytetracycline has been found to be effective in field immunisations, when used with an appropriate stabilate dilution. An alternative method that has been used involves stabilate infection and treatment with parvaquone at 20 mg/kg on day 8 (depending on the stabilate). This method can be applied where tetracyclines are not reliable, but it requires that the animal be handled more than once. A single treatment with buparvaquone at 2.5 mg/kg at the time of infection has also been shown to be effective with stabilate infections that were not controlled with a single treatment at 20 mg/kg of a long-acting formulation of tetracycline.

Once the procedure that results in a safe and effective immunising dose is established, it must be adhered to strictly in the field, or breakdown of immunisation may occur. It is also important that the stabilate dilution and drug/dose regimen be determined in the most susceptible cattle in which it is likely to be used.

2.3. Requirements for ~~authorisation/registration/licensing~~ regulatory approval

2.3.1. Manufacturing process

For ~~registration~~ regulatory approval of vaccine, all relevant details concerning manufacture of the vaccine and quality control testing (see Sections C2.2.2.1 and C2.2.2) should be submitted to the regulatory authorities. This information shall be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume.

2.3.2. Safety requirements

i) Target and non-target animal safety

Theileria parva sporozoites are not hazardous to humans, but they are infectious to bovines, and infection of naive animals for production of vaccine batches, as well as the titration experiments for dose quantification should be done in tick-free facilities. Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9. At a meeting in Malawi in 1988, the following recommendations on safety in the preparation, handling and delivery of *T. parva* infection and treatment vaccines were adopted (Anon, 1989).

ii) Reversion-to-virulence for attenuated/live vaccines and environmental considerations

The introduction of an immunising stock into an area/country from which it does not originate may result in that parasite, or a component parasite(s) of that stock, becoming established through a carrier state in cattle and transmission by ticks. The long-term effect of the introduction of new (and potentially lethal) parasites on the disease epidemiology should be considered before introduction, and should be monitored carefully following immunisation.

The characterisation of parasites in target populations should be carried out before immunisation, and at intervals following immunisation. At present the characterisation of parasite stocks with reference to vaccination relies primarily on immunisation and cross-challenge experiments in cattle. However, a number of methods for characterising parasite stocks *in vitro* have been attempted in laboratories possessing a high degree of expertise. Early studies have shown that parasite stocks that differ in MAb profile may not cross-protect, whereas stocks showing similar profiles give cross-protection ~~(Irvin & Morrison, 1987)~~. However, in more recent experiments using other *T. parva* stocks, this observation has been proven to be wrong. Another method to detect antigenic differences used T cell clones specific for parasitised cell lines, as T cell responses are believed to be important in mediating immunity against *T. parva* and the strain specificity observed in *in-vitro* killing assays reflects the *in-vivo* challenge results (~~Irvin & Morrison, 1987;~~ Taracha *et al.,* 1995). Apart from this, there are no other, simpler *in vitro* assays that correlate with protection *in vivo*. Statistically derived disease reaction index, based on parasitological, clinical and haematological measurements, was proposed for characterising levels of infectivity and virulence of different parasite stocks and assessing the impact of control intervention against theileriosis (~~Rowlands *et al*., 2000;~~ Schetters *et al*., 2010). Recently, DNA typing for characterisation of vaccine stabilates has been used and could be based on multi-locus genotyping using polymorphic antigen genes or satellite markers or a combination of both (Hemmink *et al*., 2016~~; Patel *et al*., 2011~~).

iii) Precautions (hazards)

Care should be taken during the preparation of sporozoite stabilates to avoid aerosol infection of personnel with extraneous pathogens when ticks are being ground. Those grinding ticks should be educated in the potential hazards involved; access to areas where ticks are homogenised should be restricted to specified and informed personnel; personnel should wear protective clothing, including gloves and masks; and tick grinding should be carried out in a microbiological safety cabinet (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

2.3.3. Efficacy requirements

i) Capacity to protect against naturally transmitted theileriosis

Although successful final product batch testing (see Section C.2.2.4.iii) indicates that a vaccine batch is immunogenic, it is important to note that protection against homologous challenge does not necessarily indicate that the vaccine will protect against all *T. parva* strains encountered in the field, and that the 'needle' challenge used in pen trials does not necessarily reflect a challenge delivered by ticks. This can only be assessed by controlled

field trials, which are expensive, complicated and lengthy. The need for and extent of field trials for each vaccine batch is the subject of debate and there is no agreed protocol. The least intensive approach, if veterinary authorities allow, is to closely monitor the initial 'roll-out' of the vaccine after successful final product batch testing as described above, with particular attention being given to the incidence of 'reactors' soon after vaccination and the occurrence of any 'breakthrough' cases following field exposure.

ii) Use in the field

The frozen vaccine is viably preserved in large liquid nitrogen repositories at the production facility and transported to farms in smaller liquid nitrogen containers. Field centres for storage and supply of vaccine can be set up in theileriosis-enzootic areas. The basic equipment required for field application of frozen vaccine includes a wide-mouthed jar for preparing a 38°C water bath, a thermometer for measuring the temperature of water, long forceps, face shield and temperature-resistant gloves. Application of the frozen vaccine to field cattle begins by putting on a face shield and temperature-resistant gloves. The required numbers of vials are withdrawn with the forceps from the canister of the liquid nitrogen refrigerator. When withdrawing the vials, the canister should be kept as deep as possible in the neck of the refrigerator to avoid quick warming of the remaining vials. Each withdrawn vial should be checked to ascertain that liquid nitrogen has not leaked inside. The liquid nitrogen does not alter the vaccine, but may cause the vial to explode when introduced in the water bath. Such a vial should be held at ambient temperature for 1–2 minutes to allow the nitrogen to escape and then processed in the usual way. The reported leaking of liquid nitrogen into a vial containing frozen vaccine has raised questions about the sterility of the frozen vaccine. However the system has been used for decades with no significant problem observed.

2.3.4. Breadth of immunity

Unlike *T. annulata*, where considerable cross-protection is observed among different strains in the field, a more complex situation exists for *T. parva*. Two strategies are used to try to overcome this antigenic complexity. A combination of three stocks, which provides a broad spectrum of protection, has been tested in a number of countries. Two large batches of a trivalent stabilate have been prepared by the International Livestock Research Institute (ILRI, Nairobi), the first in 1996 for the FAO and another one in 2008. These stabilates were prepared to the latest proposed standards and have been used safely and effectively in Tanzania. Further batches are being prepared at the African Union Centre for Ticks and Tick-Borne Diseases, Lilongwe, Malawi with increasing demand for the infection and treatment method of immunisation in *T. parva*-endemic areas in sub-Saharan Africa. If an immunising stabilate fails to protect against a 'breakthrough stock', this should be isolated, characterised, tested and considered for use, either alone, or as an addition to the current immunising stabilate. Another strategy is to prepare stabilates of national or local stocks for use within defined areas. This latter strategy is more costly in time and resources, but it avoids, to some extent, the introduction of new stocks into an area. With movement of cattle, there is a risk of the introduction of different stocks into an area, which may breakthrough the immunity provided by the local stock. Therefore, the use of local or introduced stocks for immunisation needs to be carefully evaluated ~~(Geysen, 2008; McKeever, 2007)~~.

The infection and treatment method of immunisation is effective provided the appropriate quality assurance measures are enforced. In the longer term, the attendant delivery problems and the risk of induction of carrier states and disease transmission, emphasise the need for the identification of protective antigens for development of subunit vaccines.

2.3.5. Duration of immunity

There have been few reports of controlled experiments to determine the duration of immunity induced by infection and treatment, in either the presence or absence of field challenge. However, ~~Burridge *et al.* (1972) established that~~ cattle ~~that had~~ survived an experimental infection (without treatment) and were subsequently maintained in an ECF-free environment, survived a lethal homologous challenge up to 43 months later.

2.3.6 Stability

If kept in liquid nitrogen, the frozen vaccine has a practically unlimited shelf life.

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NB: There is a WOAH Reference Laboratory for theileriosis (see the WOAH Web site for the most up-to-date list: http://www.woah.org/en/scientific-expertise/reference-laboratories/list-of-laboratories/). Please contact the WOAH Reference Laboratory for any further information on diagnostic tests, reagents and vaccines for theileriosis

NB: FIRST ADOPTED IN 1991. MOST RECENT UPDATES ADOPTED IN 2018.

Appendix 1: Theileriosis in cattle

Intended purpose of test: population freedom from infection, individual animal freedom from infection prior to movement, confirmation of clinical cases, prevalence of infection – surveillance

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
IFAT for *T. parva* Bovine Population: + Movement: + Clinical disease: – Surveillance: +++	Serum Glass slides with schizont whole body antigen	Reference test: No Dsp = 86% @1:40 Dsp = 95% @ 1:160 Dse = 55% @ 1:40 Dse = 28% @ 1:160	1. Herd of 105 sentinel cattle that tested negative at start and 63 calves born over surveillance period (1995–2000) 2. No true negative sample set used	See reference	1. Easy workflow 2. Sensitivity 3. Low cost 4. Low cost, sensitivity and ease of workflow allows surveillance of large populations	1. May show cross-reactivity with antibodies against *T. taurotragi*, *T. mutans* and *T.* sp. (buffalo). 2. Cell culture facilities required to produce antigen 3. Antigen is cold sensitive 4. Requires a fluorescent microscope. 5. High per cent of false negative results under endemic vs epidemic conditions. 6. Highly operator dependent 7. Low diagnostic sensitivity may not detect positive animals in small populations 8. Infected individuals from non-endemic regions not constantly challenged may test negative 9. Positive animals that present clinical disease may have prior exposure to parasites in endemic regions	Billiouw *et al.* (2005)
ELISA for *T. parva* Bovines Population: + Movement: + Clinical disease: - Surveillance: +	Serum PIM antigen	Reference test: IFAT DSe = >99% Dsp = 98%%	1. 12 experimentally infected cattle served as positive set 2. 712 serum samples from various non-endemic regions were used as negative set	See reference	1. More sensitive than IFAT 2. Antigen highly variable and may impact different geographical regions.	1. Not commercially available 2. PIM antigen may show cross-reactivity to *T.* sp. (buffalo) 3. Need spectrophotometer 4. Use monoclonal anti-bovine secondary conjugate that preclude use for buffalo 5. Antigen variability may limit testing of population freedom 6. Antigen variability may limit testing for individual movement 7. Positive animals that present clinical disease may have prior exposure to parasites in endemic regions 8. Cross-reactivity may limit surveillance capacity	Bishop *et al.* (2015) Katende *et al.* (1998)
P104 Nested-PCR for *T. parva* Bovines Buffalo Population: +++ Movement: ++ Clinical disease: ++ Surveillance: +++	EDTA-blood P104 gene	Reference test: No Dsp = nd Dse = nd	1. Two Experimentally infected animals 2. 151 field samples in endemic regions in Kenya (42.3% positive) 3. 169 field samples in endemic region in southern Sudan (36% positive)	See reference	1. More sensitive than IFAT 2. Direct detection of parasite 3. Highly specific since p104 is unique 4. High specificity and sensitivity would allow testing of populations from freedom of infection	1. Two-step PCR with potential for contamination 2. False negative values due to variation in primer regions 3. Variation in primer sequences may miss a low percentage of positive animals	Odongo *et al.* (2010)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
					5. Positive testing of animals with clinical signs may allow confirmation of clinical cases 6. High specificity and sensitivity allow accurate surveillance of disease		
18S real-time PCR for *T. parva* Bovines Buffalo Population: ++ Movement: ++ Clinical disease: ++ Surveillance: +++	EDTA-blood 18S ribosomal RNA gene	Reference test: No Dsp = nd Dse = nd	1. Gold standard positive group One buffalo; two experimentally infected cattle; 127 African buffalo from endemic regions 2. Gold standard negative group 89 cattle non-endemic region 3. Undefined infection status 107 cattle Specific results not given for groups	See reference	1. More sensitive than IFAT 2. Direct detection of parasite 3. Single step PCR that prevents contamination 4. Positive testing of animals with clinical signs may allow confirmation of clinical cases using Ct values as estimate of parasitaemia 5. High specificity and sensitivity allow accurate surveillance of disease	1. Co-amplify *T.* sp. (buffalo)-like parasites that may lead to PCR suppression and false negatives 2. Need a real-time PCR system, specifically optimised for Roche LightCycler 1.0 or 2.0 capillary-based system 3. Declaring populations free of infection in buffalo is impacted by mixed-infections in buffalo with *T.* sp. *buffalo*-like parasites 4. Declaring individuals free of infection for movement in buffalo is impacted by mixed-infections in buffalo with *T.* sp. *buffalo*-like parasites	Pienaar et *al.* (2011) Sibeko *et al.* (2008)
Hybrid 18S real-time PCR for *T. parva* Bovines Buffalo Population: +++ Movement: +++ Clinical disease: ++ Surveillance: +++	EDTA-blood 18S ribosomal RNA gene	Reference test: 18S rRNA PCR Dsp = nd Dse = nd Accreditation validation Dsp = 100 Dse = 100 Reference test: P104 nested PCR Dsp = 100% Dse = 98.3%	1. Gold standard negative sample set: 525 buffalo and cattle samples 2. Gold standard positive sample set: 850 18S PCR positive samples 3. 1036 *T.* sp. (buffalo) positive samples Accreditation validation 1. Gold standard negative sample set: 501 African buffalo from non-endemic region 2. Gold standard positive sample set: 692 African buffalo from endemic region 1. 73 Gold standard positive buffalo 2. 73 Gold standard negative buffalo	See references See references	1. More sensitive than IFAT 2. Direct detection of parasite 3. More specific than real-time PCR of Sibeko *et al.* (2008) 4. High specificity 5. High sensitivity 6. Single step PCR that prevents contamination 7. Compares well with the p104 nested PCR reference test 8. High sensitivity and specificity will allow declaration of populations free of infection 9. High sensitivity and specificity allow testing of individuals free of infection for movement 10. Positive testing of	1. Need a real-time PCR system, specifically optimised for Roche LightCycler systems	Pienaar *et al.* (2011) In-house validation for ISO17025 accreditation

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
					animals with clinical signs may allow confirmation of clinical cases using Ct values as estimate of parasitaemia 11. High specificity and sensitivity allow accurate surveillance of disease		
IFAT for *T. annulata* Bovine Population: + Movement: + Clinical disease: – Surveillance: +++	Serum Glass slides with piroplasm antigen	Reference test: No Dsp = 82% Dse = 72%	1. 70 bovines in endemic region monitored for 1 year	See references	1. Easy workflow 2. Specific 3. Relatively sensitive for surveillance purposes	1. Requires a fluorescent microscope 2. Operator dependent 3. Low diagnostic sensitivity may not detect positive animals in small populations 4. Infected individuals from non-endemic regions not constantly challenged may test negative 5. Positive animals that present clinical disease may have prior exposure to parasites in endemic regions	El Damaty *et al.* (2021)
Conventional PCR for *T. annulata* Population: ++ Movement: ++ Clinical disease: ++ Surveillance: +++	EDTA-blood 18S ribosomal RNA gene	Reference test: No Dsp = 95% Dse = 83%	1. 70 bovines in endemic region monitored for 1 year	See references	1. More sensitive than IFAT 2. Direct detection of parasite 3. Lower cost than real-time PCR 4. High specificity 5. Relative high sensitivity, high specificity and low cost allow surveillance of large populations	1. Time consuming 2. Relative low sensitivity would not allow for declaration of freedom in small populations 3. Relative low sensitivity would impact on declaration of freedom for individual freedom for movement 4. Positive testing of animals with clinical signs may allow confirmation of clinical cases if PCR amplicon is quantified to estimate parasitaemia	El Damaty *et al.* (2021)
Real-time PCR for *T. annulata* Population: ++ Movement: ++ Clinical disease: ++ Surveillance: +++	EDTA-blood 18S ribosomal RNA gene	Reference test: No Dsp = 100% Dse = nd	1. 102 cattle samples from endemic region 2. 39 cattle samples from non-endemic region	See references	1. More sensitive than IFAT 2. Direct detection of parasite 3. Single step PCR that prevents contamination 4.	1. Need a real-time PCR system 2. Unknown diagnostic sensitivity impact on assessment for testing population freedom of infection, individual freedom of infection for movement and ability to be used for surveillance 3. Positive testing of animals with clinical signs may allow confirmation of clinical cases if PCR amplicon is quantified to estimate parasitaemia	Ros-Garcia *et al.* (2012)
Multiplex real-time PCR for *T. orientalis* genotypes buffeli, chitose, ikeda, type 5 Population: +++ Movement: +++ Clinical disease:	EDTA-blood p23 gene: buffeli MPSP gene: chitose ITS-1 gene: ikeda MPSP gene: type 5	Reference test: No Dsp = 98.9% Dse = 98%	1. 154 cattle from an outbreak region	See references	1. One test to differentiate four genotypes both non-pathogenic and pathogenic 2. Sensitive and specific 3. High sensitivity, specificity and screening of wide array of strains allow for declaration of	1. Need a high resolution melt real-time PCR system 2. Mixed infections may complicate result interpretation 3. Contamination would be difficult to detect due to the multiplex design	Perera *et al.* (2015)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
++ Surveillance: +++					freedom in populations 4. High sensitivity and specificity allow declaration of freedom for individual freedom for movement 5. Positive testing of animals with clinical signs may allow confirmation of clinical cases if PCR amplicon is quantified to estimate parasitaemia 6. High sensitivity and specificity allow surveillance of populations for a variety of strains or genotypes		
Quantitative real-time PCR for *T. orientalis* Chitose and Ikeda strains Population: ++ Movement: ++ Clinical disease: ++ Surveillance: +++	EDTA-blood MPSP gene	Reference test: No Chitose/Ikeda: Dsp = 92.6%; 96.4% Dse = 89.6%; 99.4%	1. 318 cattle samples from endemic region 2. 237 samples that tested positive with different tests used for sensitivity 3. 81 samples that tested negative using prior PCR tests	See references	1. Direct detection of parasite 2. Differentiate pathogenic strains 3. High specificity 4. High sensitivity 5. Single step PCR that prevents contamination 6. Relative high sensitivity, high specificity and low cost allow surveillance of large populations	1. Need a real-time PCR system 2. Test not validated against non-pathogenic strains 3. Relative low sensitivity would not allow for declaration of freedom in small populations 4. Relative low sensitivity would impact on declaration of freedom for individual freedom for movement 5. Positive testing of animals with clinical signs may allow confirmation of clinical cases if PCR amplicon is quantified to estimate parasitaemia	Bogema *et al.* (2015)

Annexe 25. Chapitre 3.4.14. Trichomonose

RÉUNION DE LA COMMISSION DES NORMES BIOLOGIQUES
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CHAPTER 3.4.14.

TRICHOMONOSIS

SUMMARY

Description and importance of the disease: *Trichomonosis is a venereal disease of cattle caused by* Tritrichomonas foetus*, a flagellate protozoan parasite.*

Trichomonosis is ~~asymptomatic~~ subclinical in bulls, however in cows the disease is characterised by infertility, abortion, embryonic and early fetal death, fetal maceration, pyometra and vaginal discharge. The disease has a world-wide distribution and, at one time, was of major economic importance as a cause of abortion and infertility, especially in dairy cattle. The widespread use of artificial insemination in many areas of the world has contributed to reduced prevalence. Nevertheless, trichomonosis is still of importance in countries with extensive farming practices where artificial insemination is not used.

Transmission of the disease is primarily by coitus, but mechanical transmission by insemination instruments or by gynaecological examination can occur. The organism can survive in whole or diluted semen at 5°C. Bulls over 3–4 years old are the main reservoir of the parasite as they tend to be long-term carriers, whereas most cows and young bulls (*less than 3 years old*) *may clear the infection spontaneously. For these reasons samples from bulls are usually preferred for diagnosing and controlling the disease in herds.*

Identification of the agent by* in-vitro *culture and microscopy: Tritrichomonas foetus *is a flagellate, pyriform protozoan parasite, approximately 8–18 µm long and 4–9 µm wide, with three anterior and one posterior flagellae and an undulating membrane. The organisms move with a jerky, rolling motion and are seen in culture tests of preputial samples of infected bulls and vaginal washings or cervico-vaginal mucus of infected cows, or sometimes in aborted fetuses.* Tritrichomonas foetus *can be cultured* in vitro *and may be ~~viewed~~ detected by microscopy using wet mounts or stained slides. The standard diagnostic method for bulls involves the appropriate collection, examination and culture of smegma from the prepuce and penis, while in cows the preferred sample is vaginal mucus. Smegma can be collected by a variety of means including preputial lavage, brushing or scraping the preputial cavity and glans penis at the level of the fornix with a dry insemination pipette. A number of* in-vitro *culture media exist, including a commercially available field culture test kit, which supports trichomonad growth and allows direct microscopic examination.*

Identification of the agent by molecular methods: *Bovine trichomonosis ~~may~~ can also be detected by polymerase chain reaction* (*PCR*) *amplification. Both conventional and quantitative real-time PCR have been used successfully in the identification of* T. foetus *and the diagnosis of trichomonosis. Both methods have been used either in combination with culture or alone. ~~The conventional PCR has increased sensitivity when combined with culture, while the quantitative PCR has been successfully used on clinical samples. The quantitative real-time PCR has been validated both in the USA and Canada and it is now available as a commercial kit. This assay is also used routinely in Australia.~~ Various commercials kits are also available for the detection of parasite DNA directly in clinical samples.*

Serological tests: *~~Attempts have been made at developing~~ Immunological tests, including an agglutination test using mucus collected from the cervix and an antigen made from cultured organisms, ~~and these were~~ have been used as herd tests, ~~.~~ however, these tests lack sensitivity and are not ~~used~~ suitable for the individual diagnosis of trichomonosis. ELISAs developed to target whole IgG, IgG1 and IgG2 in serum and IgA in vaginal mucus are most commonly applied to confirm immune status in individual animals or populations post-vaccination. There is one report demonstrating the use of these ELISAs to confirm clinical infection in heifers.*

Requirements for vaccines: *A partially efficacious, killed whole-cell vaccine is commercially available as either a monovalent, or part of a polyvalent vaccine containing* Campylobacter *and* Leptospira. *The efficacy of several experimental whole cell vaccines and purified* T. foetus *membrane extracts demonstrate that* T. foetus *clearance rates are shorter compared with unvaccinated heifers. Subunit vaccines are less effective and recombinant vaccines have not been developed.*

A. INTRODUCTION

1. Description and importance of the disease

Trichomonosis is a bovine venereal disease caused by the flagellate protozoan parasite, *Tritrichomonas foetus*. The habitual hosts of *T. foetus* are cattle (*Bos taurus, B. indicus*). This parasite can be present in the genital tract of infected bulls, more precisely, the preputial cavity. It localises on the surface of the epithelium of the penis and on the proximal foreskin in the fornix area (BonDurant, 1997), a suitable microenvironment for facultative or microaerophilic microorganisms. In bulls trichomoniasis is subclinical, and infection can persist for the life of the bull (Collántes-Fernández *et al.*, 2018). However, shortly after infection, preputial discharge associated with small nodules on penile membranes may be detected (Dąbrowska *et al.*, 2019). For bulls older than 3–4 years, spontaneous recovery rarely occurs, resulting in a permanent source of infection in herds. In bulls under 3–4 years of age, infection may be transient. One of the proposed measures for controlling *T. foetus* infection in a herd is the use of bulls under 3–4 years of age rather than bulls older than 3–4 years (Yao, 2013).

In the infected cow, the initial lesion is a vaginitis, which, in pregnant animals, results in invasion of the cervix and uterus. Various sequelae can result, including a placentitis leading to early abortion (1–16 weeks), uterine discharge, and pyometra. In some cases, despite infection, pregnancy is not terminated by abortion and a normal, full-term calf is born. On a herd basis, cows may, following infection, exhibit irregular oestrous cycles, uterine discharge, pyometra, or early abortion (BonDurant, 1997; Skirrow & BonDurrant, 1988). Cows usually clear their infection within 90 days and acquire a short-lived immune protection to *T. foetus* for a period of at least a year and in some cases up to three years (BonDurant, 1997).

~~In some cases, despite infection, pregnancy is not terminated by abortion and a normal, full-term calf is born. On a herd basis, cows may, following infection, exhibit irregular oestrous cycles, uterine discharge, pyometra, or early abortion (BonDurant, 1997; Skirrow & BonDurrant, 1988). Cows usually clear their infection within 90 days and acquire a short-lived immune protection to T. foetus for a period of at least a year and in some cases up to three years (BonDurant, 1997).~~ Transmission of infection occurs by coitus, or by gynaecological examination of cows using contaminated instruments. It may also occur via artificial insemination (AI) as semen from infected bulls may be passively contaminated by *T. foetus* present in the preputial cavity. Therefore, all bulls must be routinely checked for the absence of *T. foetus* infection. Where AI along with diagnostic monitoring and culling of infected bulls is used, trichomonosis has been controlled; however, it is still prevalent in the Americas, Australia, South Africa and Eastern European countries where extensive farming and natural mating is practised.

2. ~~Causal~~ Taxonomical classification of the pathogen

Tritrichomonas foetus belongs to the genus *Tritrichomonas* in the family Trichomonadidae, Order Trichomonadorida. *Tritrichomonas foetus* is pyriform, 8–18 µm long and 4–9 µm wide, with three anterior and one posterior flagellae, and an undulating membrane. Live organisms move with a jerky, rolling motion, and ~~can be detected by light microscopy. Phase contrast dark field microscopy or other methods must be used to observe the details needed for identification~~ when growing in medium cultures, are usually observed in a trophozoite stage, appearing with a pyriform or ovoid shape (Collántes-Fernández *et al.*, 2018; Dąbrowska *et al.*, 2019). Under unfavourable environmental conditions, cells adopt a "defensive mechanism" and change into pseudocysts, having a spherical shape without flagellates or undulating membranes. This can be observed in post-exponential phases in laboratory cultures, but also in fresh preputial and swab samples (Dąbrowska *et al.*, 2019). Detailed morphological descriptions~~, including electron microscopy studies,~~ have been published (Benchimol *et al.*, 2021; Warton & Honigberg, 1979). *Tritrichomonas foetus* ~~has only the trophozoite stage and~~ multiplies by longitudinal binary fission; sexual reproduction is not known to occur. Three serotypes are recognised based on agglutination (Skirrow & BonDurrant, 1988): the *'belfast'* strain, reported predominantly in Europe, Africa and the USA (Gregory *et al.*, 1990); the *'brisbane'* strain described in Australia (Elder, 1964) and the *'manley'* strain, which has been reported in only a few outbreaks (Skirrow & BonDurrant, 1988)

~~The habitual hosts of *T. foetus* are cattle (*Bos taurus, B. indicus*). The bovine gastrointestinal tract hosts a number of other, commensal trichomonads e.g. *Pentatrichomonas hominis*, *Tetratrichomonas buttreyi*, *Tetratrichomonas pavlova*, *Tritrichomonas enteris* and *Pseudotrichomonas* species, which often contaminate preputial samples (Taylor *et al.*, 1994). The number of flagellae, observed under phase contrast illumination or after staining, is an important morphological characteristic that can help differentiate *T. foetus* from other bovine flagellated parasites.~~

~~However, non-*T. foetus* trichomonads are often difficult to distinguish from *T. foetus* based on culture and morphology (Taylor *et al.*, 1994).~~

~~*Tritrichomonas suis,* a commensal of pigs, and bovine *T. foetus* are indistinguishable morphologically, serologically and antigenically. The use of modern molecular techniques such as restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA analysis (RAPD), variable length repeats (VLR), and internal transcribed spacers 1 and 2 (ITS1 and 2) polymerase chain reaction methods (PCR), supports the view that these two species are identical (Tachezy *et al.*, 2002). More recently, *T. foetus* and *T. suis* were found to be identical at 9/10 loci and the use of the *T. suis* senior synonym has been suppressed in favour of *T. foetus* (Šlapeta *et al*, 2012).~~

Tritrichomonas foetus has been reported in domestic cats, horses and roe deer. Other species, such as goats, pigs, dogs, rabbits and guinea-pigs, have been experimentally infected (Levine, 1973). *Tritrichomonas foetus* has also been isolated from cats with diarrhoea and is now commonly known as the 'cat genotype' *T. foetus* (Šlapeta *et al.*, 2012). ~~*Tritrichomonas foetus* has also been reported to cause infections in humans including meningoencephalitis and peritonitis in immunocompromised and immunosuppressed individuals (Yao, 2012).~~ In humans, only three cases have been reported (to 2016) of infection by *Tritrichomonas foetus* and these were related with rare opportunistic infections in immunocompromised and immunosuppressed individuals (Suzuki *et al.*, 2016)."

~~Transmission of infection occurs by coitus, or by gynaecological examination of cows using contaminated instruments. It may also occur via artificial insemination (AI) as semen from infected bulls may be passively contaminated by *T. foetus* present in the preputial cavity. Therefore, all bulls must be routinely checked for absence of *T. foetus* infection. Where AI along with diagnostic monitoring and culling of infected bulls is used, trichomonosis has been controlled; however, it is still prevalent in the Americas, Australia, South Africa and Eastern European countries where extensive farming is still practised, and natural mating is allowed.~~

~~The site of infection in bulls is primarily the preputial cavity (BonDurant, 1997), and little or no clinical manifestation occurs. For bulls older than 3–4 years, spontaneous recovery rarely occurs, resulting in a permanent source of infection in herds. In bulls under 3–4 years of age, infection may be transient. One of the proposed measures for controlling *T. foetus* infection in a herd is the use of bulls under 3–4 years of age rather than bulls older than 3–4 years (Yao, 2013).~~

~~*Tritrichomonas foetus* is present in small numbers in the preputial cavity of infected bulls, with some concentration in the fornix and around the glans penis (BonDurant, 1997). Chronically infected bulls show no gross lesions. In the infected cow, the initial lesion is a vaginitis, which, in pregnant animals, results in invasion of the cervix and uterus. Various sequelae can result, including a placentitis leading to early abortion (1–16 weeks), uterine discharge, and pyometra. In some cases, despite infection, pregnancy is not terminated by abortion and a normal, full-term calf is born. On a herd basis, cows may, following infection, exhibit irregular oestrous cycles, uterine discharge, pyometra, or early abortion (BonDurant, 1997; Skirrow & BonDurrant, 1988). Cows usually clear their infection within 90 days and acquire a short lived immune protection to *T. foetus* for a period of at least a year and in some cases up to three years (BonDurant, 1997).~~

3. Zoonotic potential and biosafety and biosecurity requirements

Tritrichomonas foetus is not identified as a zoonotic agent. Laboratory manipulations should be performed with appropriate biosafety and containment procedures as determined by biological risk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

4. Differential diagnosis

Diseases such as campylobacteriosis, leptospirosis, brucellosis, neosporosis, chlamydiosis, bovine viral diarrhoea, infectious bovine rhinotracheitis and anaplasmosis, that may cause clinical signs including infertility, vaginitis, pyometra, abortions and vaginal discharge, should be excluded.

B. DIAGNOSTIC TECHNIQUES

A tentative diagnosis of trichomonosis as a cause of reproductive failure in a herd is based on the clinical history, signs of early abortion, repeated returns to service, or irregular oestrous cycles. Confirmation of infection depends on the demonstration of *T. foetus* in placental fluid, stomach contents of the aborted fetus, uterine washings, pyometra discharge, vaginal mucus or preputial smegma. In infected herds, the most reliable material for diagnosis is either preputial or vaginal washings or scrapings (Buller & Corney, 2013; Yao, 2013). The quantity of organisms may differ depending on the sample. There are numerous *T. foetus* cells in the aborted fetuses, in the uterus several days after abortion, and, in the vaginal mucus of recently infected cows for 12–20 days after infection. Samples collected from bulls, cows and aborted fetuses are tested by conventional methods (direct examination and culture)

or by molecular methods. The sampling techniques are the same in both cases; however, samples for culture are inoculated into transport medium or culture medium, whereas those for molecular biology can be collected either in medium or in phosphate-buffered saline (PBS) or normal saline.

Table 1. Test methods available for the diagnosis of trichomonosis and their purpose

Method	Purpose					
	Population freedom from infection[(a)]	Individual animal freedom from infection prior to movement[(b)]	Contribute to eradication policies[(c)]	Confirmation of clinical cases[(d)]	Prevalence of infection – surveillance[(e)]	Immune status in individual animals or populations post-vaccination
Detection and identification of the agent[(f)]						
Microscopy for morphological identification	+++	++	+++	+++	++	–
Conventional PCR on clinical samples	+	++	–	++	–	–
Conventional PCR in combination with culture	++	+++	–	+++	+	–
Real-time PCR	+++	+++	+++	+++	++	–
ELISA	≡	≡	≡	+++	≡	+++
Detection of an immune response						
IgG ELISA (serum), IgA ELISA (vaginal mucus)	≡	≡	≡	++	≡	+++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction.
[(a)]See Appendix 1 of this chapter for justification table for the scores given to the tests for this purpose.
[(b)]See Appendix 2 of this chapter for justification table for the scores given to the tests for this purpose.
[(c)]See Appendix 3 of this chapter for justification table for the scores given to the tests for this purpose.
[(d)]See Appendix 4 of this chapter for justification table for the scores given to the tests for this purpose.
[(e)]See Appendix 5 of this chapter for justification table for the scores given to the tests for this purpose.
[(f)]A combination of agent identification methods applied on the same clinical sample is recommended.

1. ~~Identification~~ Detection and identification of the agent

~~A tentative diagnosis of trichomonosis as a cause of reproductive failure in a herd is based on the clinical history, signs of early abortion, repeated returns to service, or irregular oestrous cycles. Confirmation of infection depends on the demonstration of *T. foetus* in placental fluid, stomach contents of the aborted fetus, uterine washings, pyometra discharge, vaginal mucus or preputial smegma. In infected herds, the most reliable material for diagnosis is either preputial or vaginal washings or scrapings (Buller & Corney, 2013; Yao, 2013).~~

~~The number of organisms varies in different situations. They are numerous in the aborted fetus, in the uterus several days after abortion and, in recently infected cows, they are plentiful in the vaginal mucus 12–20 days after infection. In the infected bull, *T. foetus* organisms are present on the mucosa of the prepuce and penis, apparently not invading the submucosal tissues. It is recommended to allow at least 1 week after the last service before taking a preputial sample.~~

1.1. ~~Sampling techniques and transport conditions~~ Collection of samples

~~A number of~~ Several techniques for collecting preputial samples from bulls or vaginal samples from cows have been described. It is important to avoid faecal contamination, as this may introduce intestinal protozoa that may be confused with *T. foetus* (Taylor *et al.*, 1994). Also, the presence of urine may

interfere with DNA detection (Clothier *et al.*, 2019). Contamination of samples should be minimised by removal of extraneous material and soiled hair from around the preputial orifice or vulva; however, cleansing of the area, particularly with disinfectants, is to be avoided, as this may reduce diagnostic sensitivity.

~~Samples collected from bulls, cows and aborted fetuses are tested by conventional methods (direct examination and culture) or by molecular methods. The sampling techniques are the same in both cases; however, samples for culture are inoculated into transport medium or culture medium, whereas those for molecular biology can be collected either in medium or in phosphate buffered saline (PBS) or normal saline.~~

1.1.1. ~~Collection of samples:~~ Bulls

Samples can be collected from bulls by three methods: ~~, namely~~ scraping, brushing or washing (Buller & Corney, 2013; Yao, 2013). When using the same sampling method, a sample can be used for the detection of bovine genital campylobacteriosis (see Chapter 3.4.4 *Bovine genital campylobacteriosis*). All three methods provide similar analytical sensitivity, independently of the diagnostic method implemented afterwards, i.e. culture or polymerase chain reaction (PCR) methods. The sampling techniques should be chosen based on the context and local conditions. For optimal sensitivity (95% or more) the first sampling should be done after a 1-week sexual rest and bulls should be sampled and tested three times at weekly intervals (Yao, 2013).

i) Scraping the preputial and penile mucosa with an artificial insemination pipette connected to a syringe or a bulb via a silicone rubber tube, is a common technique.

ii) Special brushes made of metal or plastic, may be used to collect smegma from the penis and prepuce (Yao, 2013). Plastic disposable brushes, with a hole in the tip (hollow 'brush' or 'rasper') are commercialised for this purpose. They are easy to use and fast. The tool is gently scraped along the surface of the penis and internal prepuce near the fornix. The collected smegma is rinsed into ~5 ml PBS or normal saline, or medium.

iii) Preputial lavage is still a common technique. A strong plastic tubing attached to a rubber bulb is inserted into the full length of the preputial cavity and the latter is washed with 20–30 ml PBS pH 7.2 or normal saline Collecting washes from the artificial vagina after semen collection is not recommended due to low diagnostic sensitivity (Gregory *et al.*, 1990).

~~Sampling techniques for bulls have been compared by several laboratories (reviewed in Yao, 2013). The results indicate that all three methods, i.e. brushing, scraping and lavage, provide similar analytical sensitivity, irrespective of the diagnostic method implemented afterwards, i.e. culture or PCR. Thus the sampling techniques should be chosen based on the context and local conditions.~~

~~Several laboratories have examined the effect on diagnostic sensitivity of repeated sampling (reviewed in Yao [2013]). For optimal sensitivity (95% or more) the first sampling should be done after a 1-week sexual rest and bulls should be sampled and tested three times at weekly intervals.~~

1.1.2. ~~Collection of samples:~~ Cows

Samples from cows are collected by washing the vagina, or by scraping the cervix with an artificial insemination pipette or a brush. The collected mucus is rinsed into ~ 5 ml PBS or normal saline (if PCR is intended) or in medium.

1.1.3. ~~Samples from~~ Aborted fetus

Abortions due to *T. foetus* may occur at any time during gestation from 2 months onwards, but most frequently at 3–5 months (Buller & Corney, 2013). When abortions in that period occur and *T. foetus* infection is suspected, the placenta and fetal fluids should be sampled, together with the lungs of the aborted foetus (Buller & Corney, 2013). The abomasal content is also reported to contain high numbers of *T. foetus* (Rhyan *et al.*, 1988).

1.2. Transport

The choice of sampling and diagnostic techniques should take into account several factors including the transport conditions and possibilities, and the expected duration of transport. PBS or normal saline is not a good transport medium if the samples are to be cultured (Bryan *et al.*, 1999). The use of a transportation media with nutrients and antibiotics is essential for the survival of the parasite. Therefore, there are different media used today as transportation and culture media, e.g. a thioglycolate broth media with antibiotics, Diamond's medium, or a commercial plastic pouch (Bryan *et al.*, 1999; Collántes-Fernández

et al., 2018). Under certain transport conditions, extended transport of cultures from remote areas to diagnostic laboratories, i.e. northern Australia, leads to heavily contaminated field isolates thus higher concentrations of antibiotics and fungicides are applied to isolate pure *T. foetus*. Yeast and fungi are viewed using microscopy and bacteria screened using blood agar plates (Tabor *et al.*, 2023). Note: if *T. foetus* can be visualised in heavily contaminated cultures using microscopy and if storage of pure cultures is not needed, the high doses of antibiotics are not required.

Samples for diagnosis should arrive at the laboratory, ideally within 48 hours after sample collection. ~~Where samples must be submitted to a laboratory and cannot be delivered within 24 hours, a transport medium containing antibiotics should be used (e.g. a thioglycolate broth media with antibiotics [Bryan *et al.*, 1999]) or a commercial plastic pouch. PBS or normal saline is not a good transport medium if the samples are to be cultured (Bryan *et al.*, 1999).~~ During transportation, ~~the organisms~~ samples should be protected from light and extremes of temperature~~, especially if cultures are to be undertaken in the laboratory~~. The temperature should remain above 5°C and below 37°C during transport (Bryan *et al.*, 1999). ~~Transport at 25°C followed by culture at 37°C is considered optimal for the survival and growth of *T. foetus* (Buller & Corney, 2013).~~ Freezing samples can result in negative cultures (Bryan *et al.*, 1999; Loy *et al.*, 2023), however it has been demonstrated that cooling or freezing samples for PCR analysis do not affect detection limits (Loy *et al.*, 2023).

~~In conclusion, the choice of sampling and diagnostic techniques should take into account several factors including the transport conditions and possibilities, and the expected duration of transport.~~

The use of lyophilised modified Diamond's medium in areas where cold chain storage of this medium prior to use is difficult, has been proved efficient (Rush *et al.*, 2019).

1.3. Identification of *T. foetus* by direct examination or in culture

1.3.1. Direct detection of parasites

Direct detection of parasites by microscopy immediately after sample collection, or on reception of the sample in transport medium, can be attempted (see below for examination and identification criteria). However, the organisms are often too few to allow for direct detection from the original samples. Thus, cultures should be prepared to allow multiplication of the parasites above the detection limit (around 10^4/ml; Bryan *et al.,* 1999).

1.3.2. Culture media

~~Samples should be inoculated into culture media as soon as possible after collection and thereafter incubated at 30°C–37°C, for 48–72 hours or longer, depending on the culture medium used, before being examined (see Section B.1.2.2.1). Inoculated media should not be chilled or refrigerated as this would affect survival of *T. foetus*.~~

~~1.2.2.1. Culture media~~

~~Several culture media can be used.~~ Diamond's trichomonad medium has been widely used for decades with some modifications over time ~~(Bryan *et al.*, 1999)~~. Today there are many variations from the original Diamond's medium that can be bought ready to use or prepared in a laboratory (Bryan *et al*., 1999; Clothier *et al*., 2019; Paradiso & Oyhenart, 2023; Rush *et al*., 2019). However, other culture media can be used for example ~~such as~~ the liver infusion broth medium (Lun *et al.* 2000) also named TFM medium, ~~Clausen's and Oxoid's media~~. Commercial culture kits are also available in various countries. It is important to ensure that the culture media are used before their established expiry date as many media do not have a long shelf life. In-house made media should normally be kept for no longer than 1 month at 5°C ± 3°C.

The quality of the water used is important and an antifungal can be added to the media to control yeast growth. Quality control checks including sterility checks should be carried out on all batches of media.

When liquid cultures will be further used for DNA extraction, agar can be omitted from the media due to the difficulty of harvesting pure *T. foetus* cells in the presence of the agar; a two per cent agar plug can be layered on top of the liquid medium to provide the anaerobic conditions for growth without the agar interfering with harvesting (Tabor *et al*., 2023).

~~It is important to make sure that the culture media are used before their established expiry date, as many media are not stable. In-house made media should normally be kept for no longer than 1 month at 5°C ± 3°C.~~

~~a) Composition of three commonly used culture media~~

i) Modified Diamond's Medium (Bryan *et al.,* 1999; Lun *et al.,* 2000)

Modified Diamond's Medium can be prepared as follows:

~~The modified Diamond's medium consists of:~~ 2 ~~g~~% trypticase peptone, 1 ~~g~~% yeast extract, 0.5 ~~g~~% maltose, 0.1 ~~g~~% L-cysteine hydrochloride, ~~and~~ 0.02 ~~g~~% L-ascorbic acid, ~~and is made up with 90 ml distilled water containing~~ 0.08 ~~g~~% ~~each~~ of K_2HPO_4 and 0.08% KH_2PO_4 are dissolved in distilled water. The pH of this solution is adjusted to 7.2 using NaOH or HCl~~, and adjusted to pH 7.2–7.4 with sodium hydroxide or hydrochloric acid~~. Following the addition of 0.05 ~~g~~% agar, the medium is autoclaved for 10–15 minutes at 121°C~~, allowed to cool to 49°C, and then 10 ml inactivated bovine serum (inactivated by heating to 56°C for 30 minutes), 100,000 units crystalline penicillin G and 0.1 g streptomycin sulphate are added aseptically~~.

After cooling the medium (room temperature or at least 48°C), 10% inactivated serum (56°C for 30 minutes) and antibiotics are added aseptically. Different types of inactivated serum can be used: bovine serum, fetal bovine serum (FBS), horse serum, sheep serum, are some examples (Diamond, 1957; Paradiso & Oyhenart, 2023; Rush *et al.*, 2019). Their availability and accessibility may depend on the country and laboratory. Antibiotics such as penicillin G (1000 U/ml) streptomycin sulphate (1000 µg/ml) and amphotericin B (10–25 µg/ml) are recommended for samples to be analysed. For purified cultures, the use of antibiotics can be omitted (Diamond, 1957).

The medium is aseptically dispensed in 3–10 ml aliquots into sterile vials and kept ~~16 × 125 mm screw top vials and refrigerated~~ at 4°C until use. Before use, aliquots should be pre-warmed to room temperature, or ideally, 37°C. The incorporation of agar into the medium confines contaminating organisms largely to the upper portion of the culture medium, while helping to maintain microaerophillic conditions at the bottom where the trichomonads occur in largest numbers.

ii) *Tritrichomonas foetus* medium (TFM) (based on modified Plastridge's medium)

TFM medium can be prepared as follows: 2.5% neutralised liver digest, 1% tryptose are dissolved in distilled water. The pH of this solution is adjusted to 7.4 using NaOH or HCl. Following the addition of 0.3% of Bacto agar, this medium is autoclaved for 15 minutes at 121°C. ~~The TFM consists of: 12.5 g neutralised liver digest, 5 g tryptose dissolved in 500 ml distilled water. The pH is adjusted to 7.4 with sodium hydroxide or hydrochloric acid. Following addition of 1.5 g Bacto agar, the medium is autoclaved for 15 minutes at 121°C. An antibiotic solution containing 0.75 g penicillin and 0.082 g streptomycin is prepared in 100 ml distilled water.~~ To prepare 1 litre of TFM, 500 ml basal medium is combined with 500 ml sterile inactivated (at 56°C for 30 minutes) bovine serum and 10 ml of an antibiotic solution. The antibiotics penicillin G and streptomycin sulphate are used at a final concentration of 75 µg/ml and 8.2 µg/ml respectively. This medium can be stored at –20°C.

iii) Media in a pouch

Where a combination of convenience and sensitivity is required, a combined specimen transport and culture kit may be used (BonDurant, 1997; Borchardt *et al.*, 1992). The kit consists of a clear flexible plastic pouch with two chambers. The upper chamber contains special medium into which the sample is introduced. Field samples for direct inoculation into the culture pouch would normally be collected by the preputial scraping technique (BonDurant, 1997). Samples collected by preputial washing require centrifugation before introduction of the sediment into the upper chamber. Following mixing, the medium is forced into the lower chamber, and the pouch is then sealed and incubated at 37°C. Microscopic examination for trichomonads can be done directly through the plastic pouch (Borchardt *et al.*, 1992).

~~The quality of the water used is important and an antifungal can be added to the media to control yeast growth.~~

~~Quality control checks including sterility checks should be carried out on all batches of media.~~

~~It is important to make sure that the culture media are used before their established expiry date, as many media are not stable. In house made media should normally be kept for no longer than 1 month at 5°C ± 3°C.~~

~~b) Growth characteristics in the different media~~

~~Lun et al. (2000) tested the three media described above, i.e. Diamond's, liver infusion broth medium and a commercial culture kit. They found that the three media supported the growth of T. foetus at 37°C, sixteen isolates from various geographical origins were tested. The growth characteristics of these isolates were different in the three media. For isolates grown in Diamond's the growth kinetics appeared less variable among isolates, the peak concentration tended to be reached earlier (by day 2–4 post-inoculation) and was generally higher (above 10^7 organisms per ml) than with the other two media. However, the parasites died faster after reaching the peak concentration in Diamond's medium. This has implications for monitoring of cultures (see Section B.1.2.3).~~

1.3.3. Culture inoculation

It is desirable to process samples collected by preputial wash or vaginal wash by centrifugation. If the sampling conditions in the field do not permit the use of a centrifuge, the samples should be decanted for 15–20 minutes on the bench. A volume of approximately 1 ml or less of the sediment or the pellet from the centrifugation step is then inoculated into culture media.

When using commercial sampling devices with a disposable brush, the latter is aseptically cut off directly into culture medium after sampling.

1.3.3.1. Culture conditions

Samples should be inoculated into culture media as soon as possible after collection and thereafter incubated between 30°C–37°C. A laboratory isolate of *T. foetus* should be cultured in parallel with the test samples, as a media positive control.

The optimal incubation period to reach peak concentration is between 2 days up to 7 days for modified Plastridge's medium and commercial kit media. As for modified Diamond's medium, incubation can be done between 2 days up to 5 days, however it is from day 2 up to day 4 that they reach their peak, and from day 5 (included) onwards unviable parasites start to appear in cultures (Lun *et al.*, 2000).

~~Cultures should be maintained at a temperature within the range 30°C–37°C. In no case should the incubation temperature rise above 37°C.~~

~~A known isolate of *T. foetus* should be cultured in parallel with the test samples, as a control.~~

~~It is advisable to start examining culture media microscopically at day 2–4 following inoculation, which in most media will correspond to the peak concentration.~~ If Diamond's medium is being used, a single examination at day 2, 3 or ~~—~~4 may be performed ~~as the parasites are rapidly dying out in this medium from day 5 onwards~~ (BonDurant *et al.*, 1996; Lun *et al.*, 2000). With other media supporting slower growth, such as liver broth infusion and even more so with commercial medium, it is recommended to examine the cultures at intervals until day 7 post-inoculation (Bryan *et al.*, 1999; Lun *et al.*, 2000). A single examination at day 7 is an alternative option with those media that support slower growth of *T. foetus* than Diamond's ~~(Section B.1.2.3)~~.

In conclusion, it should be emphasised that the different culture media described above are equally successful provided the culture procedures follow the general requirements and are adapted to the type of medium. Laboratories should evaluate what is most suitable for them in their own context, given their facilities and expertise, and considering the climatic, logistical, and economic considerations.

1.3.3.2. Culture cryopreservation

One method of conserving *T. foetus* cultures consists of using a freezing solution containing 10% inactivated calf serum, 80% RPMI (Roswell Park Memorial Institute) 1640 medium (without L-glutamine) and 10% dimethyl sulfoxide.

Cultures are grown for 2–3 days in media and centrifuged at 1500 ***g*** for 10 minutes. Five hundred µl of the lower layer is then added to 1 ml of freezing solution. Vials are paced in a freezing apparatus at –80°C for approximately 24 hours to cool the vials at around –1°C/minute. After this period, vials are transferred into storage boxes at –80°C or liquid nitrogen.

To recover *T. foetus* from cryopreservation, vials are placed at 30°C until the solution has just thawed, then quickly transferred into prewarmed medium. Subculture is made every 2–3 days, checking under the microscope for growth and motility; full recovery is reached in about 3–4 subcultures.

~~1.2.3. Summary of times for optimal growth and survival for different media~~

~~The optimal growth for modified Plastridge's medium, *Trichomonas* medium and commercial kit media is after 2–7 days, and the survival time in culture is 1–7 days; for modified Diamond's medium optimal growth is after 2–4 days and survival time in culture is 1–4/5 days (Buller & Corney, 2013).~~

1.3.4. *Tritrichomonas foetus* detection and identification by microscope ~~microscopic examination~~

Initial detection of organisms can be done by light microscopy, on a wet mount slide prepared directly from a drop of the sample or culture, or through the wall of a plastic pouch. When a drop of culture medium is examined, it should be carefully taken from the bottom of the tube, where the trichomonads are likely to concentrate due to the microaerophilic conditions.

The organisms may be seen under a standard compound microscope using a magnification of ×40 or ×80 initially, then ×100 or ×400. *Tritrichomonas foetus* is motile and normally exhibits jerky movements in wet preparations. Sufficient time, i.e. 2–5 minutes, should be devoted to examination of each slide, to allow the detection of parasites in low numbers and observation of the morphological features.

The pear-shaped organisms have three anterior and one posterior flagellae and an undulating membrane that extends nearly to the posterior end of the cell. They also have an axostyle that usually extends beyond the posterior end of the cell (Table 2). There are several other microorganisms similar to *T. foetus* that can also be found in cattle and that are very difficult to distinguish from *T. foetus*. It must therefore be considered that routine examination by microscopy can render false positive results (Dąbrowska *et al.*, 2019). Molecular detection in combination with culture is recommended when possible.

When relatively high numbers of organisms are present, phase-contrast microscopy ~~is~~ may be very valuable in revealing ~~these~~ *T. foetus* features and/or a rapid, Giemsa-based staining procedure ~~may~~ can also be used (Buller & Corney 2013; Lun & Gajadhar, 1999). ~~Both these techniques work best when relatively high numbers of organisms are present, especially the staining technique.~~

The staining of trichomonads is best performed using Lugol's iodine, which enhances the morphological features of the flagellae and the membrane (Lun & Gajadhar, 1999). Briefly, 1 ml of the culture containing the parasites is concentrated at high speed e.g. 16,000 ***g*** for 10 seconds. The supernatant is removed and the pellet is resuspended homogenously in the culture medium containing 10% bovine serum. A thin smear is prepared on a microscope slide using 10 µl of the suspension. The slide is air-dried and fixed for 1 minute in methyl alcohol and then stained in Lugol's iodine for 1 minute. The slide is subsequently stained with the rapid Giemsa method following the manufacturer's instructions. The slide is then washed thoroughly to remove any remaining stain and air dried before being examined at × 100 under oil-immersion (Buller & Corney, 2013). ~~The~~ Staining ~~allows~~ may help in the differentiation of *T. foetus* from other trichomonads ~~as shown~~ (see ~~in~~ Table 2).

Table 2. Morphological features of trichomonads (Buller & Corney, 2013)

Organism	Anterior flagella	Posterior flagella	Undulating membrane	Host
Tritrichomonas				
T. foetus	3	1	2–5	Cattle
T. enteris	3	1	3	Cattle
T. vaginalis	4	0	4	Human
Tetratrichomonas				

Organism	Anterior flagella	Posterior flagella	Undulating membrane	Host
T. buttreyi	3 or 4 variable length	3–5	1	Pigs, cattle
T. pavlovi	4	1	2–4	Calves
Pentatrichomonas				
P. hominis	4	1	3 waves	Human, primates, cats, dogs, cattle

1.3.5. Overall sensitivity and specificity of culture and identification

Any estimate of the diagnostic sensitivity and specificity of the culture and identification test will be dependent on the efficacy of sample collection, handling and processing, as well as the composition and quality of the culture medium.

~~In bulls, a commercial pouch kit has a sensitivity of 92% (95% confidence interval, 84–96%), while the same device used in experimentally infected young cows had an apparent sensitivity of 88% through a 10-week period after infection (Kittel *et al.*, 1998).~~

~~Estimates for Diamond's and related media have been variable, possibly due to variation in composition and preparation, but range from 78% to 99% (Skirrow & BonDurrant, 1988). Until recently, it has been assumed that the specificity of the culture test was 100%, but this is likely to be an overestimation.~~

Diagnostic results with samples from bulls using either Diamond's medium or a field kit have shown that the two methods give comparable results (Borchardt *et al.*, 1992; Bryan *et al.*, 1999; Kittel *et al.*, 1998; Lun *et al.*, 2000). It should be emphasised that not every sample taken from a particular bull known to be infected, will necessarily give a positive culture result. Even when conditions of sampling, transport, culture and identification are optimal, more than one negative sample should be obtained before there is reasonable assurance that the animal is not infected. This is the basis for most breeding regulations to specify that bulls above 6 months of age should be tested three times at weekly intervals before concluding ~~on~~ the absence of *T. foetus* infection. For bulls ~~below~~ under 6 months~~, or that~~ and those that have been kept with negative bulls only and never mated, one negative test is considered sufficient.

In females, as the infection is usually cleared within 90–95 days, it may be difficult to isolate organisms from individuals in the late stages of their infection.

The diagnosis of abortion induced by *T. foetus* may be relatively easy where an aborted foetus is recovered, because of the large number of organisms demonstrable in the fetal abomasal contents or placental fluids (Rhyan *et al.*, 1988). In addition, immunohistochemical techniques and molecular methods can be used to demonstrate ~~*Tritrichomonas*~~ *T. foetus* organisms in tissues of aborted foetuses.

1.3.6. Immunohistochemical techniques

Immunohistochemical techniques using monoclonal antibodies have been described for revealing *T. foetus* organisms in formalin-fixed tissues (Rhyan *et al.*, 1995). These techniques can be used to identify *T. foetus* in tissues from aborted fetuses (e.g. placenta and lungs).

1.4. Molecular methods ~~– detection of nucleic acids~~

PCR diagnostic frameworks are best determined for a particular region and country taking into account transport time to the laboratory, temperature of the sample, and optimised DNA extraction and PCR methods used at the laboratory. Currently, the best option is to use published protocols (adapted to the particular laboratory) or commercial *T. foetus* real-time PCR kits applied to transport medium or PBS, which guarantees fast preliminary results especially when using boiled lysates.

Several molecular methods have been described for the detection of *T. foetus* DNA prepared from cultures or directly from clinical samples. These include conventional and real-time PCR methods targeting conserved regions of the 5.8S ribosomal RNA gene and the flanking internal transcribed spacer regions (ITS). A PCR diagnostic test offers a number of potential advantages, including increased analytical sensitivity, faster diagnostic turnaround time, and the fact that the organisms in the collected

sample are not required to be intact and viable. However, attention to inhibitors must be considered when sampling (Clothier *et al.*, 2019; Collántes-Fernández *et al.*, 2018).

1.4.1. DNA Extraction

The preferred samples for DNA extraction are smegma and vaginal mucus in PBS or normal saline, transport medium and cultures.

1.4.1.1. Heat lysis

One ml of sample (vaginal mucus or smegma) is centrifuged for 5 minutes at 12,000 ***g***. The supernatant is removed and the pellet is resuspended in 500 µl RNase/DNase-free water. The suspension is heated at 95°C for 10 minutes. The lysate is centrifuged at 2000 ***g*** for 3 minutes and 5 µl of the supernatant is tested.

1.4.1.2. Magnetic beads-based extraction

Magnetic beads extraction is an automated method in which DNA can be isolated from crude samples by adding magnetic beads and without much processing. This methodology uses a robotic system and therefore, specific equipment for magnetic separation. It is sensitive and suitable for high throughput set up, where numerous samples are submitted for *T. foetus* testing. There is a variety of magnetic beads kits commercially available on the market. ~~This methodology uses a robotic system; it is sensitive and suitable for high throughput set up, where numerous samples are submitted for *T. foetus* testing.~~

1.4.1.3. Spin column method

This method uses spin columns and is more suitable for manual extraction of a small number of samples. There are a number of spin column kits available on the market and they all perform similarly.

~~The sensitivity of conventional PCRs is enhanced when using purified DNA extracts obtained by the use of commercial kits, e.g. magnetic beads or spin column methods. The real-time PCR can be used for all three extraction methods. Increased sensitivity has been observed by McMillen & Lew (2006) when using the heat lysis method, which was not suitable for conventional PCR.~~ For conventional PCR, it is best to use commercial DNA extraction kits, as the heat lysis method is not suitable. For real-time PCR, the three extraction methods can be used, but there is evidence that the heat lysis method has optimal sensitivity (McMillen & Lew, 2006).

1.4.2. Conventional PCR

Pathogen/ target gene	Primer (5'–3')	Concentration	Cycling parameters[a]
Method 1: Felleisen *et al.* (1998); GenBank Accession No.: M81842; amplicon size: 347 bp			
TFR3 TFR4	TFR3: 5'-CGG-GTC-TTC-CTA-TAT-GAG-ACA-GAA-CC-3' TFR4: 5'-CCT-GCC-GTT-GGA-TCA-GTT-TCG-TTA-A-3'	1 µM (final concentration)	40 cycles of: 94°C/30 sec and 67°C/30 sec Extension 72°C/90 sec
Method 2: Campero *et al.* (2003); GenBank Accession No.: M81842; amplicon size: 372 bp (TFR1+TFR2) and 347 bp (TFR3+TFR4)			
TFR1 TFR2 TFR3 TFR4	TFR1: 5'-TGCTTCAGTTCAGCGGGTCTTCC-3' TFR2: 5'-CGGTAGGTGAACCTGCCGTTGG-3' TFR3: 5'-CGG-GTC-TTC-CTA-TAT-GAG-ACA-GAA-CC-3' TFR4: 5'-CCT-GCC-GTT-GGA-TCA-GTT-TCG-TTA-A-3'	(not mentioned)	30 cycles of: 94°C/30 sec and 58°C/20 sec Extension 72°C/30 sec Final extension 72°C/20min

[a]A denaturation step prior to cycling has not been included.

Felleisen *et al.* (1998) described a conventional PCR that uses primers TFR3 and TFR4 to differentiate *T. foetus* from other commensal trichomonads, which are faecal contaminants of clinical samples submitted for the diagnosis of bovine trichomonosis.

~~The primers used are TFR3 and TFR4, which target a 347bp region and have the following sequences:~~

~~TFR3 – 5'-CGG-GTC-TTC-CTA-TAT-GAG-ACA-GAA-CC-3'~~
~~TFR4 – 5'-CCT-GCC-GTT-GGA-TCA-GTT-TCG-TTA-A-3'~~

~~The PCR is usually conducted for 40 cycles with a 30 second denaturation at 94°C, 30 seconds annealing at 67°C and 90 seconds extension at 72°C for 90 seconds. The final extension is performed at 72°C for 15 minutes (Felleisen *et al.*, 1998).~~

~~The use of two sets of primers together, one set amplifying DNA from the trichomonad group (TFR1 and TFR2) and the *T. foetus* specific set of primers TFR3 and TFR4 (Felleisen *et al.*, 1998) allowed the differentiation of *T. foetus* from other commensal trichomonads of the gastrointestinal tract that are often faecal contaminants of the bovine reproductive system (Campero *et al.*, 2003). *Tritrichomonas foetus* DNA is amplified by both sets of primers, while the DNA of commensal trichomonads is only amplified by the TFR1 and TFR2 primers.~~

Using pan-trichomonal primers and *T. foetus*-specific primers in a PCR assay, amplification products of 372 bp were detected in all virgin bull isolates, but only with the pan-trichomonal primers. Positive control isolates of *T. foetus* yielded amplification products of the expected size (372 and 347 bp) with the two sets of primers, respectively (Campero *et al.*, 2003).

The sequences of the generic primers TFR1 and TFR2, which amplify a 372 bp region of the internal transcribed spacer (ITS)1 and ITS2 region of the trichomonad group, are as follows:

~~TFR1 – 5' GTA GGT GAA CCT GCC GTT G 3'~~
~~TFR2 – 5' ATG CAA CGT TCT TCA TCG TG 3'~~

~~The PCR is conducted for 30 cycles with a 30 second denaturation at 94°C, followed by 20 seconds annealing at 58°C and a 30 second extension at 72°C. The final extension was performed at 72°C for 20 minutes.~~

Hayes *et al.* (2003) have also used RFLP of the amplicon generated by primers TFR1 and TFR2 to differentiate various trichomonads including *T. foetus*. The TFR3 and TFR4 primers have also been used in conjunction with DNA chelating fluorescent dyes under real-time PCR conditions for research purposes ~~rather than diagnostic screening for trichomonosis~~ (Casteriano *et al.*, 2016) or in comparison with other PCR methods (Polo *et al.*, 2022). Grahn *et al.* (2005) have also used the TFR1 and TFR2 primers in combination with a fluorophore, whereas the forward primer (TFR1) is labelled with a 6FAM fluorophore, resulting in increased sensitivity of the conventional PCR.

~~Hayes *et al.* (2003) have also used RFLP of the amplicon generated by primers TFR1 and TFR2 to differentiate various trichomonads including *T. foetus*.~~

~~A loop mediated isothermal amplification assay (LAMP) targeting *T. foetus* 5.8S rDNA has been reported with slightly higher sensitivity than the TFR3/TFR4 PCR and demonstrated increased specificity (Oyhenart *et al.*, 2013). Due to the simplicity of the assay over PCR or real-time PCR, the LAMP assay may be a cheaper alternative for low skill operators, however the assay has not been field tested at this stage.~~

1.4.3. Real-time PCR ~~or quantitative PCR~~

Real-time PCR methods are a more advantageous detection method as it combines the high specificity of end-point PCR with higher sensitivity. However, it is more expensive and requires highly trained personnel, which can be a limitation for some laboratories (Oyhenart, 2018).

Pathogen/ target gene	Primer/probe (5'–3')	Concentration	Cycling parameters[(a)]
Method 1: McMillen & Lew (2006); GenBank Accession No.: AF339736			
TFR2	TFR2: 5'-GCG-GCT-GGA-TTA-GCT-TTC-TTT-3' TFR2: 5'- GGC-GCG-CAA-TGT-GCA-T-3'	900 nM	50°C/2min to activate UDG (if used) 40 cycles of: 95°C/20 sec and 60°C/45 sec
	TrichP2: 6FAM-ACA-AGT-TCG-ATC-TTT-G-MGB	80 nM	
Method 2: Polo *et al.* (2022); GenBank Accession No.: AF466749.1			

EF1A1	Ftf_EF1A1: 5'-AGTCCGCCGCCAAATCAA-3' Rtf_EF1A1: 5' CTC TTCAACTTCGGCTGTGA-3'	0.5 µM	45 cycles of: 95°C/20 sec and 61°C/35 sec
	Ptf_EF1A1: 6-FAM-ATCATCAAGTACGGCTCAGT-MGBNFQ	0.4 µM	

A Minor Groove Binder (MGB) probe real-time PCR method described by McMillen & Lew (2006), based on the ITS-1 region within the same rDNA ~~regions~~ as the TFR3-TFR4 PCR primers, was shown to be highly specific and sensitive compared with culture/microscopy and the previously described PCR methods (McMillen & Lew, 2006).

~~Assay sensitivity was evaluated with 10-fold dilutions of known numbers of *T. foetus* cells, and compared with that for microscopy following culture and the TFR3-TFR4 PCR assay. The probe-based real-time PCR assay detected a single cell per assay directly from non-cultured smegma or vaginal mucus and was 2500-fold and 250-fold more sensitive than microscopy following selective culture respectively and 500-fold more sensitive than culture followed by the TFR3-TFR4 PCR assay. When compared with TFR3-TFR4 amplification of *T. foetus* DNA from cultures, the real-time PCR was consistently 10-fold more sensitive for smegma samples at 0, 2 and 5 days post culture. For vaginal mucus, both PCRs demonstrated equivalent sensitivities at days 0 and 2 with 10-fold increase in sensitivity for real-time PCR at day 5 post culture. The sensitivity of the TFR3-TFR4 PCR assay was 10-fold lower compared with the real-time PCR assay when testing purified DNA extracted from clinical specimens. Furthermore, the sensitivity of the real-time PCR assay improved 500-fold when using crude cell lysates, which were not suitable as template for the conventional PCR assay (McMillen & Lew, 2006). Initial evaluations of this real-time PCR method showed that from 159 Australian diagnostic specimens, 14 bulls were positive by real-time PCR (directly from clinical specimens: smegma and vaginal mucus) with only three confirmed by selective culture/microscopy detection (Fisher's exact test $p<0.001$) (McMillen & Lew 2006). The real-time PCR was designed with a MGB probe. The substitution of the MGB probe with other probes e.g. black hole quenchers or TAMRA will result in false positive results. The real-time PCR uses the following primers and probe:~~

~~TFF2 (20µM) 5' GCG GCT GGA TTA GCT TTC TTT 3'~~
~~TFR2 (20µM) 5'-GGC-GCG-CAA-TGT-GCA-T-3'~~
~~TrichP2 (5µM) 5' 6FAM-ACA AGT TCG ATC TTT G-MGB 3'~~

The real-time PCR is performed using a 25 µl reaction containing commercially available mastermix, 900 nM of TFF2 and TFR2 primers and 80 nM of TichP2 probe. ~~When using a uracyl DNA glycosilase (UDG) containing mastermix, the real-time PCR is conducted at 50°C for 2 minutes to activate the UDG and prevent any carryover contamination, followed by incubation at 95°C for 2 minutes, and then 40 cycles of denaturation at 95°C for 20 seconds and annealing/extension at 60°C for 45 seconds.~~ The fluorescence is acquired in the green channel at the end of each annealing/extension step.

Tritrichomonas mobilensis, present to date only in squirrel monkeys, can potentially pose a problem in analysis of field samples when using PCR protocols based on rDNA-ITS. This region has been identified *in silico* to be highly similar with the *T. foetus* and therefore, potentially render false positive results (Polo *et al.*, 2022).

For confirmation of clinical cases, a real-time PCR assay based on the EF1-alpha-Tf1 gene has been shown to have an analytical sensitivity of approximately 5.75 T. *foetus* genome copies (Polo *et al.*, 2022).

~~Robust evaluation of the commercial version of this real-time PCR assay has been undertaken as described by Effinger *et al.* (2014) using real-time PCR post-culture. This evaluation used 833 cultured smegma samples, which were tested by five laboratories in the USA using the commercial real-time PCR kit different DNA processing methods, PCR equipment and PCR cycling conditions. Pools consisting of one *T. foetus* positive and four negative smegma samples were also processed and 96% of the positive samples were detected in these pools. For individual sample testing an overall agreement of 95.89% was attained between the five participating laboratories. A recent study undertaken in Canada showed that real-time PCR detection of *T. foetus* in pooled preputial samples in PBS was more sensitive than culture methods. The study also demonstrated that there was no difference in the detection of *T. foetus* from known infected bulls between direct real-time PCR (from PBS), culture/microscopy or culture/real-time PCR.~~

Another real-time PCR method, which targets the gene encoding beta-tubulin 1 from *T. foetus*, has been developed. It is available on the market as commercial kits. Despite the high degree of

conservation of beta tubulin genes in trichomonads, the test is claimed to be specific and it has broad range detection of *T. foetus* isolates. However, there is only limited and unpublished data on the evaluation of these kits.

~~PCR diagnostic frameworks are best determined for a particular region and country taking into account transport time to the diagnostic laboratory, temperature of the sample, and optimised DNA extraction and PCR methods used at the diagnostic laboratory. Currently, the best option is to use published protocols (adapted to the particular laboratory) or commercial *T. foetus* real-time PCR kits applied to transport medium or PBS, which guarantees fast preliminary results especially when using boiled lysates.~~

1.4.4. Loop-mediated isothermal amplification (LAMP)

A loop mediated isothermal amplification assay (LAMP) targeting *T. foetus* 5.8S rDNA has been reported with slightly higher sensitivity than the TFR3/TFR4 PCR and demonstrated increased specificity (Oyhenart *et al.*, 2013). Due to the simplicity of the assay over PCR or real-time PCR, the LAMP assay may be a less expensive alternative for low skill operators. However, the assay has not yet been field tested.

Another LAMP assay targeting the *T. foetus* elongation factor 1 alfa (tf-ef1a1) showed even higher sensitivity. With this assay, it was possible to amplify *T. foetus* DNA without DNA purification from infected cervical vaginal mucus. This was possible by embedding a fragment of paper strip in cervical vaginal samples and then cutting and transferring into a tube containing the LAMP mix (Oyhenart, 2018). This paper strip is a chemically treated filter paper for the collection of biological samples for DNA analysis and may not be commercially available in every country. This assay has not yet been tested in positive samples from naturally infected cattle.

~~PCR diagnostic frameworks are best determined for a particular region and country taking into account transport time to the diagnostic laboratory, temperature of the sample, and optimised DNA extraction and PCR methods used at the diagnostic laboratory. Currently, the best option is to use published protocols (adapted to the particular laboratory) or commercial *T. foetus* real-time PCR kits applied to transport medium or PBS, which guarantees fast preliminary results especially when using boiled lysates.~~

1.5. Combination of culture and PCR ~~on 5-day cultures~~

Combining PCR methods and culture has been reported to yield higher sensitivity or improved specificity and has been suggested as the most cost-effective and practical approach to assess bulls before breeding (Michi *et al.*, 2016). However, it has also been reported that testing clinical samples from bulls directly by PCR is more sensitive than culture followed by PCR (McMillen & Lew, 2006), even though most laboratories process cultures as a priority.

The culture and PCR combination should be implemented in some specific situations where specificity is a problem, due to occurrence of other trichomonads that may result in false positives in culture. In view of the difficulty of distinguishing *T. foetus* from other trichomonads based on morphology, it is recommended that, whenever the facilities exist for DNA-based methods, cultures with trichomonads be systematically tested by PCR to confirm the presence of *T. foetus*.

2. Serological tests

Bulls do not develop prominent immune responses to *T. foetus*. ~~Some immunological tests have been developed for the diagnosis of bovine trichomonosis, such as mucus agglutination test and intradermal test (Rhyan *et al.*, 1999) and an antigen capture enzyme-linked immunosorbent was described more recently (BonDurant, 1997). However, these tests appear very limited in use due to low sensitivity or specificity, and thus they are not recommended for the detection of *T foetus* in individual animals.~~ ELISAs based on whole *T. foetus* cells rather than specific antigens are the most reliable and these serological tests are useful in vaccination studies to ensure IgG and IgA responses post-vaccination in serum and vaginal mucus, respectively. There is some evidence that these ELISAs can also be used for the confirmation of clinical cases in heifers. Serum whole IgG ELISA, and IgG1 and IgG2 indirect ELISAs, have demonstrated post-vaccination responses in vaccinated bulls.

~~Infected cows develop specific IgG1 and IgG2 antibodies that are present in the vaginal mucus and in serum, but these are not exploited for diagnostic purposes.~~

C. REQUIREMENTS FOR VACCINES

1. Background

There are currently no globally registered drugs for the treatment of *T. foetus* infections with the culling of positive animals the only control tool. Prevention using vaccination has been the most widely adopted method to control trichomonosis and to manage cow infertility. Whole cell vaccines for cows have been shown to offer protection and are available commercially (Corbeil, 1994) as either a monovalent vaccine or part of a polyvalent vaccine also containing *Campylobacter* and *Leptospira* spp. (BonDurant, 1997; Cobo *et al.*, 2004). ~~These products have shown efficacy in the female but not in the bull.~~

~~One example of a method of whole cell vaccine production is by growing *T. foetus* (culture VMC-84) in modified Diamond's medium (Corbeil, 1994) and freezing the culture at –20°C for 60 minutes. After thawing, a suspension of 5 × 10^7 organisms/ml in PBS is added to the CL-vaccine.~~

Several types of non-commercial vaccine approaches have been described including inactivated whole cell vaccines, subunit vaccines and fractioned cell extracts.

Inactivated whole cell vaccines have been the most studied and commercialised for the control of bovine trichomonosis. No *T. foetus* vaccine prevents *T. foetus* infection but rather they shorten the time to clear *T. foetus* infection compared with unvaccinated cattle. Several different adjuvants have been used with the most common being mineral oil followed by aluminium hydroxide in studies undertaken before 2002. More recently, a comparison of four adjuvants used with the inactivated whole *T. foetus* vaccine demonstrated that Quil A mixed with aluminium hydroxide led to the fastest clearance of *T. foetus* infection (27 days) followed by Montanide ISA 206 VG or Quil A (41 days) with aluminium hydroxide clearing at 55 days (Fuchs *et al.*, 2017). Two or three doses of inactivated vaccines every 3 to 4 weeks are the most common vaccination regimes used.

Two subunit vaccines have been described (Tf190 adhesin and TF1.17 surface antigen) with only one demonstrating efficacy following challenge. TF1.17 surface antigen (immunoaffinity purified) was adjuvanted using incomplete Freund's (IFA) and, following three doses of 100 µg 3 weeks apart, heifers were challenged 2 weeks later. Seventy-five per cent of the vaccinated heifers had cleared by week 6 in contrast to at least 10 weeks for unvaccinated controls (BonDurant *et al.*, 1993). This trial was repeated by the same group, which also compared IFA with IFA + dextran sulfate as adjuvants giving similar results (Anderson *et al.*, 1996). Subunit vaccines have not been directly compared with inactivated vaccines, but would appear to have longer parasite clearance rates and will not be discussed further here.

Although a few different versions of fractionated cell extracts have been described, *T. foetus* purified membrane extracts have demonstrated slightly better *T. foetus* clearance rates and calving rates compared with inactivated whole cell vaccines (Cobo *et al.*, 2002; Clark *et al.*, 1984). Anecdotally, early studies suggested that vaccines produced from inactivated *T. foetus* cells or fractionated proteins could be used to treat *T. foetus* positive bulls, particularly if younger than 5.5 years of age (Clark *et al.*, 1983; 1984). Subsequent studies have suggested that this is not a reliable treatment regime and is not a recommended practise (Alling *et al.*, 2018; Herr *et al.*, 1991; Tabor *et al.*, 2023).

Characteristics of a target product profile:

i) For whole cell inactivated *T. foetus* vaccines, studies have used local strains for vaccine development.

ii) Membrane fractions as *T. foetus* vaccines have limited application to date, and the use of local strains should also apply but has not been demonstrated to date.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given below and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics of the master seed

Inactivated vaccines

There are considerable variations in the nomenclature of *T. foetus* strains used in inactivated vaccine experiments, however the most common are abbreviated letters and numbers associated with the geographical origin of the isolate and number, respectively. Examples include BP-4 from

Beltsville, USA, isolated in 1954 (ATCC isolate 30003) and MT85-330.1 from Montana, USA, isolated in 1985. Most commonly the isolate is from a persistently infected bull. There are no published data reporting the maximum time for *T. foetus* culture and passages for vaccine production. Nonetheless, seed cultures are cryopreserved and thawed to produce vaccine production batches to minimise long-term culture of seeds.

Membrane fraction vaccines

Tritrichomonas foetus strains used for inactivated vaccines have also been developed as membrane fraction vaccines.

See chapter 1.1.8 for guidelines on master seeds.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

Tritrichomonas foetus seeds must be pure culture and free from extraneous bacteria, yeast, and fungi. Culture media inoculated using bovine clinical samples (mucus from vagina or preputial reproductive tracts) may require high doses of antimicrobials and anti-fungals, prior to several subculture passages to obtain pure *T. foetus* cell lines for vaccine development. Negative aerobic blood agar cultures and *T. foetus* microscopy are tools used to confirm culture purity (see Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*).

2.1.3. Validation as a vaccine strain

Suitability as a vaccine strain in cattle is demonstrated in efficacy and safety trials.

2.2. Method of manufacture

2.2.1. Procedure

Inactivated vaccines

Tritrichomonas foetus cultures are grown in suitable broth media omitting the agar from the medium and either using an agar plug or suitable gas microaerophilic conditions e.g. nitrogen. Cultures are subcultured until the desired volume and cell density is achieved, confirmed by microscopy cell counts. Cells are inactivated by formaldehyde or formalin or heating to 65°C or lyophilisation or irradiation. Cells in solution are subsequently harvested by centrifugation, washed, and resuspended at the desired cell count (vaccine dose) in PBS. The culture is then blended with adjuvant prior to filling sterile vials.

Membrane fraction vaccines

Tritrichomonas foetus cells are collected from a 24- to 48-hour culture, washed (3×) with PBS pH 7.2 by centrifugation at 7500 ***g*** for 15 minutes and suspended in homogenising buffer: PBS containing 1 mM $MgCl_2 \cdot 6H_2O$ (PBS-ClMg) and 1% phenyl-methylsulphonyl fluoride (88 mg/ml methanol). The suspension is sonicated for five rounds of 5 minutes on ice in an ultrasonicator (Sonifier 250 Branson Sonic Power, USA). The sonicate is centrifuged at 1100 ***g*** for 10 minutes and the supernatant is layered onto 10 ml of 41% (v/v) sucrose in homogenising buffer before centrifuging at 30,000 ***g*** for 150 minutes at 6°C. The membranes are collected from the interface between the buffer and the sucrose solution.

2.2.2. Requirements for ingredients

See Chapter 1.1.8 and culture methods described above.

2.2.3. In-process controls

The purity of the culture is determined at each stage of production prior to inactivation. Microscopy and bacterial agar plates are used to confirm *T. foetus* cells and absence of contaminants respectively. Inactivated cells are cultured and examined microscopically to confirm the complete inactivation. Analytical assays to determine levels of chemical inactivators are done on bulk vaccine and must be within specified limits. Production parameters must be managed to ensure that manufacturing processes are consistent between batches.

2.2.4. Final product batch tests

i) Sterility

Sterility tests are done on each batch. For the USA, these are detailed in the 9 CFR Part 113.26 (CFR USDA, 2013). Depending on the jurisdiction, the requirements may vary and need to be checked by Regulatory body of the given country or region. See also Chapter 1.1.9.

ii) Identity

The identity of the inactivated product is ensured through the master seed concept and good manufacturing controls.

iii) Safety

Batch testing would occur by inoculating cattle to confirm safety of vaccination. No withholding periods for milk or meat are necessary following vaccination, unless specified by local legislation.

iv) Batch potency

Inactivated vaccines should be tested for batch potency in a vaccination challenge trial.

2.3. Requirements for regulatory approval

2.3.1. Manufacturing process

For regulatory approval of vaccine, all relevant details concerning manufacture of the vaccine and quality control testing (see Section C.2.1 *Characteristics of the seed* and Section C.2.2 *Method of manufacture*) should be submitted to the authorities. This information shall be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume.

In-process controls are part of the manufacturing process.

2.3.2. Safety requirements

Each commercial product requires a 'Safety Data Sheet' (SDS), which should include information in regard to: hazard identification, first aid measures, accidental release measures, handling and storage, exposure controls and personal protection, physical and chemical properties, stability and reactivity, toxicological information, ecological information, disposal considerations, transport information, regulatory information, and any other information pertinent to the region or country.

i) Target and non-target animal safety

Inactivated vaccines do not pose a hazard to non-target species. Generally inactivated vaccines are safe as currently there are many inactivated veterinary vaccines on the market. It is favourable to vaccinate young bulls and heifers for protection from trichomonosis preferably before breeding.

Safety needs to be evaluated in the field setting prior to regulatory approval. This evaluation typically involves approximately three different geographical locations or husbandry conditions and many cattle. The number of cattle recommended varies on the jurisdiction and regional regulations for product approval.

ii) Reversion-to-virulence for attenuated/live vaccines and environmental considerations

Not applicable for inactivated vaccines.

iii) Precautions (hazards)

Vaccines prepared with aluminium hydroxide adjuvants may cause temporary nodules at the site of injection. Operator self-injection poses no immediate issues. Vaccines prepared with oil-based adjuvants may cause larger nodules. To avoid lasting lumps, it is important that correct vaccination procedures are followed to ensure vaccine is deposited under the skin and not into the muscle. Operator self-injection requires immediate medical attention. Safety precautions should be clearly outlined on the commercial product including the SDS, and it is recommended that experienced personnel administer vaccines to cattle.

2.3.3. Efficacy requirements

Vaccine doses usually contain 10^7 to 10^8 cells per dose with a minimum of two doses required for protection from live challenge. Vaccine challenge is either achieved by artificial challenge with live *T. foetus* cells (typically doses of 10^5–10^6 cells) originating from a strain that is different to the vaccine strain or by natural service with infected cattle. When natural service is used for vaccinated heifers, calf output is a good measure of vaccine efficacy. Trichomonosis vaccine efficacy is often measured by the time taken to clear *T. foetus* from the reproductive tract, which is measured using culture and direct real-time PCR. Note these vaccines do not eliminate *T. foetus* infection following challenge, but vaccination decreases the time taken to clear *T. foetus* compared to unvaccinated cattle.

The number of cattle needed to confirm efficacy for product registration varies between different jurisdictions and regions.

2.3.4. Vaccines permitting a DIVA strategy (detection of infection in vaccinated animals)

Not applicable to this disease.

2.3.5. Duration of immunity

Efficacy for 1 year is usually demonstrated by repeating challenge at 6–9 months post-vaccination. Annual boosts of the vaccine are subsequently recommended prior to breeding seasons.

2.3.6. Stability

Inactivated vaccines are stored at 2–8°C (refrigerated) and should not be frozen. The shelf life of the product needs to be determined by the manufacturer and clearly stated on the product.

3. Vaccines based on biotechnology

There are no biotechnology-based vaccines for bovine trichomonosis.

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NB: At the time of publication (2024) there were no WOAH Reference Laboratories for trichomonosis (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).

NB: FIRST ADOPTED IN 1991 AS TRICHOMONIASIS. MOST RECENT UPDATES ADOPTED IN 2018.

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Appendix 1: Trichomonosis
Intended purpose of test: population freedom from infection

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
Culture followed by microscopy for morphological identification +++ Bovine	Preputial smegma inoculated into pouch media	1 week after infection: Se = 72.43% Sp = 95.4% 3 consecutive weeks post-infection: Se = 80.0% Sp = 98.1%	Mature dairy bulls: 30 infected intrapreputially inoculated with *T. foetus* 49 not infected		-Basic microbiology laboratory equipment required -Findings are consistent with the recommendation of three consecutive negative results at weekly intervals before concluding on absence of *T. foetus* infection	-Does not differentiate between *T. foetus* and other trichomonads	Cobo *et al.* (2007)
PCR in combination with culture ++ Bovine	Smegma samples cultured in a pouch 37°C 5.8S rRNA Preputial scraping samples cultured in a pouch 37°C 5' Tag nuclease real-time PCR assay with MGB probe (McMillen & Lew, 2006)	Se = 95% Sp = 100% Agreement between culture PCR and analysis by microscope = 98% (kappa = 0.96; McNemar test p = 0.2) Culture: Sp = 98.8 (95 CI, 97.0–99.5%) PCR: Sp = 100% (95% CI, 98.9–100%)	Field bulls: 56 positives 110 negatives 180 bulls 150 steers		-Confirmation of positive cases after identification of trichomonads by microscopy;	-Cost of the analysis increases as it is culture + PCR	Summarell *et al.* (2018) Guerra *et al.* (2014)
PCR +++ Bovine	Preputial smegma inoculated into PBS PCR specific primers (TFR1, TFR2, TFR3 and TFR4) for the 5.8S rRNA gene and the flanking internal transcribed spacer regions ITS1 and ITS2	3 consecutive weeks (post-infection): Se = 85.0% Sp = 95.4%	Mature dairy bulls: 30 infected intrapreputially - inoculated with *T. foetus* 49 not infected		-It is possible to differentiate between *T. foetus* and other trichomonads	-False negative results can occur due to degradation of DNA/ presence of inhibitors -Requires highly trained personnel and specific molecular biology equipment	Cobo *et al.* (2007)

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Appendix 2: Trichomonosis
Intended purpose of test: individual animal freedom from infection prior to movement

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
Culture followed by microscopy for morphological identification ++ Bovine	Preputial fluid samples inoculated in Plastridge's medium	Se = 72.43% (95%probability interval: 58.79–87.25%) Sp = 95.24% (95%probability interval: 94–96.39%)	2832 mature (>4 years old) from 124 beef herds – suspected to be *T. foetus* infected		-Basic microbiology laboratory equipment required	-False negative results may be obtained if animals are tested only once -Sampling technique, conditions of storage/transport of culture media and samples may affect test sensitivity - PCR in addition to culture is recommended as a confirmatory test for positive cultures	Perez *et al.* (2006)
PCR in combination with culture ++ Bovine	Smegma samples cultured in a pouch 37°C 5.8S rRNA Preputial scraping samples cultured in a pouch 37°C 5' Tag nuclease real-time PCR assay with MGB probe (McMillen & Lew, 2006)	Se = 95% Sp = 100% Agreement between culture PCR and analysis by microscope = 98% (kappa = 0.96; McNemar test p = 0.2) Culture: Sp = 98.8 (95 CI, 97.0–99.5%) PCR: Sp = 100% (95% CI, 98.9–100%)	Field bulls: 56 positives 110 negatives 180 bulls 150 steers		-Confirmation of positive cases after identification of trichomonads by microscopy	-Cost of the analysis increases as it is culture + PCR	Summarell *et al.* (2018) Guerra *et al.* (2014)
PCR +++ Bovine	Preputial wash fluids, vaginal mucus or vaginal washing fluids Primers TFR3 and TFR4 directed to the rRNA gene units	Se = 50 parasites/ml	7 bulls with suspected *T. foetus* infection (positive by microscopy) 71 cows with abortion problems 123 healthy bulls 20 healthy cows		-Better sensitivity -This method overcomes possible problems with sensitivity when performing culture + microscopy	-False negative results can occur due to degradation of DNA -The use of these specific primers does not differentiate *T. foetus* from *T. suis* (swine) or *T. mobilensis* (squirrel monkey) -Requires highly trained personnel and specific molecular biology equipment	Felleisen *et al.* (1998)

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Appendix 3: Trichomonosis
Intended purpose of test: contribute to eradication policies

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
Culture followed by microscopy for morphological identification +++ Bovine	Preputial smegma inoculated into pouch media	1 week after infection: Se = 72.43% Sp = 95.4% 3 consecutive weeks post-infection: Se = 80.0% Sp = 98.1%	Mature dairy bulls: 30 infected intrapreputially - inoculated with *T. foetus* 49 not infected		-Basic microbiology laboratory equipment required -Findings are consistent with the recommendation of three consecutive negative results at weekly intervals before concluding on absence of *T. foetus* infection	-Does not differentiate between *T. foetus* and other trichomonads -If only one test is made, the sensibility of this test is lower than other methods	Cobo *et al.* (2007)
Real-time PCR +++ Bovine	Preputial smegma inoculated into PBS PCR specific primers (TFR1, TFR2, TFR3 and TFR4) for the 5.8S rRNA gene and the flanking internal transcribed spacer regions ITS1 and ITS2	3 consecutive weeks (post-infection): Se = 85.0% Sp = 95.4%	Mature dairy bulls: 30 infected intrapreputially inoculated with *T. foetus* 49 not infected		-It is possible to differentiate between *T. foetus* and other trichomonads	-False negative results can occur due to degradation of DNA/ presence of inhibitors -Requires highly trained personnel and specific molecular biology equipment	Cobo *et al.* (2007)

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Appendix 4: Trichomonosis
Intended purpose of test: confirmation of clinical cases

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
Culture followed by microscopy for morphological identification +++ Bovine	Preputial fluid samples inoculated in Plastridge's medium	Se = 72.43% (95%probability interval: 58.79–87.25%) Sp = 95.24% (95%probability interval: 94–96.39%)	2832 mature (>4 years old) from 124 beef herds – suspected to be *T. foetus* infected		-Basic microbiology laboratory equipment required	-False negative results may be obtained if animals are tested only once -Sampling technique, conditions of storage/transport of culture media and samples may affect test sensitivity - PCR in addition to culture is recommended as a confirmatory test for positive cultures	Perez *et al.* (2006)
PCR in combination with culture ++ Bovine	Smegma samples cultured in a pouch 37°C 5.8S rRNA Preputial scraping samples cultured in a pouch 37°C 5' Tag nuclease real-time PCR assay with MGB probe (McMillen & Lew, 2006)	Se = 95% Sp = 100% Agreement between culture PCR and analysis by microscope = 98% (kappa = 0.96; McNemar test p = 0.2) Culture: Sp = 98.8 (95 CI, 97.0–99.5%) PCR: Sp = 100% (95% CI, 98.9–100%)	Field bulls: 56 positives 110 negatives 180 bulls 150 steers		-Confirmation of positive cases after identification of trichomonads by microscopy	-Cost of the analysis increases as it is culture + PCR	Summarell *et al.* (2018) Guerra *et al.* (2014)
PCR +++ Bovine	Preputial wash fluids, vaginal mucus or vaginal washing fluids Primers TFR3 and TFR4 directed to the rRNA gene units	Se = 50 parasites/ml	7 bulls with suspected *T. foetus* infection (positive by microscopy) 71 cows with abortion problems 123 healthy bulls 20 healthy cows		-Better sensitivity -This method overcomes possible problems with sensitivity when performing culture + microscopy	-False negative results can occur due to degradation of DNA -The use of these specific primers does not differentiate *T. foetus* from *T. suis* (swine) or *T. mobilensis* (squirrel monkey) -Requires highly trained personnel and specific molecular biology equipment	Felleisen *et al.* (1998)
ELISA IgA ++ ELISA IgG1 ++ ELISA IgG2 + ELISA IgM –	Vaginal, cervical and uterine secretions, serum using whole *T. foetus* cell antigen	Reference test was culture: trichomonads disappeared from the vagina of infected heifers after 13–28 weeks, from the uterus after 12–26 weeks. Two	Four *T. foetus* vaginally inoculated heifers and two control heifers		-Vaginal samples most reliable for IgA ELISA -IgA ELISA most reliable over IgG1, IgG2 and IgM when	-Low number of heifers used in this validation	Skirrow & BonDurant (1990)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
Whole IgG ++ Bovine		control heifers were negative for the duration of the trial Specificity and Sensitivity of IgA ELISA comparable to culture Vaginal IgA and IgG1 increased significantly at weeks 7–9 and remained high for at least 24 weeks. Vaginal IgG2 and IgM responses inconsistent Cervical IgA and IgG1 increased at weeks 7–9 and IgG2 increased in ¾ heifers by 10–12 weeks. IgM inconsistent Uterine IgA in 2/4 heifers by 7–9 weeks but did not last as long as for vaginal and cervical IgA. IgG1 at 10–12 weeks. IgG2 and IgM inconsistent.			testing reproductive tract secretions -IgG1 and IgG2 responses from serum and not reproductive tract samples		
ELISA IgG1 ++ ELISA IgG2 + ELISA IgM ++ ELISA IgA ++ Bovine	Preputial samples (smegma) against *T. foetus* TF1.17 surface antigen	Reference test was *T. foetus* whole cell ELISA antigen IgG1 responses were greatest, IgA and IgM responses were approximately equal, and IgG2 responses were very low. In each case, the response in infected bulls was significantly greater than that in the controls IgG1, $p = 0.01$; IgG2, $p = 0.04$; IgM, $p = 0.04$; IgA, $p = 0.03$	32 bulls: 16 *T. foetus*-infected bulls; 16 *T. foetus* culture negative bulls		-The bull immune response may enhance immunologic protection at the local level. -Specific antibody detection in smegma using IgG1, IgM and IgA better than previous studies using total immunoglobulins	-Access to *T. foetus* TF1.17 antigen may be limited. -ELISAs using whole *T.foetus* antigen were not compared	Rhyan *et al.* (1999)

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Appendix 5: Trichomonosis
Intended purpose of test: prevalence of infection – surveillance

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
Culture followed by microscopy for morphological identification +++ Bovine	Preputial smegma inoculated into pouch media	1 week after infection: Se = 72.43% Sp = 95.4% 3 consecutive weeks post-infection: Se = 80.0% Sp = 98.1%	Mature dairy bulls: 30 infected intrapreputially - inoculated with *T. foetus* 49 not infected		-Findings are consistent with the recommendation of three consecutive negative results at weekly intervals before concluding on absence of *T. foetus* infection -Basic microbiology laboratory equipment required	-Does not differentiate between *T. foetus* and other trichomonads -If only one test is made, the sensibility of this test is lower than other methods	Cobo *et al.* (2007)
PCR in combination with culture ++ Bovine	Smegma samples cultured in a pouch 37°C 5.8S rRNA Preputial scraping samples cultured in a pouch 37°C 5' Tag nuclease real-time PCR assay with MGB probe (McMillen & Lew, 2006)	Se = 95% Sp = 100% Agreement between culture PCR and analysis by microscope = 98% (kappa = 0.96; McNemar test p = 0.2) Culture: Sp = 98.8 (95 CI, 97.0–99.5%) PCR: Sp = 100% (95% CI, 98.9–100%)	Field bulls: 56 positives 110 negatives 180 bulls 150 steers		-Confirmation of positive cases after identification of trichomonads by microscopy	-Cost of the analysis increases as it is culture + PCR	Summarell *et al.* (2018) Guerra *et al.* (2014)
Real-time PCR +++ Bovine	Preputial smegma inoculated into PBS PCR specific primers (TFR1, TFR2, TFR3 and TFR4) for the 5.8S rRNA gene and the flanking internal transcribed spacer regions ITS1 and ITS2	3 consecutive weeks (post-infection): Se = 85.0% Sp = 95.4%	Mature dairy bulls: 30 infected intrapreputially inoculated with *T. foetus* 49 not infected		-It is possible to differentiate between *T. foetus* and other trichomonads	-False negative results can occur due to degradation of DNA/ presence of inhibitors -Requires highly trained personnel and specific molecular biology equipment	Cobo *et al.* (2007)

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Appendix 6: Trichomonosis
Intended purpose of test: immune status in individual animals or populations post-vaccination

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
IgG ELISA +++ Bovine	Serum/Preputial washings/Seminal plasma using whole *T. foetus* cell antigen and membrane protein antigen used in ELISAs	Reference test was microagglutination test – serum titres less sensitive than IgG ELISA Seminal plasma: IgG detected in vaccinated bulls with variable individual responses Preputial washings: low IgG responses in 4/7 vaccinated bulls only Serum: Good IgG titres in all vaccinated bulls	11 bulls: seven vaccinated bulls (three doses *T. foetus* membranes) and four unvaccinated controls naïve to *T. foetus* – all challenged with live *T. foetus*		-ELISA more sensitive than microagglutination test -Seminal plasma not recommended when comparing challenged vaccinated and unvaccinated bulls -Serum is the most reliable sample for whole IgG ELISA	ELISA descriptions used in the publication did not always define which Immunoglobulin thus compromising interpretations	Campero *et al.* (1990)
IgG1 ELISA +++ IgG2 ELISA + IgA ELISA +++ IgM ELISA + Bovine	Serum/cervicovaginal mucus	Reference test was culture. 80% of unvaccinated heifers remained infected 8 weeks after challenge with a mean duration of 10.2 weeks compared to 3.2 weeks for 60% of the naturally challenged and 3.5 weeks for 85% of the artificially challenged vaccinated heifers, respectively Serum: IgG1 and IgA isotypes detected in vaccinated heifers Cervicovaginal mucus: IgA and IgG1 isotypes detected in vaccinated heifers	35 Heifers: 10 unvaccinated challenged with infected bulls; 10 vaccinated challenged with infected bulls; 15 vaccinated challenged with intravaginal inoculation		-In serum, IgG1 predominant isotype -In cervicovaginal mucus, IgA predominant response -Culture confirmed clearance of *T. foetus* in response to vaccination	-Results in contrast to Skirrow & BonDurant (1990) who reported no detectable IgA and IgM in serum	Gault *et al.* (1995)
Whole ELISA IgG+++	Serum	Reference test was haemolytic test and *T. foetus* culture ELISA was more sensitive and reliable than the haemolytic test.	40 heifers: 12 heifers vaccinated with whole cells, 12 vaccinated with cellular membrane vaccine, and 16 unvaccinated; challenged by service using *T.foetus* infected bull		-Whole IgG ELISA reliable and demonstrated a stronger response to the cell membrane extract vaccine over the whole cell vaccine		Cobo *et al.* (2002)
IgG ELISA using TF1.17 antigen +++	Serum, uterine and vaginal mucosal IgG to	Reference to *T. foetus* culture and real-time PCR	20 postpartum beef cows: 10 vaccinated with		-IgG responses to TF1.17 reliably detected in response	-Control unvaccinated cattle had IgG serum	Palomares *et al.* (2017)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
IgG ELISA using whole *T. foetus* antigen –/+ Bovine	whole *T. foetus* and surface antigen TF1.17	All animals were *T. foetus* negative by real-time PCR and culture prior to vaccination Serum IgG to whole *T. foetus* antigen positive in vaccinated and unvaccinated cows. No difference in IgG uterine responses to whole cell antigen Serum, vaginal and uterine IgG responses to TF1.17 higher in vaccinated cows compared with controls	commercial vaccine and 10 unvaccinated		to vaccination in serum, uterine and vaginal mucosa -Serum, uterine and vaginal mucosal IgG responses were not differentiated in vaccinated and unvaccinated control cows	responses to whole *T. foetus* antigen suggesting pre-exposure to *T. foetus* however real-time PCR and culture negative -IgG serum results following inactivated T. foetus whole cell vaccination using whole *T. foetus* ELISA antigen reported here is inconsistent with other reports	
Indirect ELISA IgG1 +++ Indirect ELISA IgG2 +++ Bovine	Serum	Reference tests were culture and real-time PCR No significant decrease in infection measured by real-time PCR and culture in the vaccine treated group IgG1 and IgG2 responses increased from weeks 2–18 in response to vaccination compared to controls.	20 *T. foetus* infected bulls: 10 treated with commercial vaccine, 10 controls		-IgG1 and IgG2 indirect ELISAs provide a good indication of vaccination responses in vaccinated *T. foetus* positive bulls	-*T. foetus* whole vaccines cannot be used as therapeutic in *T. foetus* infected bulls	Alling *et al.* (2018)

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RÉUNION DE LA COMMISSION DES NORMES BIOLOGIQUES
Paris, 9–13 septembre 2024

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SECTION 3.6.

EQUIDAE

CHAPTER 3.6.1.

AFRICAN HORSE SICKNESS (INFECTION WITH AFRICAN HORSE SICKNESS VIRUS)

SUMMARY

Description of the disease: *African horse sickness (AHS) is an infectious but noncontagious viral disease especially affecting horses, although all species of the family equidae are susceptible to infection. It is caused by an orbivirus of the family Sedoreoviridae ~~and characterised by alterations in the respiratory and circulatory functions~~. Disease, when present, varies from severe breakdown of the respiratory and circulatory systems resulting in death, to mild fever, depending on the levels of immunity and the equid species involved. ~~AHS is transmitted by at least two species of~~ Culicoides. ~~Nine different serotypes have been described.~~*

All serotypes of AHS occur in eastern and southern Africa. AHS serotypes 9, 4 and 2 have been found in North and West Africa, from where they occasionally spread into countries surrounding the Mediterranean. Examples of outbreaks that have occurred outside Africa are: in the Middle East (1959–1963), in Asia (Thailand 2020), in Spain (serotype 9, 1966, serotype 4, 1987–1990), and in Portugal (serotype 4, 1989).

Laboratory diagnosis of AHS is essential. Although the clinical signs and lesions are characteristic of the severe forms of the disease, such signs~~, they~~ can still be confused with those of other equine diseases.

As a viral disease, the laboratory diagnosis of AHS can be based on the identification of infectious virus, virus nucleic acid, viral antigens or specific antibodies. A wide variety of laboratory tests have been adapted for the detection of both AHS virus (AHSV) and specific antibodies.

Detection and identification of the agent: *a rapid and highly sensitive diagnosis is usually achieved by nucleic acid viral detection using reverse-transcription polymerase chain reaction (PCR). Nevertheless, it is particularly important to perform virus isolation and serotyping whenever AHS outbreaks occur outside the enzootic regions in order to choose a homologous serotype for the vaccine.*

AHSV can be isolated from blood collected during the early febrile stage. For virus isolation, the other tissues of choice for diagnosis are spleen, lung, and lymph nodes, collected at necropsy. Sample preparations can be inoculated in cell cultures, such as baby hamster kidney-21 (BHK-21), monkey stable (MS), African green monkey kidney (Vero) or insect cells (KC) or ~~,~~ intravenously into embryonated eggs. Virus isolates can be serotyped by a type-specific serological test such as virus

neutralisation (VN), by type-specific reverse-transcription PCR or by sequencing. Molecular typing assays can also be carried out on clinical samples.

~~Several~~ Other methods such as enzyme-linked immunosorbent assays (ELISAs) for the rapid detection of AHSV antigen in ~~blood,~~ spleen tissues and supernatant from infected cells have been developed although they are currently out of use due to their limited sensitivity. ~~Identification of AHSV RNA has also been achieved using a reverse-transcription polymerase chain reaction (PCR) method. Virus isolates can be serotyped by a type-specific serological test such as virus neutralisation (VN), by type-specific reverse-transcription PCR or by sequencing.~~

***Serological tests:** Horses that survive natural infection develop antibodies against the infecting serotype of AHSV within 8–12 days post-infection. This may be demonstrated by several serological methods, such as complement fixation test, ELISA, immunoblotting and VN. The latter test ~~is~~ can be used for serotyping.*

***Requirements for vaccines:** Attenuated (monovalent and polyvalent) live vaccines for use in horses, mules and donkeys, are currently commercially available. ~~Subunit vaccines~~ Inactivated vaccines were used in the past and recombinant approaches have been evaluated experimentally..*

A. INTRODUCTION

1. Description and impact of the disease

African horse sickness (AHS) (Peste equina africana, Peste equine) is an infectious, non-contagious arthropod-borne disease ~~of~~ especially of horses, although all equids are susceptible to infection, which is caused by a double-stranded RNA orbivirus belonging to the family Sedoreoviridae. ~~equidae, caused by a double-stranded RNA orbivirus belonging to the family *SedorReoviridae*. The genus *Orbivirus* also includes bluetongue virus and epizootic haemorrhagic disease virus, which have similar morphological and biochemical properties with distinctive pathological and antigenic properties as well as host ranges. Nine antigenically distinct serotypes of AHSV have been identified by virus neutralisation; some cross-reaction has been observed between 1 and 2, 3 and 7, 5 and 8, and 6 and 9, but no cross-reactions with other known orbiviruses occur. The virus can be inactivated at 72°C for 120 minutes (confirmed by three blind passages in the Vero cell line).~~

~~The virion is an unenveloped particle of a size around 70 nm. The genome of AHS virus (AHSV) is composed of ten double-stranded RNA segments, encoding seven structural proteins (VP1-7), most of which have been completely sequenced for AHSV serotypes 4, 6 and 9 (Roy *et al.*, 1991; Venter *et al.*, 2000; Williams *et al.*, 1998), and four nonstructural proteins (NS1, NS2, NS3, NS3A) (Grubman & Lewis, 1992; Laviada *et al.*, 1993). Proteins VP2 and VP5 form the outer capsid of the virion, and proteins VP3 and VP7 are the major inner capsid proteins. Proteins VP1, VP4 and VP6 constitute minor inner capsid proteins. The NS3 proteins are the second most variable AHSV proteins (Van Niekerk *et al.*, 2001), the most variable being the major outer capsid protein, VP2. This protein, VP2, is the determinant of AHSV serotypes and, together with VP5, the target for virus neutralisation activity (Martinez-Torrecuadrada *et al.*, 2001).~~ At least two field vectors are involved in the transmission of the virus: *Culicoides imicola* and *C. bolitinos*.

AHS is enzootic in sub-Saharan Africa, although occasional outbreaks have occurred in northern Africa (1965, 1989–1990, 2007–2010), the Middle East (1959–1961), Asia (Thailand 2020), and in Europe (Spain: 1966, 1987–1990, and Portugal: 1989) (Lu *et al.* 2020; ~~Sanchez-Vizcaíno, 2004~~).

There are four classical clinical forms of AHS: pulmonary, cardiac, mixed, and horse sickness fever. The peracute, pulmonary form occurs in fully susceptible animals and has a short course, often only a few hours, and a high mortality rate. The animal exhibits respiratory distress, an extended head and neck, and profuse sweating. Terminally, froth exudes from the nostrils. The cardiac, oedematous form has a more subacute course with mortality reaching 50%. The head and neck may show severe swelling that can extend down to the chest. Swelling of the supraorbital fossae is characteristic and may include conjunctival swelling with petechiae. Paralysis of the oesophagus may result in aspiration pneumonia and sublingual haemorrhages are always a poor prognostic sign. The mixed, acute form is most commonly seen and has features of both the cardiac and pulmonary forms. Mortality can reach 70%. Horse sickness fever is an often overlooked, mild form of the disease and is seen in partially immune horses and more resistant ~~equidae~~ equids such as ~~zebra and~~ donkeys, as well as in zebra post-experimental infection (Coetzer & Guthrie, 2005).

Clinical cases have also been described in dogs, with acute respiratory distress syndrome or sudden death. The mortality in dogs is high, and they may play a role in spread of the disease ~~(Oura, 2018)~~. Historically, infection was attributable to the consumption of infected horse meat, however more recent evidence includes the suspicion of vector-transmission (O'Dell *et al.,* 2018).

The disease has both a seasonal (late summer/autumn) and a cyclical incidence with major epizootics in southern Africa during warm-phase events, such as occurrences of El Niño ~~(Baylis *et al.*, 1999)~~. Mortality due to AHS is related to the equid species ~~of equidae~~ affected and to the strain or serotype of the virus. Among equidae, horses are the most susceptible to AHS with a mortality rate of 50–95%, followed by mules with mortality around 50%. In enzootic regions of Africa, donkeys are very resistant to AHS and experience only subclinical infections. In European and Asian countries, however, donkeys are moderately susceptible and have a mortality rate of 10%. Zebras are also markedly resistant with no clinical signs, except for fever, although this has only been observed after experimental inoculation, and they may also have an extended viraemia (up to 40 days).

~~A laboratory diagnosis is essential to establish a correct and confirmatory diagnosis. Although some clinical signs and lesions are characteristic, AHS can be confused with other diseases. For example, the supraorbital swelling, which is often present in horses with subacute AHS, is, in combination with an appropriate history, sufficient for a tentative diagnosis. Other signs and lesions are less specific for AHS, and other diseases such as equine encephalosis, equine infectious anaemia, Hendra virus, equine viral arteritis, piroplasmosis and purpura haemorrhagica should be excluded (WOAH, 2010).~~

Attenuated (monovalent and polyvalent) live vaccines for use in horses, mules and donkeys, are currently commercially available.

~~There is no evidence that humans can become infected with any field strain of AHSV, either through contact with naturally or experimentally infected animals or by virus manipulation in laboratories. Laboratory manipulations should be performed with appropriate containment determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).~~

2. Nature and classification of the pathogen

AHS is caused by African horse sickness virus (AHSV), a double-stranded RNA orbivirus belonging to the family Sedoreoviridae. The genus *Orbivirus* also includes bluetongue virus and epizootic haemorrhagic disease virus, which have similar morphological and biochemical properties with distinctive pathological and antigenic properties as well as host ranges. Nine antigenically distinct serotypes of AHSV have been identified by virus neutralisation; some cross-reaction has been observed between 1 and 2, 3 and 7, 5 and 8, and 6 and 9, but no cross-reactions with other known orbiviruses occur. The virus can be inactivated at 72°C for 120 minutes (confirmed by three blind passages in the Vero cell line).

The virion is an unenveloped particle of a size around 70 nm. The genome of AHSV is composed of ten double-stranded RNA segments, encoding seven structural proteins (VP1-7), and four nonstructural proteins (NS1, NS2, NS3, NS3A). Proteins VP2 and VP5 form the outer capsid of the virion, and proteins VP3 and VP7 are the major inner capsid proteins. Proteins VP1, VP4 and VP6 constitute minor inner capsid proteins (Roy *et al.*, 1994). The NS3 proteins are the second most variable AHSV proteins, the most variable being the major outer capsid protein, VP2. This protein, VP2, is the determinant of AHSV serotypes and, together with VP5, the target for virus neutralisation activity.

3. Zoonotic potential and biosafety and biosecurity requirements

There is no evidence that humans can become infected with any field strain of AHSV, either through contact with naturally or experimentally infected animals or by virus manipulation in laboratories. However, infection by vaccine strains of AHSV has been described in four laboratory workers of a vaccine-packing facility, but it must be stressed that the infections described occurred under particular circumstances with known encephalitogenic strains and there is no evidence to suggest that AHS virus should ordinarily be considered a human pathogen (van der Meyden *et al.*, 1992). Laboratory manipulations should be performed with appropriate containment determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

4. Differential diagnosis

A laboratory diagnosis is essential to establish a correct and confirmatory diagnosis. Although some clinical signs and lesions are characteristic, AHS can be confused with other diseases. For example, the supraorbital swelling, which is often present in horses with subacute AHS, is, in combination with an appropriate history, sufficient for a tentative diagnosis. Other signs and lesions are less specific for AHS, and other diseases such as equine encephalosis, equine infectious anaemia, Hendra virus, equine viral arteritis, piroplasmosis and purpura haemorrhagica should be excluded (WOAH, 2010).

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of African horse sickness and their purpose

Method	Purpose					
	Population freedom from infection[a]	Individual animal freedom from infection prior to movement[b] [c]	Contribute to eradication policies[d]	Confirmation of clinical cases[e]	Prevalence of infection – surveillance[f]	Immune status in individual animals or populations post-vaccination[g]
Detection and identification of the agent[h]						
Real-time RT-PCR	+	+++	+++	+++	++	–
Agarose gel-based RT-PCR	–+	+±	+±	++	+	–
Virus isolation	–	++	–	+++	–	–
Detection of immune response						
ELISA (serogroup specific based on VP7)	+++	++±	++	++	+++	++
CFT	+	+	+	+	+	+
VN	+	+	–	+	+	+++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
RT-PCR = reverse-transcription polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay;
VN = virus neutralisation; CFT = complement fixation test.

[a] See Appendix 1 of this chapter for justification table for the scores given to the tests for this purpose.
[b] See Appendix 2 of this chapter for justification table for the scores given to the tests for this purpose.
[c] For serological tests, paired samples may be required as recommended in the *Terrestrial Code* Chapter 12.1. 'Infection with African horse sickness virus', Article 12.1.7 'Recommendations for importation from AHS infected countries or zones'
[d] See Appendix 3 of this chapter for justification table for the scores given to the tests for this purpose.
[e] See Appendix 4 of this chapter for justification table for the scores given to the tests for this purpose.
[f] See Appendix 5 of this chapter for justification table for the scores given to the tests for this purpose.
[g] See Appendix 6 of this chapter for justification table for the scores given to the tests for this purpose.
[h] A combination of agent identification methods applied on the same clinical sample is recommended.

~~Several techniques are already available for AHS viral identification ranging from the rapid capture (indirect sandwich) enzyme-linked immunosorbent assay (ELISA) (Rubio *et al.*, 1998), using either polyclonal antibodies (PAbs) or monoclonal antibodies (MAbs), to the polymerase chain reaction (PCR) test, including reverse-transcription (RT) PCR for discrimination of the nine AHSV serotypes or virus isolation in cell culture.~~ If possible, more than one test should be performed to diagnose an outbreak of AHS, especially the index case. The initial test can be a polymerase chain reaction (PCR) ~~quick test such as enzyme-linked immunosorbent assay (ELISA) or polymerase chain reaction (PCR)~~, followed by virus isolation in tissue culture. Serological techniques for serogroup specific detection enzyme-linked immunosorbent assay (ELISA) can also be used as a complementary method to detect infection by the detection of seroconversion, for which paired samples should be analysed. Virus neutralisation (VN) for serotype identification, type-specific reverse-transcription (RT)-PCR or sequencing should be performed as early in the outbreak as possible so that the serotype can be identified and the correct vaccine selected.

~~At present, there are no international standards for viruses or diagnostic reagents, and there is no standard methodology for the identification of AHSV. However, a viral and antibody panel has been evaluated, and comparative studies between different ELISAs for AHSV antigen and antibody determination have been carried out in different laboratories, including in the European Union (EU) Reference Laboratory for AHS. The results have demonstrated a high level of correlation for both antigen and antibody determination with an in-house test and commercial kits. Similar studies have been conducted with several RT-PCR assays also providing a high level of correlation. Further information on comparative studies of different test methods and kits is available from the~~

~~WOAH Reference Laboratories for AHS. A very important aspect of the diagnosis is the selection of samples and their safe transportation to the laboratory.~~

At present, international standards, both viral and antibody panels, for the identification of AHSV and specific antibodies, are available at the European Union (EU) Reference Laboratory (RL) for AHS. Inactivated virus of serotypes 1–9 reference strains can be obtained from the WOAH Reference Laboratory in Spain to set up the RT-PCR detection method and establish its analytical sensitivity in comparison with real-time RT-PCR procedure (Agüero *et al.*, 2008). These materials have been distributed worldwide.

Since 2007, proficiency tests have been organised annually by the EU-RL for AHSV antibody determination and viral genome detection. Laboratories involved in AHS official control around the world have participated. A commercial blocking ELISA for AHSV antibodies detection has been widely used by participants and the results have demonstrated a good performance. Regarding viral genome detection, real-time RT-PCR methods have been widely used and similar results have been obtained with different RT-PCR assays.

A key aspect of the diagnosis is the selection of samples and their correct storing and safe transporting. Orbiviruses remain viable at 4°C in ethylene diamine tetra-acetic acid (EDTA) blood samples for weeks or at –80°C for long-term storage. Organ samples should be frozen at –80°C for 48–72 hours. Serum samples for antibody detection should be kept at –20°C if storage is required for >1 week. Both the International Air Transport Association (IATA) and the European Agreement concerning the International Carriage of Dangerous Goods by Road (ADR) categorise clinical samples and culture containing AHSV as B UN 3373 (see Chapter 1.1.3 *Transport of biological materials*).

1. Identification of the agent

1.1. Virus isolation

Unclotted whole blood collected during the early febrile stage of the disease from sick animals, as well as small pieces (2–4 g) of spleen, lung and lymph nodes from animals that have died, are the samples of choice for diagnosis. Samples should be kept at 4°C during transportation and short-term storage prior to processing.

1.1.1. Cell culture

Successful direct isolation of AHSV has been performed on baby hamster kidney (BHK-21), monkey stable (MS) and African green monkey kidney (Vero) mammalian cell lines and on *Culicoides* and mosquito insect cell lines. Blood samples collected in an appropriate anticoagulant can be used ~~un~~diluted as the inoculum. After 15–60 minutes of adsorption at ambient temperature or at 37°C, the cell cultures are washed and maintenance medium is added. Alternatively and more commonly, the blood is washed, lysed and diluted 1/10. This procedure removes unwanted antibody, which could neutralise free virus, and promotes release of virus associated with the red blood cell membranes. When tissue samples, such as spleen, lung, etc., are used, a 10% tissue suspension is prepared in phosphate buffered saline (PBS) or cell culture medium, containing antibiotics.

A cytopathic effect (CPE) may appear between 2 and 10 days post-infection with mammalian cells. Three blind passages should be performed before considering the samples to be negative. No CPE is observed in insect cells but the presence of the virus can be detected in the supernatant after 5–7 days by real-time RT-PCR. Supernatant from infected insect cells can then be passed onto mammalian cells, which will show CPE after one or two passages. Positive CPE must be confirmed by real time RT-PCR or VNT. Real-time RT-PCR results should be carefully interpreted as positive results do not necessarily indicate the presence of infectious virus.

1.2. Nucleic acid methods

1.2.1. Reverse-transcription polymerase chain reaction

RT-PCR is a highly sensitive technique that provides a rapid identification of AHS viral nucleic acid in blood and other tissues of infected animals. ~~This technique has greatly improved the laboratory diagnosis of AHS by increasing the sensitivity of detection and shortening the time required for the diagnosis.~~ The RT-PCR procedure will detect virus-specific nucleic acid after the virus is no longer viable and capable of establishing a new infection in either insects or mammalian cells. ~~Therefore, positive results do not necessarily indicate the presence of infectious virus.~~

Several agarose gel-based RT-PCR assays for the specific detection of AHSV RNA have been described targeted at viral segments 3, 7 or 8 (Aradaib, 2009; Bremer ~~et al.~~ & Viljoen, 1998;

~~Laviada *et al.*, 1997; Sakamoto *et al.*, 1994; Stone-Marschat *et al.*, 1994;~~ Zientara *et al.*, 1994). The most widely used method employs primers corresponding to the 5' end (nucleotides 1–21) and 3' end (nucleotides 1160–1179) of RNA segment 7 (coding for VP7) amplifying the complete viral segment (Zientara *et al.,* 1994).

Real-time RT-PCR methods for the highly sensitive and specific detection of AHSV RNA have been developed based on the use of a pair of primers and a labelled probe from conserved sequences of viral segments 3, 5 or 7 (Agüero *et al.*, 2008; Bachanek-Bankowska *et al.,* 2014~~; Fernández-Pinero *et al.*, 2009; Rodriguez Sanchez *et al.*, 2008~~). A duplex real-time RT-PCR has also been described that targets segments 7 and 8 of the genome (coding for ~~NS1~~ VP7 and NS2 respectively) (Quan *et al.*, 2010).

Although both gel-based and real-time RT-PCR procedures can detect reference strains from the nine virus serotypes, real-time RT-PCR provides advantages over agarose gel-based RT-PCR methods, with its faster analysis time, higher sensitivity, and suitability for high-throughput automation. Nevertheless, gel-based RT-PCR methods, particularly those amplifying long RNA fragments (~~Laviada *et al.*, 1997;~~ Zientara *et al.*, 1994), can be very useful in the further genetic characterisation of the virus by sequencing of the amplicons. In addition, it may be beneficial in laboratories without the capacity to perform real-time RT-PCR.

In 2015 the WOAH Reference Laboratories for AHS carried out an international ring trial to gather information on the performance of the different methods used in the main AHSV diagnostic laboratories. Ten different RT-PCR protocols were evaluated. Although in this trial some methods could only be tested in one or two laboratories, they produced very good results and therefore are suitable for further evaluation and validation. The study identified that the real-time RT-PCR methods of Agüero *et al.* (2008) and Guthrie *et al.* (2013) correctly detected all the representative strains included in the international ring trial with a high sensitivity in the analysis of field samples. These methods are validated for certification of individual animals prior to movement and are described below.

Details of AHSV gel-based RT-PCR and real-time RT-PCR methods are given below.

To assure a good reaction it is necessary to extract from the sample an AHSV RNA of high quality. The extraction of nucleic acids from clinical samples can be performed by a variety of in-house and commercially available methods.

1.2.2. Agarose gel-based RT-PCR procedure (Zientara *et al.,* 1994)

Denaturation of extracted RNA has to be performed prior to the RT-PCR procedure as the AHSV genome consists of double-stranded RNA. The sequences of the PCR primers used are 5'-GTT-AAA-ATT-CGG-TTA-GGA-TG-3', which corresponds to the messenger RNA polarity, and 5'-GTA-AGT-GTA-TTC-GGT-ATT-GA-3', which is complementary to the messenger RNA polarity.

All the components required for the reverse transcription and PCR are included in the reaction tube containing the denatured RNA. A one-step RT-PCR is carried out by incubating in a thermocycler as follows: 45 minutes to 1 hour at 37–55°C, 5–10 minutes at 95°C, then 40 cycles of: 94–95°C for 1 minute, 55°C for 1–1.5 minutes, 70–72°C for 2–2.5 minutes, followed by a final extension step of 7–8 minutes at 70–72°C. Analysis of the PCR products is carried out by agarose gel electrophoresis. AHS-positive samples will resolve in a 1179 base-pair band that can additionally be used as template in the sequencing reaction, using the PCR primers independently to obtain the nucleotide sequence of viral segment 7..

1.2.3. Real-time RT-PCR procedure (Agüero *et al.,* 2008)

This group-specific real-time RT-PCR has been employed with very good results by the participating national reference laboratories of the EU Member States in annual proficiency tests for the period 2009~~–2015~~–2023. Moreover, in an international ring trial organised in 2015 under the auspices of the WOAH Reference Laboratory network, it was found to be one among other top-ranking protocols.

a) Protocol

The assay targets AHSV segment 7 (VP7) and is described in Agüero *et al.* (2008). ~~It is able to detect all known AHSV types and strains currently circulating.~~ It is highly recommended to include an internal control in the PCR to detect inhibitors in the sample that may produce a false negative result. The method, which has been optimised for both VP7 and VP7 plus β-actin, is able to detect all known AHSV types and strains currently circulating.

i) RNA extraction from blood and tissue samples

Commercial kits are widely available; the RNA extraction step can be performed according to the procedures specified in each kit.

ii) Several one-step real-time RT-PCR kits are commercially available that can be used depending on local or case-specific requirements, kits used and equipment available. Some basic steps as described by Agüero *et al.* (2008) are given below. (For primers and probe sequences see Table 2).

iii) Primer stock concentration is diluted to a working concentration of 8 µM whereas probe is diluted to a working concentration 50 µM.

iv) 2.5 µl of each 8 µM primer working stock ~~8 µM~~ (final concentration 1 µM) is added to each well of the PCR plate (or tube or strip) that will contain RNA samples, positive or negative controls. The plate is held on ice.

v) 2 µl of RNA samples, including test and positive and negative controls, is added to each well.

vi) Samples are subjected to heat denaturation at 95°C for 5 minutes, followed by rapid cooling on ice for further 5 minutes.

vii) An appropriate volume of real-time one-step RT-PCR master mix for the number of samples to be tested is prepared following the manufacturer's instructions. Probe should be included in a final concentration of 0.25 µM (0.1 µl of 50 µM probe working stock~~, 50 µM~~ per sample). β-actin primers/probe should be included in a final concentration of 0.05 µM (0.1 µl of working stock 10 µM of each primer and probe per sample).

viii) 13 µl of master mix is distributed in each well on the PCR plate containing the denatured primers and RNA.

ix) The plate is placed in a real-time thermal cycler programmed with the following profile:
48°C × 25 minutes
95°C × 10 minutes
40 cycles: 95°C × 15 seconds, 55°C × 35 seconds, 72°C × 30 seconds

If reagents and thermal cycler allowing fast reactions are employed then the following program can be used:
48°C × ~~25~~ 10 minutes
95°C × 10 minutes
40 cycles: 97°C × 2 seconds, 55°C × 30 seconds
Fluorescence data are acquired at the end of the 55°C step.

Note: times and temperatures may vary and should be optimised for the reagents or kit used.

b) Interpretation of the results

The assay is considered not valid if atypical amplification curves are obtained. If this is the case, the assay must be repeated.

The assay is considered positive when a typical amplification curve is obtained and the Ct value (the number of polymerase chain reaction (PCR) cycles required for fluorescent signal to exceed the background) is lower or equal to the defined Ct threshold (35) within 40 PCR cycles (Ct≤35).

The assay is considered inconclusive when a typical amplification curve is obtained and the Ct value is higher ~~to~~ than the defined Ct threshold (35) within 40 PCR cycles (Ct≥35).

The assay is considered negative when a horizontal amplification curve is obtained and does not cross the threshold line within 40 PCR cycles and the Ct for the internal control (β-actin) is lower or equal to 32.

~~**c) Diagnostic characteristics**~~

~~i) Cut-off determination~~

~~The positive cut-off for the test method is less than 35 PCR cycles (Ct≤35).~~

~~The negative cut-off for the test method is 40 PCR cycles.~~

~~Test results between the positive and negative cut-offs (35 and 40 PCR cycles) are considered inconclusive (35 ≤ Ct ≤ 40).~~

~~ii) Diagnostic sensitivity and specificity~~

~~The diagnostic specificity (DSp) and diagnostic sensitivity (DSe) were calculated according to the procedure detailed in Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals*.~~

~~In total 186 known negatives and 132 known positive samples were analysed to estimate DSe and DSp of the Agüero AHS real-time RT-PCR method, which is higher than the minimum number required (73) for an estimated DSe and DSp of 95% allowing a 5% error. DSe and DSp were 97% and 100%, respectively. It can thus be concluded that the number of known status samples used to calculate these diagnostic parameters was sufficient to comply with WOAH requirements.~~

d) Reproducibility

Reproducibility of the Agüero real-time RT-PCR method in the international ring trial cited above was at least 93.55%, correctly identifying all positive and negative samples included in the panel. All laboratories detected dilutions of positive samples to at least 10^{-5}. Similar results have been reported in other proficiency test programmes.

~~Inactivated virus of serotypes 1–9 reference strains can be obtained from the WOAH Reference Laboratory in Spain to set up the RT-PCR detection method.~~

1.2.4. Real-time RT-PCR procedure (Guthrie *et al.,* 2013)

a) Protocol

The test method presented here is adapted from Guthrie *et al.* (2013) and is capable of detecting all known AHSV types and strains currently circulating. The assay targets AHSV segment 7 (VP7). The procedure given may require modification to accommodate individual laboratory or different RT-PCR kit requirements.

i) RNA extraction from blood and tissue samples

Commercial kits are widely available; the RNA extraction step can be performed according to the procedures specified in each kit.

ii) Kits for the one-step real-time RT-PCR are available commercially. Below are some basic steps as described by Guthrie *et al.* (2013), which can be modified depending on local or case-specific requirements, kits used and equipment available.

iii) Primer and probe mix stock solutions are made up in a 25× concentration at 5 µM for the forward and reverse primers and 3 µM for the probe.

iv) 5 µl of RNA samples, including test and positive and negative controls, are added to appropriate wells of the PCR plate (or tube or strip).

v) Samples are subjected to heat denaturation at 95°C for 2 minutes, and held on ice for at least 3 minutes.

vi) An appropriate volume of real-time one-step RT-PCR master mix for the number of samples to be tested is prepared, following the manufacturer's instructions. 1 µl of 25× primer probe mix stock solution (from step iii above) is included in the master mix to give a final concentration in each well of 200 nM for each primer and 120 nM of the probe.

vii) 20 µl of master mix is distributed in each well on the PCR plate containing the denatured RNA.

viii) The plate is placed in a real-time thermal cycler programmed for reverse transcription and cDNA amplification or fluorescence detection as suggested by the manufacturers.

The following thermal profile is an example:
48°C × 10 minutes
95°C × 10 minutes
40 cycles: 95°C × 15 seconds, 60°C × 45 seconds

Note: times and temperatures may vary and should be optimised for the reagents or kit used.

b) Interpretation of the results

Note: the positive/inconclusive/negative cut-off values shown should be validated or adjusted in individual laboratories according to the reagents and equipment in use.

Samples are classified as "AHSV positive" if the normalised fluorescence for the AHSV real-time RT-PCR assay exceeds a 0.1 threshold within 36 PCR cycles in all replicates of a sample.

Samples are classified as "AHSV Inconclusive" if the normalised fluorescence for the AHSV real-time RT-PCR assay exceeds a 0.1 threshold between 36 and 40 PCR cycles in any replicate of a sample.

Samples are classified as "AHSV negative" if the normalised fluorescence for the AHSV assay did not exceed a 0.1 threshold within 40 PCR cycles in all replicates of a sample and if the normalised fluorescence for the internal positive control assay exceeded a 0.1 threshold within 33 PCR cycles.

~~c) Diagnostic characteristics~~

~~i) Cut-off determination~~

~~The positive cut-off for the test method is less than 36 PCR cycles.~~

~~The negative cut-off for the test method is 40 PCR cycles.~~

~~Test results between the positive and negative cut-offs (36 and 40 PCR cycles) are considered inconclusive.~~

~~ii) Diagnostic sensitivity and specificity~~

~~The DSe and DSp of the AHSV real-time RT-PCR for detection of AHSV nucleic acid in whole blood samples were estimated by comparison with virus isolation using a two-test two-population Bayesian latent class model that allowed for conditional dependence (correlation) among test results. A total of 503 equine blood samples collected from individual horses with pyrexia and one or more clinical signs typical of AHS were used to represent AHS suspect cases. Blood samples were also collected from two separate healthy populations of horses (503 and 98 horses each, respectively) that were not vaccinated against AHS and that were highly unlikely to have been exposed to natural infection with AHSV; these samples were used to represent AHS negative cases.~~

~~The median diagnostic specificity of the test method exceeded 99.9%.~~

~~The median diagnostic sensitivity of the test method exceeded 97.8%.~~

c) Reproducibility

In the international ring trial cited above the Guthrie FRET probe real-time RT-PCR method demonstrated sensitivity in excess of 88.1%, a specificity of 100%, correctly identifying all positive and negative samples included in the panel. All laboratories detected dilutions of positive samples to at least 10^{-5}.

Table 2. Comparison of the real-time RT-PCR methods of Agüero et al. (2008) and Guthrie et al. (2013)

	Agüero *et al.* (2008)	**Guthrie *et al.* (2013)**
Target	Group specific (VP7)	Group specific (VP7)
Primers (5'-3')	CCA-GTA-GGC-CAG-ATC-AAC-AG	AGA-GCT-CTT-GTG-CTA-GCA-GCC-T
	CTA-ATG-AAA-GCG-GTG-ACC-GT	GAA-CCG-ACG-CGA-CAC-TAA-TGA
Probe (5'-3')	FAM-GCT-AGC-AGC-CTA-CCA-CTA-MGB	FAM-TGC-ACG-GTC-ACC-GCT-MGB
Annealing temperature	55°C	60°C
Number of amplification cycles	40	40

	Agüero *et al.* (2008)	Guthrie *et al.* (2013)
~~Analytical sensitivity (LOD)~~	~~Dilution 10^{-5} of a viral suspension of AHSV-4 reference strain with a titre of $10^{6.3}$ $TCID_{50}$/ml, which corresponded to a Ct of 34.3±0.5 ($10^{1.3}$ $TCID_{50}$/ml)~~	~~Dilution of 3.02×10^{-6} of a AHSV positive blood samples, with corresponding Ct of 35.71~~
~~Diagnostic specificity~~	~~100%~~	~~99.9%.~~
~~Diagnostic sensitivity~~	~~97%~~	~~97.8%.~~

1.3. AHSV typing

Until recently, the VN test has been the method of choice for typing as well as the 'gold' standard test for identifying AHSV isolated from the field using type-specific antisera (Verwoerd, 1979). This technique takes 5 or more days before results are obtained. The development of type-specific gel-based RT-PCR (Maan *et al.,* 2011~~; Sailleau *et al.,* 2000~~; Van Schalkwyk *et al.,* 2019), and real-time RT-PCR using hybridisation probes (Koekemoer, 2008) targeting AHSV Seg-2 for identification and differentiation of AHSV genotypes, as well as the more recently described type-specific real-time RT-PCR assays based on the use of labelled DNA probes–MGB probes (Bachanek-Bankowska *et al.*, 2014; Villalba *et al.*, 2024; Weyer *et al.,* 2015) provides a rapid typing method for AHSV in tissue samples and blood. In comparison to VN tests, these methods can be used to ~~very~~ significantly increase the speed and reliability of detection and identification ~~(compared with VN tests)~~ of the nine serotypes of AHSV. ~~Type-specific real-time RT-PCR assays based on the use of labelled DNA probes–MGB probes have been developed recently by Bachanek-Bankowska *et al.*, 2014, Weyer *et al.,* 2015).~~

However, the genetic variation that may appear over time in the AHSV genome, ~~in particular~~ specifically in the VP2 coding region, where specific primers/probes for typing assays have to be designed, makes the detection of all genetic variants within each serotype by this type of technique difficult. Therefore, although molecular methods ~~are able to~~ can rapidly type AHSV in many positive field samples, VN should be kept as the gold standard for serotyping AHSV isolates.

2. Serological tests

Indirect and competitive blocking ELISAs using either soluble AHSV antigen or a recombinant protein VP7 (~~Hamblin *et al.*, 1990;~~ Laviada *et al*., 1992; Maree & Paweska, 2005~~; Wade-Evans *et al.,* 1993~~) have proved to be good methods for the detection of anti-AHSV group-reactive antibodies, especially for large-scale investigations ~~(Rubio *et al.*, 1998). Both of these tests have been recognised by the European Commission (2002).~~ The competitive blocking ELISA can also be used for testing wildlife as species-specific anti-globulin is not required with this method. An immunoblotting test has also been adapted for anti-AHS antibody determination (Laviada *et al*., 1992), which is especially suitable for small numbers of sera. The complement fixation (CF) test has been widely used, but some sera are anti-complementary, particularly donkey and zebra sera.

2.1. Blocking enzyme-linked immunosorbent assay

The competitive blocking ELISA technique detects specific antibodies against AHSV, present in any equine species. VP7 is the main antigenic protein within the molecular structure of AHSV and it is conserved across the nine AHSV serotypes. An MAb directed against VP7 is used in this test, allowing high sensitivity and specificity. Moreover, other species of the family Equidae (e.g. donkeys, zebra, etc.) can be tested thus preventing the problem of specificity experienced occasionally using the indirect ELISAs. VP7 recombinant antigen is non-infectious, which provides a high level of security ~~(European Commission, 2002)~~.

The principle of this test is to block the specific reaction between the recombinant VP7 protein absorbed on an ELISA plate and a conjugated MAb against VP7. AHSV antibodies present in a suspect serum sample will block this reaction. A decrease in the amount of colour is evidence of the presence of AHSV antibodies in the serum sample.

The competitive blocking ELISA is commercially available. The reproducibility of the test was assessed in an international ring trial (Durán-Ferrer *et al.,* 2019).

2.1.1. Test procedure

The test described here is an example of a blocking ELISA.

i) *Solid phase:* coat 96-well ELISA plates with 50–100 ng of recombinant AHSV-4 VP7 diluted in carbonate/bicarbonate buffer, pH 9.6. Incubate overnight at 4°C.

ii) Wash the plates three times with PBS 0.1× containing 0.135 M NaCl and 0.05% (v/v) Tween 20 (washing solution). Gently tap the plates onto absorbent material to remove any residual wash.

iii) *Test samples:* serum samples to be tested, and positive and negative control sera (if not ready to use by kit manufacturer), are diluted 1/5 in diluent containing 0.35 M NaCl, 0.05% Tween 20; and 0.1% Kathon, 100 µl per well. Incubate for 1 hour at 37°C.

iv) Wash the plates five times with PBS 0.1× containing 0.135 M NaCl and 0.05% (v/v) Tween 20 (washing solution). Gently tap the plates onto absorbent material to remove any residual wash.

v) *Conjugate:* dispense 100 µl/well of horseradish peroxidise-conjugated MAb anti-VP7 at optimal dilution in a suitable diluent. The MAb and diluent may be included in commercial kits. Incubate for 30 minutes at 37°C.

vi) Wash the plates as described in step iv.

vii) *Substrate/chromogen:* add 100 µl/well substrate/chromogen solution, e.g. ABTS (2,2'-azino-di-[3-ethyl-benzothiazoline]-6-sulphonic acid) 5 mg/ml diluted 1/10 in 0.1 M phosphate/ citrate buffer, pH 4, containing 0.03% H_2O_2, and incubate for 10 minutes at room temperature.

Colour development is stopped by adding 100 µl/well of 2% (w/v) of SDS. Alternative chromogen systems may be used (e.g. tetramethyl benzidine).

viii) Read the plates at 405 nm.

ix) *Validation of the assay:* positive control lower than 0.2 and negative control higher than 1.0.

x) *Interpretation of results:* determine the blocking percentage (BP) of each sample by applying the following formula:

$$BP = \frac{\text{Abs (Control Neg)} - \text{Abs (sample)}}{\text{Abs (Control Neg)} - \text{Abs (Control Pos)}} \times 100$$

Samples showing BP value lower than 45% are considered negative for antibodies to AHSV. Samples showing BP value higher than 50% are considered positive for antibodies to AHSV. Samples with BP value between 45% and 50% are considered doubtful and must be retested. If the result is the same, resample and test 2 weeks later.

2.2. Indirect enzyme-linked immunosorbent assay

The recombinant VP7 protein has been used as antigen[4] for AHSV antibody determination with a high degree of sensitivity and specificity (Laviada *et al.,* 1992; Maree & Paweska, 2005). Other advantages of this antigen are its stability and its lack of infectivity. The conjugate used in this method is a horseradish peroxidase anti-horse gamma-globulin reacting with horse, mules and donkeys. The method described by Maree & Paweska (2005) uses protein G as conjugate that also reacts with zebra serum. The indirect ELISA is recently also commercially available.

2.2.1. Test procedure

There are several test procedures described; this is an example of one AHS indirect ELISA procedure.

i) *Solid phase:* Coat 96-well ELISA plates with recombinant AHSV-4 VP7 diluted in carbonate/bicarbonate buffer, pH 9.6. Incubate overnight at 4°C.

ii) Wash the plates five times with distilled water containing 0.01% (v/v) Tween 20 (washing solution). Gently tap the plates on to absorbent material to remove any residual wash.

iii) Block the plates with PBS, pH 7.2 + 5% (w/v) skimmed milk, 200 µl/well, for 1 hour at 37°C.

iv) Remove the blocking solution and gently tap the plates on to absorbent material.

[4] ~~The antigen can be provided on request by the Centro de Investigación en Sanidad Animal (CISA), Spain. The delivery time is 4–6 months.~~

v) *Test samples:* Serum samples to be tested, and positive and negative control sera, are diluted 1/25 in PBS + 5% (w/v) skimmed milk + 0.05% (v/v) Tween 20, 100 µl per well. Incubate for 1 hour at 37°C. For titration, add twofold dilution series from 1/25 (100 µl/well), one serum per plate column, in duplicate columns, and do the same with positive and negative controls. Incubate for 1 hour at 37°C.

vi) Wash the plates as described in step ii.

vii) *Conjugate:* Dispense 100 µl/well of horseradish peroxidase conjugated anti-horse gamma-globulin diluted in PBS + 5% milk + 0.05% Tween 20, pH 7.2. Incubate for 1 hour at 37°C.

viii) Wash the plates as described in step ii.

ix) *Chromogen/Substrate:* Add 200 µl/well of chromogen/substrate solution (10 ml 80.6 mM DMAB [3-(dimethylamino) benzoic acid] + 10 ml 1.56 mM MBTH [3-methyl-2-benzothiazolinone hydrazone] + 5 µl H_2O_2). Colour development is stopped by adding 50 µl of 3 N H_2SO_4 after approximately 5–10 minutes (before the negative control begins to be coloured). Other chromogens such as ABTS, tetramethyl benzidine or orthophenyldiamine can also be used.

x) Read the plates at 600 nm, ~~(or~~ 620 nm, or 450nm, depending on the chromogen used~~)~~.

xi) *Interpretation of results:* Calculate the cut-off value by adding 0.06 to the value of the negative control. (0.06 is the standard deviation derived with a group of 30 negative sera) Test samples giving absorbance values lower than the cut-off are regarded as negative. Test samples giving absorbance values greater than the cut-off + 0.15 are regarded as positive. Test samples giving intermediate absorbance values are doubtful and a second technique must be employed to confirm the result.

2.3. Complement fixation

The CF test has been used extensively in the past, but currently its use is decreasing and has been replaced in many laboratories by ELISA as a screening technique. This progressive replacement is because of the higher sensitivity and degree of standardisation of ELISA as well as a significant number of sera with anti-complementary activity. Nevertheless, the CF test is a useful tool in endemic areas for the demonstration and titration of group-specific IgM antibodies against AHSV notably following a recent infection or vaccination.

2.3.1. Reagents

Veronal buffered saline containing 1% gelatin (VBSG).

ii) Serum samples, free from erythrocytes, must be heat inactivated: horse serum at 56°C, zebra serum at 60°C and donkey serum at 62°C, for 30 minutes.

iii) The antigen is a sucrose/acetone extract of AHSV-infected mouse brain. The control antigen is uninfected mouse brain, extracted in the same way. In the absence of an international standard serum, the antigen should be titrated against a locally prepared positive control serum. In the test, four to eight units are used. The antigen may also be obtained by inoculation of the virus in suitable cell culture (see Section B.1 above).

iv) The complement is a normal guinea-pig serum.

v) The haemolysin is a hyperimmune rabbit serum against sheep red blood cells (SRBCs).

vi) The SRBCs are obtained by aseptic puncture of the jugular vein and preserved in Alsever's solution[2] or sodium citrate.

vii) The haemolytic system (HS) is prepared by diluting the haemolysin to contain two haemolytic doses and using this to sensitise washed SRBCs. The SRBCs are standardised to a 3% concentration.

viii) *Control sera:* A positive control serum is obtained locally and validated. Serum from a healthy antibody-negative horse is used as the negative control serum.

[2] 20.5 g dextrose (114 mM), 7.9 g sodium citrate $2H_2O$ (27 mM), 4.2 g NaCl (71 mM), H_2O to 1 litre. Adjust to pH with 1 M citric acid.

2.3.2. Test procedure

i) The reaction is performed in 96-well round-bottom microtitre plates in a final volume of 100 µl/well or in tubes if the macro-technique is used, at 4°C for 18 hours.

ii) All the sera, samples and controls are diluted 1/5 in VBSG and 25 µl of each serum is added in duplicate. A twofold dilution series of each serum is done from 1/5 to 1/~~180~~ 160.

iii) Add 25 µl of the antigen diluted according to the previous titration.

iv) Add 25 µl of the complement diluted according to a previous titration.

v) Incubate at 4°C for 18 hours.

vi) 25 µl of HS is added to all wells on the microtitre plate.

vii) The plate is incubated for 30 minutes at 37°C.

viii) Plates are then centrifuged at 200 ***g***, and the wells are scored for the presence of haemolysis. Control of sera, complement, antigen and HS are used

ix) Results are read using 50% haemolysis as the end point. The inverse of the highest dilution of serum specifically fixing complement with the CF antigen is called the titre.

x) A titre of 1/10 or more is positive, under 1/10 is negative.

2.4. Virus neutralisation serology ~~(VN)~~

Serotype-specific antibody can be detected using the VN serology test (House *et al.*, 1990). The VN ~~test~~ serology may have additional value in epidemiological surveillance and transmission studies, mainly in endemic areas where multiple serotypes are likely to be present.

2.4.1. VN serology test procedure

i) From a starting dilution of 1/5, serial twofold dilutions of the test sera are made in a cell-culture grade flat-bottomed 96-well microtitre plate, using cell culture medium as diluent. For each sample two wells are used at each dilution. Control positive and negative sera should also be included in each batch of tests. An equal volume (e.g. 25 µl) of a stock of AHSV containing 100 $TCID_{50}$ (50% tissue culture infective dose) is added to each well.

ii) Serum/virus mixtures are incubated for 60 minutes at 37°C 5% CO_2 and 95% humidity prior to the addition of 0.1 ml of Vero cell suspension (200,000 cells/ml) to each test well.

iii) A back titration of virus stock is prepared for each test using four wells per tenfold dilution, 25 µl per well. Test plates are incubated at 37°C, 5% CO_2, 95% humidity for 4–5 days, until the back titration indicates that the stock virus contains 30–300 $TCID_{50}$.

iv) After incubation for 4–5 days, the test is read using an inverted microscope. Wells are scored for the presence or absence of CPE. The presence of CPE in the wells containing the serum sample indicates that the tested serum does not contain specific neutralising antibodies against the virus ~~in the assay that cannot neutralise the virus~~, therefore producing cell lysis with the consequent destruction of the cell layer.

By contrast, the absence of CPE in the wells containing the serum sample indicates that the tested serum does contain specific neutralising antibodies against the virus ~~in the assay that can neutralise the virus~~, therefore maintaining intact the cell layer.

v) Alternatively, the plates are then fixed and stained in a solution of 0.15% (w/v) crystal violet in 2% (v/v) glutaraldehyde and rinsed or they may be fixed with 70% ethanol and stained with 1% basic fuchsin.

vi) The 50% end-point titre of the serum is calculated by the Spearman–Kärber method and expressed as the negative $\log_{10}$.

C. REQUIREMENTS FOR VACCINES

1. Background

1.1. Rationale and intended use of the product

Polyvalent or monovalent live attenuated AHS vaccines, based on the selection in Vero cell culture of genetically stable macroplaques, have been used for the control of AHSV in and out of Africa ~~(Erasmus, 1976; Sanchez Vizcaíno, 2004)~~. Polyvalent vaccines are commercially available.

It must be noted that detection of viral genome in animals vaccinated with live attenuated AHS vaccines has been described up to 16 weeks after vaccination (Weyer *et al.*, 2017).

An inactivated monovalent (serotype 4) AHSV vaccine based on virus purification and inactivation with formalin was produced commercially in the early 1990s (House *et al*., 1992), but is not commercially available at the present time. More recently, inactivated vaccines have been produced and successfully used against all serotypes (Rodríguez et al., 2020). Subunit AHSV vaccines based on serotype 4 outer capsid protein VP2 and VP5 plus inner capsid protein VP7, derived from single and dual recombinant baculovirus expression vectors have been used experimentally in different combinations to immunise horses (Martinez *et al*., 1996). The protective efficacy of VP2 in a subunit vaccine was also evaluated (Scanlen *et al*., 2002). However, these vaccines are not commercially available. Other examples of experimental new generation vaccines described but currently not commercially available, include virus-vectored vaccines (e.g. modified vaccinia Ankara virus: Alberca *et al.*, 2014), plant-derived virus-like particles (O'Kennedy *et al.*, 2024) as well as entry-competent replicative-abortive (ECRA) and disabled infection single animal (DISA) reverse genetics vaccine platforms (Van Rijn *et al.*, 2018).

2. Outline of production and minimum requirements for conventional vaccines

At present only the live attenuated AHS vaccines (polyvalent or monovalent) are commercially available. Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

3. Live attenuated African horse sickness vaccine

3.1. Characteristics of the seed

3.1.1. Biological characteristics

The seed virus is prepared by selection in Vero cells of genetically stable large plaques from low passage levels of AHSV. The plaque mutants are then further multiplied by three passages in Vero cells. A large quantity of this antigen is lyophilised and stored at –20°C as seed stock antigen.

3.1.2. Quality criteria

The seed virus must be shown to be free of contaminating viruses, bacteria and mycoplasmas by the appropriate techniques. The serotype identity of the seed virus is confirmed.

3.2. Method of manufacture

3.2.1. Procedure

At the onset of a production run, working antigens are produced from the seed stock antigen in either BHK-21 or Vero cell cultures. The working antigens are tested for sterility, purity and identity and should contain at least 1×10^6 plaque-forming units (PFU)/ml of infectious virus.

3.2.2. Requirements for substrates and media

Roller bottle cultures of Vero or BHK-21 cells are grown using gamma-irradiated bovine serum in the growth medium. Once the cultures are confluent, the medium is poured off and the cells are seeded with the working antigens. After 1 hour, maintenance medium is added to the cultures. Incubation is continued at 37°C for 2–3 days. When the CPE is advanced, both cells and supernatant medium are harvested. The products from the same serotype are pooled and stored at 4°C..

3.2.3. In-process control

The pooled harvests of the individual serotypes are tested for sterility and assayed for infectivity by plaque titration on Vero cell cultures. The minimum acceptable titre is 1×10^6 PFU/ml.

Finally, two multivalent vaccines are constituted by mixing equal volumes of serotypes 1, 3, 4 and 2, 6, 7, 8 respectively. Serotypes 5 and 9 are not included in vaccine formulations. A monovalent type can also be prepared. After addition of suitable stabiliser, the vaccine is distributed in 1.0 ml volumes into glass vials and freeze-dried.

3.2.4. Final product batch test

i) Sterility

Following lyophilisation, five bottles of vaccine are selected at random and tested for sterility by internationally accepted methods. Tests for sterility and freedom from contamination of biological materials intended for veterinary use are given in chapter 1.1.9.

ii) Safety

Innocuity of a vaccine is determined by the inoculation of reconstituted vaccine into mice (0.25 ml intraperitoneally), guinea-pig (1.0 ml intraperitoneally), and a horse (5.0 ml subcutaneously). All the animals are observed daily for 14 days. The rectal temperature of the horse is taken twice daily for 14 days and should never exceed 39°C.

iii) Batch potency

Potency is largely based on virus concentration in the vaccine.

The minimum immunising dose for each serotype is about 1×10^3 PFU/dose. The infectivity titre of the final product is assayed by plaque titration in Vero cell cultures and should contain at least 1×10^5 PFU/dose. The horse used for safety testing is also used for determining the immunogenicity of a vaccine.

Serum samples are collected on the day of vaccination and 21 days later, and are tested for neutralising antibodies against each serotype by the plaque-reduction test using twofold serum dilutions and about 100 PFU of virus. The horse should develop a neutralising antibody titre of at least 20 against at least three of the four serotypes in the quadrivalent vaccine.

3.3. Requirements for regulatory approval ~~authorisation~~

No specific guideline is described for AHS vaccine. However, a guideline is described in the EU for Bluetongue virus under exceptional circumstances that could probably be used for AHS virus. This guideline includes the minimum date requirements for the ~~authorisation~~ regulatory approval under exceptional circumstances for vaccine production for emergency use against bluetongue virus (Regulation EC N°726/2004, in particular Articles 38, 39 and 43 thereof and Article 26 of Direction 2001/82/EC), including guidance measures to facilitate the rapid inclusion of new or different virus serotypes.

4. Vaccines based on biotechnology

4.1. Vaccines available and their advantages

None is available commercially. Experimental ~~subunit~~ vaccines developed by different approaches have been described (Section C.1.1 *Rationale and intended use of the product*).

4.2. Special requirements for biotechnological vaccines, if any

None.

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NB: There are WOAH Reference Laboratories for African horse sickness (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3). Please contact the WOAH Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for African horse sickness

NB: FIRST ADOPTED IN 1991. MOST RECENT UPDATES ADOPTED IN 2019.

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Appendix 1: African horse sickness
Intended purpose of test: population freedom from infection

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
Identification of the agent							
Real-time RT-PCR ± Equids	EDTA blood/tissue (VP7)	(1) Dse 97% DSp 100% (2) Dse > 99.9% DSp > 97.8%	(1) 186 known negatives and 132 known positive samples (2) 503 equine blood samples collected from suspect cases; two separate healthy populations of horses (503 and 98 horses) were used as negative cases	(1) Validation report done at LCV (EURL), sent to WOAH and published in WOAH *Bulletin* nº 2016-1 (2) Validation report done at CVR (WOAH-RL), sent to WOAH and published in Guthrie *et al.* (2013)	- High sensitivity, specificity and reproducibility - High throughput - Minimal risk of cross contamination - Applicable to uninfectious samples allowing viral genome to be detected even after the viraemic window as well as in poor condition or inactivated samples	- Expensive equipment - Expensive reagents - Surviving animals present shorter periods of viraemia in comparison with antibodies - Detection of viral genome in animals vaccinated with live attenuated AHS vaccines has been described up to 16 weeks after vaccination	(1) Agüero *et al.* (2008) (2) Guthrie *et al.* (2013) Quan *et al.* (2010)
Agarose gel-based RT-PCR +	EDTA blood/tissue (VP7)	Not available	Not available		- Less expensive instruments than those used in real-time RT-PCR - High sensitivity, specificity and reproducibility - Applicable to uninfectious samples	- Longer analysis time than real-time RT-PCR - Not suitable for high throughput analysis - Risk of cross contamination -Surviving animals present shorter periods of viraemia in comparison with antibodies	Zientara *et al.* (1994)
Virus isolation –	EDTA blood/tissue	DSe ≥ 50% DSp > 99%	- 91 BTV positive blood samples collected from naturally infected animals during BTV outbreaks in Spain in 2007–2018 (validation done jointly for all Orbiviruses) - 12 tissue samples from horses experimentally infected with AHSV-9	Dossier for Verification of the Orbivirus isolation carried out at LCV (EURL)	- Detection of infectious virus - Getting available isolates for further studies	- Time consuming - Laborious - Requirement for samples from viraemic period - Requirement for well conserved samples - Biosafety laboratory consideration BLS3 - Requirement for cell cultures - Low sensitivity	

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
Detection of immune response							
ELISA (serogroup specific based on VP7) +++ Equids	Serum (antibodies against VP7)	(1) DSe ≥ 95% DSp > 99% (2) DSe > 99% Dsp > 99%	(1, 2) Goat, sheep and guinea-pig sera from animals inoculated with AHS1-AHS9 (1) Nine sera from horse naturally infected and positive by VN (1) 152 sera from horses vaccinated and serotyped by VN (1, 2) Sera from horses from free areas (512, 1015) (1) 24 bovine pos to BTV, 20 horse sera pos to EA and nine deer sera pos to EHDV (1) Seven sera from the WOAH reference collection, from different equidae species and in different levels of positivity (1) Seven sera from two experimentally infected horses with AHS9 (1) 70 samples from the EU-RL proficiency tests organised from 2014 to 2020 (1) 129 pos sera from vaccinated or experimentally or naturally infected Equidae, and 57 neg sera from naïve equidae	(1, 2) Manufacturer's validated method certificates (1) Dossier for Verification of blocking ELISA for detection of antibodies against African horse sickness virus carried out at LCV (EU-RL). Stage 3 of validation (PT) published in Durán-Ferrer *et al.* (2019)	- High sensitivity and specificity - Commercially available (both B-ELISA and I-ELISA) - Cost-effective - VP7 is highly conserved among serotypes -Long lasting of presence of antibodies in affected animals	- Unable to differentiate vaccinated from infected animals - Unable to differentiate serotype specificity of the antibodies	(1) B-ELISA INGEZIM AHSV Compac Plus 2.0 (Gold Standard Diagnostics); Durán-Ferrer *et al.* (2019) (2) I-ELISA IDScreen African horse sickness indirect (IDVET)
VN + Equids	Serum (neutralising serotype-specific antibodies against VP2)	DSe: > 95% (infected animals) DSp > 99%	(1) 18 sera from sheep experimentally inoculated with AHS1–AHS9 (2) Seven sera from two experimentally infected horses with AHS9	Dossier for Verification of the Orbivirus Seroneutralisation Test carried out at LCV (EU-RL)	- Allows serotyping from serum samples	- Unable to differentiate vaccinated from infected animals - Depends on the availability of live virus strains of all serotypes and susceptible cultured cells - Cross-reactions may appear between serotypes - Time consuming - Laborious - Biosafety laboratory consideration BLS3	
CFT + Equids	Serum	Not available	Not available	Not available	- Useful in recent infections or vaccination	- High number of sera presenting anticomplementary activity -Less sensitivity than ELISA	

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
						-Less degree of standardisation than ELISA	

Appendix 2: African horse sickness
Intended purpose of test: individual animal freedom from infection prior to movement*

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
Identification of the agent							
Real-time RT-PCR +++ Equids	EDTA blood/tissue (VP7)	(1) Dse 97% DSp 100% (2) Dse > 99.9% DSp > 97.8%	(1) 186 known negatives and 132 known positive samples (2) 503 equine blood samples collected from suspect cases; two separate healthy populations of horses (503 and 98 horses) were used as negative cases	(1) Validation report done at LCV (EURL), sent to WOAH and published in WOAH *Bulletin* n° 2016-1 (2) Validation report done at CVR (WOAH-RL), sent to WOAH and published in Guthrie *et al.* (2013)	- High sensitivity, specificity and reproducibility - Rapid technique - Minimal risk of cross contamination - Applicable to uninfectious samples allowing viral genome to be detected even after the viraemic window as well as in poor condition or inactivated samples	- Expensive equipment - Expensive reagents - Surviving animals present shorter periods of viraemia in comparison with antibodies - Detection of viral genome in animals vaccinated with live attenuated AHS vaccines has been described up to 16 weeks after vaccination - Discordant results are possible in the case of weak positive samples	(1) Agüero *et al.* (2008) (2) Guthrie *et al.* (2013) Quan *et al.*, (2010)
Agarose gel-based RT-PCR ++	EDTA blood/tissue (VP7)	Not available	Not available		- Less expensive instruments than those used in real-time RT-PCR - High sensitivity, specificity and reproducibility - Rapid technique - Applicable to uninfectious samples	- Longer analysis time than real-time RT-PCR - Risk of cross contamination -Surviving animals present shorter periods of viraemia in comparison with antibodies	Zientara *et al.* (1994)
Virus isolation –+	EDTA blood/tissue	DSe ≥ 50% DSp > 99%	- 91 BTV positive blood samples collected from naturally infected animals during BTV outbreaks in Spain in 2007–2018 (validation done jointly for all Orbiviruses) - 12 tissue samples from horses experimentally infected with AHSV-9	Dossier for Verification of the Orbivirus isolation carried out at LCV (EURL)	- Detection of infectious virus	- Time consuming - Laborious - Requirement for well conserved samples - Biosafety laboratory consideration BLS3 - Requirement for cell cultures - Low sensitivity	
*Detection of immune response							

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
ELISA (serogroup specific based on VP7) +++ Equids	Serum (antibodies against VP7)	(1) DSe ≥ 95% DSp > 99% (2) DSe > 99% Dsp > 99%	(1, 2) Goat, sheep and guinea-pig sera from animals inoculated with AHS1-AHS9 (1) Nine sera from horse naturally infected and positive by VN (1) 152 sera from horses vaccinated and serotyped by VN (1, 2) Sera from horses from free areas (512, 1015) (1) 24 bovine pos to BTV, 20 horse sera pos to EA and nine deer sera pos to EHDV (1) Seven sera from the WOAH reference collection, from different equidae species and in different levels of positivity (1) Seven sera from two experimentally infected horses with AHS9 (1) 70 samples from the EU-RL proficiency tests organised from 2014 to 2020 (1) 129 pos sera from vaccinated or experimentally or naturally infected equidae, and 57 neg sera from naïve equidae	(1, 2) Manufacturer's validated method certificates (1) Dossier for Verification of blocking ELISA for detection of antibodies against African horse sickness virus carried out at LCV (EU-RL). Stage 3 of validation (PT) published in Durán-Ferrer *et al.* (2019)	- High sensitivity and specificity - Commercially available (both B-ELISA and I-ELISA) - Cost-effective - VP7 is highly conserved among serotypes -Long lasting of presence of antibodies in affected animals	- Unable to differentiate vaccinated from infected animals - Seroconversion takes place after at least 8–12 d.p.i.	(1) B-ELISA INGEZIM AHSV Compac Plus 2.0 (Gold Standard Diagnostics): Durán-Ferrer *et al.* (2019) (2) I-ELISA IDScreen African horse sickness indirect (IDVET)
VN + Equids	Serum (neutralising serotype-specific antibodies against VP2)	DSe: > 95% (infected animals) DSp > 99%	(1) 18 sera from sheep experimentally inoculated with AHS1–AHS9 (2) Seven sera from two experimentally infected horses with AHS9	Dossier for Verification of the Orbivirus Seroneutralisation Test carried out at LCV (EU-RL)	- Allows serotyping from serum samples	- Unable to differentiate vaccinated from infected animals - Depends on the availability of live virus strains of all serotypes and susceptible cultured cells - Cross-reactions may appear between serotypes - Time consuming - Laborious - Biosafety laboratory consideration BLS3	
CFT + Equids	Serum	Not available	Not available	Not available	- Useful in recent infections or vaccination	- High number of sera presenting anticomplementary activity	

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
						-Less sensitivity than ELISA -Less degree of standardisation than ELISA	

*For serological tests, paired samples may be required as recommended in the *Terrestrial Code* Chapter 12.1. 'Infection with African horse sickness virus', Article 12.1.7 'Recommendations for importation from AHS infected countries or zones'

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Appendix 3: African horse sickness
Intended purpose of test: contribute to eradication policies

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
Identification of the agent							
Real-time RT-PCR +++ Equids	EDTA blood/tissue (VP7)	(1) Dse 97% DSp 100% (2) Dse > 99.9% DSp > 97.8%	(1) 186 known negatives and 132 known positive samples (2) 503 equine blood samples collected from suspect cases; two separate healthy populations of horses (503 and 98 horses) were used as negative cases	(1) Validation report done at LCV (EURL), sent to WOAH and published in WOAH *Bulletin* nº 2016-1 (2) Validation report done at CVR (WOAH-RL), sent to WOAH and published in Guthrie *et al.* (2013)	- High sensitivity, specificity and reproducibility - High throughput - Rapid technique - Minimal risk of cross contamination - Applicable to uninfectious samples allowing viral genome to be detected even after the viraemic window as well as in poor condition or inactivated samples	- Expensive equipment - Expensive reagents - Surviving animals present shorter periods of viraemia in comparison with antibodies - Detection of viral genome in animals vaccinated with live attenuated AHS vaccines has been described up to 16 weeks after vaccination	(1) Agüero *et al.* (2008) (2) Guthrie *et al.* (2013) Quan *et al.*, (2010)
Agarose gel-based RT-PCR ++	EDTA blood/tissue (VP7)	Not available	Not available		- Less expensive instruments than those used in real-time RT-PCR - High sensitivity, specificity and reproducibility - Rapid technique - Applicable to uninfectious samples	- Longer analysis time than real-time RT-PCR - Not suitable for high throughput analysis - Risk of cross contamination -Surviving animals present shorter periods of viraemia in comparison with antibodies	Zientara *et al.* (1994)
Virus isolation –	EDTA blood/tissue	DSe ≥ 50% DSp > 99%	- 91 BTV positive blood samples collected from naturally infected animals during BTV outbreaks in Spain in 2007–2018 (validation done jointly for all Orbiviruses) - 12 tissue samples from horses experimentally infected with AHSV-9	Dossier for Verification of the Orbivirus isolation carried out at LCV (EURL)	- Detection of infectious virus - Getting available isolates for further studies	- Time consuming - Laborious - Requirement for samples from viraemic period - Requirement for well conserved samples - Biosafety laboratory consideration BLS3 - Requirement for cell cultures - Low sensitivity	

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
Detection of immune response							
ELISA (serogroup specific based on VP7) ++ Equids	Serum (antibodies against VP7)	(1) DSe ≥ 95% DSp > 99% (2) DSe > 99% Dsp > 99%	(1, 2) Goat, sheep and guinea-pig sera from animals inoculated with AHS1-AHS9 (1) Nine sera from horse naturally infected and positive by VN (1) 152 sera from horses vaccinated and serotyped by VN (1, 2) Sera from horses from free areas (512, 1015) (1) 24 bovine pos to BTV, 20 horse sera pos to EA and nine deer sera pos to EHDV (1) Seven sera from the WOAH reference collection, from different equidae species and in different levels of positivity (1) Seven sera from two experimentally infected horses with AHS9 (1) 70 samples from the EU-RL proficiency tests organised from 2014 to 2020 (1) 129 pos sera from vaccinated or experimentally or naturally infected equidae, and 57 neg sera from naïve equidae	(1, 2) Manufacturer's validated method certificates (1) Dossier for Verification of blocking ELISA for detection of antibodies against African horse sickness virus carried out at LCV (EU-RL). Stage 3 of validation (PT) published in Durán-Ferrer *et al.* (2019)	- High sensitivity and specificity - Commercially available (both B-ELISA and I-ELISA) - Cost-effective - VP7 is highly conserved among serotypes -Long lasting of presence of antibodies in affected animals	- Unable to differentiate vaccinated from infected animals - Unable to differentiate serotype specificity of the antibodies - Seroconversion takes place after at least 8–12 d.p.i.	(1) B-ELISA INGEZIM AHSV Compac Plus 2.0 (Gold Standard Diagnostics): Durán-Ferrer *et al.* (2019) (2) I-ELISA IDScreen African horse sickness indirect (IDVET)
VN – Equids	Serum (neutralising serotype-specific antibodies against VP2)	DSe: > 95% (infected animals) DSp > 99%	(1) 18 sera from sheep experimentally inoculated with AHS1–AHS9 (2) Seven sera from two experimentally infected horses with AHS9	Dossier for Verification of the Orbivirus Seroneutralisation Test carried out at LCV (EU-RL)	- Allows serotyping from serum samples	- Unable to differentiate vaccinated from infected animals - Depends on the availability of live virus strains of all serotypes and susceptible cultured cells - Cross-reactions may appear between serotypes - Time consuming - Laborious - Biosafety laboratory consideration BLS3	

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
CFT + Equids	Serum	Not available	Not available	Not available	- Useful in recent infections or vaccination	- High number of sera presenting anticomplementary activity -Less sensitivity than ELISA -Less degree of standardisation than ELISA	

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Appendix 4: African horse sickness
Intended purpose of test: confirmation of clinical cases

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
Identification of the agent							
Real-time RT-PCR +++ Equids	EDTA blood/tissue (VP7)	(1) Dse 97% DSp 100% (2) Dse > 99.9% DSp > 97.8%	(1) 186 known negatives and 132 known positive samples (2) 503 equine blood samples collected from suspect cases; two separate healthy populations of horses (503 and 98 horses) were used as negative cases	(1) Validation report done at LCV (EURL), sent to WOAH and published in WOAH *Bulletin* n° 2016-1 (2) Validation report done at CVR (WOAH-RL), sent to WOAH and published in Guthrie *et al.* (2013)	- High sensitivity, specificity and reproducibility - High throughput - Rapid technique - Minimal risk of cross contamination - Applicable to uninfectious samples allowing viral genome to be detected even after the viraemic window as well as in poor condition or inactivated samples	- Expensive equipment - Expensive reagents - Surviving animals present shorter periods of viraemia in comparison with antibodies - Detection of viral genome in animals vaccinated with live attenuated AHS vaccines has been described up to 16 weeks after vaccination	(1) Agüero *et al.* (2008) (2) Guthrie *et al.* (2013) Quan *et al.*, (2010)
Agarose gel-based RT-PCR ++	EDTA blood/tissue (VP7)	Not available	Not available		- Less expensive instruments than those used in real-time RT-PCR - High sensitivity, specificity and reproducibility - Rapid technique - Applicable to uninfectious samples	- Longer analysis time than real-time RT-PCR - Not suitable for high throughput analysis - Risk of cross contamination -Surviving animals present shorter periods of viraemia in comparison with antibodies	Zientara *et al.* (1994)
Virus isolation +++	EDTA blood/tissue	DSe ≥ 50% DSp > 99%	- 91 BTV positive blood samples collected from naturally infected animals during BTV outbreaks in Spain in 2007–2018 (validation done jointly for all Orbiviruses) - 12 tissue samples from horses experimentally infected with AHSV-9	Dossier for Verification of the Orbivirus isolation carried out at LCV (EURL)	- Detection of infectious virus - Getting available isolates for further studies	- Time consuming - Laborious - Requirement for samples from viraemic period - Requirement for well conserved samples - Biosafety laboratory consideration BLS3 - Requirement for cell cultures - Low sensitivity	

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
Detection of immune response							
ELISA (serogroup specific based on VP7) + Equids	Serum (antibodies against VP7)	(1) DSe ≥ 95% DSp > 99% (2) DSe > 99% Dsp > 99%	(1, 2) Goat, sheep and guinea-pig sera from animals inoculated with AHS1-AHS9 (1) Nine sera from horse naturally infected and positive by VN (1) 152 sera from horses vaccinated and serotyped by VN (1, 2) Sera from horses from free areas (512, 1015) (1) 24 bovine pos to BTV, 20 horse sera pos to EA and nine deer sera pos to EHDV (1) Seven sera from the WOAH reference collection, from different equidae species and in different levels of positivity (1) Seven sera from two experimentally infected horses with AHS9 (1) 70 samples from the EU-RL proficiency tests organised from 2014 to 2020 (1) 129 pos sera from vaccinated or experimentally or naturally infected equidae, and 57 neg sera from naïve equidae	(1, 2) Manufacturer's validated method certificates (1) Dossier for Verification of blocking ELISA for detection of antibodies against African horse sickness virus carried out at LCV (EU-RL). Stage 3 of validation (PT) published in Durán-Ferrer *et al.* (2019)	- High sensitivity and specificity - Commercially available (both B-ELISA and I-ELISA) - Cost-effective - VP7 is highly conserved among serotypes	- Unable to differentiate vaccinated from infected animals - Unable to differentiate serotype specificity of the antibodies - Seroconversion takes place after at least 8–12 d.p.i.	(1) B-ELISA INGEZIM AHSV Compac Plus 2.0 (Gold Standard Diagnostics); Durán-Ferrer *et al.* (2019) (2) I-ELISA IDScreen African horse sickness indirect (IDVET)
VN + Equids	Serum (neutralising serotype-specific antibodies against VP2)	DSe: > 95% (infected animals) DSp > 99%	(1) 18 sera from sheep experimentally inoculated with AHS1–AHS9 (2) Seven sera from two experimentally infected horses with AHS9	Dossier for Verification of the Orbivirus Seroneutralisation Test carried out at LCV (EU-RL)	- Allows serotyping from serum samples	- Unable to differentiate vaccinated from infected animals - Depends on the availability of live virus strains of all serotypes and susceptible cultured cells - Cross-reactions may appear between serotypes - Time consuming - Laborious - Biosafety laboratory consideration BLS3	

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
CFT + Equids	Serum	Not available	Not available	Not available	- Useful in recent infections or vaccination	- High number of sera presenting anticomplementary activity -Less sensitivity than ELISA -Less degree of standardisation than ELISA	

Appendix 5: African horse sickness
Intended purpose of test: prevalence of infection – surveillance

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
Identification of the agent							
Real-time RT-PCR ++ Equids	EDTA blood/tissue (VP7)	(1) Dse 97% DSp 100% (2) Dse > 99.9% DSp > 97.8%	(1) 186 known negatives and 132 known positive samples (2) 503 equine blood samples collected from suspect cases; two separate healthy populations of horses (503 and 98 horses) were used as negative cases	(1) Validation report done at LCV (EURL), sent to WOAH and published in WOAH *Bulletin* n° 2016-1 (2) Validation report done at CVR (WOAH-RL), sent to WOAH and published in Guthrie *et al.* (2013)	- High sensitivity, specificity and reproducibility - High throughput - Rapid technique - Minimal risk of cross contamination - Applicable to uninfectious samples allowing viral genome to be detected even after the viraemic window as well as in poor condition or inactivated samples	- Expensive equipment - Expensive reagents - Surviving animals present shorter periods of viraemia in comparison with antibodies - Detection of viral genome in animals vaccinated with live attenuated AHS vaccines has been described up to 16 weeks after vaccination	(1) Agüero *et al.* (2008) (2) Guthrie *et al.* (2013) Quan *et al.*, (2010)
Agarose gel-based RT-PCR +	EDTA blood/tissue (VP7)	Not available	Not available		- Less expensive instruments than those used in real-time RT-PCR - High sensitivity, specificity and reproducibility - Rapid technique - Applicable to uninfectious samples	- Longer analysis time than real-time RT-PCR - Not suitable for high throughput analysis - Risk of cross contamination -Surviving animals present shorter periods of viraemia in comparison with antibodies	Zientara *et al.* (1994)
Virus isolation –	EDTA blood/tissue	DSe ≥ 50% DSp > 99%	- 91 BTV positive blood samples collected from naturally infected animals during BTV outbreaks in Spain in 2007–2018 (validation done jointly for all Orbiviruses) - 12 tissue samples from horses experimentally infected with AHSV-9	Dossier for Verification of the Orbivirus isolation carried out at LCV (EURL)	- Detection of infectious virus - Getting available isolates for further studies	- Time consuming - Laborious - Requirement for samples from viraemic period - Requirement for well conserved samples - Biosafety laboratory consideration BLS3 - Requirement for cell cultures - Low sensitivity	

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
Detection of immune response							
ELISA (serogroup specific based on VP7) +++ Equids	Serum (antibodies against VP7)	(1) DSe ≥ 95% DSp > 99% (2) DSe > 99% Dsp > 99%	(1, 2) Goat, sheep and guinea-pig sera from animals inoculated with AHS1-AHS9 (1) Nine sera from horse naturally infected and positive by VN (1) 152 sera from horses vaccinated and serotyped by VN (1, 2) Sera from horses from free areas (512, 1015) (1) 24 bovine pos to BTV, 20 horse sera pos to EA and nine deer sera pos to EHDV (1) Seven sera from the WOAH reference collection, from different equidae species and in different levels of positivity (1) Seven sera from two experimentally infected horses with AHS9 (1) 70 samples from the EU-RL proficiency tests organised from 2014 to 2020 (1) 129 pos sera from vaccinated or experimentally or naturally infected equidae, and 57 neg sera from naïve equidae	(1, 2) Manufacturer's validated method certificates (1) Dossier for Verification of blocking ELISA for detection of antibodies against African horse sickness virus carried out at LCV (EU-RL). Stage 3 of validation (PT) published in Durán-Ferrer *et al.* (2019)	- High sensitivity and specificity - Commercially available (both B-ELISA and I-ELISA) - Cost-effective - VP7 is highly conserved among serotypes	- Unable to differentiate vaccinated from infected animals - Unable to differentiate serotype specificity of the antibodies - Seroconversion takes place after at least 8–12 d.p.i.	(1) B-ELISA INGEZIM AHSV Compac Plus 2.0 (Gold Standard Diagnostics); Durán-Ferrer *et al.* (2019) (2) I-ELISA IDScreen African horse sickness indirect (IDVET)
VN + Equids	Serum (neutralising serotype-specific antibodies against VP2)	DSe: > 95% (infected animals) DSp > 99%	(1) 18 sera from sheep experimentally inoculated with AHS1–AHS9 (2) Seven sera from two experimentally infected horses with AHS9	Dossier for Verification of the Orbivirus Seroneutralisation Test carried out at LCV (EU-RL)	- Allows serotyping from serum samples	- Unable to differentiate vaccinated from infected animals - Depends on the availability of live virus strains of all serotypes and susceptible cultured cells - Cross-reactions may appear between serotypes - Time consuming - Laborious - Biosafety laboratory consideration BLS3	

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
CFT + Equids	Serum	Not available	Not available	Not available	- Useful in recent infections or vaccination	- High number of sera presenting anticomplementary activity -Less sensitivity than ELISA -Less degree of standardisation than ELISA	

853
854

Appendix 6: African horse sickness
Intended purpose of test: immune status in individual animals or populations post-vaccination

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
Identification of the agent							
Real-time RT-PCR = Equids	EDTA blood/tissue (VP7)	(1) Dse 97% DSp 100% (2) Dse > 99.9% DSp > 97.8%	(1) 186 known negatives and 132 known positive samples (2) 503 equine blood samples collected from suspect cases: two separate healthy populations of horses (503 and 98 horses) were used as negative cases	(1) Validation report done at LCV (EURL), sent to WOAH and published in WOAH *Bulletin* n° 2016-1 (2) Validation report done at CVR (WOAH-RL), sent to WOAH and published in Guthrie *et al.* (2013)	- None for this purpose	- Not suitable for this purpose	(1) Agüero *et al.* (2008) (2) Guthrie *et al.* (2013) Quan *et al.*, (2010)
Agarose gel-based RT-PCR –	EDTA blood/tissue (VP7)	Not available	Not available		- None for this purpose	- Not suitable for this purpose	Zientara *et al.* (1994)
Virus isolation –	EDTA blood/tissue	DSe ≥ 50% DSp > 99%	- 91 BTV positive blood samples collected from naturally infected animals during BTV outbreaks in Spain in 2007–2018 (validation done jointly for all Orbiviruses) - 12 tissue samples from horses experimentally infected with AHSV-9	Dossier for Verification of the Orbivirus isolation carried out at LCV (EURL)	- None for this purpose	- Not suitable for this purpose	
Detection of immune response							
ELISA (serogroup specific based on VP7) ++ Equids	Serum (antibodies against VP7)	(1) DSe ≥ 95% DSp > 99% (2) DSe > 99% Dsp > 99%	(1, 2) Goat, sheep and guinea-pig sera from animals inoculated with AHS1-AHS9 (1) Nine sera from horse naturally infected and positive by VN (1) 152 sera from horses vaccinated and serotyped by VN (1, 2) Sera from horses from free areas (512, 1015) (1) 24 bovine pos to BTV, 20 horse sera pos to EA and nine deer sera pos to EHDV (1) Seven sera from the WOAH	(1, 2) Manufacturer's validated method certificates (1) Dossier for Verification of blocking ELISA for detection of antibodies against African horse sickness virus carried out at LCV (EU-RL). Stage 3 of validation (PT) published in Durán-Ferrer *et al.* (2019)	- High sensitivity and specificity - Commercially available (both B-ELISA and I-ELISA) - Cost-effective - VP7 is highly conserved among serotypes -Long lasting of presence of antibodies in affected animals	- Unable to differentiate serotype specificity of the antibodies - Seroconversion takes place after at least 8–12 d.p.i.	(1) B-ELISA INGEZIM AHSV Compac Plus 2.0 (Gold Standard Diagnostics); Durán-Ferrer *et al.* (2019) (2) I-ELISA IDScreen African horse sickness indirect (IDVET)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
			reference collection, from different Equidae species and in different levels of positivity (1) Seven sera from two experimentally infected horses with AHS9 (1) 70 samples from the EU-RL proficiency tests organised from 2014 to 2020 (1) 129 pos sera from vaccinated or experimentally or naturally infected Equidae, and 57 neg sera from naïve, equidae				
VN +++ Equids	Serum (neutralising serotype-specific antibodies against VP2)	DSe: > 95% (infected animals) DSp > 99%	(1) 18 sera from sheep experimentally inoculated with AHS1–AHS9 (2) Seven sera from two experimentally infected horses with AHS9	Dossier for Verification of the Orbivirus Seroneutralisation Test carried out at LCV (EU-RL)	- Allows serotyping from serum samples	- Depends on the availability of live virus strains of all serotypes and susceptible cultured cells - Cross-reactions may appear between serotypes - Time consuming - Laborious - Biosafety laboratory consideration BLS3	
CFT + Equids	Serum	Not available	Not available	Not available	- Useful in recent infections or vaccination	- High number of sera presenting anticomplementary activity -Less sensitivity than ELISA -Less degree of standardisation than ELISA	

Annexe 27. Chapitre 3.6.5. Anémie infectieuse des équidés

RÉUNION DE LA COMMISSION DES NORMES BIOLOGIQUES
Paris, 9–13 septembre 2024

CHAPTER 3.6.5.

EQUINE INFECTIOUS ANAEMIA

SUMMARY

Equine infectious anaemia (*EIA*) *is a persistent viral infection of equids. The causative agent, EIA virus* (*EIAV*) *is a lentivirus in the family* Retroviridae*, subfamily* Orthoretrovirinae*. Other members of the genus* Lentivirus *include: bovine immunodeficiency virus; caprine arthritis encephalitis virus; feline immunodeficiency virus; human immunodeficiency virus 1; human immunodeficiency virus 2; simian immunodeficiency virus; ~~and~~ maedi/visna virus, Jembrana disease virus, and Puma lentivirus. Although EIA may be suspected ~~on~~ in the ~~basis~~ presence of clinical signs and pathological lesions, confirmation of infection ~~requires further serological and molecular-based testing~~ is usually by serology. Infected horses remain viraemic carriers for life and, with very rare exceptions, yield a positive serological test result. Although antibody levels fluctuate, EIAV infection generates a persistent antibody response. All equids older than 12 months that test seropositive are identified as virus carriers. In young equids less than 12 months of age, positive serological reactions can be due to maternal antibodies; therefore, the EIA status may have to rely solely on molecular techniques. As virus reservoirs, infected equids ~~are~~ represent a transmission risk to other equids. The virus is primarily blood-borne. Biting flies are mechanical vectors for the virus in nature and infection is often spread via iatrogenic routes.*

Identification of the agent: *EIAV cannot be differentiated clinically from a number of other aetiological agents of haemolytic anaemic fever syndromes and systemic equine diseases. Diagnosis of EIAV infection is laboratory dependent and based on the demonstration of a specific antibody response, virus isolation, or detection of viral nucleic acid.*

Virus can be isolated by inoculating suspect blood ~~into a susceptible horse or~~ onto leukocyte cultures prepared from susceptible horses. Recognition of infection in experimentally challenged horses may be made based on ~~the basis of~~ clinical signs, haematological changes, positive serological reactions and/or detection of the virus by molecular techniques. Successful virus isolation in ~~horse leukocyte cultures~~ equine monocyte-derived macrophages (eMDMs), derived from peripheral blood mononuclear cells (PBMCs), need to be confirmed by the detection of specific EIA antigen, by immunofluorescence assay, polymerase chain reaction-based techniques, or by the inoculation of culture fluids into susceptible horses. Virus isolation is rarely attempted due to the time, difficulty and expense involved.

Serological tests: *Agar gel immunodiffusion* (*AGID*) *tests and enzyme-linked immunosorbent assays* (*ELISAs*), *are simple, reliable serological tests for the demonstration of EIAV infection. The AGID tests should be used to confirm positive ELISA results. An immunoblot may be used in case of discrepancy in the results. Antibody levels are highly variable~~,~~ and fluctuate due to the changing nature of the virus and the immune status of the infected subject. EIA antigens can be prepared from infected ~~tissue~~ cell cultures or by using recombinant DNA technology. A variety of licensed and validated commercial test kits is available.*

Colloidal gold immunochromatographic strip (CGICG) test has demonstrated potential for the diagnostic of EIA.

Requirements for vaccines: *An attenuated live vaccine was developed in the early 1970s and used extensively in China (People's Rep. of) between 1975 and 1990. Numerous other methods of vaccine production have since been attempted with ~~variable results~~ limited success. The strategy for EIA control has shifted from vaccination to quarantine to avoid the interference of vaccinal antibodies with diagnostic tests. There are no vaccines currently available.*

A. INTRODUCTION

1. Description of disease and aetiology

Equine infectious anaemia (EIA) occurs worldwide and causes significant losses to the equine industry (Li *et al.*, 2023). EIA is a WOAH notifiable disease. The infection, formerly known as swamp fever, is limited to equids including horses, donkeys, mules and hinny (Wang *et al.*, 2023), though studies on the presence of the infection in zebras are scarce. Many cases remain clinically ~~unapparent~~ inapparent. The disease is characterised by recurrent febrile episodes, thrombocytopenia, anaemia, rapid loss of weight, cachexia, jaundice, and oedema of the lower parts of the body (Li *et al.*, 2023). If death does not result from one of the acute clinical attacks, a chronic stage develops, and the infection tends to become inapparent. The incubation period is normally 1–3 weeks, but may be as long as 3 months or more. Some horses remain subclinical until they experience some form of stress (Harrold *et al.*, 2020) or may never show outward clinical signs. In acute cases, lymph nodes, spleen and liver are hyperaemic and enlarged. Histologically, these organs are infiltrated with nests of immature lymphocytes and plasma cells. Kupffer cells in the liver often contain haemosiderin or erythrocytes. The enlarged spleen may be felt on rectal examination. Differential diagnoses include equine viral arteritis (Chapter 3.6.9), *Anaplasma phagocytophilum, Piroplasmosis, leptospirosis, severe strongyliosis* and other causes of oedema, fever, anaemia, or thrombocytopaenia/ ecchymoses.

EIA virus (EIAV) is in the genus *Lentivirus* in the family *Retroviridae*, subfamily *Orthoretrovirinae*. Other members of the genus include: bovine immunodeficiency virus; caprine arthritis encephalitis virus; feline immunodeficiency virus; human immunodeficiency virus 1; human immunodeficiency virus 2; simian immunodeficiency virus; ~~and~~ maedi/visna virus; Jembrana disease virus and Puma lentivirus.

Once a horse is infected with EIAV, its blood remains infectious for the remainder of its life and the horse can potentially transmit the infection to other horses ~~(Choevers & McGuire, 1985)~~. Transmission occurs by transfer of blood or contaminated secretions from an infected horse. In nature, spread of the virus is most likely via interrupted feeding of bloodsucking horseflies (*Tabanidae*) and stable flies (Dantas Malossi *et al.*, 2020) on an ~~clinically ill~~ infected horse and then on susceptible horses. Transmission by haematophagous flies is mechanical as it does not involve EIAV replication in their tissues (Fideles Resende *et al.*, 2022). Transmission can also occur by the iatrogenic transfer of blood ~~through the use of~~ using contaminated blood products, needles, syringes, IV administration sets or other equipment. Infection can also be transmitted through the congenital route or body secretions, e.g. semen, sweat, urine (Romo-Sáenz *et al.*, 2021). No effective treatment or vaccine is currently available (Dantas Malossi *et al.*, 2020. ~~*In utero* infection of the fetus may occur (Kemen & Coggins, 1972).~~ The ~~virus titre~~ viral load is higher in the plasma of horses with clinical signs and the risk of transmission is higher from these animals than the carrier animals with a lower v~~virus titre~~ viral load. ~~However~~, Studies in mules ~~indicate infected animals~~ have demonstrated a lack of correlation between viral load and serological reactivity. Naturally infected asymptomatic mules with positive enzyme-linked immunosorbent assay (ELISA) ~~and indeterminate~~ but negative or equivocal agar gel immunodiffusion (AGID) test results ~~can~~ may have high ~~viral~~ virus nucleic acid loads ~~at the same level as animals with strong antibody responses, and are therefore equally likely as a potential source of transmission (Scicluna *et al.*, 2013)~~ in the plasma and could potentially contribute to the maintenance and spread of infection.

EIAV is not considered a risk for human health. Laboratory manipulations should be carried out at an appropriate biosafety and containment level determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

B. DIAGNOSTIC TECHNIQUES

AGID tests (Coggins *et al.*, 1972) and ELISAs ~~(Suzuki *et al.*, 1982)~~ are accurate, reliable tests for the detection of EIA in horses, except for animals in the early stages of infection and foals of infected dams (~~McConnico *et al.*, 2000;~~ United States Department of Agriculture [USDA], 2007). In other rare circumstances, misleading results may occur when the level of virus circulating in the blood during an acute episode of the disease is sufficient to bind available antibody, and if initial antibody levels never rise high enough to be detectable ~~(Toma, 1980)~~. Although the ELISA will detect antibodies somewhat earlier and at lower concentrations than the AGID test, positive ELISAs are confirmed using the AGID test. This is due to false-positive results that have been noted with indirect ELISAs. The AGID test is specific, thus has the advantage of distinguishing

between EIA and non-EIA antigen–antibody reactions. Nonspecific reactions in AGID may occur with antigen derived from the spleen of infected animals or equine dermal cell cultures that might contain other cellular or host-derived proteins with consequent nonspecific precipitation lines with antibodies present in the tested serum against non-EIAV antigens. Recombinant technology for the production of EIAV antigens can obviate AGID nonspecific reactions (Alvarez *et al.*, 2007). Competitive ELISA developed using specific monoclonal antibodies against a common epitope on viral P26 protein showed high specificity and sensitivity (Hu *et al.*, 2023). A colloidal gold immunochromatographic (CGICG) fast test strip was developed with good specificity, sensitivity, stability, and repeatability, which provides a tool for point-of-care testing for the primary screening of EIAV antibodies (Zhang *et al.*, 2024). Discrepancies between testing methods or tests with questionable results can be further evaluated by immunoblot testing (Issel *et al.,* ~~1999;~~ 2013; Rusvai *et al.*, 2009).

Table 1. Test methods available for the diagnosis of equine infectious anaemia and their purpose

Method	**Purpose**					
	Population freedom from infection[(a)]	Individual animal freedom from infection prior to movement[(b)]	Contribute to eradication policies[(c)]	Confirmation of clinical cases[(d)]	Prevalence of infection – surveillance[(e)]	Immune status in individual animals or populations (post-vaccination)
Identification of the agent[(f)]						
PCR	–	+/–	–	+/–	–	–
Virus isolation	–	–	–	+	–	–
Detection of immune response						
AGID	++	++	++	++	++	–
ELISA	+++	+++	+++	+++	+++	–
~~Immunoblot~~	–	~~++~~	~~++~~	~~++~~	–	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; AGID = agar gel immunodiffusion; ELISA = enzyme-linked immunosorbent assay.
[(a)] See Appendix 1 of this chapter for justification table for the scores given to the tests for this purpose.
[(b)] See Appendix 2 of this chapter for justification table for the scores given to the tests for this purpose.
[(c)] See Appendix 3 of this chapter for justification table for the scores given to the tests for this purpose.
[(d)] See Appendix 4 of this chapter for justification table for the scores given to the tests for this purpose.
[(e)] See Appendix 5 of this chapter for justification table for the scores given to the tests for this purpose.
[(f)] A combination of agent identification methods applied on the same clinical sample is recommended.

1. Identification of the agent

1.1. Virus isolation and identification

Virus isolation is usually not necessary to make a diagnosis.

Isolation of the virus from suspect horses may be made by inoculating their blood (plasma, serum, leukocytes) or spleen homogenates onto ~~leukocyte~~ equine monocyte-derived macrophages (eMDMs) derived from peripheral blood mononuclear cells (PBMCs) cultures prepared from horses free of infection (Fidalgo-Carvalho *et al.*, 2009). Virus production in cultures can be confirmed by detection of specific EIA antigen by ELISA ~~(Shane *et al.*, 1984)~~, by immunofluorescence assay ~~(Weiland *et al.*, 1982)~~, or by molecular tests (Cook *et al.*, 2002; Dong *et al.*, 2012). Virus isolation is rarely attempted because of the difficulty of growing horse leukocyte cultures and poor adaptability of some strains to cell culture (Ma *et al.*, 2014).

1.2. Polymerase chain reaction

A nested polymerase chain reaction (PCR) assay to detect EIA proviral DNA from the peripheral blood of horses has been described (Nagarajan & Simard, ~~2001~~ 2007). The nested PCR method is based on primer sequences

from the gag region of the proviral genome. It has proven to be a sensitive technique to detect field strains of EIAV in white blood cells of EIA infected horses; the lower limit of detection is typically around 10 genomic copies of the target DNA (Nagarajan & Simard, ~~2001;~~ 2007). A real-time reverse-transcriptase PCR assay has also been described (Cook *et al.,* 2002). Other real-time PCR methods are based on primer sequences from the exon 1 tat genomic portion (118 bp) that has a limit of detection of the EIAV tat RNA transcript of 1 genomic copy (Scicluna *et al.*, 2013). Another real-time PCR amplifies a fragment between the tat and the gag genes (Li *et al.*, 2023). To increase the diagnostic sensitivity, PCR methods for detecting viral RNA and proviral DNA should be used. To confirm the results of these very sensitive assays, it is recommended that duplicate samples of each diagnostic specimen be processed. Because of the risk of cross contamination, it is also important that proper procedures are followed (see Chapter 1.1.5 *Quality management in veterinary testing laboratories* and Chapter 1.1.6 V*alidation of diagnostic assays for infectious diseases of terrestrial animals*). It should be noted that primer mismatches with circulating virus, possibly caused by the high rate of mutation in the virus, may cause a failure of PCR to detect virus (~~Cappelli *et al.*, 2011;~~ Li *et al.*, 2023; ~~Quinlivan *et al.*, 2007;~~ Scicluna *et al.*, 2013).

The following are some of the circumstances where the PCR assay maybe used for the detection of EIAV infection in horses:

i) Conflicting results on serologic tests;

ii) Suspected infection but negative or questionable serologic results;

iii) Complementary test to serology for the confirmation of positive results;

iv) Confirmation of early infection, ~~before~~ when the serum antibodies to EIAV ~~develop~~ are not detectable;

v) ~~Ensuring~~ In addition to serology, to ensure that horses that are ~~to be~~ used for antiserum or vaccine production or as blood donors are free of EIAV;

vi) Confirmation of the status of a foal from an infected mare.

2. Serological tests

Due to the persistence of EIAV in infected equids, detection of serum antibody to EIAV confirms the diagnosis of EIAV infection. An efficient diagnostic algorithm in terms of sensitivity and specificity can be adopted, such as the one based on a three-tiered diagnostic system (Issel *et al.*, 2013; Scicluna *et al.*, 2013). This procedure involves initial screening by ELISA (Tier 1) with test-positive samples confirmed by the AGID (Tier 2) and, in the case of ELISA positive/AGID negative results, final verification by Immunoblotting (IB) (Tier 3).

2.1. Agar gel immunodiffusion test

The AGID test detects precipitating antibody produced in response to EIAV infection. Specific reactions are indicated by precipitation lines between the EIA antigen and the test serum and confirmed by their identity with the reaction between the antigen and the positive standard serum.

Reagents for AGID are available commercially from several companies. Alternatively, AGID antigen and reference serum may be prepared as described below.

2.1.1. Preparation of antigen

Specific EIA antigen may be prepared from the spleen of acutely infected horses (Coggins *et al*., 1973), from infected equine tissue culture ~~(Malmquist *et al*., 1973)~~, from a persistently infected canine thymus cell line ~~(Bouillant *et al*., 1986)~~, or from proteins expressed in bacteria or baculovirus using the recombinant DNA technique ~~(Archambault *et al*., 1989; Kong *et al*., 1997)~~. Preparation from infected cultures or from recombinant DNA techniques gives a more uniform result than the use of spleen cells and allows for better standardisation of reagents.

~~To obtain a satisfactory antigen from spleen, a horse must be infected with a highly virulent strain of EIAV. The resulting incubation period should be 5–7 days, and the spleen should be collected 9 days after inoculation, when the virus titre is at its peak and before any detectable amount of precipitating antibody is produced. Undiluted spleen pulp is used in the immunodiffusion test as antigen (Coggins *et al*., 1973). Extraction of antigen from the spleen with a saline solution and concentration with ammonium sulphate does not give as satisfactory an antigen as selection of a spleen with a very high titre of EIA antigen.~~

~~Alternatively,~~ Equine fetal kidney or dermal cells or canine thymus cells are infected with a strain of EIAV adapted to grow in tissue culture (American Type Culture Collection, or Chinese strain adapted to equine fetal dermal cells). Virus is collected from cultures by precipitation with 8% polyethylene glycol or by

pelleting by ultracentrifugation. The diagnostic antigen, p26, is released from the virus by treatment with detergent or ether ~~(Malmquist *et al.*, 1973)~~. EIAV core proteins, expressed in bacteria, i.e. *E. coli* or baculovirus (Alvarez *et al.*, 2007; Scicluna *et al.*, 2019), are commercially available and find practical use as high quality antigens for serological diagnosis (Bannai *et al.* 2023).

The p26 is an internal structural protein of the virus that is coded for by the *gag* gene. The p26 is more antigenically stable among EIAV strains than the virion glycoproteins gp45 and gp90 ~~(Montelaro *et al.*, 1984)~~. There is evidence of strain variation in the p26 amino acid sequence; however, there is no evidence to indicate that this variation influences any of the serological diagnostic tests ~~(Zhang *et al.*, 1999)~~.

2.1.2. Preparation of standard antiserum

A known positive ~~anti~~serum may be collected from a horse previously experimentally or naturally infected with EIAV. This serum should yield a single dense precipitation line that is specific for EIA, as demonstrated by a reaction of identity in comparison with a known standard positive reference serum. It is essential to balance the antigen and antibody concentrations ~~in order~~ to ensure the optimal sensitivity of the test. Reagent concentrations should be adjusted ~~to form~~ by cross-testing serial dilutions of antigen and serum to obtain a narrow precipitation line approximately equidistant between the two wells containing ~~antigen and serum~~ the antigen and the standard positive reference serum (Chapter 2.2.6 *Selection and use of reference samples and panels*). A list of WOAH-approved International Standard Reagents is also available[1]).

2.1.3. Test procedure (Association Française de Normalisation [AFNOR], 2000; Coggins *et al.*, 1973~~; Pearson & Coggins, 1979~~)

i) Immunodiffusion reactions are carried out in a layer of agar in plastic Petri dishes as glass dishes can result in slippage. For Petri dishes that are 100 mm in diameter, 15–17 ml of 1% Noble agar in 0.145 M borate buffer (9 g H_3BO_3, plus 2 g NaOH per litre), pH 8.6 (± 0.2) is used. A metal punch is used to create several "rosettes," each of six wells surrounding a centre well of the same diameter. The wells are 5.3 mm in diameter and 2.4 mm apart. Each well must contain the same volume of reagent and should be completely but not over-filled.

ii) The antigen is placed in the central well and the standard antiserum is placed in alternate exterior wells. Serum samples for testing are placed in the remaining three wells.

iii) The dishes are maintained at room temperature in a humid environment (18°C–26°C recommended).

iv) After 24 ~~–~~ and 48 hours the precipitation reactions are examined over a narrow beam of intense, oblique light and against a black background, with also the help of a magnification. The reference lines should be clearly visible at 24 hours, and at that time, any test sera that are strongly positive may also have formed lines of identity with those between the standard reagents. A weakly positive reaction may take 48 hours to form and is indicated by a slight bending of the standard serum precipitation line between the antigen well and the test serum well. For EIA AGID, the bending caused by a weak positive reaction looks like a very small hook or rounding into the sample well. Sera with high precipitating antibody titres will form a complete line of identity or may form broader precipitin bands that break into the sample well or tend to be diffuse (see Figure 1). Such reactions can be confirmed as specific for EIA by dilution at 1/2 ~~or ¼~~ and subsequent serial dilutions prior to retesting; these then give a more distinct line of identity. Sera devoid of EIA antibody will not form precipitation lines and will have no effect on the reaction lines of the standard reagents. Nonspecific precipitation lines may occur. These nonspecific lines can cross the control lines, typically showing no line of identity with the control lines.

v) Interpretation of the results: Horses that are in the early stages of an infection may not give a positive serological reaction in an AGID test. Such animals should be bled again after 3–4 weeks, or, in case of clinical signs at least a week after their appearance. To make a diagnosis in a young foal, it may be necessary to determine the antibody status of the dam. If the mare passes EIA antibody to the foal through colostrum, then a period of 6 months or longer after birth must be allowed for the maternally-derived antibody to wane. Sequential testing of the foal at monthly intervals may be useful to observe the decline in maternal antibody. To conclude that the foal is not infected, a negative result must be obtained (following an initial positive result) at least ~~2~~ 3 months after separating the foal from contact with the EIA positive mare or any other positive horse. It should be noted that maternal antibodies can often be detected for up to 12 months of age, therefore alternative diagnostic methods should be considered, for example PCR could be used to determine the presence/absence of EIA virus in the blood of the foal.

[1] https://www.woah.org/en/what-we-offer/veterinary-products/reference-reagents/

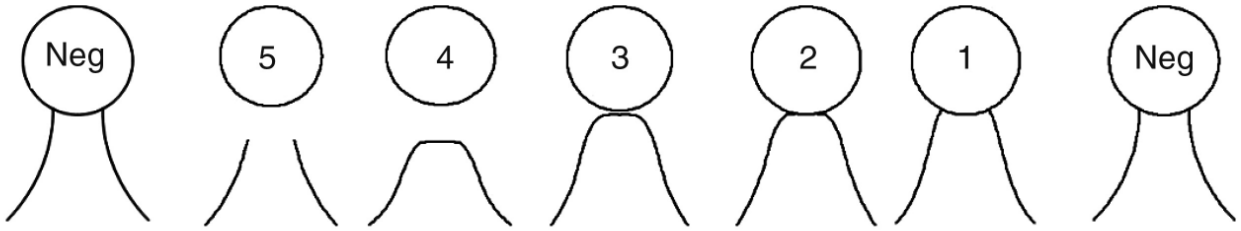

Fig. 1. Reactions in AGID test for EIAV. From: Issel et al. (2013).

2.2. Enzyme-linked immunosorbent assay

Several diagnostic test kits for EIA, including AGID and ELISA, are licensed in various countries for the diagnosis of equine infectious anaemia and are available internationally (Hu *et al.*, 2023; Nardini *et al.*, 2017). The ELISAs generally target antibody produced against the p26 core protein antigen but may also have a second target antibody produced against the gp45 antigen. These antigens may also be synthetic fusion proteins or recombinant antigens. Typical ELISA protocols are used. Both indirect and competitive ELISAs are used for disease surveillance. If commercial ELISA materials are not available, a non-competitive ELISA using p26 antigen purified from cell culture material may be employed ~~(Shane *et al.*, 1984)~~.

A positive test result by ELISA should be confirmed using the AGID test because false-positive results have been noted with the ELISA. The results can also be confirmed by the immunoblotting technique. A standard antiserum for immunodiffusion, which contains detectable antibody, is available from the WOAH Reference Laboratories[2]. This standard should not be used as the reference for minimum detection limits for the ELISA reaction. Uniform methods for EIA control have been published (USDA, 2007).

2.3. Immunoblotting test

Immunoblotting (IB) is characterised by both high sensitivity and specificity and is the alternative confirmatory assay for ELISA/AGID discordant results. In the IB, the immunological reactivity to EIAV antigens, as in the case of using the whole virus, p26, gp45 and gp90 adsorbed on the IB membrane, can be used to define the serological status of the animal for EIA: a p26 positive band together with at least one of the other antigen defines a subject as serologically positive for EIAV (Issel *et al.*, 2013; Scicluna *et al.* 2019). This test is not commercially available and has not been the subjected to an international proficiency test; this test is only available at WOAH Reference Laboratory in Italy.

2.4. Colloidal gold immunochromatographic test

Colloidal gold immunochromatographic test (CGICG) is a method that has been widely used for disease screening and surveillance, especially in human diseases such as COVID-19. In the CGICG methods, the P26 and gp45 are major antigens used to detect EIAV antibodies. The CGICG test for EIAV antibody detection showed high sensitivity and specificity, and can be concluded within 15 minutes in on-site detection (Alvarez *et al.*, 2010; Zhang *et al.*, 2024), thereby providing an alternative method for disease surveillance.

C. REQUIREMENTS FOR VACCINES

Inactivated and subunit EIAV vaccines were tested in different laboratories and proved to protect infections of homologous prototype strains only. An attenuated live vaccine, developed in the early 1970s, was extensively used in China (People's Rep. of) between 1975 and 1990 and was effective in controlling the prevalence of EIA. With low prevalence since 1990, the strategy for EIA control has shifted from vaccination to quarantine to avoid the interference of vaccine antibodies with diagnostic tests that are not yet capable to differentiate vaccinated and infected subjects.

[2] https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3

Although no safety concerns arose with the use of attenuated EIAV vaccine in China, it should be noted that, like other lentiviruses, EIAV is highly mutable and can integrate into host genomes (Lin *et al.*, 2020; Liu *et al.*,2017). The use of a live EIAV vaccine should be considered only after a thorough risk assessment.

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NB: There are WOAH Reference Laboratories for equine infectious anaemia:
(please consult the WOAH Web site:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3)
Please contact the WOAH Reference Laboratories for any further information on
diagnostic tests and reagents for equine infectious anaemia

NB: FIRST ADOPTED IN 1989. MOST RECENT UPDATES ADOPTED IN 2019.

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Appendix 1: Equine infectious anaemia
Intended purpose of test: population freedom from infection

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
PCR =	Blood Plasma Organs (spleen, liver) Exon 1 tat Blood samples LTR-tat	For Exon 1 tat: Analytical sensitivity (LOD) = 1 copy target/µl Analytical specificity = 44 exon 1 tata amplicons sequenced For LTR-tat: Analytical sensitivity (LOD) = 10 copy target/µl Analytical specificity = 40 LTR-tat amplicons sequenced	For Exon 1 tat: 5 Mules plasma (all positive) 37 positive equine bloods 16 positive equine plasma For LTR-tat: 53 blood samples (40 positive sequenced and 13 negative) See reference	See reference	For Exon 1 tat and LTR tat 1. High analytical sensitivity 2. High analytical specificity	For Exon 1 tat and LTR tat 1. Relatively low turnover 2. High costs 3. Requires specific laboratory equipment 4. Requires skilled laboratory personnel 5. Potential low diagnostic specificity due to the high genetic variability of the virus	Scicluna *et al.* (2013)
AGID ++	Serum recombinant p26	Reference test: IB Specificity 100% Sensitivity 59.4%	548 samples positive in a screening ELISA subsequently analysed by AGID and IB at the WOAH RL, Italy	To be published	1. High specificity 2. High sensitivity in case of populations not previously tested 3. Low cost 4. Minimal laboratory equipment required	1. Low sensitivity in case of early infection or low antibody levels 2. Low turnover 3. Results are not readily available 4. In case of low levels of reactivity, interpretation of results depends on technical expertise	
ELISA +++	Serum, recombinant p26, chimeric recombinant p26/gp45	Reference test were: AGID, IB **Recombinant p26:** Dsp: 73.2% (CI: 67.6–78.1%) Dse: 100% (CI: 91.6–100%) 50% inhibition as determined by ROC analysis. **Recombinant p26/gp45:** Dsp: 99.3% (CI: 96.8-99.8%) Dse: 100% (CI: 100-100%) 50% inhibition as determined by ROC analysis. **bELISA:** Reference tests were AGID Dsp = 100% Dse = 97.0% Accuracy 98.3%	**Recombinant p26:** 324 Field sera analysed by immunoblot (52 positive, 272 negative). 96 Sera of horses experimentally vaccinated 1,102 field sera analysed by AGID test (857 positive and 245 negative) Intralaboratory test with nine laboratories **Recombinant p26/gp45:** 96 Sera of horses experimentally vaccinated 615 field sera analysed by AGID test **bELISA:** 1129 negative samples were taken from different farms in China which were free from	See reference	1. High sensitivity 2. Serological ELISAs are commercially available in many countries, together with in house ELISAs (see references) 3. A comparative analysis of some commercial and in house ELISAs reported that their diagnostic performances are comparable (see reference) 4. Rapid to carry out with an immediate result output 5. Low cost	1. Specific laboratory equipment required 2. Considering the diagnostic specificity, positive samples should be confirmed using AGID and, in case of discordant results, in IB 3. Generally, low sensitivity during early stages of infection 4. The bELISA will of course only test positive from the onset of antibodies in the sera, and will miss sera without antibodies, which can occur when sera are collected from early-stage infections in horses 5. False-positive results have been noted with some ELISAs	Nardini *et al.* (2016) Nardini *et al.* (2017) Scicluna *et al.* (2018) Naves *et al.* (2019) Dominguez *et al.* (2021) Hu *et al.* (2023) Russi *et al.* (2023)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
			EIA 18 positive sera, 45 negative sera, and 353 test serum samples from Argentina 3 standard positive sera 15,900 serum samples of either horses or donkeys collected from different provinces of China		6. Target antigen used is usually highly conserved 7. High turnover 8. No cross reactivity with other known viruses 9. Detection sensitivity and specificity of the bELISA were higher than those of either AGID or cELISA 10. bELISA detects the disease much earlier than does AGID 11. bELISA has not produced a single false-positive result		

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Appendix 2: Equine infectious anaemia
Intended purpose of test: individual animal freedom from infection prior to movement

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
PCR +/–	Blood Plasma Organs (spleen, liver) Exon 1 tat Blood samples LTR-tat	For Exon 1 tat: Analytical sensitivity (LOD) = 1 copy target/µl Analytical specificity = 44 exon 1 tata amplicons sequenced For LTR-tat: Analytical sensitivity (LOD) = 10 copy target/µl Analytical specificity = 40 LTR-tat amplicons sequenced	For Exon 1 tat: 5 Mules plasma (all positive) 37 positive equine bloods 16 positive equine plasma For LTR-tat: 53 blood samples (40 positive sequenced and 13 negative) See reference	See reference	For Exon 1 tat and LTR tat 1. High analytical sensitivity 2. High analytical specificity	For Exon 1 tat and LTR tat 1. Requires specific laboratory equipment 2. Skilled personnel required 3. Potential low diagnostic specificity due to the high genetic variability of the virus	Scicluna *et al.* (2013)
AGID ++	Serum recombinant p26	Reference test: IB Specificity 100% Sensitivity 59.4%	548 samples positive in a screening ELISA subsequently analysed by AGID and IB at the WOAH RL, Italy	To be published	1. High specificity 2. Low cost 3. Minimal laboratory equipment required	1. Low sensitivity in case of early infection or low antibodies levels 2. Results are not readily available 3. In case of low levels of reactivity, interpretation of results depends on technical expertise	
ELISA +++	Serum recombinant p26 chimeric recombinant p26/gp45	Reference test were: AGID, IB **Recombinant p26:** Dsp: 73.2% (CI: 67.6–78.1%) Dse: 100% (CI: 91.6–100%) 50% inhibition as determined by ROC analysis. **Recombinant p26/gp45:** Dsp: 99.3% (CI: 96.8-99.8%) Dse: 100% (CI: 100-100%) 50% inhibition as determined by ROC analysis. **bELISA:** Reference tests were AGID Dsp = 100% Dse = 97.0% Accuracy 98.3%	**Recombinant p26:** 1. 324 Field sera analysed by immunoblot (52 positive, 272 negative). 2. 96 Sera of horses experimentally vaccinated 3. 1,102 field sera analysed by AGID test (857 positive and 245 negative) 4. Intralaboratory test with nine laboratories **Recombinant p26/gp45:** 1. 96 Sera of horses experimentally vaccinated 3. 615 field sera analysed by AGID test **bELISA:** 1129 negative samples were taken from different farms in	See reference	1. High sensitivity 2. Serological ELISAs are commercially available in many countries, together with in house ELISAs (see references) 3. A comparative analysis of some commercial and in house ELISAs reported that their diagnostic performances are comparable (see reference) 4. Rapid to carry out with an immediate result output	1. Specific laboratory equipment required 2. Considering the diagnostic specificity, positive samples should be confirmed using AGID and, in case of discordant results, in IB 3. Generally, low sensitivity during early stages of infection 4. The bELISA will of course only test positive from the onset of antibodies in the sera, and will miss sera without antibodies, which can occur when sera are collected from early-stage infections in horses 5. False-positive results have been noted with some ELISAs	Nardini *et al.* (2016) Nardini *et al.* (2017) Scicluna *et al.* (2018) Naves *et al.* (2019) Dominguez *et al.* (2021) Hu *et al.* (2023) Russi *et al.* (2023)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
			China which were free from EIA 18 positive sera, 45 negative sera, and 353 test serum samples from Argentina 3 standard positive sera 15,900 serum samples of either horses or donkeys collected from different provinces of China (People's Rep. of)		5. Low cost 6. Target antigen used is usually highly conserved 7. High turnover 8. No cross reactivity with other known viruses 9. Detection sensitivity and specificity of the bELISA were higher than those of either AGID or cELISA 10. bELISA detects the disease much earlier than does AGID 11. bELISA has not produced a single false-positive res		

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Appendix 3: Equine infectious anaemia
Intended purpose of test: contribute to eradication policies

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
PCR =	Blood Plasma Organs (spleen, liver) Exon 1 tat Blood samples LTR-tat	For Exon 1 tat: Analytical sensitivity (LOD) = 1 copy target/µl Analytical specificity = 44 exon 1 tata amplicons sequenced For LTR-tat: Analytical sensitivity (LOD) = 10 copy target/µl Analytical specificity = 40 LTR-tat amplicons sequenced	For Exon 1 tat: 5 Mules plasma (all positive) 37 positive equine bloods 16 positive equine plasma For LTR-tat: 53 blood samples (40 positive sequenced and 13 negative) See reference	See reference	For Exon 1 tat and LTR tat 1. High analytical sensitivity 2. High analytical specificity 3. Historical experimental infection studies in susceptible equids indicated that positive results correlated with infectivity	For Exon 1 tat and LTR tat 1. Relatively low turnover 2. High costs 3. Requires specific laboratory equipment 4. Requires skilled laboratory personnel 5. Potential low diagnostic specificity due to the high genetic variability of the virus	Scicluna *et al.* (2013)
AGID ++	Serum recombinant p26	Reference test: IB Specificity 100% Sensitivity 59.4%	548 samples positive in a screening ELISA subsequently analysed by AGID and IB at the WOAH RL, Italy	To be published	1. High specificity 2. High sensitivity in the initial stages of the intended purpose 3. Low cost 4. Minimal laboratory equipment required	1. Low sensitivity in case of early infection or low antibody levels 2. Low turnover 3. Results not readily available 4. In case of low levels of reactivity, interpretation of results depends on technical expertise	
ELISA +++	Serum recombinant p26 chimeric recombinant p26/gp45	Reference test were: AGID, IB **Recombinant p26:** Dsp: 73.2% (CI: 67.6–78.1%) Dse: 100% (CI: 91.6–100%) 50% inhibition as determined by ROC analysis. **Recombinant p26/gp45:** Dsp: 99.3% (CI: 96.8-99.8%) Dse: 100% (CI: 100-100%) 50% inhibition as determined by ROC analysis. **bELISA:** Reference tests were AGID Dsp = 100% Dse = 97.0% Accuracy 98.3%	**Recombinant p26:** 1. 324 Field sera analysed by immunoblot (52 positive, 272 negative). 2. 96 Sera of horses experimentally vaccinated 3. 1,102 field sera analysed by AGID test (857 positive and 245 negative) 4. Intralaboratory test with 9 laboratories **Recombinant p26/gp45:** 1. 96 Sera of horses experimentally vaccinated 3. 615 field sera analysed by AGID test **bELISA:**	See reference	1. High sensitivity 2. Serological ELISAs are commercially available in many countries, together with in house ELISAs (see references) 3. A comparative analysis of some commercial and in house ELISAs reported that their diagnostic performances are comparable (see reference) 4. Rapid to carry out with an immediate result output 5. Low cost 6. Target antigen used is usually highly conserved	1. Specific laboratory equipment required 2. Considering the diagnostic specificity, positive samples should be confirmed using AGID and, in case of discordant results, in IB 3. Generally, low sensitivity during early stages of infection 4. The bELISA will of course only test positive from the onset of antibodies in the sera, and will miss sera without antibodies, which can occur when sera are	Nardini *et al.* (2016) Nardini *et al.* (2017) Scicluna *et al.* (2018) Naves *et al.* (2019) Dominguez *et al.* (2021) Hu *et al.* (2023) Russi *et al.* (2023)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
			1129 negative samples were taken from different farms in China which were free from EIA 18 positive sera, 45 negative sera, and 353 test serum samples from Argentina 3 standard positive sera 15,900 serum samples of either horses or donkeys collected from different provinces of China (People's Rep. of)		7. High turnover 8. No cross reactivity with other known viruses 9. Detection sensitivity and specificity of the bELISA were higher than those of either AGID or cELISA 10. bELISA detects the disease much earlier than does AGID 11. bELISA has not produced a single false-positive res	collected from early-stage infections in horses 5. False-positive results have been noted with some ELISAs	

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Appendix 4: Equine infectious anaemia
Intended purpose of test: confirmation of clinical cases

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
PCR ±	Blood Plasma Organs (spleen, liver) Exon 1 tat Blood samples LTR-tat	For Exon 1 tat: Analytical sensitivity (LOD) = 1 copy target/µl Analytical specificity = 44 exon 1 tata amplicons sequenced For LTR-tat: Analytical sensitivity (LOD) = 10 copy target/µl Analytical specificity = 40 LTR-tat amplicons sequenced	For Exon 1 tat: 5 Mules plasma (all positive) 37 positive equine bloods 16 positive equine plasma For LTR-tat: 53 blood samples (40 positive sequenced and 13 negative) See reference	See reference	For Exon 1 tat and LTR tat 1. High analytical sensitivity 2. High analytical specificity	For Exon 1 tat and LTR tat 1. Relatively low turnover 2. High costs 3. Requires specific laboratory equipment 4. Requires skilled laboratory personnel 5. Potential low diagnostic specificity due to the high genetic variability of the virus	Scicluna *et al.* (2013
Virus isolation ±	Plasma, serum, leukocytes or spleen homogenates. EIA virus	Data not available	Data not available	Data not available	Useful for the study of field strains	1. Low sensitivity 2. Skilled personnel required 3. Time consuming, not suitable for high turnover	Fidalgo-Carvalho *et al.* (2009) Ma *et al.* (2014)
AGID ++	Serum recombinant p26	Reference test: IB Specificity 100% Sensitivity 59.4%	548 samples positive in a screening ELISA subsequently analysed by AGID and IB at the WOAH RL, Italy	To be published	1. High specificity 2. High sensitivity in the initial stages of the intended purpose 3. Low cost 4. Minimal laboratory equipment required	1. Low sensitivity in case of early infection or low antibody levels 2. Low turnover 3. Results not readily available 4. In case of low levels of reactivity, interpretation of results depends on technical expertise	
ELISA +++	Serum recombinant p26 chimeric recombinant p26/gp45	Reference test were: AGID, IB **Recombinant p26:** Dsp: 73.2% (CI: 67.6–78.1%) Dse: 100% (CI: 91.6–100%) 50% inhibition as determined by ROC analysis. **Recombinant p26/gp45:** Dsp: 99.3% (CI: 96.8-99.8%)	**Recombinant p26:** 1. 324 Field sera analysed by immunoblot (52 positive, 272 negative). 2. 96 Sera of horses experimentally vaccinated 3. 1,102 field sera analysed by AGID test (857 positive and 245 negative) 4. Intralaboratory test with 9 laboratories	See reference	1. High sensitivity 2. Serological ELISAs are commercially available in many countries, together with in house ELISAs (see references) 3. A comparative analysis of some commercial and in house ELISAs reported	1. Specific laboratory equipment required 2. Considering the diagnostic specificity, positive samples should be confirmed using AGID and, in case of discordant results, in IB 3. Generally, low sensitivity during early stages of infection 4. The bELISA will of course only test positive from the	Nardini *et al.* (2016) Nardini *et al.* (2017) Scicluna *et al.* (2018) Naves *et al.* (2019) Dominguez *et al.* (2021) Hu *et al.*

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
		Dse: 100% (CI: 100-100%) 50% inhibition as determined by ROC analysis. **bELISA:** Reference tests were AGID Dsp = 100% Dse = 97.0% Accuracy 98.3%	**Recombinant p26/gp45:** 1. 96 Sera of horses experimentally vaccinated 3. 615 field sera analysed by AGID test **bELISA:** 1129 negative samples were taken from different farms in China which were free from EIA 18 positive sera, 45 negative sera, and 353 test serum samples from Argentina 3 standard positive sera 15,900 serum samples of either horses or donkeys collected from different provinces of China (People's Rep. of)		that their diagnostic performances are comparable (see reference) 4. Rapid to carry out with an immediate result output 5. Low cost 6. Target antigen used is usually highly conserved 7. High turnover 8. No cross reactivity with other known viruses 9. Detection sensitivity and specificity of the bELISA were higher than those of either AGID or cELISA 10. bELISA detects the disease much earlier than does AGID 11. bELISA has not produced a single false-positive res	onset of antibodies in the sera, and will miss sera without antibodies, which can occur when sera are collected from early-stage infections in horses 5. False-positive results have been noted with some ELISAs	(2023) Russi *et al.* (2023)

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Appendix 5: Equine infectious anaemia
Intended purpose of test: prevalence of infection – surveillance

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
PCR ±	Blood Plasma Organs (spleen, liver) Exon 1 tat Blood samples LTR-tat	For Exon 1 tat: Analytical sensitivity (LOD) = 1 copy target/µl Analytical specificity = 44 exon 1 tata amplicons sequenced For LTR-tat: Analytical sensitivity (LOD) = 10 copy target/µl Analytical specificity = 40 LTR-tat amplicons sequenced	For Exon 1 tat: 5 Mules plasma (all positive) 37 positive equine bloods 16 positive equine plasma For LTR-tat: 53 blood samples (40 positive sequenced and 13 negative) See reference	See reference	For Exon 1 tat and LTR tat 1. High analytical sensitivity 2. High analytical specificity	For Exon 1 tat and LTR tat 1. Relatively low turnover 2. High costs 3. Requires specific laboratory equipment 4. Requires skilled laboratory personnel 5. Potential low diagnostic specificity due to the high genetic variability of the virus	Scicluna *et al.* (2013
AGID ++	Serum recombinant p26	Reference test: IB Specificity 100% Sensitivity 59.4%	548 samples positive in a screening ELISA subsequently analysed by AGID and IB at the WOAH RL, Italy	To be published	1. High specificity 2. High sensitivity in the initial stages of the intended purpose 3. Low cost 4. Minimal laboratory equipment required	1. Low sensitivity in case of early infection or low antibody levels 2. Low turnover 3. Results not readily available 4. In case of low levels of reactivity, interpretation of results depends on technical expertise	

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
ELISA +++	Serum recombinant p26 chimeric recombinant p26/gp45	Reference test were: AGID, IB **Recombinant p26:** Dsp: 73.2% (CI: 67.6–78.1%) Dse: 100% (CI: 91.6–100%) 50% inhibition as determined by ROC analysis. **Recombinant p26/gp45:** Dsp: 99.3% (CI: 96.8-99.8%) Dse: 100% (CI: 100-100%) 50% inhibition as determined by ROC analysis. **bELISA:** Reference tests were AGID Dsp = 100% Dse = 97.0% Accuracy 98.3%	**Recombinant p26:** 1. 324 Field sera analysed by immunoblot (52 positive, 272 negative). 2. 96 Sera of horses experimentally vaccinated 3. 1,102 field sera analysed by AGID test (857 positive and 245 negative) 4. Intralaboratory test with 9 laboratories **Recombinant p26/gp45:** 1. 96 Sera of horses experimentally vaccinated 3. 615 field sera analysed by AGID test **bELISA:** 1129 negative samples were taken from different farms in China which were free from EIA 18 positive sera, 45 negative sera, and 353 test serum samples from Argentina 3 standard positive sera 15,900 serum samples of either horses or donkeys collected from different provinces of China (People's Rep. of)	See reference	1. High sensitivity 2. Serological ELISAs are commercially available in many countries, together with in house ELISAs (see references) 3. A comparative analysis of some commercial and in house ELISAs reported that their diagnostic performances are comparable (see reference) 4. Rapid to carry out with an immediate result output 5. Low cost 6. Target antigen used is usually highly conserved 7. High turnover 8. No cross reactivity with other known viruses 9. Detection sensitivity and specificity of the bELISA were higher than those of either AGID or cELISA 10. bELISA detects the disease much earlier than does AGID 11. bELISA has not produced a single false-positive res	1. Specific laboratory equipment required 2. Considering the diagnostic specificity, positive samples should be confirmed using AGID and, in case of discordant results, in IB 3. Generally, low sensitivity during early stages of infection 4. The bELISA will of course only test positive from the onset of antibodies in the sera, and will miss sera without antibodies, which can occur when sera are collected from early-stage infections in horses 5. False-positive results have been noted with some ELISAs	Nardini *et al.* (2016) Nardini *et al.* (2017) Scicluna *et al.* (2018) Naves *et al.* (2019) Dominguez *et al.* (2021) Hu *et al.* (2023) Russi *et al.* (2023)

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CHAPTER 3.8.2.

SMALL RUMINANT LENTIVIRUSES (CAPRINE ARTHRITIS/ENCEPHALITIS & MAEDI-VISNA)

SUMMARY

Caprine arthritis/encephalitis (*CAE*) and *maedi-visna* (*MV*) *are persistent lentivirus infections of goats and sheep. They are often grouped together as the small ruminant lentiviruses* (*SRLVs*). *Maedi-visna is also known as ovine progressive pneumonia* (*OPP*). *Phylogenetic analyses comparing nucleotide sequences of MV virus* (*MVV*) *and CAE virus* (*CAEV*) *have demonstrated that these are closely related lentiviruses. One source of CAEV and MVV transmission is colostrum and milk. The source of horizontal transmission in the absence of lactation remains unknown; however, faeces and lung fluids are known to harbour infectious virus. Ovine lentiviruses have been identified in most of the sheep-rearing countries of the world, with the notable exceptions of Australia and New Zealand. The distribution of CAEV is highest in industrialised countries, and seems to have coincided with the international movement of European breeds of dairy goats. Clinical and subclinical MV and CAE are associated with progressive, mononuclear cell inflammatory lesions in the lungs, joints, udder and central nervous system. Indurative mastitis is common in both host species, and its economic significance may be underestimated. Laboured breathing associated with emaciation caused by progressive pneumonitis is the predominant feature in clinically affected sheep, whereas polyarthritis is the main clinical sign in goats. However, most lentivirus-infected sheep and goats are largely subclinical , but remain persistent carriers of virus and are capable of transmitting infection via colostrum or milk and respiratory secretions. The most practical and reliable approach to confirming a diagnosis of MV or CAE is a combination of serology and clinical evaluation. Although serology represents the most cost-effective method of diagnosing infection in persistently infected, clinically normal animals, it should be understood that testing errors can occur. The frequency of error depends on several factors including but not limited to: 1*) *the assay format, 2*) *the homology between the strain of virus used in the assay and the strains of virus present in the tested populations, and 3*) *the viral antigen used in the assay*.

***Detection and* identification of the agent:** *Virus isolation can be attempted from live clinical or subclinical cases by co-cultivating peripheral blood or milk leukocytes with appropriate ovine or caprine cell cultures, such as choroid plexus* (*MVV*) *or synovial membrane* (*CAEV*) *cells. Virus isolation is very specific but has variable sensitivities. Following necropsy, virus isolation is most readily accomplished by establishing explant cultures of affected tissues, e.g. lung, choroid plexus, synovial membrane, spleen or udder. Also, alveolar macrophages may be obtained from the lung at post-mortem and co-cultivated with susceptible cells. The cytopathic effects are characteristic, consisting of the appearance of refractile stellate cells and syncytia. The presence of MVV or CAEV can be confirmed by immunolabelling methods, reverse transcription activity and electron microscopy.*

***Nucleic acid ~~recognition~~ detection methods:** Many standard and a few quantitative polymerase chain reaction* (*PCR*) *assays for detecting MV and CAE provirus have been described and are used routinely in many laboratories for the rapid detection, quantitation, and identification of the small*

ruminant lentivirus strains. Cloning and/or sequencing of PCR products is the most direct method to confirm the specificity of PCR results.

Serological tests: *Most infected sheep and goats possess detectable specific antibodies that can be assayed by a number of different serological tests. The two most commonly used are the agar gel immunodiffusion test and the enzyme-linked immunosorbent assay (ELISA). Western blot analysis and radio-immunoprecipitation are also performed, but only in specialised laboratories. A milk antibody assay may be appropriate in dairy goat herds. The time required for seroconversion following infection can be relatively prolonged and unpredictable, being measured in months rather than in weeks. However, after seroconversion, the antibody response usually persists and antibody-positive sheep and goats are regarded as virus carriers.*

Requirements for vaccines: *There are no vaccines* available.

A. INTRODUCTION

Caprine arthritis/encephalitis (CAE) of goats and maedi-visna (MV) of sheep are persistent virus infections caused by closely related lentiviruses (~~Minguijón *et al.*, 2015;~~ Peterhans *et al*., 2004). Maedi-visna is also known as ovine progressive pneumonia (OPP). Sheep can be ~~experimentally~~ infected with CAE and goats can be ~~experimentally~~ infected with MV. In addition, phylogenetic analyses comparing nucleotide sequences of MV virus (MVV) and CAE virus (CAEV) show clear indications of the existence and epidemiological importance of cross-species transmission between sheep and goats without demonstrating clearly that one virus has emerged from the other (Shah *et al*., 2004a; 2004b). Despite the fact that MVV and CAEV are still classified as two different viral species, there is an increasing consensus among the scientific community to consider them as two different genotypes (A and B, respectively) of a larger group of viruses, named small ruminant lentiviruses (SRLV), displaying different genetic, antigenic and biological properties.

MV and CAE are characterised by lifelong persistence of the causal agent in host monocytes and macrophages, and a variable length of time between infection and induction of a serologically detectable antiviral antibody response. Most infected sheep and goats do not exhibit clinical disease, but remain persistently infected and are capable of transmitting virus (Adams *et al*., 1983; Crawford *et al*., 1980).

Maedi-visna is an Icelandic name that describes two of the clinical syndromes recognised in MV virus (MVV)-infected sheep. 'Maedi' means 'laboured breathing' and describes the disease associated with a progressive interstitial pneumonitis, and 'visna' means 'shrinkage' or 'wasting', the signs associated with a paralysing meningoencephalitis. Whereas progressive lung disease is the primary finding with MVV infection, chronic polyarthritis, with synovitis and bursitis is the primary clinical outcome of CAEV infection. Encephalitis occurs primarily in kids aged between 2 and 6 months following CAEV infection, though more rare compared with when first recognised in the early 1980s. Thus ~~but~~ careful differential diagnoses need to be conducted to rule out other syndromes or infections in kids. Indurative mastitis occurs in both syndromes. The lungs of sheep affected by MV do not collapse when removed from the thorax and often retain the impression of the ribs. The lungs and lymph nodes increase in weight (up to 2–3 times the normal weight). The lesions are uniformly distributed throughout the lungs, which are uniformly discoloured or mottled grey-brown in colour and of a firm texture. Diagnosis of CAEV and MVV induced respiratory disease was reviewed by Chakraborty *et al.* (2014). Udders affected by MV are diffusely indurated and associated lymph nodes may be enlarged.

When MV or CAE is the suspected cause of clinical disease, confirmation of the diagnosis can be achieved by a combination of clinical evaluation, detection and identification of the viruses, or by serology and, when necessary, histological examination of appropriate tissues collected at necropsy. Important tissues to examine include lung for progressive interstitial pneumonitis, brain and spinal cord for meningoencephalitis, udder for indurative mastitis, affected joints and synovium for arthritis, and kidney for vasculitis ~~(Crawford & Adams, 1981)~~. The nature of the inflammatory reaction in each site is similar, consisting of an interstitial, mononuclear cell reaction, sometimes with large aggregates of lymphoid cells and follicle formation.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of caprine arthritis/encephalitis and maedi-visna and their purpose

Method	Purpose					
	Population freedom from infection[a]	Individual animal freedom from infection prior to movement[b, c]	Contribute to eradication policies[d]	Confirmation of clinical cases[e]	Prevalence of infection – surveillance[f]	Immune status in individual animals or populations post-vaccination[g]
Detection and identification of the agent						
Virus isolation	–	–	–	+	–	–
Antigen detection	–	–	–	+	–	–
PCR	+	+	++	++±	++	–
Detection of immune response						
AGID	+	++[h] ±	++[h]	++ ±	+++	± –
~~CFT~~	~~–~~	~~–~~	~~–~~	~~–~~	~~–~~	~~–~~
ELISA	+++	+++[h]	+++[h]	++±	+++	± –
~~VN~~	~~–~~	~~–~~	~~–~~	~~–~~	~~–~~	~~+++~~ –
~~IFAT~~	~~–~~	~~–~~	~~–~~	~~–~~	~~–~~	~~–~~

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
PCR = polymerase chain reaction; AGID = agar gel immunodiffusion; ~~CFT = complement fixation test;~~
ELISA = enzyme-linked immunosorbent assay~~; VN = virus neutralisation; IFAT = indirect fluorescent antibody test~~.
[a] See Appendix 1 of this chapter for justification table for the scores given to the tests for this purpose.
[b] See Appendix 2 of this chapter for justification table for the scores given to the tests for this purpose.
[c] A combination of agent identification method and detection of immune response is recommended.
[d] See Appendix 3 of this chapter for justification table for the scores given to the tests for this purpose.
[e] See Appendix 4 of this chapter for justification table for the scores given to the tests for this purpose.
[f] See Appendix 5 of this chapter for justification table for the scores given to the tests for this purpose.
[g] No vaccines available for small ruminant lentiviruses.
[h] Samples from animals under 6 months of age are not recommended for testing due to maternal antibody interference, especially if heat-treated colostrum is used for eradication purposes.

1. Detection and identification of the agent

Isolation and characterisation of MVV or CAEV would not normally be attempted for routine diagnostic purposes. Due to the persistent nature of these infections, the establishment of a positive antibody status is sufficient for the ~~identification~~ detection of virus carriers. However, due to a late seroconversion after infection, negative serology may occur in recently infected animals.

There are two approaches to the isolation of MVV and CAEV: one for use with the live animal, and the second for use with necropsy tissues.

1.1. Isolation from the live animal

1.1.1. Maedi-visna virus

The MV provirus DNA is carried in circulating monocytes and tissue macrophages. Virus isolation from the live animal therefore requires the establishment of leukocyte preparations, with aseptic precautions, from peripheral blood or milk during lactation, culturing them together with indicator cells. Sheep choroid plexus (SCP) cells are commonly used for this purpose. These indicator cells can be prepared as primary explant cultures from fetal or new-born virus-free lambs, and their number can be multiplied over three to four passages for storage in liquid nitrogen. The recovered

SCP cells are suitable for co-cultivation for up to 10 or 15 passages. Although the cells continue to grow well thereafter, their susceptibility to MVV may become reduced.

Leukocyte preparations can be made from peripheral blood as buffy coats by the centrifugation at 1000 ***g*** of heparinised, ethylenediamine tetra-acetic acid (EDTA) or citrated samples for 15 minutes. The cells are aspirated, suspended in Hanks' balanced salt solution (HBSS), and further purified by centrifugation at 400 ***g*** on to a suitable cushion of density medium for 40 minutes. The interface cells are spin-washed once or twice in HBSS at 100 ***g*** for 10 minutes, and the final cell pellet is resuspended in medium to a concentration of approximately 10^6 cells/ml; cells are generally cultured for 10–12 days in Teflon bags and are then added to a washed monolayer of slightly subconfluent SCP cells in a flask with an area of 25 cm^2.

Leukocytes can be similarly deposited from milk by centrifugation, when they are spin-washed, resuspended and finally added to SCP monolayer cultures.

These cultures are maintained at 37°C in a 5% CO_2 atmosphere, changing the medium and passaging as necessary. They are examined for evidence of a cytopathic effect (CPE), which is characterised by the appearance of refractile stellate cells with dendritic processes accompanied by the formation of syncytia. The cultures should be maintained for several weeks before being discarded as uninfected. Once a CPE is suspected, cover-slip cultures should be prepared. These are fixed, and evidence of viral antigen is sought by immunolabelling, usually by means of indirect fluorescent antibody or by the use of indirect immunoperoxidase methods. In addition, the cells of any suspect monolayers are deposited by centrifugation, and preparations are made for the identification of any characteristic lentivirus particles by transmission electron microscopy. Reverse transcriptase in the supernatant of the cell culture is indicative of the presence of retroviruses.

1.1.2. Caprine arthritis/encephalitis virus

The same principles that apply to the isolation of MVV also apply to the isolation of CAEV. CAEV was originally isolated by explantation of synovial membrane from an arthritic goat ~~(Crawford & Adams, 1981)~~. With live CAEV-infected goats, peripheral blood, milk, and possibly joint fluid aspirate represent the most suitable specimens from which leukocyte preparations can be established. Goat synovial membrane (GSM) cells are suitable indicator cells. If a CPE is suspected, tests for detection of viral antigen should be carried out, as described above.

1.2. Isolation from necropsy tissues

1.2.1. Caprine arthritis/encephalitis virus and maedi-visna virus

Samples of suspect tissues, collected as fresh as possible, such as lung, synovial membranes, udder, ~~etc.~~ spleen, are collected aseptically into sterile HBSS or cell culture medium and minced finely (the smaller the better) in a Petri dish using scalpel blades. Approximately 10–20 fragments are resuspended with 10 ml of medium (i.e. Dulbecco's modified Eagle's medium) supplemented with 2× antibiotic/antimycotic solution, transferred into a 14 ml tube and incubated for 3 hours at 37°C in a humid 5% CO_2 atmosphere. Sedimented fragments are then resuspended into 10 ml of medium with 1× antibiotic/antimycotic solution and 10% FBS and ~~Individual fragments are collected by Pasteur pipette and~~ transferred to 25 cm^2 flasks ~~approximately 20–30 fragments per flask, and a drop of growth medium is placed carefully on each~~. The flasks are then incubated ~~at 37°C in a humid 5% CO_2 atmosphere,~~ and left undisturbed for ~~a few~~ 3–5 days to allow the individual explants to adhere to the plastic. Partial fresh medium changes can be ~~added with care~~ made every 3 days until a confluent monolayer is obtained (usually 10–15 days), after which ~~rafts of cells will gradually grow out from the fragments. When there is sufficient cell out-growth,~~ the cultures are trypsin dispersed ~~to allow the development of cell monolayers~~ and propagated at weekly intervals. Paired culture wells (i.e. 24 wells microplate) can be fixed and Giemsa stained to facilitate ~~These can be examined~~ examination for CPE at each passage, and any suspected virus growth is confirmed in the same way as for the co-cultivations.

Adherent macrophage cultures are easy to establish from lung-rinse material (post-mortem broncho-alveolar lavage) and can be tested for virus production by serology, electron microscopy, or reverse transcriptase assay within 1–2 weeks. Virus isolations can be done by co-cultivation of macrophages and SCP or GSM cells as described for leukocytes above.

1.3. Nucleic acid ~~recognition~~ detection methods

Real-time PCR

Pathogen/ target gene	Primer/probe (5'–3')	Concentration	Cycling parameters[a]
Method[b][c]: Schaer *et al.* (2022); GenBank Accession No.: M33677			
Geno A LTR-gag	RTMVVLTRgag-F: GGG-GAC-GCC-TGA-AGT-RAG-GTA-A RTMVVLTRgag-R: YTT-GAG-CTC-RGG-GTA-YCC-CTT RTMVVLTRgag-P: FAM-CTT-TGA-GCC-TTG-CKT-CGC-CAT-GTC-T-TAMRA	900 nM 900 nM 200nM	40 cycles of: 95°C/15 sec and 60°C/60 sec
Geno B LTR-gag	RTCAELTRgag-F: CTG-RAG-GAG-TAM-GGT-AAG-TRA-CTC-TGC RTCAELTRgag-R: TTG-ATR-CAT-TTK-TCS-AKC-TCA-GGA-TAA RTCAELTRgag-P: FAM-CCG-GAG-ACT-TGC-CTC-GCC-ATG-TC-TAMRA	900 nM 900 nM 200nM	40 cycles of: 95°C/15 sec and 60°C/60 sec

[a]A denaturation step prior to cycling has not been included.
[b]IUPAC (International Union of Pure and Applied Chemistry) codes were used to indicate degenerated primers. [c]This method has been proposed as a second nested amplification step after a first conventional PCR, see reference for details.

Conventional PCR

Pathogen/ target gene	Primer (5'–3')	Concentration	Cycling parameters[a]
Method 1: Extramiana *et al.* (2002); GenBank Accession No.: M10608, amplicon size: 291[bp]			
SRLV-LTR	LTRs: TGA-CAC-AGC-AAA-TGT-AAC-CGC-AAG	0.5 µM	SRLV-LTR

[a]A denaturation step prior to cycling has not been included.

Nucleic acid ~~recognition~~ detection methods may be used for the detection, quantitation, and identification of MV and CAE proviral DNA using the standard polymerase chain reaction (PCR) followed by Southern blotting, *in situ* hybridisation, or cloning and/or sequencing of the PCR product (Alvarez *et al*., 2006; Extramiana et al., 2002 ~~Herrmann-Hoesing *et al*., 2007; Johnson *et al*., 1992~~). Standard PCR techniques for the detection of MV and CAE proviral DNA in cells and tissues are routinely used in many laboratories and are generally used as supplemental tests for determining infection status of those animals that cannot be definitively diagnosed by serology ~~(Deandres *et al*., 2005)~~. Real-time or quantitative PCR techniques are used in some laboratories and these tests, in addition to determining infection status, also quantify the amount of MV or CAE provirus in an animal (Alvarez *et al*., 2006; De Regge & Cay, 2013~~; Herrmann-Hoesing *et al*., 2007~~). Furthermore, molecular techniques such as PCR, cloning and sequencing also provide knowledge on a country's or region's specific MV and CAE strains, which may influence which serological assay and corresponding MV or CAE antigen to use. Phylogenetic analyses of MV and CAE proviral DNAs from SRLV strains throughout the world have suggested that in some areas, MV may have naturally infected goats, and CAE may have naturally infected sheep (Shah *et al*., 2004a; 2004b). Recently, loop-mediated isothermal amplification (LAMP) was applied to the detection of CAE provirus (Balbin *et al.,* 2014). ~~LAMP uses 4–6 primers that amplify 6–8 regions of the target gene (Notomi *et al.*, 2000).~~ In the future, molecular diagnostic tests along with phylogenetic analyses of MV and CAE provirus may be used to track transmission. Sensitivity of PCR methods may be influenced by the high genetic heterogeneity of SRLV and some primer sets have been developed for detection of specific viral genotype/subtypes (De Regge & Cay, 2013; Schaer *et al.*, 2022).

An important issue in the use of PCR is specificity. Because of the possibility of amplifying unrelated sequences from the host's genomic DNA (false positives), the amplified product should be checked by either hybridisation, restriction endonuclease digestion patterns, or sequencing. Sequencing provides the best proof of specificity in the validation of PCR-based tests and is recommended by the WOAH (see Chapter 2.1.2 *Biotechnology advances in the diagnosis of infectious diseases*). ~~Sensitivity of PCR-based tests can be improved by the use of nested PCR, but specificity of the nested PCR test should be checked using hybridisation, restriction endonuclease digestion patterns, or sequencing methods.~~ A high throughput sequencing approach is becoming a useful tool in specialised laboratories to characterise viral isolates at the full genome level (Colitti *et al.*, 2019).

2. Serological tests

Ovine and caprine lentivirus infections are frequently persistent, so antibody detection is a valuable serological tool for identifying virus carriers. The close antigenic relationship between MVV and CAEV does not extend to detection of heterologous antibody in some serological assays (Knowles *et al*., 1994).

The assays most commonly used to serologically diagnose the presence of a small ruminant lentivirus infection are agar gel immunodiffusion (AGID) and the enzyme-linked immunosorbent assay (ELISA). AGID was first developed and reported in 1973 (Terpstra & De Boer, 1973), and the ELISA was first developed and reported in 1982 (Houwers *et al*., 1982). The AGID is specific, reproducible and simple to perform, but experience is required for reading the results. The ELISA is economical, quantitative and can be automated, thus making it useful for screening large numbers of sera. The sensitivity and specificity of both the AGID assay and ELISA depend upon the virus strain used in the assay, viral antigen preparation, and the standard of comparison assay. Western blot analysis and/or radio-immunoprecipitation are the standards of comparison used to access sensitivity and specificity of new AGID tests and ELISAs.

2.1. Agar gel immunodiffusion

There are two MV and CAE viral antigens of major importance in routine serology, a viral surface envelope glycoprotein commonly referred to as SU or gp135, and a nucleocapsid protein referred to as CA or p28. These are both conserved in an antigen preparation consisting of medium harvested from infected cell cultures and concentrated approximately 50-fold by dialysis against polyethylene glycol. As an example the WLC-1 strain of MV virus is commonly used in the AGID assay in the United States ~~(Cutlip *et al*., 1977).~~[1] ~~–~~and a Canadian MV field strain is used for AGID tests in Canada (Simard & Briscoe, 1990b).

It is important to recognise that the sensitivity of the AGID test for detecting anti-CAEV antibody is dependent on both the virus strain and the viral antigen used (~~Adams & Gorham, 1986; ~~Knowles *et al*., 1994). It was demonstrated that an AGID test with CAEV gp135 afforded greater sensitivity than an AGID test with CAEV p28 ~~(Adams & Gorham, 1986)~~. Also, it was shown that when compared with radio-immunoprecipitation, the sensitivity of the AGID test for anti-CAEV antibody was 35% greater using CAE virus antigen over using MV virus antigen (Knowles *et al*., 1994). The most likely explanation for this difference in sensitivity between the CAE and MV virus antigen for the detection of anti-CAEV antibody is that although the radio-immunoprecipitation assay requires only the binding of a single epitope by antibody to obtain a positive result, precipitation in an agar gel requires multiple epitope–antibody interactions. Although the MV and CAE viruses have 73–74.4% nucleotide sequence identity in the envelope gene, this amount of identity may not be sufficient to produce sufficient antibody to CAE and MV mutually common epitopes resulting in undetectable antibody/antigen precipitin lines using MV virus antigen. When the appropriate antigen is used, the AGID test performance is high. When compared with immunoprecipitation, the AGID for the detection of anti-CAEV antibody, if CAEV antigen was used, had 92% sensitivity and 100% specificity (Knowles *et al*., 1994). In addition, the AGID for detection of anti-MVV antibody, if MVV antigen was used, had 99.3 and 99.4% sensitivity and specificity, respectively.

In adult persistently MVV-infected sheep and CAEV-infected goats, the predominant immunoprecipitating antibody response is directed against gp135 antigen. An anti-p28 response is usually present at a lower titre than the anti-gp135 response in persistently infected adult small ruminants using immunoprecipitation. In some CAEV-infected goats there is evidence to suggest that an anti-gp135 antibody response is produced, in the absence of an anti-p28 response and vice versa, in a proportion of individuals (Rimstad *et al*., 1994). Hence, for validation of a test, standard sera producing both anti-gp135 and anti-p28 precipitin lines are required.

The gel medium is 0.7–1% agarose in 0.05 M Tris buffer, pH 7.2, with 8.0% NaCl. The test is conveniently performed in plastic Petri dishes, or in 10 cm^2 plastic trays. The pattern and size of the wells will determine the number of sera tested per plate. Various well patterns can be adopted, but a hexagonal arrangement with a central well is usual: for example, a pattern with alternating large (5 mm in diameter) and small (3 mm in diameter) peripheral wells, 2 mm apart and 2 mm from a central antigen well that is 3 mm in diameter. The large peripheral wells are used for test sera and the small ones for standard sera. A weak positive control must be included in each test. The plates are incubated overnight at 20–25°C in a humid chamber, and then examined for precipitin lines. Plates may be incubated at 2–8°C for another 24 hours to enhance the precipitin lines.

[1] ~~This virus has been distributed by Dr Howard Lehmkuhl, National Animal Disease Center, United States Department of Agriculture, P.O. Box 70, Ames, Iowa, USA.~~

An important consideration is the need for experienced personnel to interpret the AGID. Interpretation of AGID results is dependent on the antigen used. Examples of AGIDs with different antigen preparations and a guide for interpretation of the results can be found in Adams *et al.* (1983).

2.2. Enzyme-linked immunosorbent assay

Currently, there are over 30 different ELISAs reported for the detection of anti-MVV or anti-CAEV antibodies in the sera of sheep or goats, respectively ~~(Deandres *et al.*, 2005)~~. Most of these ELISAs are indirect ELISAs (I-ELISA) although there are three reported competitive ELISAs (C-ELISA) using monoclonal antibodies (MAbs) (Herrmann *et al*., 2003~~; Houwers & Schaake, 1987~~). Half of I-ELISAs use purified whole virus preparations for antigen whereas the other half use recombinant protein and/or synthetic peptide antigens. Some I-ELISAs use a combination of both CAEV-like and MVV-like antigens. A few of the I-ELISAs have shown high sensitivity and specificity against a standard of comparison, western blot analysis or radio-immunoprecipitation (Rosati *et al*., 1994; Saman *et al*., 1999). When compared with radio-immunoprecipitation, one C-ELISA has shown high sensitivity and specificity both in sheep and goats in the USA suggesting that this one test can be used for both MVV and CAEV US surveillance (Herrmann *et al*., 2003). ELISAs have been used for several years in some European countries in control and eradication schemes for MVV in sheep and CAEV in goats (~~Motha & Ralston, 1994;~~ Pépin *et al*., 1998), AGID is useful to confirm positive ELISA results due to its high specificity. A number of both I-ELISAs and C-ELISAs are commercially available.

Whole-virus antigen preparations are produced by differential centrifugation of supernatants from infected cell cultures and detergent treatment of purified virus, and are coated on microplates (~~Dawson *et al*., 1982;~~ Simard & Briscoe, 1990a; Zanoni *et al*., 1994). Whole-virus preparations should contain both gp135 and p28. Recombinant antigens or synthetic peptides are usually produced from whole or partial segments of the gag or envelope genes and may be used in combination (~~Power *et al*., 1995;~~ Rosati *et al*., 1994; Saman *et al*., 1999). Thus, recombinant gag or envelope gene products fused with glutathione S-transferase fusion protein antigen that have been produced in Escherichia coli provide a consistent source of antigen for international distribution and standardisation.

The ELISA technique is also applicable to colostrum or milk, and some studies have evaluated paired serum and milk samples. Because colostrum and milk are sources of CAEV transmission, the testing of milk samples for anti-CAEV or anti-MVV antibody would not provide timely information for the prevention of transmission, especially to offspring from the immediate gestation. An I-ELISA based on a variable and immunodominant epitope of the capsid antigen has been proposed as a serotyping method for discrimination between SRLV genotypes (Nogarol *et al*., 2019).

The ELISA is performed at room temperature (~25°C) and is easy to perform in laboratories that have the necessary equipment (microplate reader) and reagents. It is convenient for large-scale screening, as it is a reliable and quantitative technique for demonstrating small ruminant lentiviruses (SRLVs) antibodies in sheep and goats. It requires relatively pure antigen. One disadvantage of several ELISAs is that many have not been validated against a standard of comparison such as western blot analysis or radio-immunoprecipitation. The test method should be validated in accordance with Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals* using a standard of comparison such as western blot analysis or radio-immunoprecipitation. To date, only one ELISA has met these testing standards (Zanoni *et al*., 1994).

For I-ELISA, wells of the microplate are coated with antigen. Diluted serum samples are added to the wells and react to antigens bound to the solid support. Unbound material is removed by washing after a suitable incubation period. Conjugate (e.g. horseradish-peroxidase-labelled anti-ruminant Ig) reacts with specific antibodies bound to the antigen. Unreacted conjugate is removed by washing after a suitable incubation period. Enzyme substrate is added. The rate of conversion of substrate is proportional to the amount of bound antibodies. The reaction is terminated after a suitable time and the amount of colour development is measured spectrophotometrically. ~~A disadvantage of the I-ELISA is that test sera typically need to be diluted 1/50 or greater in order to lower the number of false positives.~~ Some I-ELISAs require a biphasic test format, including a negative antigen well, to minimise false positive reactions.

Specific MAbs have been used in a C-ELISA for SRLVs to capture gp135 or p28 as antigen (Frevereiro *et al*., 1999; Herrmann *et al*., 2003~~; Houwers & Schaake, 1987; Ozyoruk *et al*., 2001~~). C-ELISA overcomes the problem of antigen purity, as the specificity of this test depends on the MAb epitope. For C-ELISA, sample sera containing anti-SRLV antibodies inhibit binding of enzyme–labelled MAb to SRLV antigen coated on the plastic wells. Binding of the enzyme-labelled MAb conjugate is detected by the addition of enzyme substrate and quantified by subsequent colour product development. Strong colour development indicates little or no blockage of enzyme-labelled MAb binding and therefore the absence of SRLV antibodies in sample sera. In contrast, weak colour development due to the inhibition of the enzyme-labelled MAb binding to the antigen on the solid phase indicates the presence of SRLV

antibodies in sample sera. The format of the C-ELISA requires that serum antibodies must bind to or bind in close proximity to the specific MAb epitope. C-ELISA is less suitable than I-ELISA for antibody detection in milk, due to lower IgG concentration.

2.2.1. Materials and reagents

Microtitre plates with 96 flat-bottomed wells, freshly coated or previously coated with SRLV antigen; microplate reader (equipped with 405, 450, 490 and 620 nm filters); 37°C humidified incubator; 1-, 8- and 12-channel pipettes with disposable plastic tips; microplate shaker (optional); fridge; freezer.

Positive and negative control sera; conjugate (e.g. ruminant anti-immunoglobulin labelled with peroxidase); tenfold concentration of diluent (e.g. phosphate buffered saline/Tween); distilled water; 10× wash solution; substrate or chromogen (e.g. ABTS [2,2'-azino-bis-(3-ethylbenzo-thiazoline-6-sulphonic acid)] or TMB [3,3',5,5'-tetramethylbenzidine]); stop solution (e.g. detergent, sulfuric acid).

2.2.2. Indirect ELISA: test procedure

i) Dilute the serum samples, including control sera, to the appropriate dilution (e.g. 1/20) and distribute 0.1–0.2 ml per well (in duplicate if biphasic ELISA). Control sera are positive and negative sera provided by the manufacturer and an internal positive reference serum from the laboratory in order to compare the titres between different tests.

ii) Cover the plate with a lid and incubate at room temperature or 37°C for 30–90 minutes. Empty the contents and wash three times in washing solution at room temperature.

iii) Add the appropriate dilution of freshly prepared conjugate to the wells (0.1 ml per well). Cover each plate and incubate as in step ii. Wash again three times.

iv) Add 0.1 ml of freshly prepared or ready-to-use chromogen substrate solution to each well (e.g. ABTS in citrate phosphate buffer, pH 5.0, and 30% H_2O_2 solution [0.1 µl/ml]).

v) Shake the plate; after incubation, stop the reaction by adding stopping solution to each well (e.g. 0.1 ml sulphuric acid).

vi) Read the absorbance of each well with the microplate reader at 405 nm (ABTS) or 450–620 nm (TMB). The absorbance values will be used to calculate the results.

vii) Interpretation of the results

For commercial kits, interpretations and validation criteria are provided with the kit.

Interpretation criteria should be developed and validated for the individual procedures and reagents used in the laboratory. The following is given as an example:

Calculate the mean absorbance (Ab) of the sample serum and of the positive (Ab_{pos}) and negative (Ab_{neg}) control sera, and for each serum, calculate the percentage:

$$\frac{Ab - Ab_{neg}}{Ab_{pos} - Ab_{neg}} \times 100$$

if a test sample has mean absorbance of <30% it is classed as negative, 30-40% is classed as doubtful, and >40% as positive.

2.2.3. Competitive ELISA: test procedure

i) Add 0.05 ml of undiluted serum and positive/negative controls to antigen-coated plate.

ii) Incubate for 1 hour at room temperature.

iii) Empty the plate and wash the plate three with diluted wash solution.

iv) Add 0.05 ml of diluted MAb ~~antibody~~ peroxidase conjugate to each well. Mix well and incubate for 30 minutes at room temperature.

v) After the 30-minute incubation, empty the plate and repeat the washing procedure described in step iii.

vi) Add 0.05 ml of substrate solution (ex: TMB) to each well. Mix and cover plate with aluminium foil. Incubate for 20 minutes at room temperature. Do not empty wells.

vii) Add 0.05 ml of stop solution to each well. Mix. Do not empty wells.

viii) Immediately after adding the stop solution, the plate should be read on a plate reader (620, 630 or 650 nm).

ix) Interpretation of results

Interpretation criteria should be developed and validated for the individual procedures and reagents used in the laboratory. The following is given as an example:

Calculation: 100 – [(Sample Ab × 100)/(Mean negative control Ab)] = % inhibition.

For goats, if a test sample causes >33.2% inhibition, it is positive; if a test sample causes <33.2% inhibition, it is negative. For sheep, if a test sample causes >20.9% inhibition, it is positive; if a test sample causes <20.9% inhibition, it is negative.

C. REQUIREMENTS FOR VACCINES

There are no vaccines available.

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NB: At the time of publication (2025) there were no WOAH Reference Laboratories for caprine arthritis/encephalitis & maedi-visna (please consult the WOAH Web site for the current list: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3.

NB: CAPRINE ARTHRITIS/ENCEPHALITIS FIRST ADOPTED IN 1990; MAEDI-VISNA FIRST ADOPTED 1989. MOST RECENT UPDATES ADOPTED IN 2017.

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Appendix 1: Caprine arthritis/encephalitis & maedi-visna
Intended purpose of test: population freedom from infection

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
PCR +	Blood (PBMC)	Reference was agreement of AGID or ELISA DSe 84% (Blood samples), DSp 100% (Extramiana *et al.*, 2002) Reference was agreement of two serological tests and western blot DSe 75,52%, DSp 100% (Schaer *et al.*, 2022)	115 sheep (Spain): blood, milk cells, target tissues Different European countries 221 sheep and 69 goats 396 sheep	See references	High specificity Moderate sensitivity	Expensive Require equipped laboratory Some protocols were developed for a specific genotype (i.e. MVV-like)	Extramiana *et al.* (2002) Schaer *et al.* (2022)
AGID +	Serum p28 and/or gp135	Reference test was immunoprecipitation DSe 91%, DSp 100% (Knowles *et al.*, 1994) Reference test was agreement of four of six serological tests and real-time PCR Sheep DSe 35-100%, DSp 100% Goat DSe 75-87%, DSp 100% (Michiels *et al.*, 2018)	218 goat serum (111 negative and 117 positive) 51 positive and 502 negative sheep sera 24 positive and 370 negative goat sera	See references	Easy to perform Does not require equipped laboratory High specificity Low-cost reagents Commercially available	Time consuming Low sensitivity Not suitable for pooled sera or milk testing Commercially available antigens are derived from few, well culture adapted, strains	Knowles *et al.* (1994) Michiels *et al.* (2018)
ELISA +++	Serum, plasma, milk Gag and Env antigen One or more genotype-derived antigen Indirect or competitive	Reference test was immunoprecipitation DSe 100%, DSp 96,4% (Herrmann *et al.*, 2003) Reference test was agreement of four of six serological tests and real-time PCR Sheep DSe up to 100%, DSp up to 99,6% Goat DSe up to 100%, DSp up to 100% (Michiels *et al.*, 2018) Reference test was AGID and western blot DSe 99,4%, DSp 99,3% (Saman *et al.*, 1999) Reference was agreement of two serological tests, western blot and nested PCR DSe 92,2%, DSp 98,9% (Schaer *et al.*, 2022)	US 200 goat sera (140 negative and 60 positive) Belgium 51 positive and 502 negative sheep sera 24 positive and 370 negative goat sera Different European countries 2336 sheep different European countries 221 sheep and 69 goats	See references	Easy to perform High sensitivity Commercially available Indirect ELISA suitable for pooled sera or milk	Moderate specificity C-ELISA not suitable for pooled sera or milk testing Genotype specific ELISA less sensitive to detect heterologous infection (especially genotype B antigen for genotype A infection)	Herrmann *et al.* (2003) Michiels *et al.* (2018) Saman *et al.* (1999) Schaer *et al.* (2022)

Appendix 2: Caprine arthritis/encephalitis & maedi-visna
Intended purpose of test: individual animal freedom from infection prior to movement

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
PCR +	Blood (PBMC)	Reference was agreement of two serological tests and western blot DSe 75.52%, DSp 100% (Schaer *et al.*, 2022)	Different European countries 221 sheep and 69 goats	See reference	High specificity Moderate sensitivity May detect very early stage of infection	Expensive Require equipped laboratory Less sensitive than antibody response detection	Schaer *et al.* (2022)
AGID ++	Serum p28 and gp135	Reference test was immunoprecipitation DSe 91%, DSp 100% (Knowles *et al.*, 1994)	218 goat serum (111 negative and 117 positive)	See reference	Easy to perform Does not require equipped laboratory High specificity Low-cost reagents Commercially available	Time consuming Low sensitivity especially for heterologous genotype antibody detection. Commercially available antigens are derived from few, well culture adapted, strains	Knowles *et al.* (1994)
ELISA +++	Serum, plasma, milk Gag and Env antigen One or more genotype-derived antigen Indirect or competitive	Reference test was immunoprecipitation DSe 100%, DSp 96,4% (Herrmann *et al.*, 2003) Reference test was agreement of four out of six serological tests and real-time PCR Sheep DSe up to 100%, DSp up to 99,6% Goat DSe up to 100%, DSp up to 100% (Michiels *et al.*, 2018) Reference test was AGID and western blot DSe 99,4%, DSp 99,3% (Saman *et al.*, 1999) Reference was agreement of two serological tests, western blot and nested PCR DSe 92,2%, DSp 98,9% (Schaer *et al.*, 2022)	US 200 goat sera (140 negative and 60 positive) Belgium 51 positive and 502 negative sheep sera 24 positive and 370 negative goat sera Different European countries 2336 sheep Different European countries 221 sheep and 69 goats	See references	Easy to perform High sensitivity Commercially available	Moderate specificity Genotype specific ELISA less sensitive to detect heterologous infection (especially genotype B antigen for genotype A infection)	Herrmann *et al.* (2003) Michiels *et al.* (2018) Saman *et al.* (1999) Schaer *et al.* (2022)

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Appendix 3: Caprine arthritis/encephalitis & maedi-visna
Intended purpose of test: contribute to eradication policies

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
PCR ++	Blood (PBMC) Useful targets: LTR, gag, pol	Reference was agreement of AGID or ELISA DSe 84% (PBMC), DSp 100% (Extramiana *et al.*, 2002) Reference was agreement of two serological tests and western blot DSe 75,52%, DSp 100% (Schaer *et al.*, 2022)	115 sheep (Spain): blood, milk cells, target tissues Different European countries 221 sheep and 69 goats 396 sheep	See references	High specificity Moderate sensitivity Sequence analysis of PCR targeting gag gene offers useful information to select appropriate serological test based on SRLV genotype/s in the population	Expensive Require equipped laboratory Some protocols were developed for a specific genotype (i.e. MVV-like)	Extramiana *et al.* (2002) Schaer *et al.* (2022) Shah *at al.* (2004a)
AGID ++	Serum p28 and/or gp135	Reference test was immunoprecipitation DSe 91%, DSp 100% (Knowles *et al.*, 1994) Reference test was agreement of four of six serological tests and real-time PCR Sheep DSe 35-100%, DSp 100% Goat DSe 75-87%, DSp 100% (Michiels *et al.*, 2018)	218 goat serum (111 negative and 117 positive) 51 positive and 502 negative sheep sera 24 positive and 370 negative goat sera	See references	Easy to perform Does not require equipped laboratory High specificity Low-cost reagents Commercially available	Time consuming Low sensitivity Not suitable for pooled sera or milk testing Commercially available antigens are derived from few, well culture adapted, strains Not practical for large number of samples	Knowles *et al.* (1994) Michiels *et al.* (2018)
ELISA +++	Serum, plasma, milk Gag and Env antigen One or more genotype-derived antigen Indirect or competitive	Reference test was immunoprecipitation DSe 100%, DSp 96,4% (Herrmann *et al.*, 2003) Reference test was agreement of four out of six serological tests and real-time PCR Sheep DSe up to 100%, DSp up to 99,6% Goat DSe up to 100%, DSp up to 100% (Michiels *et al.*, 2018) Reference test was AGID and western blot DSe 99,4%, DSp 99,3% (Saman *et al.*, 1999) Reference was agreement of two serological tests, western blot and nested PCR DSe 92,2%, DSp 98,9% (Schaer *et al.*, 2022)	US 200 goat sera (140 negative and 60 positive) Belgium 51 positive and 502 negative sheep sera 24 positive and 370 negative goat sera Different European countries 2336 sheep Different European countries 221 sheep and 69 goats	See references	Easy to perform High sensitivity Commercially available Suitable for high throughput screening Indirect ELISA suitable for pooled sera or milk	Moderate specificity C-ELISA not suitable for pooled sera or milk testing Genotype specific ELISA less sensitive to detect heterologous infection (especially genotype B antigen for genotype A infection)	Herrmann *et al.* (2003) Michiels *et al.* (2018) Saman *et al.* (1999) Schaer *et al.* (2022)

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Appendix 4: Caprine arthritis/encephalitis & maedi-visna
Intended purpose of test: confirmation of clinical cases

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
Virus isolation +	Tissues (spleen, mammary gland, lymph nodes, lung, choroid plexus, synovial membrane, skin) Peripheral blood mononuclear cells, milk epithelial cells.	Reference was gross and histopathological lesions and RT activity on tissue culture supernatant.	16 sheep 26 goats	See reference	Allows downstream characterisation (antigenic and genetic) High specificity High sensitivity	Requires fresh tissues or cells Time consuming Requires equipped laboratory and tissue culture skills Expensive	Colitti *et al.* (2019)
Antigen detection ±	Tissues (lung, udder, synovial membrane) Immunohistochemistry	Reference was virus isolation and PCR	Six sheep and six goats	No robust diagnostic validation studies have been published for this purpose	No particular advantage over other direct methods	Requires equipped laboratory and histopathological skills Requires broad reactive Mabs	Grego *et al.* (2002)
PCR +++	Blood (PBMC), tissues	Reference was agreement of AGID or ELISA DSe 94% (combined tissue + PBMC), DSp 100% (Extramiana *et al.*, 2002) Reference was gross and histopathological lesions and virus isolation (Colitti *et al.*, 2019).	115 sheep (Spain): blood, milk cells, target tissues 16 sheep 26 goats	Many validation studies have been carried out using field samples, viral field isolates or strains and a subset of samples from clinically affected sheep or goats. Although from the latter set no robust data are available in each study, there is a consensus to consider the usefulness of PCR-based methods for confirmation of clinical cases (necropsy tissues)	High specificity (especially if combined with amplicon sequencing) Moderate sensitivity	Expensive Require equipped laboratory Some protocols were developed for a specific genotype (i.e. MVV-like)	Extramiana *et al.* (2002) Colitti *et al.* (2019) Alvarez *et al.* (2006) De Regge & Cay (2013) Shah *et al.* (2004a)
AGID ++	Serum p28 and gp135	Reference test was immunoprecipitation DSe 91%, DSp 100% (Knowles *et al.*, 1994) Reference test was agreement of four of six serological tests and real-time PCR	218 goat serum (111 negative and 117 positive) no data available for clinically affected goats 51 positive and	No robust diagnostic validation studies have been published for this purpose	Easy to perform Does not require equipped laboratory High specificity Good sensitivity (clinical stage of infection)	Time consuming Commercially available antigens are derived from few, well culture adapted, strains	Knowles *et al.* (1994)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
			502 negative sheep sera 24 positive and 370 negative goat sera		Low-cost reagents Commercially available		
ELISA ++	Serum Gag and Env antigen (TM antigen)	Reference test was arthritic index, RIPA, WB (Bertoni *et al.*, 1994) Reference was virus isolation	190 Swiss goat (108 clinically affected) 17 goats and three sheep (Italy)	See references	Easy to perform High sensitivity Commercially available Anti-TM antibodies have been specifically associated to clinical stage of infection in arthritic goats	Moderate specificity Genotype specific ELISA less sensitive to detect heterologous infection (especially genotype B antigen for genotype A infection)	Bertoni *et al.* (1994) Colitti *et al.* (2019)

Appendix 5: Caprine arthritis/encephalitis & maedi-visna
Intended purpose of test: prevalence of infection – surveillance

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
PCR ++	Blood (PBMC) Milk (MEC)	Reference was agreement of two serological tests and western blot DSe 75.52%, DSp 100% (Schaer *et al.*, 2022) Reference was agreement with antibody detection on paired samples (Adjadj *et al.*, 2019)	Different European countries 221 sheep and 69 goats 396 sheep 231 goat blood and milk samples (Belgium)	See references	High specificity Moderate sensitivity	Expensive Require equipped laboratory Some protocols were developed for a specific genotype (i.e. MVV-like) Milk PCR less sensitive than blood PCR; Blood PCR less sensitive than antibody detection	Schaer *et al.* (2022) Adjadj *et al.* (2019)
AGID +++	Serum p28 and/or gp135	Reference test was immunoprecipitation DSe 91%, DSp 100% (Knowles *et al.*, 1994) Reference test was agreement of four of six serological tests and real-time PCR Sheep DSe 35-100%, DSp 100% Goat DSe 75-87%, DSp 100% (Michiels *et al.*, 2018)	218 goat serum (111 negative and 117 positive) 51 positive and 502 negative sheep sera 24 positive and 370 negative goat sera	See reference	Easy to perform Does not require equipped laboratory High specificity Low-cost reagents Commercially available	Time consuming Low sensitivity Not suitable for pooled sera or milk testing Commercially available antigens are derived from few, well culture adapted, strains	Knowles *et al.* (1994) Michiels *et al.* (2018)
ELISA +++	Serum, plasma, milk Gag and Env antigen One or more genotype-derived antigen Indirect or competitive	Reference test was immunoprecipitation DSe 100%, DSp 96.4% (Herrmann *et al.*, 2003) Reference test was agreement of four of six serological tests and real-time PCR Sheep DSe up to 100%, DSp up to 99.6% Goat DSe up to 100%, DSp up to 100% (Michiels *et al.*, 2018) Reference test was AGID and western blot	US 200 goat sera (140 negative and 60 positive) Belgium 51 positive and 502 negative sheep sera 24 positive and 370 negative goat sera Different European countries 2336 sheep 196 goat milk samples, 188 sheep milk samples Different European countries 221 sheep and 69 goats	See references	Easy to perform High sensitivity Commercially available Indirect ELISA suitable for pooled sera or milk	Moderate specificity C-ELISA not suitable for pooled sera or milk testing Genotype specific ELISA less sensitive to detect heterologous infection (especially genotype B antigen for genotype A infection)	Herrmann *et al.* (2003) Michiels *et al.* (2018) Saman *et al.* (1999) Brinkhof *et al.* (2010) Schaer *et al.* (2022)

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
		DSe 99.4%, DSp 99.3% (Saman *et al.*, 1999) Reference was antibody detection on paired blood serum samples Goat RSe 98%, RSp 100% (K=0.98) Sheep RSe 97%, RSp 98% (K=0.95 (Brinkhof *et al.*, 2010) Reference was agreement of two serological tests, western blot and nested PCR DSe 92.2%, DSp 98.9% (Schaer *et al.*, 2022)					

Annexe 29. Chapitre 3.8.5. Avortement enzootique des brebis (Chlamydiose ovine) (infection à *Chlamydia abortus*)

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CHAPTER 3.8.5.

ENZOOTIC ABORTION OF EWES (OVINE CHLAMYDIOSIS) (INFECTION WITH *CHLAMYDIA ABORTUS*)

SUMMARY

***Description and importance of the disease**: Ovine chlamydiosis, also known as enzootic abortion of ewes (EAE) or ovine enzootic abortion (OEA), is caused by the bacterium* Chlamydia abortus. *Chlamydial abortion typically occurs in the last 2–3 weeks of pregnancy with the appearance of stillborn lambs and inflamed placentas. However, infection can also result in the delivery of full-term stillborn lambs or weak lambs that do not survive longer than 48 hours. Infected ewes can also give birth to healthy lambs. There are rarely any predictive signs that abortion is going to occur, although behavioural changes and a vulval discharge can be observed in the last 48 hours of pregnancy.*

Diagnosis of enzootic abortion depends on the detection of antigen or nucleic acid of the causative agent in the products of abortion or vaginal excretions of freshly aborted females. A humoral antibody response may be detected following abortion. Goats as well as sheep and, less commonly, cattle, pigs, horses and wild ruminants, can be affected. Chlamydiosis of small ruminants caused by C. abortus *is zoonotic and the organism must be handled with appropriate biosafety precautions. Pregnant women are particularly at risk.*

***Detection and identification of the agent:** The basis for a positive diagnosis of infection with* C. abortus *depends on a history of abortion in sheep or goats (often in late pregnancy), evidence of purulent to necrotising placentitis with vasculitis, and the demonstration of ~~large numbers of~~ the organism in affected placentae (usually present in large numbers) by ~~quantitative~~ polymerase chain reaction (PCR) or antigen tests or in stained smears. The still moist fleece of fetuses or their abomasal content or vaginal swabs of females that have freshly aborted are also useful. It is important to distinguish cotyledonary damage caused by* Toxoplasma gondii *and, in stained smears, to be aware of the morphological similarities between* C. abortus *and* Coxiella burnetii*, the agent of Q fever.*

Chlamydial organisms in tissues and smears can be detected by staining, or antigen-detection methods (immunohistochemistry or immunofluorescence), whereas chlamydial DNA can be detected by PCR-based methods ~~including real-time PCR and DNA microarray~~. Some of these methods are available in commercial kit form.

Chlamydia abortus *can be isolated only in living cells; thus facilities for growth in cell cultures or chicken embryos, with appropriate biohazard containment, are required.*

***Serological tests:** A rise in antibody titre to* C. abortus*, which can be detected by enzyme-linked immunosorbent assay (ELISA), is common after abortion or stillbirth, but this does not occur in every case.* ~~Chlamydia abortus *shares common antigens with other* Chlamydia *species and some Gram-negative bacteria, so that the complement fixation (CF) test or crude ELISAs are not specific and no longer recommended.~~ *Serological screening during the period after parturition helps to identify infected flocks, to which control*

measures can then be applied. Serological tests to differentiate between vaccinated and naturally infected sheep or goats (DIVA tests) are not currently available.

Requirements for vaccines: *Inactivated and live vaccines are available that have been reported to prevent abortion and to reduce excretion. ~~They assist in control of the disease but will not eradicate it.~~*

A. INTRODUCTION

1. Description and impact of the disease

Ovine chlamydiosis (enzootic abortion of ewes [EAE] or ovine enzootic abortion [OEA]) is caused by the bacterium *Chlamydia abortus*. Chlamydial abortion in late pregnancy causes serious economic loss in many sheep-rearing areas of the world, except Australia and New Zealand, particularly where flocks are closely congregated during the parturient period (Aitken & Longbottom, 2007~~; Longbottom & Coulter, 2003~~). Abortion typically occurs in the last 2–3 weeks of pregnancy with the appearance of stillborn lambs and grossly inflamed placentas. Infection can also result in the delivery of full-term stillborn lambs and weak lambs that generally fail to survive beyond 48 hours. It is also not uncommon in multiple births for an infected ewe to produce one dead lamb and one or more weak or healthy lambs. Infection is generally established in a 'clean' (immunologically naïve) flock through the introduction of infected replacements and results in a small number of abortions in the first year, followed by an 'abortion storm' in the second year that can affect around 30% of ewes.

Infected animals show no clinical illness prior to abortion, although behavioural changes and a vulval discharge may be observed in ewes within the last 48 hours of pregnancy. Pathogenesis commences around day 90 of gestation coincident with a phase of rapid fetal growth when chlamydial invasion of placentomes produces a progressively diffuse inflammatory response, thrombotic vasculitis and tissue necrosis. Milder changes occur in the fetal liver and lung and, in cases with severe placental damage, there may be evidence of hypoxic brain damage (Buxton *et al*., 2002; Longbottom *et al*., 2013). Abortion probably results from a combination of impairment of materno-fetal nutrient and gaseous exchange, disruption of hormonal regulation of pregnancy and induced cytokine aggression (Entrican, 2002). Infected ewes shed vast numbers of infective *C. abortus* at the time of abortion or parturition, particularly in the placenta and uterine discharges, thus providing an infection source. Ewes having aborted do not usually abort again from *C. abortus* infection.

Chlamydial abortion also occurs to a similar extent in goats and, less frequently, cattle, pigs, horses and wild ruminants may be affected. In sheep, abortion in late pregnancy with expulsion of necrotic fetal membranes are diagnostic indicators.

2. Nature and classification of the pathogen

As a member of the *Chlamydiaceae* family, *Chlamydia abortus* has a biphasic developmental life cycle with two morphoforms: extracellular infectious elementary bodies (EBs) and intracellular metabolically active reticulate bodies (RBs). Replication takes place within a membrane-bound vacuole. The bacteria obtain essential nutrients by hijacking intracellular organelles and redirecting transport vesicles.

The closest phylogenetically related species of *C. abortus* is the primarily avian pathogen *C. psittaci*, while *C. pecorum* also resides in ruminant hosts. Both should be considered for a differential diagnosis. Recently, the *C. abortus* species has been expanded to include not only genotypes from ruminants but also avian genotypes with unknown pathogenicity that were detected in poultry and wild birds (Zaręba-Marchewka *et al.*, 2021).

~~Taxonomically, the family *Chlamydiaceae* comprises a group of Gram-negative, obligate intracellular bacteria within the single genus *Chlamydia*, which includes eleven species: *C. trachomatis* (humans), *C. suis* (swine), *C. muridarum* (mouse and hamster), *C. psittaci* (avian), *C. felis* (cat), *C. abortus* (sheep, goat and cattle), *C. caviae* (guinea-pig), *C. pecorum* (sheep, cattle and, koala), *C. pneumoniae* (humans), *C. avium* and *C. gallinaceae* (both in birds) (Sachse *et al*., 2015) as well as two candidate species named *Candidatus* Chlamydia ibidis and *Candidatus* Chlamydia sanzinia (Taylor-Brown *et al*., 2016; Vorimore *et al*., 2013).~~

~~Infected ewes shed vast numbers of infective C. abortus at the time of abortion or parturition, particularly in the placenta and uterine discharges, thus providing an infection source. Ewes having aborted do not usually abort again from C. abortus infection. Recent evidence suggests that the proportion of infected ewes is reduced at the subsequent breeding season and only low levels of chlamydial DNA are detected during the periovulation period and at lambing, so that this would not have significant impact on the epidemiology (Gutierrez *et al.*, 2011; Livingstone *et al.*, 2009).~~

3. Zoonotic risk and biosafety requirements

Human infection may be acquired from infected products of abortion or parturition or from carelessly handled laboratory cultures of the organism, with manifestations ranging from subclinical infection to acute influenza-like illness. Cultures and

potentially infected tissues should be handled with appropriate biosafety and containment procedures as determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*). Authenticated cases of human placentitis and abortion caused by *C. abortus* of ovine/caprine origin indicate that pregnant women are at special risk and should not be exposed to sources of infection (Borel & Sachse, 2022; ~~Longbottom & Coulter, 2003; Sillis & Longbottom, 2011~~ Turin *et al.*, 2022).

4. Differential diagnosis

Specific experience is needed to distinguish the diffuse pattern of necrosis and inflammation caused by *C. abortus* infection from necrosis caused by *Toxoplasma gondii,* which is limited to the cotyledons. Differentiation from other infectious causes of abortion, such as brucellosis (see Chapter 3.1.4), ~~coxiellosis~~ Q fever (see Chapter 3.1.18) or other bacterial pathogens (*Campylobacter* ~~[see Chapter 3.10.4], *Listeria* [see Chapter 3.10.5], *Salmonella*~~ [see Chapter 3.10.~~7~~ 3]), can be achieved by conducting further agent-specific diagnostic tests. ~~Recently,~~ Other chlamydial species, such as *C. pecorum* and *C. psittaci,* have been implicated as abortigenic agents in ruminants (Berri *et al*., 2009~~; Lenzko *et al.*, 2011~~).

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of enzootic abortion of ewes and their purpose

Method	Purpose					
	Population freedom from infection[(a)]	Individual animal freedom from infection prior to movement[(b)]	Contribute to eradication policies[(c)]	Confirmation of clinical cases[(d)]	Prevalence of infection – surveillance[(e)]	Immune status in individual animals or populations (post-vaccination)[(f)]
Detection and identification of the agent[(g)]						
Stained smears	–	–	–	+	–	–
Bacterial isolation	–	–	–	++	–	–
Antigen detection by IHC	–	–	–	++	+	–
Conventional PCR	–	–	–	++ ~~±~~	+ ~~±~~	–
Real-time PCR	–	–	-	+++	++	–
Detection of immune response						
~~CFT~~	~~±~~	~~±~~	~~±~~	~~±~~	~~±~~	~~±~~
ELISA	+++	++	+++	++	+++	+++
CFT	±	±	±	±	±	±

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
IHC = immunohistochemisty; PCR = polymerase chain reaction; CFT = complement fixation test;
ELISA = enzyme-linked immunosorbent assay.
[(a)] See Appendix 1 of this chapter for justification table for the scores given to the tests for this purpose.
[(b)] See Appendix 2 of this chapter for justification table for the scores given to the tests for this purpose.
[(c)] See Appendix 3 of this chapter for justification table for the scores given to the tests for this purpose.
[(d)] See Appendix 4 of this chapter for justification table for the scores given to the tests for this purpose.
[(e)] See Appendix 5 of this chapter for justification table for the scores given to the tests for this purpose.
[(f)] See Appendix 6 of this chapter for justification table for the scores given to the tests for this purpose.
[(g)] A combination of agent identification methods applied on the same clinical sample is recommended.

1. Identification of the agent

1.1. Smears

Where the clinical history of the flock and the character of lesions in aborted placentae suggest enzootic abortion, a diagnosis can be attempted by microscopic examination of smears made from affected chorionic villi or adjacent chorion. Smears are stained according to modified Machiavello, Giemsa, *Brucella* differential, or modified Ziehl–Neelsen (Stamp *et al.*, 1950). In positive cases stained by the latter method and examined under a high-power microscope, large numbers of small (300 nm) coccoid elementary bodies are seen individually or in clumps stained red against the blue background of cellular debris. Under dark-ground illumination, the elementary bodies appear pale green. Fluorescent antibody tests (FATs) using a specific antiserum or monoclonal antibody may be used for identification of *C. abortus* in smears. However, polymerase chain reaction (PCR)-based tests are superior to stained or FAT smears regarding sensitivity and specificity and should therefore be applied if available. Stained smears might be useful as an initial screening test, but confirmation by molecular methods is highly recommended ~~due to inferior sensitivity of staining and lack of species specificity~~.

If placental material is not available, smears may be prepared from vaginal swabs of ewes that have aborted within the previous 24 hours, or from the moist fleece of a freshly aborted or stillborn lamb that has not been cleaned by its mother, or from the abomasal content of the aborted or stillborn lamb. In general, such preparations contain fewer organisms than placental smears.

In terms of morphology and staining characteristics, *C. abortus* resembles *Coxiella burnetii* (see chapter 3.1.17 *Q fever*), which, in some circumstances, may provoke abortion and which causes Q fever in humans. Care must be taken to differentiate between these two organisms in cases lacking a good history or evidence of chlamydia-induced placental pathology.

1.2. Isolation of the agent – cell culture

Cell culture is the method of choice for isolation of the organism. The causative agent of ovine chlamydiosis is zoonotic and thus isolation and identification procedures must be carried out with appropriate biosafety and containment procedures as determined by biorisk analysis (see chapter 1.1.4).

Tissue samples, such as cotyledons, placental membranes, fetal lung or liver, or vaginal swabs, that may be subject to delay before laboratory isolation, should be maintained in a suitable transport medium in the interim period. For optimal recovery, such samples should be stored frozen, preferably at –80°C. The most satisfactory medium is sucrose/phosphate/glutamate or SPG medium (sucrose [74.6 g/litre], KH_2PO_4 [0.52 g/litre], K_2HPO_4 [1.25 g/litre], L-glutamic acid [0.92 g/litre]) supplemented with bovine serum albumin – fraction V (1 g/litre), antibiotics (streptomycin and gentamycin are suitable, but not penicillin), and a fungal inhibitor. A tissue-to-medium ratio of 1:10 is commonly employed. Alternatively, approximately 1 g of tissue can be ground with sterile sand in 8 ml of transport medium.

Chlamydia abortus of ovine origin can be isolated in a variety of cell types. McCoy, Buffalo Green Monkey (BGM) or baby hamster kidney (BHK) cells are most commonly used. For confirmatory diagnosis, cultured cell monolayers are suspended in growth medium at a concentration of 2×10^5 cells/ml. Aliquots of 2 ml of the suspension are dispensed into flat-bottomed vials <u>or 24-well plates</u>, each containing a single 12 mm coverslip. Confluent coverslip monolayers are achieved after incubation at 37°C for 24 hours. The growth medium is removed and replaced with 2 ml of test inoculum, which is then centrifuged at 2500–3500 ***g*** for 30–60 minutes onto the coverslip monolayer and incubated at 37°C and 5% CO_2 for 2 hours. The inoculum is removed and replaced with serum-free or cycloheximide (0.5 µg/ml) containing tissue culture medium, and then incubated at 37°C for 2–3 days. The coverslip monolayers are fixed in methanol and stained using Giemsa or Gimenez procedures (~~Arens & Weingarten, 1981;~~ Gimenez, 1964), or are detected by immunofluorescence using species- or genus-specific antibodies (Sachse *et al.,* 2009). After methanol fixation, infected cultures contain basophilic (Giemsa) or eosinophilic (Gimenez) fluorescent intracytoplasmic inclusions. Similar procedures are used in culturing *C. abortus* for antigen preparation.

1.3. Isolation of the agent – chicken embryos

Test samples are prepared as 10% suspensions in nutrient broth containing streptomycin (not penicillin) (200 µg/ml); 0.2 ml of suspension is inoculated into the yolk sac of 6- to 8-day old embryos, which are then further incubated at 37°C. Infected embryos die between 4 and 13 days after inoculation. Smears prepared from their vascularised yolk sac membranes reveal large numbers of elementary bodies.

1.4. Antigen detection in tissue sections

In histopathological sections, antigen detection can be performed using commercially available anti-*Chlamydiaceae* antibodies directed against lipopolysaccharide (LPS) or MOMP (major outer membrane protein) (Borel *et al.*, 2006). Immunohistochemistry is an indispensable tool to show the association of chlamydial agent and pathological lesions in tissues. Genus- or species-specific antibodies in combination with streptavidin–biotin

are used to detect the chlamydial antigen within histological lesions of the placenta or inner organs (mostly lung and liver) of aborted fetuses (Sachse *et al*., 2009).

~~Intracellular chlamydial inclusions can be demonstrated by Giemsa staining of thin (≤4 µm) sections taken from target tissues that have been suitably fixed in fluids such as Bouin or Carnoy. However, unambiguous immunological staining procedures as described above are more suitable.~~

1.5. Detection of DNA by conventional PCR~~,~~ and real-time PCR ~~and DNA microarray~~

Amplification of chlamydial DNA by PCR for verifying the presence of chlamydiae in biological samples is the method of choice because of high sensitivity and specificity of PCR. Conventional PCR protocols for *C. abortus* DNA detection target the 16S–23S rRNA region (Everett & Andersen, 1999) or *pmp* genes (Laroucau *et al*., 2001) and can be combined with restriction fragment length polymorphism (RFLP) analysis for discriminating between amplified DNA sequences originating from *C. abortus, C. psittaci* and *C. pecorum*.

Real-time PCR ~~has become~~ is now the preferred method in diagnostic laboratories due to its high specificity, rapidity, high throughput and ease of standardisation (Sachse *et al.,* 2009). ~~A hierarchical approach is recommended including~~ Different methods are available either with a *Chlamydiaceae*-specific screening PCR based on the sequences of 23S rRNA (Ehricht *et al*., 2006), ~~and, in positive cases,~~ followed by a *C. abortus*-specific PCR assay based on sequences of the outer membrane protein (*omp*A) (Livingstone *et al*., 2009; Pantchev *et al*., 2009) or other *C. abortus*-specific target genes for species identification, or with direct detection of *C. abortus*. Commercial tests for the detection of *C. abortus* are available ~~or DNA microarray hybridisation assays (Sachse *et al*., 2005). Both real-time PCR and DNA microarray have been validated for the direct detection and identification of organisms from clinical samples (Borel *et al.,* 2008; Pantchev *et. al*., 2010)~~.

PCR assays in combination with RFLP analysis or HRM (high resolution melting) analysis have been developed with the aim of differentiating naturally infected ~~from vaccinated~~ animals from animals vaccinated ~~from vaccinated~~ with the commercial live attenuated vaccine (DIVA) (Laroucau *et al*., 2010; Vorimore *et al*., 2012~~; Wheelhouse *et al.,* 2010~~).

Table 2. Examples of validated published real-time PCR assays for screening and ~~specification~~ species identification of *C. abortus*

Reference	**Ehricht *et al.* (2006)**	**Livingstone *et al.* (2009)**	**Pantchev *et al.* (2009)**
Specificity	*Chlamydiaceae*	*C. abortus*	*C. abortus*
Target	23S rRNA	*omp*A	*omp*A
Amplicon size	111 bp	86 bp	82 bp
Primer forward 5'–3'	CTG-AAA-CCA-GTA-GCT-TAT-AAG-CGG-T	GCG-GCA-TTC-AAC-CTC-GTT	GCA-ACT-GAC-ACT-AAG-TCG-GCT-ACA
Primer reverse 5'–3'	ACC-TCG-CCG-TTT-AAC-TTA-ACT-CC	CCT-TGA-GTG-ATG-CCT-ACA-TTG-G	ACA-AGC-ATG-TTC-AAT-CGA-TAA-GAG-A
Probe 5'–3'	FAM-CTC-ATC-ATG-CAA-AAG-GCA-CGC-CG-TAMRA	FAM-TGT-TAA-AGG-ATC-CTC-CAT-AGC-AGC-TGA-TCA-G-TAMRA	FAM-TAA-ATA-CCA-CGA-ATG-GCA-AGT-TGG-TTT-AGC-G-TAMRA
Cycling conditions	95°C/10 minutes 45 × (95°C/15 seconds, 60°C/60 seconds)	95°C/10 minutes 45 × (95°C/15 seconds, 60°C/60 seconds)	95°C/10 minutes 45 × (95°C/15 seconds, 60°C/60 seconds)

2. Serological tests

Sheep and goats are generally tested serologically within 3 months of abortion or parturition. Infection is evident through *C. abortus*-specific antibody response principally during active placental invasion by the pathogen in the last month of gestation and following the bacteraemia that often accompanies abortion. Consequently, serum collected after abortion will reveal an elevated antibody titre resulting from current or previous infection. None of the serological tests available to date can differentiate vaccination titres from those acquired as a result of natural infection (DIVA tests).

2.1. ELISA

Several ELISAs are commercially available for *Chlamydia* diagnosis in ewes (~~overview in~~ O'Neill *et al.*, 2018; Sachse *et al.,* 2009). Care must be taken to select an appropriate ELISA for each diagnostic problem considering different specificities and sensitivities. ~~LPS or EB (elementary body)~~ Crude antigen-based ELISAs cannot differentiate between animals infected with *C. pecorum* and *C. abortus,* but were proven to be more sensitive primary screening tools for EAE compared with the complement fixation (CFT) (Bommana *et al.*, 2019). ~~Specific detection of anti *C. abortus* antibodies can be accomplished by the use of ELISAs based on synthetic peptides of MOMP, recombinant MOMP (Salti Montesanto *et al.*, 1997), or POMP90 (polymorphic outer membrane protein) (Longbottom *et al.*, 2002; Wilson *et al.*, 2009). Most recently, a new indirect ELISA based on POMP90 has been commercialised and shown to be both sensitive and specific for *C. abortus*, in particular in differentiating animals infected with *C. pecorum* (Anon, 2015; Essig & Longbottom, 2015).~~ Specific *C. abortus* ELISAs based on recombinant proteins are commercialised and allow differentiation of animals infected with *C. pecorum* (Wilson *et al.*, 2009).

2.2. Complement fixation test

~~Complement fixation (~~CFT~~)~~ has traditionally been the most widely used procedure for detecting EAE. However, antigenic cross-reactivity between *C. abortus* and *C. pecorum, which is endemic in* ~~small ruminants~~ *many countries*, as well as with some Gram-negative bacteria (e.g. *Acinetobacter*), can give rise to false-positive CFT results. This is because chlamydial antigen contains LPS as an immunodominant component, which is common to all *Chlamydiaceae* species. Furthermore, the CFT has been shown to be less sensitive than alternative tests. Therefore, CFT is no longer recommended as the method of choice for serological diagnosis of EAE, but might be used for herd diagnosis when no alternative tools are available and the limitations mentioned above are taken into consideration.

Antigen is prepared from heavily infected yolk sac membranes obtained from chicken embryos that have been inoculated in the same manner as for isolation of the organism from field material. The preparation of the antigen should be carried out in a biosafety cabinet with the appropriate biosecurity precautions to prevent human infection (see chapter 1.1.4). Chopped and ground membranes are suspended in phosphate buffer, pH 7.6, at the rate of 2 ml per g membrane. After removal of crude debris, the supernatant fluid is centrifuged at 10,000 ***g*** for 1 hour at 4°C, the deposit is resuspended in a small volume of saline, and a smear of this is examined to ensure a high yield of chlamydiae. The suspension is held in a boiling water bath for 20 minutes, or is autoclaved, and sodium azide (0.3%) is added as a preservative. Antigen may also be prepared from cell cultures infected with *C. abortus*. Infected monolayers are suspended in phosphate buffer, pH 7.6, and the cells are disrupted by homogenisation or ultrasonication. Gross debris is removed and subsequent procedures are as for the preparation of antigen from infected yolk sacs. In either case, CFTs with standardised complement and antisera will establish the optimal working dilution for each batch of antigen. Antigen for CF testing of ruminant sera is commercially available.

Samples are tested at twofold dilutions from 1/32 to 1/512. CF titres are expressed as the highest serum dilution giving 50% or less haemolysis: 50% haemolysis is graded 2+, and 0% haemolysis is graded 4+. A titre of 4+ at a dilution of 1/32 or greater is assumed to be positive, whereas a titre of 2+ at a dilution of 1/32 is assumed to be equivocal (Stamp *et al.*, 1950).

C. REQUIREMENTS FOR VACCINES

1. Background

1.1. Rationale and intended use of the product

Currently, two types of vaccine (inactivated and attenuated live vaccines) are available commercially, to be administered intramuscularly or subcutaneously at least 4 weeks before breeding to aid in the prevention of abortion. A multi-component recombinant vaccine against *C. abortus* remains a future goal of chlamydial vaccine research (Longbottom & Livingstone, 2006).

Inactivated vaccines can be prepared from infected yolk sacs or cell cultures (Jones *et al.*, 1995) and incorporate whole organisms or fractions of them (Tan *et al.*, 1990) using the appropriate biosecurity precautions to prevent human infection (see chapter 1.1.4). Operator care should be observed in handling commercial inactivated vaccines that incorporate mineral oil-based adjuvants, as self-injection can result in severe local inflammation and tissue necrosis. The commercial live attenuated vaccine is based on a chemically induced temperature-sensitive mutant strain (strain 1B) of the organism that grows at 35°C but not at 39.5°C, the body temperature of sheep (Rodolakis, 1986). This vaccine is supplied lyophilised and must be reconstituted in diluent immediately before administration. Operator care should be observed in handling and administering this live vaccine, particularly by immunocompromised individuals and pregnant women. Importantly, the live vaccine must not be given to animals

being treated with antibiotics, particularly tetracyclines. Inactivated vaccines are safe for administration during pregnancy, whereas live vaccines cannot be used in pregnant animals.

Both types of vaccine have a role to play in controlling disease, but neither confers absolute protection against challenge or completely reduces the shedding of infective organisms. However, vaccinates exposed to infection do experience significantly lower abortion rates and reduced excretion of chlamydiae for at least two to three lambings after vaccination. It has been claimed that the live vaccine could be an aid to eradication of disease (Nietfeld, 2001). In addition, the live vaccine strain 1B has been detected in the placentas of vaccinated animals that have aborted as a result of OEA, suggesting a possible role for the vaccine in causing disease. and genomic studies proved the lack of attenuation (Longbottom *et al.*, 2018 ~~Wheelhouse *et al.*, 2010~~). However, ~~but despite this~~ the use of the live vaccine remains the most effective method of protecting from the disease (Essig & Longbottom, 2015~~; Stuen & Longbottom, 2011~~).

Vaccine stored under refrigeration (5±3°C) should remain stable for at least 1 year. No firm data are available, but revaccination is recommended every 1–3 years, according to the exposure risk.

2. Outline of production and minimum requirements for conventional inactivated vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

One or more ovine abortion isolates that consistently grow productively in the chosen substrate are suitable, and an early passage of the seed stock can be established. Alternatively, an isolate that has been adapted to the chicken embryo by multiple passage (>100) can be used. Although adaptation to the embryo may diminish the isolate's virulence for sheep, there is no evidence that such change reduces its protective efficacy as an inactivated vaccine.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

Before inoculation of ~~large numbers of~~ embryos or cell cultures, the viability and freedom from contamination (e.g. other pathogens, fungi, mycoplasma, toxins, etc.) of seed stock should be verified. It may be convenient to collect the total harvest in separate manageable lots. In this case, the infectivity of an aliquot of each lot should be separately titrated to ensure that each matches the requirements (see below). Store under refrigeration.

2.2. Method of manufacture

2.2.1. Procedure

For production, cell monolayers or chicken embryos are infected with *C. abortus*. Once the final harvest suspension is obtained, an aliquot is removed for titration of its infectivity. The bulk is treated with formalin to a final concentration of 4%, and stored until sterility tests confirm complete inactivation.

2.2.2. Requirements for substrates and media

The inactivated harvest is centrifuged and resuspended in phosphate buffered saline containing 0.2% formalin to a volume representing a preinactivation infectivity titre of approximately 10^8 infectious units/ml. Usually, the aqueous suspension is blended with an oil adjuvant, either directly or after precipitation by potassium alum ($AlK[SO_4]_2.12\ H_2O$). A preservative, such as 0.01% thiomersal, may also be added.

2.2.3. In-process controls

The main requirements are to ensure adequate growth of *C. abortus*, avoidance of extraneous infection of the culture substrate, completeness of inactivation and biohazard awareness by process workers.

2.2.4. Final product batch tests

Each separate batch of manufactured vaccine should be tested for sterility, safety and potency.

i) Sterility and purity

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9.

ii) Safety

Subcutaneous inoculation into two or more seronegative sheep of twice the standard dose of manufactured vaccine should elicit no systemic reaction, but oil-adjuvant vaccines can cause a nonharmful swelling at the inoculation site.

iii) Batch potency

At present, potency is judged by the occurrence of a serological response in previously unvaccinated sheep given 1 ml of vaccine subcutaneously. Blood samples taken before and 28 days after vaccination are compared. Ultimately, potency has to be determined by a controlled vaccination-challenge study or field performance. No *in-vitro* correlation of protective efficacy has yet been established.

2.3. Requirements for authorisation

2.3.1. Safety requirements

See Chapter 1.1.8 *Principles of veterinary vaccine production*.

2.3.2. Efficacy requirements

See chapter 1.1.8.

2.3.3. Stability

See chapter 1.1.8.

3. Vaccines based on biotechnology

3.1. Vaccines available and their advantages

No biotechnology-based vaccines are currently in use for this disease.

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NB: There are WOAH Reference Laboratories for enzootic abortion of ewes
(please consult the WOAH Web site:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for enzootic abortion of ewes

NB: FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2018.

Appendix 1: Enzootic abortion of ewes
Intended purpose of test: population freedom from infection

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
CFT + Ruminant	Serum antibodies against *C. abortus*	N/A	N/A	See reference	Inexpensive	Low sensitivity and specificity Limited commercial availability of antigen	Vretou *et al.* (2007); Bommana *et al.* (2019)
ELISA +++ Ruminant	Serum antibodies against *C. abortus*	N/A	N/A	See reference	Commercially available High sensitivity Rapid and simple	Different specificities	Vretou *et al.* (2007); Wilson *et al.* (2009); O'Neill *et al.* (2018); Bommana *et al.* (2019)

Appendix 2: Enzootic abortion of ewes
Intended purpose of test: individual animal freedom from infection prior to movement

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
CFT + Ruminant	Serum antibodies against *C. abortus*	N/A	N/A	See reference	Inexpensive	Low sensitivity and specificity Limited commercial availability of antigen	Vretou *et al.* (2007); Bommana *et al.* (2019)
ELISA ++ Ruminant	Serum antibodies against *C. abortus*	N/A	N/A	See reference	Commercially available High sensitivity Rapid and simple	Different specificities	Vretou *et al.* (2007); Wilson *et al.* (2009); O'Neill *et al.* (2018); Bommana *et al.* (2019)

Appendix 3: Enzootic abortion of ewes
Intended purpose of test: contribute to eradication policies

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
CFT + Ruminant	Serum antibodies against *C. abortus*	N/A	N/A	See reference	Inexpensive	Low sensitivity and specificity Limited commercial availability of antigen	Vretou *et al.* (2007); Bommana *et al.* (2019)
ELISA +++ Ruminant	Serum antibodies against *C. abortus*	N/A	N/A	See reference	Commercially available High sensitivity Rapid and simple	Different specificities	Vretou *et al.* (2007); Wilson *et al.* (2009); O'Neill *et al.* (2018); Bommana *et al.* (2019)

Appendix 4: Enzootic abortion of ewes
Intended purpose of test: confirmation of clinical cases

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
Stained smears ± Ruminant	Placenta and fetal tissue *C. abortus* bacteria	N/A	N/A	N/A	Simple and inexpensive	Low to intermediate sensitivity Depends on experienced staff	
Bacterial isolation ++ Ruminant	Placenta and fetal tissue, vaginal swabs *C. abortus* isolate	N/A	N/A	N/A	Gold standard Obtaining isolates for further characterisation	Low to intermediate sensitivity Cumbersome and long lab procedures Depends on experienced staff	
Antigen detection by IHC ++ Ruminant	Placenta and fetal tissue *C. abortus* antigen	N/A	N/A	N/A	Enables association of Chlamydia presence with tissue lesions	Intermediate sensitivity	
Conventional PCR ++ Ruminant	Placenta and fetal tissue, vaginal swabs *C. abortus* DNA	N/A	N/A	See reference	High specificity	Intermediate sensitivity Time consuming	Laroucau *et al.* (2001)
Real-time PCR +++ Ruminant	Placenta and fetal tissue, vaginal swabs *C. abortus* DNA	N/A	N/A	See reference	High sensitivity and specificity Rapid	Needs expensive lab equipment	Pantchev *et al.* (2009); Livingstone *et al.* (2009)
CFT ± Ruminant	Serum Antibodies against C. abortus	N/A	N/A	See reference	Inexpensive	Low sensitivity and specificity Limited commercial availability of antigen	Vretou *et al.* (2007); Bommana *et al.* (2019)
ELISA ++ Ruminant	Serum Antibodies against *C. abortus*	N/A	N/A	See reference	Commercially available High sensitivity Rapid and simple	Different specificities	Vretou *et al.* (2007); Wilson *et al.* (2009); O'Neill *et al.* (2018); Bommana *et al.* (2019)

Appendix 5: Enzootic abortion of ewes
Intended purpose of test: prevalence of infection – surveillance

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
Antigen detection by IHC ± Ruminant	Placenta and fetal tissue *C. abortus* antigen	N/A	N/A	N/A	Enables association of Chlamydia presence with tissue lesions	Intermediate sensitivity	
Conventional PCR ± Ruminant	Placenta and fetal tissue, vaginal swabs *C. abortus* DNA	N/A	N/A	See reference	High specificity	Intermediate sensitivity Time consuming	Laroucau *et al.* (2001)
Real-time PCR ++ Ruminant	Placenta and fetal tissue, vaginal swabs *C. abortus* DNA	N/A	N/A	See reference	High sensitivity and specificity Rapid	Needs expensive laboratory equipment	Pantchev *et al.* (2009); Livingstone *et al.* (2009)
CFT ± Ruminant	Serum Antibodies against C. abortus	N/A	N/A	See reference	Inexpensive	Low sensitivity and specificity Limited commercial availability of antigen	Vretou *et al.* (2007); Bommana *et al.* (2019)
ELISA +++ Ruminant	Serum Antibodies against *C. abortus*	N/A	N/A	See reference	Commercially available High sensitivity Rapid and simple	Different specificities	Vretou *et al.* (2007); Wilson *et al.* (2009); O'Neill *et al.* (2018); Bommana *et al.* (2019)

Appendix 6: Enzootic abortion of ewes
Intended purpose of test: Immune status in individual animals or populations post-vaccination

Test with score and species	Sample type and target analytes	Accuracy	Test population used to measure accuracy	Validation report	Advantages: expert opinion	Disadvantages: expert opinion	References
CFT + Ruminant	Serum antibodies against *C. abortus*	N/A	N/A	See reference	Inexpensive	Low sensitivity and specificity Limited commercial availability of antigen	Vretou *et al.* (2007); Bommana *et al.* (2019)
ELISA +++ Ruminant	Serum antibodies against *C. abortus*	N/A	N/A	See reference	Commercially available High sensitivity Rapid and simple	Different specificities	Vretou *et al.* (2007); Wilson *et al.* (2009); O'Neill *et al.* (2018); Bommana *et al.* (2019)

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CHAPTER 3.8.12.

SHEEP POX AND GOAT POX

* * *

C. REQUIREMENTS FOR VACCINES

1. Background

1.1. Rationale and intended use of the product

Vaccines are an important tool for controlling sheep pox and goat pox (SPP and GTP) spread in endemic regions and they can be helpful for controlling accidental outbreaks in free regions, although such outbreaks can be controlled without the use of vaccines when other control measures such as stamping out, movement restriction, cleaning and disinfection, etc., are strictly implemented and followed.

A variety of live attenuated and inactivated capripoxvirus vaccines has been used to provide protection against SPP and GTP and these were often derived by attenuating or inactivating local pathogenic strains (Davies & Mbugwa, 1985; Ramyar & Hessami, 1967). Nowadays most commercially available vaccines are based on live attenuated SPP and GTP virus strains. Live attenuated vaccines based on different SPPV strains (e.g. RM65, Romania, Bäkirkoy) and GTPV strains (e.g. Mysore, Gorgan, Uttharkashi) have shown to provide good protection in experimental and field conditions when used as homologous vaccines in sheep and goats, respectively (Bhanuprakash *et al.*, 2022; Hamdi *et al.*, 2021; Oreiby *et al.*, 2022; Tuppurainen *et al.*, 2017; Yogisharadhya *et al.*, 2011). These vaccines have a good safety profile with fever and local swelling at the vaccination site as the most often reported side effects. Live attenuated SPPV-based vaccines provide a protection of at least 112 months (Kitching, 2003) and a GTP vaccine based on the Uttharkashi strain was recently reported to provide protection against challenge for up to 52 months (Bhanuprakash *et al.*, 2022).

Also some heterologous live attenuated vaccines based on lumpy skin disease virus (LSDV) strains have been described. The best known example is the Kenyan sheep and goat pox vaccine virus (KSGP) O240 which ~~A variety of attenuated live and inactivated capripoxvirus vaccines has been used to provide protection against sheeppox and goatpox. All strains of capripoxvirus of ovine, caprine or bovine origin examined so far share a major neutralising site, so that animals recovered from infection with one strain are resistant to infection with any other strain (Capstick, 1961). Consequently, it is possible to use a single strain of capripoxvirus to protect both sheep and goats against all field strains of virus, regardless of whether their origin was in Asia or Africa (Kitching *et al.*, 1986; Kitching & Taylor, 1985). However, field evidence suggests some strains are quite host specific and are used only in sheep against SPPV and only in goat against GTPV. A number of strains of capripoxvirus have had widespread use as live vaccines (Davies & Mbugwa, 1985), for example the Romanian and RM-65 strains used mainly in sheep and the Mysore and Gorgan strains used in goats. The real identity of the commonly used Kenyan sheep and goat pox vaccine virus (KSGP) 0240~~ was ~~recently~~ shown to actually be LSDV (Tuppurainen *et al.*, 2014). Vaccines based on this strain should be used carefully as this strain has shown to have residual virulence due to insufficient attenuation (Hamdi *et al.*, 2021). The limited information available furthermore indicates that heterologous vaccines provide less protection than homologous vaccines (Hamdi *et al.*, 2020).

None of the vaccines currently available have DIVA[1] capacity. When vaccination is envisioned, it is important to select vaccines with proven safety and efficacy and perform an independent batch control whereby the identity, purity and potency of the vaccine are evaluated. ~~Virus strain identity and attenuation properties must be ascertained and taken into consideration when selecting vaccine strains for use in cattle, sheep and goats. The protective dose depends on the vaccine strain used. Immunity in sheep and goats against capripox following vaccination with the 0240 strain lasts over a year and the Romanian strain gave protection for at least 30 months.~~

~~Killed vaccines produced from tissue culture contain only the intracellular mature virion form of the virus, and lack the less robust but biologically crucial extracellular enveloped virion form. As a result, the vaccine does not stimulate immunity against the extracellular enveloped virion, resulting in poor protection. Killed capripox vaccines provide, at best, only temporary protection.~~

2. Outline of production and minimum requirements for conventional vaccines

~~General requirements set for the facilities used for the production of vaccines and for the documentation and record keeping throughout the whole manufacturing process are described in Chapter 1.1.8 *Principles of veterinary vaccine production*. The documentation should include the standard operating procedures (SOP) for the method of manufacture and each step for the testing of cells and reagents used in the process, each batches and the final product.~~

~~2.1. Characteristics of the seed~~

~~2.1.1. Biological characteristics~~

~~A strain of capripoxvirus used for vaccine production must be accompanied by a history describing its origin and tissue culture or animal passage. It must be safe to use in all breeds of sheep and goats for which it is intended, including pregnant and young animals. It must be non transmissible, remain attenuated after further tissue culture passage, and provide complete protection against challenge with virulent field strains for a minimum of 1 year. A quantity of master seed vaccine virus should be prepared and stored in order to provide a consistent working seed for regular vaccine production.~~

~~2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)~~

~~Each master seed must be tested to ensure its identity and shown to be free from adventitious viruses, in particular pestiviruses, such as border disease and bovine viral diarrhoea virus, and free from contamination with bacteria, fungi and/or mycoplasmas. The general procedures for sterility or purity tests are described in chapter 1.1.9. The master seed must also be safe and produce no clinical reaction in all breeds of sheep or goats when given by the recommended route and stimulate complete immunity to capripox in all breeds of sheep and goats for at least 1 year. The necessary safety and potency tests are described in Section C.2.2.4 *Final product batch tests*.~~

The production of vaccines, including SPP/GTP vaccines, starts within research and development (R&D) facilities where vaccine candidates are produced and tested in preclinical studies to demonstrate the quality, safety and efficacy of the product.

Minimum requirements for different production stages of veterinary vaccines are available in different chapters of the *Terrestrial Manual*. These are intended to be used in combination with country-specific regulatory requirements for vaccine production and release. Here we outline the most important requirements for the production of live and inactivated homologous SPP/GTP vaccines. Full requirements are available in Chapter 1.1.8 *Principles of veterinary vaccine production*, Chapter 2.3.3 *Minimum requirements for the organisation and management of a vaccine manufacturing facility* and Chapter 2.3.4 *Minimum requirements for the production and quality control of vaccine*, and other regulatory documentation.

2.1. Quality assurance

Facilities for manufacturing SPP/GTP vaccines should operate in line with the concepts of good laboratory practice (GLP) and good manufacturing practice (GMP) to produce high-quality products. Quality risk management and quality control with adequate documentation management, as an integral

[1] DIVA: detection of infection in vaccinated animals

part of the production process, have to be in place. In case some activities of the production process are outsourced, those should also be appropriately defined, recorded and controlled.

The vaccine production process (Outline of Production) should be documented in a series of standard operating procedures (SOPs), or other documents describing the manufacturing of each batch and the final product (including starting materials to be used, manufacturing steps, in-process controls and controls on the final product). Detailed requirements for documentation management in the process of vaccine production are available in Chapter 2.3.3.

A completed Outline of Production is to be enclosed in a vaccine candidate dossier and used for the evaluation of the production process and product by regulatory bodies.

2.2. Process validation

The dossier with the enclosed Outline of Production for the vaccine candidate has to be submitted for regulatory approval, so it can be assessed and approved by the Competent Authority to ensure compliance with local regulatory requirements. Among others, data on quality, safety, and efficacy will be assessed. The procedures necessary to obtain these data are described in the subsequent sections.

National regulatory authorities might also require official control authority re-testing (check testing) of final products and batches in government laboratories or an independent batch quality control by a third party.

3. Requirements for SPP/GTP vaccine candidates and batch production

3.1. Requirements for starting materials

Live attenuated vaccines (LAV) and inactivated vaccines (IV) for SPP/GTP are produced using the system of limited and controlled passages of master seed and working seed virus and cell banks with a specified maximum. This approach aims to prevent possible changes of properties of seed virus and cells that might arise from repeated passaging.

3.1.1. Master seed virus

Master seed virus is a quantity of virus of uniform composition derived from an original isolate, passaged for a documented number of times and distributed into containers at one time and stored adequately to ensure stability (via freezing or lyophilisation). Selection of master seed viruses (MSVs) should ideally be based on their ease of growth in cell culture, virus yield, and in accordance with the regional epidemiological importance. Also, measures to minimise transmissible spongiform encephalopathies (TSE) contamination should be taken into account (see Section C.3.5.1 *Purity tests*).

For each seed strain selected for SPP/GTP vaccine production, the following information should be provided:

- Historical record: geographical origin, animal species from which the virus was recovered, isolation procedure, tissue culture or animal passage history
- Identity: species and strain identification using DNA sequencing
- Purity: the absence of bacteria, fungi, mycoplasma, and other viruses (see Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*)
- Safety (overdose, one/repeated dose tests, and reversion to virulence tests) (see Section C.3.3 *Vaccine safety*)
- Efficacy data, linked to a specified (protective) dose (see Section C.3.4 *Vaccine efficacy*)
- Stability

Each master seed strain selected for production of live attenuated vaccines must remain attenuated after further passage in animals (see Section C.3.3. *Vaccine safety*), produce minimal clinical reaction when given via the recommended route, provide complete protection against challenge with virulent field strains, and is preferably not transmissible.

A quantity of master seed virus should be prepared and stored to be further used for the preparation of working seeds and production seeds. Working seed viruses may be expanded in

one or more (but, limited) cell culture passages from the master seed stock and used to produce vaccine batches. This approach and limitation of seed virus passaging will assist in maintaining uniformity and consistency in production.

3.1.2. Master cell stocks

The production process of SPP/GTP vaccines preferably employs an established master cell stock (MCS) system with defined lowest and highest cell passage to be used to grow the vaccine virus. Primary cells derived from normal tissues can be used in the production process, but the use of primary cells has an inherently higher risk of introducing extraneous agents compared with the use of established (well characterised) cell lines and should be avoided where alternative methods of producing effective vaccines exist. For each MCS, manufacturers should demonstrate:

- MCS identity
- genetic stability by subculturing from the lowest to the highest passage used for production
- stable MCS karyotype with a low level of polyploidy
- freedom from oncogenicity or tumorigenicity by using *in-vivo* studies using the highest cell passage that may be used for production
- purity of MCSs from extraneous bacteria, fungi, mycoplasma, and viruses
- implemented measures to lower TSE contamination risk (see Section C.3.5.1 *Purity tests*).

~~2~~3.2. Method of vaccine manufacturing

~~The method of manufacture should be documented as the Outline of Production.~~

~~2.2.1. Procedure~~

3.2.1. SPP/GTP vaccine batch production

As already mentioned in the first paragraphs of Section C, all steps undertaken in the production of vaccine batches should be described and documented in the Outline of Production. The production of LAV and IV against SPP/GTP starts with the inoculation of the required number of working vials of seed virus reconstituted in an appropriate medium onto a suitable primary cell line grown in suspension or monolayer in the exponential growth phase. At the time highest viral loads are present sonication or repeated freeze-thawing are used to release the intracellular virus from the cytoplasm. The lysate may then be clarified using centrifugation to remove cellular debris with the retention of supernatant.

An aliquot of the virus suspension is titrated to check the virus titre. For LAV, the virus-containing suspension is diluted to attain the dose at which the vaccine candidate will be evaluated or to at least the determined protective dose for approved vaccines and is then mixed with a suitable protectant and transferred to individually labelled bottles or bags for storage at low temperatures such as –80°C, or for freeze–drying. Commercially available vaccines mostly contain a minimum dose of $10^{2.5}$ $TCID_{50}$ (median tissue culture infective dose)/animal. A written record of all the procedures followed must be kept for all vaccine batches.

~~Vaccine seed should be lyophilised and stored in 2 ml vials at –20°C. It may be stored wet at –20°C, but when wet, is more stable at –70°C or lower. The virus should be cultured in primary or secondary LT or LK cells of wool sheep origin for maximum yield. Vero cells may also be used with suitably adapted strains.~~

~~Vaccine batches are produced on fresh monolayers of secondary LT or primary LK cells. A vial of seed virus is reconstituted with GMEM or another appropriate medium and inoculated on to an LT or LK monolayer that has been previously washed with warm PBS, and allowed to adsorb for 15 minutes at 37°C before being overlaid with additional GMEM. After 4–6 days, there will be extensive (80–90%) CPE. The culture should be examined for any evidence of nonspecific CPE, medium cloudiness or change in medium pH. The culture is freeze–thawed three times, the suspension removed and centrifuged at 600 ***g*** for 20 minutes. A second passage may be required to produce sufficient virus for a production batch. Live vaccine may be produced on roller bottles.~~

~~The procedure is repeated and the harvests from individually numbered flasks are each mixed separately with an equal volume of sterile and chilled 5% lactalbumin hydrolysate and 10% sucrose, and transferred to individually numbered bottles for storage at –20°C. Prior to storage, 0.2 ml is removed from each bottle for sterility control. An additional 0.2 ml is removed for virus~~

~~titration; 2 ml pools composed of 0.2 ml samples taken from ten bottles are used. A written record of all the procedures must be kept for all vaccine batches.~~

~~Inactivated vaccines are produced, usually from unattenuated field strains of capripoxvirus, grown in tissue culture as described above, inactivated with 0.03% formaldehyde, and mixed with an equal volume of alhydrogel as adjuvant. Formaldehyde is no longer considered to be a suitable inactivant for certain viral vaccines because its mode of action cannot be guaranteed to be totally effective in inactivating all the live virus. This has not been fully investigated for capripoxvirus.~~

3.2.2. Inactivation process for inactivated SPP/GTP vaccines

Unlike LAV, inactivated vaccines contain inactivated antigens in combination with adjuvants to strengthen the induced immune response after administration. The titres of the virus in preparations intended for inactivation can vary between different manufacturers and production processes, but should ensure the induction of an efficient immune response that provides complete clinical protection.

To monitor the inactivation process and the level of antigen inactivation, samples are taken at regular intervals during inactivation and titrated. Inactivation conditions and the length of initial and repeated exposure should be documented in detail as one or more factors during the process could influence the outcome. The inactivation kinetics should reach a predefined target e.g. one remaining infectious unit per million doses (1×10^{-6} infectious units/dose) as suggested by APHIS (2013). The confirmatory testing of inactivation is performed on each vaccine lot and represents an important part of the inactivation process monitoring. In addition to all the procedures mentioned above, the inactivation procedure and tests demonstrating that antigen inactivation is complete and consistent must additionally be documented in the Outline of Production.

3.3. Vaccine safety

During the vaccine development process, vaccine safety must be evaluated in the target animal (target animal batch safety test – TABST) to demonstrate the safety of the dose intended for regulatory approval. The animals used in the safety testing should be representative (species, age and category [lambs, ewes, ram; kid, does, buck]) for all the animals for which the vaccine is intended. Vaccinated and control groups are appropriately acclimatised, housed and managed in line with animal welfare standards. Animal suffering has to be eliminated or reduced and euthanasia is recommended in moribund animals.

Essential parameters to be evaluated in safety studies are local and systemic reactions to vaccination, including local reactions at the site of administration, fever, and effect on milk production. The effect of the vaccine on reproduction needs to be evaluated where applicable.

A part of the safety evaluation of LAV and IV can be performed during the efficacy trials (see Section C.3.4 *Vaccine efficacy*) by measuring local and systemic responses following vaccination and before challenge.

Guidelines for safety evaluation are provided by the European Medicine Agency (EMEA) in VICH GL44: TABST for LAV and IV (EMEA, 2009). Safety aspects of LAV and IV against SPP/GTP to be evaluated are:

3.3.1. Overdose test for LAV

Local and systemic responses should be measured following an overdose test whereby 10× the maximum vaccine titre is administered. If the maximum vaccine titre is not specified, 10× the minimum vaccine titre can be applied in multiple injection sites. Ideally, the 10× dose is dissolved in the 1× dose volume of the adjuvants or diluent. Generally, eight animals per group should be used (EMEA, 2009).

3.3.2. One dose and repeat dose test

This aims to test the safety of the vaccine dose applied in the vaccination regime intended for regulatory approval. LAV require one dose per year, while inactivated vaccines require a booster dose in addition to the primary dose. The minimal recommended interval between administrations is 14 days.

Generally, eight animals per group should be used unless otherwise justified (EMEA, 2009). For each target species, the most sensitive breed, age and sex proposed on the label should be used. Seronegative animals should be used. In cases where seronegative animals are not reasonably available, alternatives should be justified.

3.3.3. Reversion to virulence tests

The test carried out should be consistent with VICH GL41 (Examination of live veterinary vaccines in target animals for absence of reversion to virulence, 2008[2]). Live attenuated vaccines inherently carry the risk of vaccine virus reverting to virulence when repeated passages in a host species could occur due to shedding and transmission from vaccinated animals to contact animals. LAV vaccines should therefore be tested for non-reversion to virulence by means of passage studies. Vaccine virus (MSV, not the finished vaccine) is inoculated in a group of target animals of susceptible age via the natural route of infection or the route that is most likely to result in infection. The vaccine virus is subsequently recovered from tissues or excretions and is used directly to inoculate a further group of animals. After not less than four passages (see chapter 1.1.8), i.e. use of a total of five groups of animals, the re-isolate must be fully characterised, using the same procedures used to characterise the master seed virus.

3.3.4. Environmental consideration

This includes the evaluation of the ability of LAV vaccines to be shed, to spread and to infect contact target and non-target animals, and to persist in the environment.

3.4. Vaccine efficacy

Data enclosed in the vaccine candidate dossier should support the efficacy of the vaccine in each animal species for each vaccination regimen that is described in the product label recommendation. This includes studies regarding the onset of protection when claims for onset are made and for the duration of immunity. Efficacy studies should be conducted with the vaccine candidate that has been produced at the highest passage level permitted for vaccine production as specified in the Outline of Production.

Efficacy (and safety) should be demonstrated in vaccination–challenge studies using representative (by species, age and category) seronegative healthy animals for which the vaccine is intended and which are tested negative for standard viral pathogens.

An example of a vaccination-challenge test set-up is outlined here. The group numbers mentioned can be varied if statistically justified. Thirteen animals are placed in a high containment large animal unit and are divided into two groups:

- single/repeated dose test group (n=8) – animals inoculated with the vaccine dose and route intended for regulatory approval (in case of an IV, a booster dose should follow primary vaccination after minimum 14 days).
- control group (n=5) – non-vaccinated animals

Throughout the *in-vivo* study, all animals are clinically examined and rectal temperatures recorded. Blood, serum and swab samples are regularly collected and subjected to laboratory testing. On day 21 after the vaccination with a LAV or after the booster vaccination for an IV, the animals in both groups are challenged with a known virulent SPP/GTP strain. The challenge virus solution should be of known titre and tested free from extraneous viruses. Both intradermal or intranasal challenge models have been used (Philips *et al.*, 2024 [in prep]; Wolf *et al.*, 2022). The intranasal challenge model mimics the natural infection route most closely. Depending on the model, doses of challenge virus ranging from 10^3 $TCID_{50}$/animal (intradermal) to 10^6 $TCID_{50}$/animal (intranasal) have been applied.

The clinical response following challenge is recorded over a period of 14 days. No clinical signs should occur in the vaccinates, other than a local reaction at the site of inoculation. At least one animal in the unvaccinated control group should develop the typical clinical signs of disease. Based on experiences from previous experiments, most animals show clinical signs which can be different in intensity, depending on the challenge strain and dose. Subclinical infections are not typically seen.

Clinical and laboratory results will enable assessment of the safety and efficacy of the vaccine candidate and the induced immune responses. Serum samples collected at different time points during the trial can be examined to study seroconversion against selected viral diseases that could have contaminated the vaccine.

[2] https://www.ema.europa.eu/en/documents/scientific-guideline/vich-gl41-target-animal-safety-examination-live-veterinary-vaccines-target-animals-absence-reversion_en.pdf.

3.5. Batch/serial tests before release for distribution

Quality tests on MSV and safety and efficacy tests on vaccine candidates are performed during the evaluation process for new vaccines. Once vaccines are approved to be used in the field, it remains important to verify the quality of each vaccine batch produced. An independent batch quality control assessment may be warranted or requested by national or international regulatory authorities.

3.5.1. Purity test

Purity is defined by the absence of different contaminants (bacteria, fungi, mycoplasma, and other viruses; see full details in chapter 1.1.9) in the vaccine and its associated diluent/adjuvants. Virus isolation and bacterial culture tests can be used to show freedom from live competent replicating microorganisms, but molecular methods are more rapid and sensitive, but positives can be caused by genome fragments and non-infectious microorganisms.

Besides the contaminants mentioned above, manufacturers should demonstrate implemented measures to minimise the risk of TSE contamination in ingredients of animal origin such as:

- all ingredients of animal origin in production facilities are from countries recognised as having the lowest possible risk of bovine spongiform encephalopathy
- tissues or other substances used are themselves recognised as being of low or nil risk of containing TSE agents.

3.5.2. Identity tests

In addition to identity tests performed on the MSV, the identity tests on final batches aim to demonstrate the presence of only the selected capripoxvirus species and strain in the vaccine as indicated in the Outline of Production and the absence of other strains or members of the genus and any other viral contaminant that might arise during the production process. Identity testing could be assured by using appropriate tests (e.g. PCRs, sanger sequencing, NGS).

3.5.3. Potency tests

Standard requirements for potency tests can be found in CFR Title 9 part 113 (2024), in the European Pharmacopoeia (2012), and in this *Terrestrial Manual*.

3.5.3.1. Live vaccines

The potency of LAV against SPP/GTP can be measured by means of virus titration. The virus titre must, as a rule, be sufficiently greater than that shown to be protective in the efficacy test for the vaccine candidate. This will ensure that at any time prior to the expiry date, the titre will be at least equal to the evaluated protective titre. The titres of currently available commercial live attenuated vaccines range between $10^{2.5}$ and 10^{3} infectious units/dose (Tuppurainen *et al.*, 2021).

3.5.3.2. Inactivated vaccines

For inactivated SPP/GTP vaccines, potency tests are performed using vaccination–challenge efficacy studies in animal hosts (see Section C.3.4. *Vaccine efficacy*).

3.5.4. Safety/efficacy

Safety and efficacy testing is undertaken during the evaluation process of the vaccine candidate, and also needs to be performed on a number of vaccine batches until robust data are generated in line with international and national regulations. Afterwards, when using a seed lot system in combination with strict implementation of GMP standards and depending on local regulations, TABST could be waived as described in VICH50 and VICH55, providing the titer has been ascertained using potency testing. Batches or serials are considered satisfactory if local and systemic reactions to vaccination are in line with those described in the dossier of the vaccine candidate and product literature.

3.5.4.1. Field safety/efficacy tests

Field testing of two or more batches should be performed on all animal categories for which the product is indicated before release of the product for general use (see chapter 1.1.8). The aim of these studies is to demonstrate the safety and efficacy of the product under normal field conditions of animal care and use in different geographical locations where different factors may influence product performance. A protocol for safety/efficacy testing in

the field has to be developed with defined observation and recording procedures. However, it is generally more difficult to obtain statistically significant data to demonstrate efficacy under field conditions. Even when properly designed, field efficacy studies may be inconclusive due to uncontrollable outside influences.

3.5.4.2. Duration of Immunity

The duration of immunity (DOI) following vaccination should be demonstrated via challenge or the use of a validated serology test. Efficacy testing at the end of the claimed period of protection should be conducted in each species for which the vaccine is indicated or the manufacturer should indicate that the DOI for that species is not known. Likewise, the manufacturer should demonstrate the effectiveness of the recommended booster regime in line with these guidelines, usually by measuring the magnitude and kinetics of the observed serological response.

4. Post-market studies

4.1. Stability

Stability testing shall be carried out as specified in Annex II of Regulation (EU) 2019/6 and in the Ph. Eur. 0062: Vaccines for veterinary use, on not less than three representative batches providing this mimics the full-scale production described in the application. At the end of shelf-life, sterility has to be re-evaluated using sterility testing or by showing container closure integrity. Multiple batches of the vaccine should be re-titrated periodically throughout the shelf-life period to determine the vaccine stability.

4.2. Post-marketing surveillance

After release of a vaccine, its performance under field conditions should continue to be monitored by competent authorities and by the manufacturer itself. Not all listed adverse effects may show up in the clinical trials performed to assess safety and efficacy of the vaccine candidate due to the limited number of animals used. Post-marketing surveillance studies can also provide information on vaccine efficacy when used in normal practice and husbandry conditions, on duration of induced immunity, on ecotoxicity, etc.

First, a reliable reporting system should be in place to collect consumer complaints and notifications of adverse reactions. Secondly, post-marketing surveillance should be established to investigate whether the reported observations are related to the use of the product and to identify, at the earliest stage, any serious problem that may be encountered from its use and that may affect its future uptake. Vaccinovigilance should be an on-going and integral part of all regulatory programmes for SPPV/GTPV vaccines, especially for live vaccines.

~~**2.2.2. Requirements for substrate and media**~~

~~The specification and source of all ingredients used in the manufacturing procedure should be documented and the freedom from extraneous agents: bacteria, fungi, mycoplasma and any other viruses should be tested. The detailed testing procedure is described in the chapter 1.1.9. The use of antibiotics must meet the requirements of the licensing authority.~~

~~**2.2.3. In-process controls**~~

~~i) Cells~~

~~Cells should be obtained from the testis or kidney of a healthy young lamb from a scrapie-free flock of a wool sheep breed. During cultivation, cells must be observed for any evidence of CPE, and for normal morphology (predominantly fibroblastic). They can usually be passaged successfully up to ten times. When used for vaccine production, uninfected control cultures should be grown in parallel and maintained for at least three additional passages for further observation. They should be checked for the presence of noncytopathic strains of bovine virus diarrhoea or border disease viruses by immunofluorescence or immunoperoxidase techniques. If possible, cells should be prepared and screened prior to vaccine production and stocked in 1–2 ml aliquots containing 2 × 10^7 cells/ml in sterile 10% DMSO (dimethyl sulphoxide) and 90% FBS (fetal bovine serum) solution stored in liquid nitrogen.~~

ii) Serum

Bovine serum used in the growth or maintenance medium must be free from transmissible spongiform encephalopathies (TSEs) and antibody to capripoxvirus, and tested for contamination with pestivirus or any other viruses, extraneous bacteria, mycoplasma or fungi.

iii) Medium

Medium must be tested free from contamination with pestivirus or any other viruses, extraneous bacteria, mycoplasma or fungi.

iv) Virus

Seed virus and final vaccine must be titrated in tissue culture tubes or microtitre plates. Vaccine samples must be examined for the presence of adventitious viruses including cytopathic and noncytopathic strains of pestivirus, and should be mixed with a high-titre capripoxvirus-immune serum that has tested negative for antibody to pestivirus to prevent the vaccine virus itself interfering with the test. The vaccine bulk can be held at –20°C or below until all sterility tests and titrations have been completed, at which time it should be freeze-dried in 1 ml aliquots in vials sufficient for 100 doses. The vaccine harvest diluted with lactalbumin hydrolysate and sucrose should have a minimum titre $\log_{10}$ 4.5 $TCID_{50}$ per ml after freeze drying, equivalent to a field dose of $\log_{10}$ 2.5 $TCID_{50}$. A further titration is carried out on five randomly chosen vials of the freeze-dried preparation to confirm the titre.

2.2.4. Final product batch tests

i) Sterility/purity

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9.

ii) Safety

The safety studies should be demonstrated by statistically valid vaccination studies using seronegative young sheep and goats of known susceptibility to capripox virus. The procedure described is suitable for vaccine strains such as 0240 that are equally immunogenic in both sheep and goats. The choice of target animal should be adapted for strains with a more restricted host preference.

iii) Potency

Potency tests must be undertaken if the minimum immunising dose of the virus strain is not known. This is usually carried out by comparing the titre of a virulent challenge virus on the flanks of vaccinated and control animals. Following vaccination, the flanks of at least three animals and three controls are shaved of wool or hair. $\log_{10}$ dilutions of the challenge virus are prepared in sterile PBS and six dilutions are inoculated intradermally (0.1 ml per inoculum) along the length of the flank; four replicates of each dilution are inoculated down the flank. An oedematous swelling will develop at possibly all 24 inoculation sites on the control animals, although preferably there will be little or no reaction at the four sites of the most dilute inocula. The vaccinated animals should develop an initial hypersensitivity reaction at sites of inoculation within 24 hours, which should quickly subside. Small areas of necrosis may develop at the inoculation site of the most concentrated challenge virus. The macule/papule is measured at between 8 and 10 days post-challenge. The titre of the challenge virus is calculated for the vaccinated and control animals; a difference of $\log_{10}$ titre > 2.5 is taken as evidence of protection.

2.3. Requirements for authorisation

2.3.1. Safety requirements

i) Target and non-target animal safety

The vaccine must be safe to use in all breeds of sheep and goats for which it is intended, including young and pregnant animals. It must also be non-transmissible, remain attenuated after further tissue culture passage.

Safety tests should be carried out on the final product of each batch as described in Section C.2.2.4.

The safety of the vaccine in non-target animals must have been demonstrated using mice and guinea-pigs as described in Section C.2.2.4. There should be no evidence of pathology caused by the vaccine.

ii) Reversion to virulence for attenuated/live vaccines

The selected final vaccine should not revert to virulence during a further passages in target animals.

iii) Environmental consideration

Attenuated vaccine should not be able to perpetuate autonomously in cattle, sheep or goat populations. Vaccines using the 0240 strain should not be used in *Bos taurus* breeds. Strains of capripoxvirus are not a hazard to human health. There are no precautions other than those described above for sterility and freedom from adventitious agents.

2.3.2. Efficacy requirements

i) For animal production

The efficacy of the vaccine must be demonstrated in vaccination challenge experiment under laboratory conditions. As described in Section C.2.2.4.

Once the potency of the particular strain being used for vaccine production has been determined in terms of minimum dose required to provide immunity, it is not necessary to repeat this on the final product of each batch, provided the titre of virus present has been ascertained.

ii) For control and eradication

Vaccination is the only effective way to control the sheep pox and goat pox outbreaks in endemic countries. Unfortunately, currently no marker vaccines allowing the differentiation of infected from vaccinated animals are available.

Immunity to virulent field virus following vaccination of sheep or goats with the 0240 strain lasts over 1 year, and protection against generalised infection following intradermal challenge lasts at least 3 years and is effective lifelong. The duration of immunity produced by other vaccine strains should be ascertained in both sheep and goats by undertaking controlled trials in an environment in which there is no possibility of field strains of capripoxvirus confusing the results. The inactivated vaccines provide immunity for less than 1 year, and for the reasons given at the beginning of this section, may not give immunity to the form of capripoxvirus usually associated with natural transmission.

2.3.3. Stability

All vaccines are initially given a shelf life of 24 months before expiry. Real time stability studies are then conducted to confirm the appropriateness of the expiry date. Multiple batches of the vaccine should be re-titrated periodically throughout the shelf life to determine the vaccine variability.

Properly freeze-dried preparations of capripox vaccine, particularly those that include a protectant, such as sucrose and lactalbumin hydrolysate, are stable for over 25 years when stored at –20°C and for 2–4 years when stored at 4°C. There is evidence that they are stable at higher temperatures, but no long-term controlled experiments have been reported. The inactivated vaccines must be stored at 4°C, and their shelf life is usually given as 1 year.

No preservatives other than a protectant, such as sucrose and lactalbumin hydrolysate, are required for the freeze-dried preparation.

3. Vaccines based on biotechnology

3.1. Vaccines available and their advantages

Currently, no recombinant vaccines for capripoxviruses are commercially available. However, a new generation of capripox vaccines is being developed that uses the capripoxvirus genome as a vector for the genes of other ruminant pathogens such as peste des petits ruminants (PPR) virus (Berhe *et al.*, 2003; Tuppurainen *et al.*, 2014).

3.2. Special requirements for biotechological vaccines, if any

Not applicable.

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NB: There are WOAH Reference Laboratories for sheep pox and goat pox (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for sheep pox and goat pox

NB: FIRST ADOPTED IN 1989. MOST RECENT UPDATES ADOPTED IN 2024.

RÉUNION DE LA COMMISSION DES NORMES BIOLOGIQUES
Paris, 9–13 septembre 2024

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CHAPTER 3.9.2.

CLASSICAL SWINE FEVER (INFECTION WITH CLASSICAL SWINE FEVER VIRUS)

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B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of classical swine fever and their purpose

Method	Purpose					
	Population freedom from infection[(b) (c) (e) (f)]	Individual animal freedom from infection prior to movement[(a) (b) (c) (e) (f)]	Contribute to eradication policies[(c) (d) (e) (f)]	Confirmation of clinical cases[(a) (b) (c) (d) (f)]	Prevalence of infection – surveillance[(b) (e) (f)]	Immune status in individual animals or populations post-vaccination[(e) (f)]
Detection of the agent[(g)]						
Virus isolation	–	+	–	+++	–	–
RT-PCR	+	+++	++	+++	++	–
ELISA (antigen)	++	+	+	+	–	–
FAT	–	–	+	+	–	–
Detection of immune response						
ELISA (antibody)	+++	++~~+~~	+++	–	+++	+++
VN (FAVN or NPLA)	+	+++[(h)]	++	++	+++	+++

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
RT-PCR = reverse-transcriptase polymerase chain reaction;
ELISA = enzyme-linked immunosorbent assay; FAT = fluorescent antibody test;
VN = virus neutralisation; FAVN = fluorescent antibody virus neutralisation; NPLA = neutralising peroxidase-linked assay.
[(a)] See Appendix 1 of this chapter for justification table for the scores given to the tests for this purpose.
[(b)] See Appendix 2 of this chapter for justification table for the scores given to the tests for this purpose.
[(c)] See Appendix 3 of this chapter for justification table for the scores given to the tests for this purpose.
[(d)] See Appendix 4 of this chapter for justification table for the scores given to the tests for this purpose.
[(e)] See Appendix 5 of this chapter for justification table for the scores given to the tests for this purpose.
[(f)] See Appendix 6 of this chapter for justification table for the scores given to the tests for this purpose.
[(g)] A combination of agent detection methods applied on the same clinical sample is recommended.
[(h)] Specifically for confirmation of antibody ELISA positive cases.

* * *

C. REQUIREMENTS FOR VACCINES

1. Background

CSF has severe clinical and socio-economic consequences for pig production worldwide. The control of the disease is usually a national responsibility~~, and~~. While in many countries vaccination is carried out as part of a national control programme under the auspices of the veterinary authority, in other countries prophylactic vaccination has been banned for several decades (e.g. USA, Canada, countries of the European Union) or compulsory vaccination against CSF has been stopped more recently. It is generally accepted that the goal of CSF eradication is to be free of CSF without vaccination. Nevertheless, the availability of safe and efficacious vaccines remains an important tool for the control and early steps of eradication of CSF.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements. Varying additional requirements relating to quality, safety and efficacy will apply in particular countries or regions for manufacturers to obtain regulatory approval ~~an authorisation or licence~~ for a veterinary vaccine.

Wherever live CSFV is handled, the appropriate biosecurity procedures and practices should be used. The CSF vaccine production facility should meet the requirements for containment outlined in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*.

The optimal CSF vaccine should have the following general characteristics: short- and long-term safety for target and non-target species (especially for oral vaccines), stability, rapid induction of a stable, preferably life-long immunity, efficacy against all strains and genotypes of field viruses, full clinical protection and protection against carrier states, prevention of horizontal and vertical transmission. Furthermore, marker vaccines will have to be accompanied by reliable discriminatory tests. Manufacture should provide for consistency of production and validation.

Modified live vaccines (MLVs) based on several attenuated virus strains (e.g. C-strain, Thiverval, PAV-250, GPE-, K-strain) are most widely used, and many of them have proven to be both safe and efficacious. In addition, E2 subunit vaccines produced in baculovirus or other systems are available. Inactivated whole virus vaccines are presently not available.

Information regarding these vaccines can be found in review publications (~~Blome *et al.*, 2017b;~~ Ganges *et al.,* 2020; Postel *et al.,* 2018).

Early CSF marker vaccines were based on recombinant CSFV glycoprotein E2 expressed in insect cells. Pigs vaccinated with these subunit vaccines do not produce antibodies against other CSFV proteins (e.g. E^{ms}), allowing reliable differentiation between vaccinated and infected animals (DIVA). These vaccines are safe and have the advantage of greater thermal stability. However, ~~compared with animals vaccinated with live vaccines, two major disadvantages of these subunit vaccines were~~ a significantly delayed onset of immunity and ~~two inoculations were required~~ a requirement for an additional inoculation were major drawbacks compared with live vaccines. While a single administration of the E2 subunit vaccine was able to prevent clinical signs and mortality, along with reduced transmission following challenge infection, it did not prevent transplacental transmission. Some novel vaccine candidates have shown promising improvements compared with early generation E2 subunit vaccines, including clinical and virological protection as early as 7 days after a single dose vaccination. A comprehensive immunisation policy based on commercial E2 vaccine, implemented in China (People's Rep. of) since 2018, includes vaccination of breeding farms for the purpose of eradication and fattening pigs (Gong *et al.,* 2019; Zhou *et al.,* 2021). Nevertheless, to provide solid protection against transplacental transmission, two doses of vaccine are required (Ganges *et al.,* 2020).

New generations of marker vaccines ~~are also being~~ have been developed and a chimeric pestivirus encompassing the E2 from CSFV in a ruminant pestivirus backbone has been granted regulatory approval for use in domestic pigs by the European Medicines Agency (EMA) and has also been authorised for use in a USA vaccine bank.

This vaccine, CP7_E2alf, possesses many characteristics of an optimal vaccine including: genetic stability; ~~innocuousness~~ safety for target and relevant non-target species; absence of vaccine virus transmission to in-contact animals or shedding through urine, faeces or semen; rapid onset of protection following a single intramuscular injection; duration of immunity for at least 6 months; and protection against different CSFV genotypes (Blome *et al.,* 2017a). The CP7_E2alf vaccine capacity to prevent transplacental transmission after a single vaccine dose has been proven in pregnant sows against a moderately virulent CSFV strain (Henke *et al.,* 2018). The protection from transplacental transmission against ~~CSFV~~ highly virulent strains remains to be evaluated.

Different DIVA strategies are available by using serological methods (e.g. ELISA) or genome detection methods (e.g. RT-PCR). An opinion published by the European Food Safety Authority (EFSA, 2008) ~~demonstrated~~ proposed that the combination of a vaccine that uses the C-strain with RT-PCR to detect viral genome in slaughtered animals can be successfully used in a vaccination-to-live strategy (Zhao *et al*, 2008). See also Section B.1.1.4 of this chapter.

For the approved chimeric vaccine CP7_E2alf, the use of a ruminant pestivirus backbone may provide~~s~~ serological differentiation by the use of CSFV E^{rns}-ELISAs (Meyer *et al.,* 2017; Pannhorst *et al.,* 2015) or by a recently published system for detecting CSFV E^{rns} IgG antibody in swine serum and oral fluid (Panyasing *et al.,* 2023). However, evaluation of two such assays for DIVA capability revealed that test specificity may be compromised by infection with ruminant pestiviruses, resulting in an induction of cross-reactive antibodies (Meyer *et al.,* 2017; 2018~~;~~ ~~Pannhorst *et al.,* 2015~~). There is therefore still room for improvement with respect to marker vaccines and their companion diagnostic tests. Information regarding the current state-of-the-art with respect to CSFV vaccine candidates was reviewed by ~~Blome *et al.*, 2017b~~ Coronado *et al.* (2021) and Ganges *et al.* (2020).

CSF vaccines are used in different epidemiological settings and situations. Most countries free of the disease have adopted a control strategy without prophylactic vaccination but established legal provisions for emergency vaccination scenarios. In endemic situations, vaccination is mainly used to lower the impact of the disease or as a first step in an eradication programme. During epidemic incidents in previously free areas, emergency vaccination can be an additional tool to control and eradicate the disease and DIVA vaccines promise a valuable additional tool in this context.

Moreover, oral vaccination of affected wild boar populations may be considered. These different scenarios and the different systems of pig production may require different vaccine characteristics or may influence the focus of requirements.

2. Outline of production and minimum requirements for conventional live vaccines and modified live marker vaccines

Conventional live vaccines based on attenuated CSFV strains have a high level of safety and efficacy. However, they do not provide a possibility to differentiate infected from vaccinated animals. In contrast, live marker vaccines based on genetic technologies follow the DIVA strategy based on specific accompanying differential diagnostic tests. Several studies reviewed in detail by Coronado *et al.* (2021) and Ganges *et al.* (2020) proposed live marker vaccine candidates. The most frequently explored strategy is based on chimeric pestiviruses, in which one or more glycoprotein-encoding regions are exchanged between a CSFV strain and a heterologous pestivirus. Another strategy is the introduction of genetic marker(s) into CSFV strains.

This chapter provides the minimum requirements for live vaccines, both conventional and modified live marker vaccines, with special requirements for all live marker vaccines added where necessary. A detailed description of each production stage should be recorded in the manufacturing process documentation (see Chapter 1.1.8, Section *Documentation of the manufacturing process and record keeping* and Chapter 2.3.3 *Minimum requirements for the organisation and management of a vaccine manufacturing facility*, Section 3. *Rules governing documentation*).

2.1. Characteristics of the seed

~~CSF vaccines prepared in live animals do not follow WOAH animal welfare principles. Their production and use should be discontinued.~~

2.1.1. Biological characteristics of the master seed

i) Conventional MLVs

Conventional MLVs are produced from CSFV strains that have been attenuated by serial passages either in cell cultures or in a suitable host species not belonging to the family *Suidae*. Production is carried out in cell cultures, based on a seed-lot system. CSF vaccines prepared in live animals do not follow WOAH animal welfare principles. Their production and use is no longer recommended. Master seed viruses (MSVs) for MLVs should be selected and produced, based on their ease of growth in cell culture, virus yield and stability.

The exact source of the underlying CSFV isolate, ~~its sequence,~~ and the passage history must be recorded. Including the full genome sequence in the manufacturing process documentation is encouraged.

ii) Live marker vaccines

The origin and characteristics of the starting materials, including the parental recipient and donor virus strains and added or deleted sequences, should be recorded.

For vaccines containing live genetically modified viruses, the final construct is considered a master seed virus (MSV). The data on genetic modifications, and characteristics of the MSV, as well as details of the construction procedure, should be recorded in the manufacturing process documentation. Biological properties as well as genetic stability of the final construct should be demonstrated. Additionally, the risk of changing the tropism or virulence due to the genetic alterations shall be addressed.

For vaccines designed to allow DIVA, sufficient data on the accompanying diagnostic test should be provided to allow adequate assessment of the DIVA properties.

2.1.2. Quality criteria

Only MSVs that have been established as sterile, pure (free of extraneous agents as described in Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use* and those listed by the appropriate ~~licensing~~ regulatory approval authorities) and immunogenic, should be used for vaccine virus (working seed viruses and vaccine batches) production. Live vaccines must be shown not to cause disease or other adverse effects in target animals injected in accordance with chapter 1.1.8 (section on *Safety tests* [for live attenuated MSVs]).

Identity of the MSV has to be confirmed using appropriate methods ensuring unequivocal identification of the strain (e.g. through the use of specific MAbs or vaccine strain-specific genome detection methods).

2.1.3. Validation as vaccine strain

The vaccine derived from the MSV must be shown to be satisfactory with respect to safety and efficacy.

Even if pigs are not known for susceptibility to transmissible spongiform encephalopathy (TSE) agents, consideration should also be given to minimising the risk of transmission by ensuring that TSE risk materials are not used as the source of the virus or in any of the media used in virus propagation.

The vaccine virus in the final product should generally not differ by more than five passages from the master seed lot. The commercial vaccine should be produced in batches in lyophilised form as a homogeneous product.

2.2. Method of manufacture

2.2.1. Procedure

The virus is used to infect an established cell line. Such cell culture should be proven to be free from contaminating microorganisms and shall comply with the requirements in chapter 1.1.8.

Regardless of the production method, the substrate should be harvested under aseptic conditions and may be subjected to appropriate methods to release cell-associated virus (e.g. freeze–thaw cycles). The harvest can be further processed by filtration and other methods. A stabiliser may be added as appropriate. The vaccine is homogenised before lyophilisation to ensure a uniform batch/serial.

2.2.2. Requirements for ingredients

All ingredients used for vaccine production should be in line with requirements in chapter 1.1.8.

2.2.3. In-process controls

In-process controls will depend on the protocol of production: they include virus titration of bulk antigen and sterility tests.

2.2.4. Final product batch/serial test

i) Sterility

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9.

ii) Identity

Appropriate methods (specific antibodies or specific genome detection methods) should be used for confirmation of the identity of the vaccine virus.

iii) Residual moisture

The level of moisture contained in desiccated products should be measured as described in chapter 1.1.8.

iv) Safety

Batch safety testing is to be performed unless consistent safety of the product is demonstrated and approved in the ~~registration~~ regulatory approval dossier and the production process is approved for consistency in accordance with the standard requirements referred to in chapter 1.1.8.

This final product batch safety test is conducted to detect any abnormal local or systemic adverse reactions.

For batch/serial safety testing, use two healthy piglets, 6–10 weeks old, that do not have antibodies against pestiviruses. Administer to each piglet by a recommended route a tenfold dose of the vaccine. Observe the piglets daily for at least 14 days. The vaccine complies with the test if no piglet shows notable signs of disease or dies from causes attributable to the vaccine.

v) Batch/serial potency

Virus titration is a reliable indicator of vaccine potency once a relationship has been established between the level of protection conferred by the vaccine in pigs and titre of the modified live vaccine *in vitro*. Batch/serial potency tests are required for each batch of the product prior to release. Appropriate release titre should correlate with the results of challenge efficacy studies, and as a rule it should be sufficiently greater to ensure efficiency of the vaccine throughout its shelf-life.

In the absence of a demonstrated correlation between the virus titre and protection, an efficacy test will be necessary (see Section C.2.3.3).

2.3. Requirements for regulatory approval ~~authorisation/registration/licensing~~

2.3.1. Manufacturing process

For regulatory approval of a vaccine, all relevant details concerning preparation of MSV, manufacture of the vaccine and quality control testing (see Sections C.2.1 and C.2.2) should be submitted to the authorities. This information shall be provided from three consecutive vaccine batches originating from the same MSV, with a volume not less than 1/3 of the typical industrial batch volume.

The in-process controls are part of the manufacturing process.

2.3.2. Safety requirements

For the purpose of gaining regulatory approval, the following safety tests should be performed satisfactorily.

Vaccines should be tested for any pathogenic effects on healthy pigs, and in sows to evaluate the safety in pregnant animals and their offspring.

i) Safety in young animals

Carry out the test for each recommended route of application using in each case piglets not older than the minimum age recommended for vaccination. Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine.

Use no fewer than eight piglets of 6–8 weeks of age that do not have antibodies against pestiviruses. Administer to each piglet a quantity of the vaccine virus equivalent to not less than ten times the maximum virus titre likely to be contained in 1 dose of the vaccine. Observe the piglets daily for at least 14 days. The body temperature of each vaccinated piglet is measured on at least the 3 days preceding administration of the vaccine, at the time

of administration, 4 hours after and then daily for at least 14 days. The vaccine complies with the test if the average body temperature increase for all piglets does not exceed 1.50°C, no piglet shows a temperature rise greater than 1.50°C for a period exceeding 3 consecutive days, and no piglet shows notable signs of disease or dies from causes attributable to the vaccine.

Blood samples are taken at 7 days after vaccination and tested for leukopenia. The average white blood cell (WBC) count should exceed 7×10^6 cells/ml.

In addition, the vaccines in their commercial presentation should be tested for safety in the field (see chapter 1.1.8, section on *Field tests* [*safety and efficacy*]).

ii) Safety test in pregnant sows and test for transplacental transmission

Carry out the test with vaccination by a recommended route using no fewer than eight healthy sows or gilts of the same age and origin, between the 55th and 70th days of gestation, that do not have antibodies against pestiviruses. Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine.

Administer to each sow a quantity of the vaccine virus equivalent to not less than the maximum virus titre likely to be contained in 1 dose of the vaccine. Clinical observation of animals is carried out daily until farrowing. Blood samples should be taken from newborn piglets before ingestion of colostrum.

The test is invalid if the vaccinated sows do not seroconvert before farrowing. The vaccine virus complies with the test if no abnormalities in the gestation or in the piglets are noted. No sow or gilt shows notable signs of disease or dies from causes attributable to the vaccine.

Vaccine virus or antibodies against CSFV must not be present in blood samples from newborn piglets.

iii) Non-transmissibility

Keep together for the test no fewer than 12 healthy piglets, 6–10 weeks old and of the same origin, that do not have antibodies against pestiviruses. When using the MSV to produce vaccines, the number of passages should be minimised. ~~Use vaccine virus at the least attenuated passage level that will be present between the master seed lot and a batch of the vaccine.~~ Administer by a recommended route to no fewer than six piglets a quantity of the vaccine virus equivalent to not less than the maximum virus titre likely to be contained in 1 dose of the vaccine.

Maintain no fewer than six piglets as contact controls. The mixing of vaccinated piglets and contact piglets is done 24 hours after vaccination.

After 45 days, kill all piglets humanely. Carry out appropriate tests on the piglets to detect antibodies against CSFV and on the control piglets to detect CSFV in the tonsils. The vaccine complies with the test if antibodies are found in all vaccinated piglets and if no antibodies and no virus are found in the control piglets.

iv) Reversion to virulence

The test carried out should be consistent with VICH GL41 (Examination of live veterinary vaccines in target animals for absence of reversion to virulence, 2008[1]).

The test for increase in virulence consists of the administration of the vaccine virus from the master seed lot or one or two passages above to piglets that do not have antibodies against pestiviruses.

This protocol is repeated five times with at least two healthy 6–10 weeks old piglets of the same age and origin per each passage.

Administer tested virus to each of two ~~healthy~~ piglets ~~free of antibodies to pestiviruses, 6–10 weeks old,~~ by a recommended route, a quantity of the vaccine virus equivalent to not less than the maximum virus titre likely to be contained in 1 dose of the vaccine. Collect an appropriate quantity of blood from each piglet daily between day 2 and day 7 after administration of the vaccine virus, and pool the samples taken on the same day. Then kill

[1] https://www.ema.europa.eu/en/documents/scientific-guideline/vich-gl41-target-animal-safety-examination-live-veterinary-vaccines-target-animals-absence-reversion_en.pdf.

the piglets and take the tonsils of both of them, pool the tonsils and prepare a 10% suspension in PBS, pH 7.2 kept at 4°C or at –70°C for longer storage. The euthanasia may be performed earlier than 7 days post-vaccination if the existing data on the dynamics of infection and virus distribution support this approach. If the optimal euthanasia time point needs to be determined, it is suggested to include more piglets into the first passage and kill them at different time points (between 4 and 7 days post-inoculation). Based on the maximal level of the virus in tonsils, an optimal time point for euthanasia is determined and adjusted accordingly for further passages.

~~At the same time, the presence of CSF antigens is confirmed at each passage. Blood and pooled tonsillar tissue are used to inoculate two further pigs of the same age and origin by the same route as before.~~

~~Administer 2 ml of the pooled material (blood and tonsillar tissue) with the highest virus titre by a recommended route~~ The presence of CSFV antigen is quantified in daily blood pools and pooled tonsils from each passage. Then, 2 ml of the sample consisting of tonsils and pooled blood from the day with the highest virus level is administered to each of two other piglets~~ of the same age and origin~~ by a recommended route. If no virus is found, repeat the administration once again with the same material and another ~~two~~ batch of piglets. If no virus is found at this point, end the process here. If, however, virus is found, carry out a second series of passages by administering 2 ml of positive material by a recommended route to each of two other piglets of the same age and origin.

Carry out this passage operation no fewer than four times (in total five groups from the start of the test should be vaccinated), verifying the presence of the virus at each passage in blood and tonsils. Care must be taken to avoid contamination by the virus from previous passages.

The vaccine virus complies with the test if no indication of increasing virulence (monitored by clinical observations) of the maximally passaged virus compared with the unpassaged virus is observed.

If virus is not recovered at any passage level in the first and second series of passages, the vaccine virus also complies with the test.

v) Special requirements for live marker vaccines

Before the release of veterinary products based on genetically modified microorganisms it is necessary to perform a risk assessment to evaluate the impact on the human and animal environment. Special attention of the risk assessment should be paid to potential environmental consequences, the characteristics of the vaccine organism and its tissue tropism, human health risk, animal health risk for both target and non-target animals, persistence in the environment and increase in virulence (see Chapter 1.1.8 Sections 7.1.2 *Increase in virulence tests* and 7.1.3 *Assessing risk to the environment*).

2.3.3. Efficacy requirements

i) Protective dose

Vaccine efficacy is estimated in vaccinated animals directly, by evaluating their resistance to live virus challenge and is expressed by the number of 50% protective doses (PD_{50}) for pigs contained in the vaccine dose.

The test consists of a vaccination/challenge trial in piglets aged 6–10 weeks using different dilutions of the vaccine in question and five piglets per dilution. An additional group of two piglets of the same age and origin are used as controls. All animals have to be free from antibodies against pestiviruses prior to the trial. Each group of piglets, except the control group, is vaccinated with an appropriate dilution of the reconstituted vaccine (e.g. 1/40 and 1/160 using a suitable buffer solution.

Fourteen days after the single injection of vaccine, challenge the piglets by a suitable route with a dose of a virulent strain of CSFV that kills at least 50% of the non-vaccinated piglets in less than 21 days. Observe the piglets for 21 days and record the body temperature 3 days before challenge and daily after challenge for 21 days. The PD_{50} content of the vaccine is calculated from the number of animals protected in each group using the Spearman-Kärber method.

The test is invalid if less than 50% of the control piglets display typical signs of serious infection with CSFV, and die, and if less than 100% of the control piglets show clinical signs of disease within the 21 days following challenge.

The vaccine complies with the test if the minimum dose corresponds to not less than 100 PD_{50}.

In addition, protection from virus replication conferred by the vaccine should be evaluated in the blood and tissues from vaccinated animals using the protocols previously described in Sections B.1.1.3 and B.1.1.4.

ii) Protection against transplacental infection

Use eight sows that do not have antibodies against pestiviruses, randomly allocated to either the vaccine group (n = 6) or the control group (n = 2).

Between the 34th and 49th day of gestation, all sows allocated to the vaccine group are vaccinated once with 1 dose of vaccine containing not more than the minimum titre stated on the label. Three weeks after vaccination, all eight sows are challenged by a suitable route with a dose of virulent strain of CSFV that would be sufficient to kill at least 50% of non-vaccinated piglets in less than 21 days.

Just before farrowing, the sows are killed humanely and their fetuses are examined for CSFV. Serum samples from sows and fetuses are tested for the presence of antibodies against CSFV. Isolation of CSFV is carried out from blood of the sows (collected 7 and 9 days after challenge and at euthanasia), and from homogenised organ material (tonsils, spleen, kidneys, lymph nodes) of the fetuses.

The real-time RT-PCR assay can be performed following the methodology described in Section B.1.1.4., although always in correlation with the virus isolation test. The test is valid if virus is found in at least 50% of the fetuses from the control sows (excluding mummified fetuses).

The vaccine complies with the test if no virus is found in the blood of vaccinated sows and in fetuses from the vaccinated sows, and antibodies against CSFV should not be found in the serum of the fetuses from the vaccinated sows.

In addition, where appropriate, the vaccines should be tested for efficacy in the field (see chapter 1.1.8, section on *Field tests* [*safety and efficacy*]).

2.3.4. Duration of immunity

As part of the ~~authorisation~~ regulatory approval procedure the manufacturer should demonstrate the duration of immunity of a given vaccine by either challenge or the use of a validated alternative test, at the end of the claimed period of protection.

At least ten vaccinated pigs are each inoculated with an amount of virus corresponding to 10^5 PID_{50} (median pig infectious dose) of a virulent strain of CSFV and observed for 3 weeks. The vaccinated animals have to remain healthy, only the controls should die.

The duration of immunity after vaccination against CSF shall not be less than 6 months.

2.3.5. Stability

The stability of all vaccines should be demonstrated as part of the shelf-life determination studies for ~~authorisation~~ regulatory approval.

The period of validity of lyophilised CSF vaccine should be shown to be at least 1 year.

~~3. Requirements for other vaccines~~

~~3.1. Oral vaccine~~

2.4. Special requirement for oral vaccines

~~3.1.~~2.4.1. Background

The most widely applied concept of oral bait vaccination of wild boar against CSF, including bait design and immunisation scheme was developed, evaluated, and optimised by Kaden *et al.* (2010). Currently used oral ~~The respective~~ vaccines are conventional MLVs, but live marker vaccines also have the potential to be used as oral vaccines. Immunisation occurs by uptake of the oral vaccine through the lymphoid tissues of the oral mucosa and tonsils, where expression of virus stimulates the immune system (Kaden *et al.,* 2000; 2010).

Safety is of paramount consideration for oral vaccine use, not only for the target animals, but for the environment (see chapter 1.1.8) and other species that may come in contact with the vaccine.

~~3.1.~~2.4.2. Outline of production and minimum requirements for vaccines

In addition to the outline of production described for injectable vaccines above, the following specific requirements must be met:

i) Method of manufacture

a) Final product batch/serial test

After combining all of the ingredients, the final blend contains the definitive formulation that is usually used in liquid form. The last step in production of a batch/serial is filling the final blend into blisters/capsules to be included in baits or filling directly into the bait. This final batch/serial is tested as described for the injectable vaccines, with the following differences:

- Residual moisture test

  The residual moisture test does not apply if the oral vaccine is presented in liquid form.

- Safety

  Administer orally by syringe to each piglet a volume corresponding to ten oral doses as indicated by the manufacturer.

ii) Requirements for regulatory approval ~~authorisation/registration/licensing~~

In addition to the requirements described for injectable vaccines, the following specific requirements must be met.

a) The bait

The bait is an integral part of the product and should ideally meet the following criteria:

- Designed for and attractive to the target species and adapted to the mode of distribution.
- Keep its form and shape under a wide range of temperature and weather conditions.
- Ingredients are non-harmful, comply with animal feed standards and should not interfere with vaccine activity.
- Feature a labelling system with a public warning and identification of the product.

b) Safety requirements

For all the tests the liquid vaccine is administered orally with a syringe (not in the final bait formulation) to ensure that each animal receives the full dose.

- Precaution hazards

  The release of oral vaccines in the environment shall comply with the requirements in chapter 1.1.8.

c) Efficacy requirements

Efficacy should be proven using the liquid vaccine administered by syringe to ensure that each animal receives the full dose. Proof-of-concept studies for the final formulation (vaccine integrated into bait) should be provided.

3.~~2.~~ Recombinant E2 glycoprotein-based vaccines

3.2.~~2.~~1. Background

~~As described in Guideline 3.3 Section E,~~ Conventional, live attenuated CSF vaccines have a rapid onset of immunity and are effective at preventing transmission of infection (Ganges *et al.,* 2020; Postel *et al.,* 2018), but have the disadvantage that it is not possible using serological methods (e.g. ELISA) to differentiate infected pigs from those that have merely been vaccinated. Commercial E2 subunit vaccines (Marker vaccine) have a slower onset of immunity and reduce, but may not completely prevent, viral shedding and transplacental infection. However, these vaccines enable a DIVA strategy to be followed thereby facilitating a 'vaccination to live' strategy.

The vaccine only elicits antibodies against the E2 glycoprotein and therefore antibodies against other CSFV antigens, such as the E^{RNS} antigen, can be used as markers of infection. New E2-based formulations, with improved efficacy and/or produced in non-baculovirus based expression systems have been (Suarez *et al.,* 2017), or are being developed (~~Blome *et al.*, 2017b;~~ Coronado *et al.,* 2021; Ganges *et al.,* 2020; Gong *et al.,* 2019).

3.2.~~2.~~ Outline of production

A detailed description of each production stage should be recorded in the manufacturing process documentation (see Chapter 1.1.8, Section on *Documentation of the manufacturing process and record keeping* and Chapter 2.3.3, Section 3. *Rules governing documentation*).

~~i)~~3.2.1. Characteristics of the seed

E2 subunit marker vaccine is prepared by the use of *Baculovirus* or other expression system expressing the E2 antigen of CSFV. The vaccine therefore does not contain any CSFV while the baculo (vector) virus is chemically inactivated.

~~a~~i) Biological characteristic of the master seed

Production is carried out in insect cell cultures, based on a seed-lot system.

Selection of MSVs should ideally be based on their ease of growth in cell culture, virus yield and stability.

The exact source of the isolate including its sequence and passage history should be recorded.

~~b~~ii) Quality criteria

Only MSVs that have been established as sterile and pure (free of extraneous agents as described in chapter 1.1.9 and those listed by the appropriate ~~licensing~~ regulatory approval authorities), and immunogenic, shall be used for preparing the vaccine virus production.

Appropriate methods (specific antibodies or specific genome detection methods) should be used for confirmation of the identity of the MSV.

~~c~~iii) Validation as a vaccine ~~strain~~

The vaccine prepared from the MSV is shown to be satisfactory with respect to safety and efficacy for the swine for which it is intended.

In accordance with chapter 1.1.8, consideration should also be given to minimising the risk of transmission of TSE agents by ensuring that TSE risk materials are not used as the source of the virus or in any of the media used in virus propagation.

The vaccine virus/expression system used to produce the final product should not differ by more than five passages from the material used for validating the seed lot. The commercial vaccine is inactivated for residual baculovirus and adjuvanted.

ii)3.2.2. Method of manufacture

a) Procedure

The baculovirus is used to infect an established insect cell line or alternative expression cell lines are established. Such cell culture should be proven to be free from contaminating microorganisms and shall comply with requirements in chapter 1.1.8.

Regardless of the production method, the substrate should be harvested under aseptic conditions and may be subjected to appropriate methods to release cell-associated virus. The harvest can be further processed by filtration and other methods. Inactivation of residual baculovirus is performed, preferably using a first order inactivant. The antigen is homogenised before formulation with adjuvant.

b) Requirements for ingredients

All ingredients used for vaccine production should be in line with requirements in chapter 1.1.8.

c) In-process controls

Infectivity, sterility and antigenic mass are monitored. After inactivation a test for innocuity is carried out on every batch of antigen. The cells used to test for absence for residual live baculovirus are the same cell line used for production or potentially equally or more sensitive cells.

d) Final product batch/serial test

a) Sterility

Must comply with chapter 1.1.8.

b) Identity

The identity test is performed by a specific MAb-based virus neutralisation against CSFV or an appropriate molecular identification. Sera prepared to be used for identity testing should not be prepared using the homologous vaccine virus or baculovirus expressed subunit antigen but from another source. This test may be combined with the potency test (see below).

c) Safety and prove of marker concept

Batch safety testing is to be performed unless consistent safety of the product is demonstrated and approved in the ~~registration~~ regulatory approval dossier and the production process is approved for consistency in accordance with the standard requirements referred to in chapter 1.1.8.

This final product batch safety test is conducted to detect any abnormal local or systemic adverse reactions.

For batch/serial safety testing, use two healthy piglets, 6–10 weeks old, that do not have antibodies against pestiviruses. Administer to each piglet by a recommended route a double dose of the formulated vaccine. Observe the piglets daily for at least 14 days for local and systems reactions to vaccination. After 14 days they are each injected with a second single dose of vaccine.

Any adverse reaction attributable to the vaccine should be assessed and may prevent acceptance of the batch. The vaccine should elicit antibodies against CSFV E2 but not against CSFV-E^{RNS} antigen.

d) Batch/serial potency

Induction of specific anti-E2 antibodies in vaccinated pigs can be used to confirm the potency of each batch once the titre has been correlated with the results of the efficacy test.

~~iii)~~3.2.3. Requirements for regulatory approval ~~authorisation /registration/ licensing~~

~~a~~i) Manufacturing process

See Section C.2.3.1.

~~b~~ii) Identity

The identity test is performed by virus neutralisation using immune sera against CSFV. Sera prepared to be used for identity testing should not be prepared using the homologous vaccine virus or baculovirus expressed subunit antigen but from another source.

~~c~~iii) Safety requirements

a) Safety in young animals

For the purposes of gaining regulatory approval, a trial batch of vaccine should be tested for local and systemic toxicity by each recommended route of administration in eight piglets of 6–8 weeks of age. Single-dose and repeat-dose tests using vaccines formulated to contain the maximum permitted payload should be conducted. The repeat dose test should correspond to the primary vaccination schedule (e.g. two injections) plus the first revaccination (i.e. a total of three injections). The animals are observed for local and systemic reaction to vaccination for no fewer than 14 days after each injection. Any undue reaction attributable to the vaccine should be assessed and may prevent acceptance of the vaccine. It has to be proven that the vaccine does not elicit antibodies against CSFV-E^{RNS} antigen.

b) Safety in pregnant sows

For the purpose of gaining regulatory approval a trial batch of vaccine should be tested for local and systemic toxicity by each recommended route of administration corresponding to the primary vaccination schedule (e.g. two injections) in eight pregnant sows. The sows are observed for local and systemic reactions to vaccination. The observation period must last until parturition to examine any harmful effects during gestation or on progeny. Any undue reaction attributable to the vaccine should be assessed and may prevent acceptance of the vaccine. It has to be proven that the vaccine does not elicit antibodies against CSFV-E^{RNS} antigen.

~~d~~iv) Efficacy requirements

a) Protective dose

Vaccine efficacy is estimated in animals vaccinated according to the manufacturer's recommendation, following the methods described in Section C.2.3.3.

b) Protection against transplacental infection

The trial vaccine should comply with the test described in Section C.2.3.3.

~~e~~v) Duration of immunity

As part of the ~~authorisation~~ regulatory approval procedure the manufacturer should demonstrate the duration of immunity (see Section C.2.3.4).

~~f~~vi) Stability

The stability of all vaccines should be demonstrated as part of the shelf-life determination studies for ~~authorisation~~ regulatory approval. The period of validity of a batch of biotechnology-based CSF vaccine should be shown to be at least 1 year (see Section C.2.3.5).

4. Other biotechnology-based vaccines

In search of an ideal vaccine, multiple studies were undertaken and several other approaches were proposed, including viral vector and replicon vaccines, immunogenic CSFV peptides or DNA vaccines (reviewed by Coronado *et al.*, 2021; Ganges *et al.*, 2020; Postel *et al.*, 2018). The regulatory approval of such candidates should take into account categorisation and special requirements provided in chapters 1.1.8 and 2.3.4, including thorough risk analysis for their environmental release.

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NB: There are WOAH Reference Laboratories for classical swine fever (please consult the WOAH web site for the most up-to-date list: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3). Please contact WOAH Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for classical swine fever

NB: FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2022.

Appendix 1: Classical swine fever

Intended purpose of test: detection of the agent – individual animal freedom from infection prior to movement; confirmation of clinical cases

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	Additional comments regarding application/utility	References
Virus isolation Confirmation of clinical cases: +++ Individual animal freedom from infection prior to movement: +	Organ suspensions, anticoagulated, whole blood, leukocytes, sera, plasma, swab samples and semen			See references	High sensitivity High specificity after immunostaining or detection of viral genome Direct method for detection of infectious virus Further characterisation of the virus isolate (e.g. virulence, antigenic relatedness) possible	Slow and labour-intensive method Specificity requires specific detection of viral antigen or viral genome Not suitable for large-scale diagnostics and screening Good sample quality is needed to reduce cytotoxic effects as well as bacterial contamination Requires expertise High biosafety level needed	Time consuming and laborious method, not fit for surveillance or other population-based use Important for collecting virus strains for further characterisation	Ganges *et al.* (2020); Handel *et al.* (2004); Dewulf *et al.* (2004); Uttenthal *et al.* (2003)

Appendix 2: Classical swine fever

Intended purpose of test: detection of the agent – population freedom from infection; individual animal freedom from infection prior to movement; contribute to eradication policies; confirmation of clinical cases; prevalence of infection – surveillance

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	Additional comments regarding application/utility	References
RT-PCR Individual animal freedom from infection prior to movement and Confirmation of clinical cases: +++ Contribute to eradication policies and Prevalence of infection – surveillance: ++ Population freedom from infection: ±	Blood, serum, tissue (kidney, spleen, tonsil, lymph nodes, thymus, intestine, lung, bone marrow), swab samples, faeces, semen, cells or supernatant of infected cell culture			See references	Highest sensitivity and specificity compared with other methods Fast results Low sample amount High throughput possible Genetic DIVA possible Larger PCR products can be used for molecular analysis and genetic typing Detection of RNA genome at early and late time points after infection possible, even in absence of detectable infectious virus less affected by sample quality	Expensive equipment and consumables needed Well-trained laboratory staff needed Cool chain for some reagents Risk of cross-contamination due to high sensitivity Conventional PCR: lower sensitivity compared with real-time PCR Conventional and SYBR-green based PCR: lower specificity compared to probe-based real-time PCR	Not suitable to identify previously infected animals after clearing the virus	Hoffmann *et al.* (2005); Leifer *et al.* (2011); Depner *et al.* (2006); Risatti *et al.* (2005); Haines *et al.* (2013); Petrov *et al.* (2014)

Appendix 3: Classical swine fever

Intended purpose of test: detection of the agent – population freedom from infection; individual animal freedom from infection prior to movement; contribute to eradication policies; confirmation of clinical cases

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	Additional comments regarding application/utility	References
ELISA (antigen detection) Population freedom from infection: ++ Individual animal freedom from infection prior to movement, Contribute to eradication policies and Confirmation of clinical cases: ±	Samples from animals with clinical signs or pathological lesions of disease: leukocytes, serum, plasma, non-coagulated blood as well as organ suspensions			See references	Screening of large sample numbers in a short time Method easy to handle Automated processing Commercially available	Less sensitive than other antigen detection methods Sensitivity is significantly better on blood samples from piglets than from adult pigs Low specificity Additional devices necessary	Not suitable to identify previously infected animals after clearing the virus	Ganges *et al.* (2020); Dewulf *et al.* (2004); Uttenthal *et al.* (2003)

Appendix 4: Classical swine fever

Intended purpose of test: detection of the agent – contribute to eradication policies; confirmation of clinical cases

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	Additional comments regarding application/utility	References
FAT Contribute to eradication policies and Confirmation of clinical cases: ±	Cryostat sections of tonsils, spleen, kidney, lymph nodes or distal portions of the ileum			See references	Quick and direct visualisation of antigens in tissue Good sensitivity and specificity	Relatively high risk of false (positive and negative) results, requires specialised equipment and expertise Highly dependent on antibodies conjugate quality Often very high background, inconclusive interpretation	Requires post-mortem collection of samples (possible to use tonsils in living animals)	Bouma *et al.* (2001)

Appendix 5: Classical swine fever

Intended purpose of test: detection of immune response – population freedom from infection; individual animal freedom from infection prior to movement; contribute to eradication policies; prevalence of infection – surveillance; immune status in individual animals or populations post-vaccination

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	Additional comments regarding application/utility	References
ELISA (antibody detection) Population freedom from infection, Contribute to eradication policies, Prevalence of infection – surveillance, and Immune status in individual animals or populations post-vaccination: +++ Individual animal freedom from infection prior to movement: ++	Serum or plasma samples			See references	Screening of large sample numbers in a short time Method easy to handle and automate Commercially available	Possible cross-reactions with other pestiviruses so confirmation with comparative assay such as NPLA required Additional devices necessary	Detects animals with previous contact with CSFV after clearing the virus Negative results in early stages of disease, until detectable level of antibodies is produced Some options for DIVA application alongside DIVA vaccines available	Ganges *et al.* (2020)

Appendix 6: Classical swine fever

Intended purpose of test: detection of immune response – population freedom from infection; individual animal freedom from infection prior to movement; contribute to eradication policies; confirmation of clinical cases; prevalence of infection – surveillance; immune status in individual animals or populations post-vaccination

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	Additional comments regarding application/utility	References
VN (FAVN or NPLA) Individual animal freedom from infection prior to movement (specifically for confirmation of antibody ELISA positive cases). Prevalence of infection – surveillance, and Immune status in individual animals or populations post-vaccination: +++: Confirmation of clinical cases and Contribute to eradication policies: ++ Population freedom from infection: ±	Serum or plasma samples (alternative sample material: meat juice, exudates of body cavities)			See references	Discrimination of CSFV-specific antibodies from antibodies resulting from infections with BVDV, BDV and other ruminant pestiviruses Highly sensitive and specific method Detection of low antibody titres possible Method can always be quickly adapted to the circulating CSFV isolate or closely related isolates, as well as vaccine strains that were applied Allows quantification of immune response	Early time points of infection are not safely detected Slow and labour-intensive method High biosecurity level needed Potential cytotoxic effects have a negative impact on the evaluation of samples containing a low antibody titre. This is to be expected in animals, which are at an early stage of infection/onset of clinical signs Not suitable for large-scale diagnostics Requires expertise Knowledge on CSFV or other pestiviruses circulating in region important for appropriate strain selection	Time consuming and laborious method, not fit for surveillance or other population-based use Used as a confirmatory method to resolve potential false positive or cross-reactions in antibody ELISA	Ganges *et al.* (2020); Terpstra *et al.* (1984)

Annexe 32. Chapitre 3.9.8. Maladie vésiculeuse du porc

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CHAPTER 3.9.8.

SWINE VESICULAR DISEASE

SUMMARY

Description and importance of the disease*: Swine vesicular disease (SVD) is a contagious disease of pigs, caused by an enterovirus and characterised by vesicles on the coronary bands, heels of the feet and occasionally on the lips, tongue, snout and teats. Strains of SVD virus (SVDV) vary in virulence, and the disease may be subclinical, mild or severe, the latter usually only being seen when pigs are housed on abrasive floors in damp conditions. The main importance of SVD is that it can be clinically indistinguishable from foot and mouth disease (FMD), and any outbreaks of vesicular disease in pigs must be assumed to be FMD until investigated by laboratory tests and proven otherwise. However, subclinical infection has been the most frequent condition observed during recent years.*

Detection and identification of the agent: *Where a vesicular condition is seen in pigs, the ~~demonstration by enzyme-linked immunosorbent assay (ELISA) of SVD viral antigen~~ detection of SVDV RNA by reverse transcription polymerase chain reaction (RT-PCR) in a sample of lesion material or vesicular fluid ~~is sufficient for a positive diagnosis~~ strongly supports a positive diagnosis. Other tests such as antigen-detection enzyme-linked immunosorbent assay (ELISA) ~~If the quantity of lesion material submitted is not sufficient (less than 0.5 g), or if the test results are negative or inconclusive, a more sensitive test, such as the reverse transcriptase polymerase chain reaction (RT-PCR)~~ or virus isolation (VI) in porcine cell cultures, may be used. If any inoculated cultures subsequently develop a cytopathic effect, the demonstration of SVD viral antigen in the cultures by ELISA or viral RNA by RT-PCR will ~~suffice~~ be required to make a positive diagnosis. Subclinical infection may be detected by random sampling of pen-floor faeces tested for identification of SVD virus using RT-PCR or VI tests.*

Serological tests: *Serological tests can be used to help confirm clinical cases as well as to identify subclinical infections. Specific antibody to SVDV can be identified using ELISA for screening and the microneutralisation test for confirmation. A small proportion (up to 0.1%) of normal, uninfected pigs will react positively in serological tests for SVD. The reactivity of these singleton reactors is transient ~~so that they can be differentiated from infected pigs by resampling of the positive animal and its cohorts~~ requiring resampling of the non-negative animal and its cohorts to eliminate the possibility that these pigs are infected with SVDV.*

Diagnostic and standard reagents are available from reference laboratories.

Requirements for vaccines: *There are currently no commercial vaccines available against SVD.*

A. INTRODUCTION

Swine vesicular disease (SVD) can be a subclinical, mild or severe vesicular condition depending on the strain of virus involved, the route and dose of infection, and the husbandry conditions under which the pigs are kept. When it occurs clinically, SVD can be indistinguishable from foot and mouth disease (FMD) and therefore a differential diagnosis and laboratory investigation is an urgent requirement. However, ~~during the present century,~~ the most recent outbreaks of SVD have been less severe or with no clinical signs and infection has been detected when samples are tested for serosurveillance programmes or for export certification.

After its first detection in 1966, the disease occurred with epidemics in eastern and western Europe (during the 1970s and 1980s), and was also detected in East Asia. Since then, SVD has only been sporadically reported, mainly from Italy where its circulation ~~is investigated and~~ has been controlled through a virological and serological surveillance plan. The most recent detection of SVDV in Italy was in 2015, and no serologically positive pigs have been identified since 2017. No further SVD outbreaks have been reported elsewhere in Europe or in any other country globally.

The incubation period for SVD is between 2 and 7 days, after which a transient fever of up to 41°C may occur. Vesicles then develop on the coronary band, typically at the junction with the heel. These may affect the whole coronary band resulting in loss of the hoof. More rarely, vesicles may also appear on the snout, particularly on the dorsal surface, on the lips, tongue and teats, and shallow erosions may be seen on the knees. Affected pigs may be lame and off their feed for a few days. Abortion is not a typical feature of SVD. Recovery is usually complete in 2–3 weeks (Loxam & Hedger, 1983). Affected pigs may excrete virus from the nose and mouth and in the faeces up to 48 hours before the onset of clinical signs. Most virus is produced in the first 7 days after infection, and virus excretion from the nose and mouth normally stops within 2 weeks. Virus may continue to be shed for up to 3 months in the faeces, though under usual circumstances virus is detectable in faeces only up to 1 month. The SVD virus (SVDV) is extremely resistant to inactivation in the environment, and is stable in the pH range 2.5–12.0 (Mann, 1981). This is in contrast to the FMD virus, which is very labile outside the pH range 6.0–8.0.

Because SVD may be mild or subclinical, it is essential when submitting samples from suspect clinical cases that serum samples from both the suspect pigs and other apparently unaffected animals in the group be included. It is possible for SVD to circulate unnoticed until it affects a particularly susceptible group. Therefore, in order to ascertain how long infection has been present, it is necessary to look for seroconversion to SVDV in apparently healthy animals. The identification of the isotype of the immunoglobulins (M or G) to SVDV may also help to ascertain the time of exposure to infection.

SVDV has been classified as a porcine variant of a human coxsackievirus B5 (*Enterovirus B*), in the family *Picornaviridae*. All isolates are classified in a single serotype, with four distinguishable antigenic/genomic variants (Brocchi *et al*., 1997), which evolved sequentially in different time-periods, except for the third and fourth variants that were co-circulating in Italy during 1992–1993. All SVDVs occurring since then diverge from a common origin and cluster in a unique antigenic/genomic lineage corresponding to the fourth and most recent group; however, two genomic sub-lineages are distinguishable within it (Knowles *et al*., 2007). Antigenically and genetically, SVDV is closely related to the human virus coxsackievirus B5 and it has been suggested that it arose through recombination with another human enterovirus, coxsackievirus A9 (Bruhn *et al.,* 2015). A second adaptation of a human enterovirus to cause vesicular disease in pigs was reported in Russia in 1975 involving coxsackievirus B4, which is serologically distinct from SVDV/CV-B5 (Lomakina *et al.,* 2016).

There are reports of seroconversion to SVDV in laboratory workers handling the agent. However, clinical disease in humans is reported to be mild, with the exception of a single case of meningitis associated with SVDV infection, and there have been no reported cases of seroconversion or disease in farmers or veterinarians working with infected pigs. Under experimental conditions, it has not been possible to show transmission of coxsackievirus B5 between pigs. Laboratory manipulations should be carried out at an appropriate biosafety and containment level determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of swine vesicular disease and their purpose

Method	**Purpose**					
	Population freedom from infection	Individual animal freedom from infection prior to movement[(a) (b)]	Contribute to eradication policies[(a) (b)]	Confirmation of clinical cases[(a) (b)]	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection and identification of the agent						
Virus isolation	–	+	+	+++	–	–
RT-PCR	–	+++	+++	+++	–	–
ELISA for antigen detection	–	–	–	+++	–	–

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement[(a) (b)]	Contribute to eradication policies[(a) (b)]	Confirmation of clinical cases[(a) (b)]	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of immune response						
Virus neutralisation	+	+++	+	+	+	–
Competitive ELISA for Ab screening	+++	+++	+++	+	+++	–
ELISA for IgG and IgM identification	+	+++	+++	+	+	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
RT-PCR = reverse-transcriptase polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay.
NOTE: Selection of assays suited for different purposes should take into account the different kinetics of the diagnostic targets (agent and antibodies) during infection.
[(a)]See Appendix 1 of this chapter for justification table for the scores given to the tests for this purpose.
[(b)]See Appendix 2 of this chapter for justification table for the scores given to the tests for this purpose.

1. Detection and identification of the agent

Any vesicular condition in pigs could represent an FMD infection and differential diagnosis between FMD and other vesicular conditions, including SVD, is necessary. The diagnosis of SVD requires the facilities of a specialised laboratory. Countries that lack such a facility should send samples for investigation to a WOAH Reference Laboratory for SVD.[1].

For clinical cases, the detection of SVDV antigens or genome ~~of SVDV~~ by means of enzyme-linked immunosorbent assay (ELISA), ~~and~~ reverse-transcription polymerase chain reaction (RT-PCR) ~~has the same diagnostic value as~~ or virus isolation can be used. Due to their speed, ELISA and RT-PCR make suitable screening tests. However, virus isolation is the reference method and should be used if a positive ELISA or RT-PCR result is not associated with the detection of clinical signs of disease, the detection of seropositive pigs, or a direct epidemiological connection with a confirmed outbreak. Any cytopathic effect (CPE) observed in the inoculated cultures needs to be tested by ELISA or RT-PCR to demonstrate that SVDV is present.

If there are clinical signs, investigation should start with the examination of a 10% suspension of lesion material in phosphate buffered saline (PBS) or tissue culture medium and antibiotics. Faecal samples are the specimen of choice for the detection of virus where subclinical SVD is suspected. Faecal samples can be collected from individual pigs or from the floor of premises suspected to contain, or to have contained, pigs infected with SVD. The level of virus in faeces is usually insufficient for detection by ELISA and the use of RT-PCR and/or virus isolation is required. A significant proportion of faecal samples inoculated into cell cultures will give rise to the growth of other enteroviruses. These can be differentiated from SVDV by ELISA or RT-PCR, but they may also outgrow SVDV that is present, and give rise to false negative results. Therefore, RT-PCR is more sensitive than virus isolation when applied to faecal samples.

1.1. Preparation of samples

1.1.1. Lesion material

Samples should be transported in phosphate-buffered saline (PBS) with antibiotics, mixed with glycerol (1/1), pH 7.2–7.6. A suspension is prepared by grinding the sample in sterile sand in a sterile pestle and mortar with a small volume of PBS or tissue culture medium and antibiotics. Further medium should be added to obtain approximately a 10% suspension. This is clarified by centrifugation at 2000 ***g*** for ~~20~~ 10 30 minutes and the supernatant is harvested.

[1] Please consult the WOAH web site for the most up-to-date list: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3

1.1.2. Faecal samples

Faecal material (approximately 20 g) is resuspended in a minimal amount of tissue culture medium or phosphate buffer (0.04 M phosphate buffer or PBS). The suspension is homogenised by vortexing and clarified by centrifugation at 2000 ***g*** for ~~20~~ 10–30 minutes; the supernatant is harvested and filtered through a 0.45 µm filter.

1.2. Virus isolation

A portion of the clarified epithelial or faecal suspension is inoculated on to monolayers of the porcine kidney cell line IB-RS-2 ~~cells~~ or other susceptible porcine cell line, grown in appropriate containers (25 cm^2 flasks, rolling tubes, 24-, 12-, 6-well plates). Generally, SVDV will grow in cells of porcine origin only. For differential diagnosis (e.g. FMD) in case of clinical lesions, ~~bovine~~ other validated cell culture systems ~~should~~ may also be employed in parallel. ~~Generally, SVDV will grow in cells of porcine origin only.~~ Tissue culture medium containing antibiotic is supplemented with 10% bovine serum for cell growth, and with 1–3% bovine serum ~~for maintenance, and with antibiotics~~ when test samples are inoculated to the cultures.

Cultures are examined daily. If a cytopathic effect (CPE) is observed, the supernatant fluid is harvested and virus identification is performed by ELISA and SVDV-specific ~~(or other appropriate test, e.g.~~ RT-PCR~~)~~. Negative cultures ~~are~~ may be blind-passaged after 48 or 72 hours, and observed for a further 2–3 days. If no CPE is evident after the second passage, the sample is recorded "NVD" (no virus detected). When isolating virus from faeces in which the amount of virus present may be low, a third tissue culture passage may be required.

1.3. Immunological methods

1.3.1. Enzyme-linked immunosorbent assay

The detection of SVD viral antigen by an indirect sandwich ELISA has replaced the complement fixation test as the method of choice. The test has the same format as that used for FMD diagnosis. Wells of ELISA plates are coated with rabbit antiserum to SVDV. This is the capture serum. Test sample suspensions are added and incubated. Appropriate controls are also included. Guinea-pig anti-SVD detection serum is added at the next stage followed by rabbit anti-guinea-pig serum conjugated to horseradish peroxidase. Extensive washing is carried out between each stage to remove unbound reagents. A positive reaction is indicated if there is a colour reaction on the addition of chromogen (for example orthophenylenediamine) and substrate (H_2O_2). With strong positive reactions this will be evident to the naked eye, but results can also be read spectrophotometrically at the appropriate wavelength, in which case an absorbance reading ≥0.1 above background indicates a positive reaction. As an alternative to guinea-pig and rabbit antisera, suitable monoclonal antibodies (MAbs) can be used, coated to the ELISA plate as the capture antibody and peroxidase conjugated as detector antibody. For example, a simple sandwich ELISA performed with MAb 5B7 as both catching and conjugated/detector antibody, that represents also the reference method for the serological competitive ELISA, is suited for the detection of SVD viral antigen.

A MAb-based ELISA can also be used to study antigenic variation among strains of SVDV. Tissue-culture grown viral strains are trapped by a rabbit hyperimmune antiserum to SVDV adsorbed to the solid phase. Appropriate panels of MAbs are then reacted and the binding of MAbs to field strains is compared with the binding of MAbs to the parental strains. Strong binding indicates the presence of epitopes shared between the parental and the field strains (Brocchi *et al*., 1997).

1.4. Nucleic acid recognition methods

1.4.1. Reverse-transcriptase-polymerase chain reaction

Reverse-transcriptase followed by the PCR (RT-PCR) is a useful method to detect SVD viral genome in a variety of samples from clinical and subclinical cases. Several methods have been described (~~Benedetti *et al*., 2010;~~ Callens & De Clercq, 1999; Fallacara *et al*., 2000; Hakhverdyan *et al*., 2006; Lin *et al*., 1997; McMenamy *et al*., 2011; Nunez *et al*., 1998; Reid *et al*., 2004a; 2004b; Vangrysperre & De Clercq, 1996), employing different techniques for RNA extraction, targeting different parts of the SVDV genome and using different approaches to detect the DNA products of amplification. However, in a comparative study on positive faecal samples from many different SVD outbreaks, the one-step RT-PCR incorporating a real-time format with a DNA-binding fluorescent dye ~~Benedetti *et al*., 2010~~ Pezzoni *et al*., 2020) had the best diagnostic performance, with the capability to reveal all the circulating genomic sub-lineages, compared with two real-time RT-PCR assays targeting the 5'-untranslated region (Reid *et al.,* 2004a; 2004b), and an RT-loop-mediated isothermal amplification (LAMP) assay (Blomström *et al*., 2008).

The method reported below describes an RNA immune-extraction protocol and a ~~one step~~ real-time RT-PCR protocol targeting SVDV *3D* region, which codes for the RNA-polymerase. NOTE: The set of primers described below can also be used for conventional RT-PCR.

To isolate RNA, the immunocapture technique using a SVDV-specific MAb has been shown to be particularly effective in the case of faecal samples (Fallacara *et al.*, 2000): it enables enrichment and purification of the SVDV, usually present in low concentration in faeces, with efficient removal of potential reaction inhibitors. NOTE: As an alternative to immunocapture, RNA extraction can be performed using suitable commercially available kits based on chaotropic salt lysis and silica RNA affinity.

~~This method is suitable for laboratories without equipment for real-time detection of DNA amplification products, but where such facilities are available an approach such as that described by Reid *et al.* (2004a; 2004b) offers advantages in terms of ease of use and reduced risk of laboratory contamination by PCR products.~~

1.4.2. RNA immune-extraction

i) RNA Immune-extraction: Coat wells of an ELISA plate with a saturating solution of MAb 5B7 (200 µl/well, diluted in carbonate-bicarbonate buffer) by overnight incubation at 4°C. Wash plates three times with PBS. Use plates immediately or store at –20°C for up to 2–3 weeks, or more if stabilised.

ii) Distribute each sample (faeces suspension) into three wells of the 5B7-coated plate (200 µl/well, 600 µl of sample in total).

iii) After incubation for 1 hour at 37°C with very slow shaking, wash wells three times with PBS. Washing is performed manually, in order to avoid cross-contamination between wells.

iv) RNA is extracted from each sample by adding approximately 100 µl/well of lysis buffer (4 M guanidine thiocyanate, 25 mM sodium citrate, pH 7, 0.5% Sarkosyl). Incubate wells for 3–5 minutes and recover the sample from the three wells (300–350 µl total), and transfer into a single tube.

v) RNA is then precipitated by adding a mixture of 750 µl of absolute ethanol and 35 µl of 3 M sodium acetate (pH 5.2); vials are vortexed and incubated at –20°C for a minimum of 1 hour (prolonged overnight precipitation at –20°C may also be suitable).

vi) Centrifuge the sample at 15,500–16,000 ***g*** for 30 minutes at 4°C, after which a pellet should be visible which should be washed with 500 µl of 70% cold ethanol (centrifuged at 15,500–16,000 ***g*** for 10 minutes at 4°C) and dried.

vii) Resuspend the RNA pellet in 20 µl of DEPC water, or commercially available RNase-free water.

NOTE: As an alternative to immunocapture, RNA extraction can be performed using suitable commercially available kits based on chaotropic salt lysis and silica RNA affinity.

1.4.2.1. One step real-time RT-PCR (with a double-stranded DNA-binding fluorescent dye)

The following protocol (and volumes of each of the specific reagents) for the ~~conventional~~ real-time RT-PCR may need to be adjusted to suit particular reagents used.

i) Assemble the reaction mix (20 µl is the final volume for each test sample)

~~5× RT-PCR buffer~~	~~5 µl~~
2× Master Mix buffer including a DNA-binding fluorescent dye	
dNTPs and DNA polymerase	12.5 µl
~~dNTP mix (10 mM each dNTP)~~	~~1 µl~~
pSVDV-SS4 Forward Primer 10 pmol/ul*	1.25 µl
pSVDV-SA2 Reverse Primer 10 pmol/ul*	1.25 µl
~~RNAse inhibitor~~	~~0.25 µl (equivalent to 5 U)~~
RT-Mix	0.25 µl
Rnase free water	~~10.75~~ 4.75 µl

*Primer sequences: pSVDV-SS4 5'-TTC-AGA-ATG-ATT-GCA-TAT-GGG-G-3' and pSVDV-SA2 5'-TCA-CGT-TTG-TCC-AGG-TTA-CY-3'

ii) Add 5 µl of each template RNA to 20 µl reaction mix.

iii) Run the following program in a thermal cycler:

i) 1 cycle at 50°C for 30 minutes (reverse transcription step)

ii) 1 cycle at 95°C for 15 minutes (initial activation step)

iii) 40 cycles, each composed of 94°C for ~~20~~ 15 seconds (denaturation), ~~60~~ 58°C for ~~20~~ 30 seconds (annealing), 72°C for ~~45~~ 30 seconds (extension), 77°C for 15 seconds (detection).

~~One cycle at 72°C for 10 minutes (final extension).~~

For the melting cycle:

iv) 1 cycle at 72°C for 10 1 minute and increasing temperature from 72°C to 95°C, by incremental steps of 0.5°C for 5 seconds each. Specific amplification products for SVDV generate melting curves with a peak within the temperature range 79.5–82.5°C.

~~iv) Mix a 20 µl aliquot of each sample with 4 µl of loading dye and load onto a 2% agarose gel containing an appropriate DNA intercalating dye. After electrophoresis, a positive result is indicated by the presence of a 154 bp fragment of the SVDV RNA polymerase (3D) encoding region in the gel. Alternatively, gel can be stained after electrophoresis to reduce contamination of equipment by soaking in a staining solution~~

~~**1.4.2.2. Real-time RT-PCR**~~

~~This test can also be adapted to the format of real-time RT-PCR with dedicated reagents/kits, in the presence of a suitable DNA stain, using the following adjusted programme in a real-time PCR cycler:~~

~~i) 1 cycle at 50°C for 30 minutes (reverse transcription step)~~

~~ii) 1 cycle at 95°C for 15 minutes (initial activation step)~~

~~iii) 40 cycles, each composed of 94°C for 15 seconds (denaturation), 58°C for 30 seconds (annealing), 72°C for 30 seconds (extension), 77°C for 15 seconds (detection).~~

~~For the melting cycle:~~

~~iv) 1 cycle at 72°C for 1 minute and increasing temperature from 72°C to 95°C, by incremental steps of 0.5°C for 5 seconds each. Specific amplification products for SVDV generate melting curves with a peak within the temperature range 79.5–82.5°C.~~

1.4.3. Sequence analyses

Comparative analysis of sequences of the viral genome is useful to establish relationships between isolates of SVDV. By sequencing the 1D region, which codes for the major structural protein VP1, ~~or the~~ 3D region or whole genome sequence (Pezzoni *et al.*, 2021), it has been possible to group strains of SVDV according to their sequence homology, and epidemiologically to relate strains causing disease in different locations or at different times (Brocchi *et al.*, 1997). Databases of *1D* and *3D* gene sequences of SVDVs are held at the WOAH Reference Laboratory, Pirbright, UK and the WOAH Reference Laboratory, Brescia, Italy, respectively. Further sequences (including those for complete SVDV genomes) are available via the International Nucleotide Sequence Database Collaboration (including GenBank, ENA, and DDBJ).

2. Serological tests

Serological assays are used in the laboratory confirmation of outbreaks, for serological surveillance and for export certification of pigs. SVD is often diagnosed solely on the evidence of serological tests. Because of the subclinical or mild nature of the disease, it is often first suspected following routine serology for disease surveillance or export certification. The virus neutralisation (VN) test, the double immunodiffusion test, the radial immunodiffusion test, the counter immunoelectrophoresis test and the ELISA have all been described for the detection of antibodies to SVDV (Brocchi *et al.*, 1995; Donaldson *et al.*, 1983; Golding *et al.*, 1976; Yang *et al.*, 2020). However, the VN test and the ELISA are the only techniques commonly used. The VN test is the accepted confirmatory test, but has the disadvantage that it takes 2–3 days to complete and requires tissue culture facilities and handling of live virus in appropriate biosafety and containment facilities as determined by biorisk analysis (see chapter 1.1.4). The ELISA is quicker and simpler. A small proportion of sera from animals with no previous exposure to SVDV may react positively in serological tests for antibody to SVDV. The 5B7 MAb competitive ELISA (MAC-ELISA) is a reliable technique for detecting SVD antibody (Brocchi *et al.*, 1995; Heckert *et al.*, 1998) and similar results have been obtained with other ELISAs (Chenard *et al.*, 1998; Ko *et al.*, 2005). Results from a small proportion, 0.2%–0.4%, of sera from normal pigs are borderline or positive by the MAC-ELISA and should be retested by the VN test. Up to approximately 50% of these sera will also be positive by the VN test (i.e. 0.1–0.2% of the original population). Animals that test positive by ELISA but negative by VN test can be regarded as uninfected. Repeat samples should be collected from animals positive in both tests and from cohorts. A constant or declining titre in the positive animal and the absence of antibody to SVDV in cohorts confirms the status of the positive animal as a 'singleton reactor'. The factors responsible for 'singleton reactors' are unknown. Serological cross-reactivity with SVDV might arise due to infection with another, as yet unidentified, picornavirus or may be due to other non-specific factors present in the serum. Identification of the isotype of antibody present in positive sera (Brocchi *et al.*, 1995) can be helpful as sera from 'singleton' reactors usually contain exclusively IgM and do not convert to IgG (De Clercq, 1998). IgM/IgG isotype-specific ELISAs are also helpful in assessing the time of infection in the pig or on the infected premises. The presence of IgM, alone or together with IgG, is

evidence of recent infection and indicative of virus shedding, while detection of IgG alone suggests an older exposure to infection (Brocchi *et al*., 1995).

2.1. Virus neutralisation

The quantitative VN microtest for antibody to SVDV is performed using IB-RS-2 cells (or suitable susceptible porcine cells) in flat-bottomed tissue-culture grade microtitre plates.

Virus is grown on IB-RS-2 cell monolayers and stored at –20°C after the addition of an equal volume of glycerol. SVDV has been found to be stable under these conditions for at least 1 year. The sera are inactivated at 56°C for 30 minutes before testing. A suitable medium is Eagle's complete medium/LYH with antibiotics.

The test is an equal volume test in 50 µl volumes:

i) Starting from a 1/4 dilution, sera are diluted in a twofold dilution series across the plate, two rows of wells per serum and a volume of 50 µl.

ii) Previously titrated virus is added; each 50 µl unit volume of virus suspension contains about 100 $TCID_{50}$ (50% tissue culture infective dose).

iii) Controls include at least ~~a weak positive serum and a negative serum,~~ a cell control, a medium control and a virus titration used to calculate the actual virus titre used in the test. Inclusion of a weak positive serum and a negative serum can also help to interpret the results.

iv) Incubate at 37°C for 1 hour with the plates covered.

v) A cell suspension at 10^6 cells/ml is prepared in medium containing 10% bovine serum for cell growth. 50 µl of cell suspension is added to each well.

vi) Plates are sealed with pressure-sensitive tape and incubated at 37°C for ~~2~~ 1–3 days. Alternatively, the plates may be covered with loosely fitting lids and incubated in an atmosphere of 5% carbon dioxide at 37°C for 2–3 days.

vii) Microscopic readings are feasible after ~~48~~ 24–72 hours; the plates may be finally fixed and stained on the third day. Fixation is ~~effected~~ carried out with 10% formalin/saline for 30 minutes; staining is done by immersion in 0.05% methylene blue in 10% formalin for 30 minutes. The plates are rinsed in tap water. Positive readings are blue-stained cell sheets (where the virus has been neutralised and the cells remain intact), whilst empty wells (where virus has not been neutralised) are read as negative.

viii) Interpretation of the results

The test is considered to be valid when the amount of virus ~~actually~~ used per well (virus dose) is between $10^{1.5}$ and $10^{2.5}$ $TCID_{50}$, and the positive standard sera are within twofold of their expected titre. Titres are expressed as the final dilution of serum present in the serum/virus mixture where 50% of wells are protected. Laboratories should establish their own cut-off titres by reference to both results from a negative population and standard reagents available from the WOAH Reference Laboratories, in particular the low-positive serum defining the lowest level of antibodies that Laboratories should consistently score positive.

2.2. Enzyme-linked immunosorbent assay

Commercial kits are available for antibody detection in pig ~~specimens~~ sera. In the ELISA developed by Brocchi *et al.* (1995), the SVD viral antigen is trapped to the solid phase using the MAb 5B7. The ability of test sera to inhibit the binding of peroxidase-conjugated MAb 5B7 to the trapped antigen is then evaluated. Finally, the amount of conjugated MAb bound is detected by the addition of substrate and chromogen.

i) ELISA plates are coated with 50 µl/well of MAb 5B7 at a saturating dilution in carbonate/bicarbonate buffer, pH 9.6, by overnight incubation at 4°C.

ii) The plates are washed three times with PBS containing 0.05% Tween 20, and 50 µl of SVD antigen (SVDV grown in IB-RS-2 cells, clarified, filtered and BEI-inactivated) at a predetermined optimal dilution, is added to each well. The optimal dilution of antigen is determined by checkerboard titrations of antigen and conjugated MAb that define the working dilution giving an absorbance on the upper part of the linear region of the antigen titration curve (between 1.5 and 2.0 optical density units). Plates are then incubated for 1 hour at 37°C.

iii) After three additional washes, 50 µl of diluted test sera (inactivation is irrelevant) and control sera are incubated with the trapped antigen for 1 hour at 37°C. Sera can be tested at a single dilution (1/7.5) or titrated. In the latter case, three-fold dilutions of sera are obtained directly in ELISA wells by adding 10 µl of serum to 65 µl of buffer (1/7.5 dilution) then transferring 25 µl to sequential wells containing 50 µl of buffer,

mixing, and finally discarding 25 µl. For spot-test, the screening dilution 1/7.5 is obtained by adding 7µl of each test serum (and control sera) to 45 µl of buffer previously distributed into wells.

iv) After incubation for 1 hour, 25 µl of an optimal dilution of peroxidase-conjugated MAb 5B7 (see step ii above) is added to each well and the plates are incubated at 37°C for a further 1 hour.

v) After a final series of washes, the colorimetric reaction is developed by distributing 50 µl per well of the substrate solution (for example 0.5 mg/ml orthophenylene-diamine in phosphate/citrate buffer, pH 5, containing 0.02% H_2O_2).

vi) The reaction is stopped after 10 minutes by adding 50 µl of 2N H_2SO_4. The absorbance is read at the appropriate wavelength using a microplate reader.

Antigen, sera and conjugate are diluted in PBS, pH 7.4, containing 0.05% Tween 20 and 1% yeast extract; the dilution buffer for sera contains, in addition, 1.0% mouse serum (or ~~alternatively~~ another source of murine immunoglobulins) to prevent nonspecific binding of pig serum to MAb 5B7 either coated to the plate or conjugated to peroxidase.

vii) *Controls:* Four wells on each plate containing all reactants except test serum confirm the maximum absorbance reading for the antigen; negative pig serum; a low positive standard pig serum; optionally, a strong positive pig serum at four dilutions, previously calibrated in order to give ≥50% inhibition (see step viii below) at the highest dilution.

viii) Interpretation of the results: Reactions are expressed as the percentage inhibition by each test serum of the MAb reaction with the SVD antigen. Sera are considered to be positive when producing an inhibition ≥80% at the 1/7.5 dilution; negative when producing an inhibition <70% at the 1/7.5 dilution; doubtful when producing an inhibition ≥70% and <80% at the 1/7.5 dilution. The second dilution (1/22.5) ~~provides an indication of~~ indicates the level of antibodies: strongly positive sera show >80% inhibition at both 1/7.5 and 1/22.5 dilutions, while sera registering >80% inhibition at the 1/7.5 dilution but <50% inhibition at the 1/22.5 dilution are considered to be low positive or borderline. All positive, borderline and doubtful sera should be confirmed using the VN test.

STANDARD REFERENCE SERA FOR SVD SEROLOGY

The WOAH Reference Laboratory, Pirbright, UK maintains a panel of reference sera that have been extensively validated by the National SVD Reference Laboratories of the Member States of the European Union. This panel includes the low-positive serum defining the lowest level of antibodies that should consistently provide a positive result by ELISA and Virus Neutralisation ~~(RS01-04-94 or equivalent)~~. Positive sera equivalent to these reference standards and Mab 5B7 are available at the WOAH Reference Laboratory, Brescia, Italy.

C. REQUIREMENTS FOR VACCINES

No commercial SVD vaccines are currently available.

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NB: There are WOAH Reference Laboratories for swine vesicular disease (please consult the WOAH Web site: https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3). Please contact the WOAH Reference Laboratories for any further information on diagnostic tests and reagents for swine vesicular disease

NB: FIRST ADOPTED IN 1989. MOST RECENT UPDATES ADOPTED IN 2018.

Appendix 1: Swine vesicular disease

Intended purpose of test: Identification of the agent – individual animal freedom from infection prior to movement; contribute to eradication policies; confirmation of clinical cases

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
RT-PCR swine	Epithelial lesions, faeces Analyte: virus genomic target (3D)	Reference tests were isolation of SVDV in cell cultures or a positive result in RT-PCR combined with other evidence of infection (serology or epidemiological correlation) Dsp=100% Dse= 100%	78 faecal samples, collected from 78 different SVD outbreaks that occurred in Italy during 1997–2014. 300 field faecal samples originating from SVDV-free regions plus many thousands of faecal samples tested over several years as part of the successful eradication plan in Italy	See reference	High analytical and diagnostic sensitivity Faster results than virus isolation		Pezzoni *et al.* (2021)
Virus Isolation swine	Epithelial lesions, faeces Analyte: live virus	Historically considered the reference method.			Enables recovery of live virus, potentially useful for subsequent studies for virus characterization	A significant proportion of faecal samples inoculated into cell cultures will give rise to the growth of other enteroviruses. Inoculation of cells with faecal samples may result in bacterial contamination, despite the use of antibiotics in the cell culture media Requires biocontainment conditions Time-consuming	
ELISA for antigen detection swine	Epithelial lesions Analyte: virus antigen	The MAb-based test was evaluated for the ability of selected monoclonal/polyclonal antibodies to recognise and capture SVDV in ELISA	77 European SVDV isolates collected up to 1994 and representative of the four antigenic variants of the virus were recognised by the 5B7 MAb selected for the antigen-ELISA (Brocchi *et al.*, 1997), as well as all SVD viruses isolated later on	See reference	Since based on specific SVD poly/monoclonal antibodies, the test is highly specific Easy to perform especially in poorly equipped laboratories Faster results than virus isolation Useful to complement virus isolation for the identification of SVDV in case of cytopathic effect	Lower analytical sensitivity than RT-PCR and virus isolation Applicable only on epithelial lesions	Brocchi *et al.* (1997)

Appendix 2: Swine vesicular disease

Intended purpose of test: Detection of immune response – population freedom from infection; individual animal freedom from infection prior to movement; contribute to eradication policies; confirmation of clinical cases; prevalence of infection –surveillance

Test with score and species	Sample type and target analytes	Accuracy	Test population	Validation report	Advantages	Disadvantages	References
Competitive ELISA for Ab screening swine	Sera	Dsp and Dse were calculated using field samples of known origin. Dsp=99.55% Dse= 100% The reference test used in the study was VNT Correlation between VNT and competitive ELISA in positive sera titration r=0.81, p<0.001	**Field sera:** 1) 5371 pig sera collected from 1060 SVDV-free herds 2) Positive field sera (*n* = 300) collected in Italy from different herds notified as SVDV outbreaks **Experimental sera**: 148 sera were collected from 12 pigs experimentally infected with SVDV UKG27/72 or SVDV Italy R1076/1992 and sequentially collected up to 1 or 2 months after infection	See reference	Easy to perform, suitable for large-scale application or automation Ideal for surveillance, control, and eradication	Singleton reactors occurrence need to be checked with VNT, IgG and IgM ELISA (and resampling)	Brocchi *et al.* (1995)
Virus neutralisation swine	Sera	It is accepted as the confirmatory test for the detection of antibodies to SVDV	See the sera description below	See reference	It detects neutralising antibodies	It requires 1–3 days for execution Maintenance and use of live virus and tissue culture cells, thus biocontainment restriction procedures are required A small proportion of sera remains false-positive in VNT when used as a confirmatory test (singleton reactors, 0,1-0.2% of a naive population)	Brocchi *et al.* (1995) DeClercq (1998)
ELISA for IgG and IgM identification swine	Sera	The methods have provided evidence of the different chronology of IgM and IgG production VNT and competitive ELISA were used as reference tests	See the experimental sera description for competitive ELISA	See reference	Can differentiate between IgG and IgM Can identify different stages of infection Can confirm the results of the competitive ELISA and VNT Can be helpful to confirm singleton reactors	For positivity confirmation, both IgG and IgM tests have to be carried out simultaneously.	Brocchi *et al.* (1995)

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SECTION 3.9.

SUIDAE

CHAPTER 3.9.1.

AFRICAN SWINE FEVER (INFECTION WITH AFRICAN SWINE FEVER VIRUS)

SUMMARY

African swine fever (*ASF*) *is an infectious disease of domestic and wild pigs of all breeds and ages, caused by ASF virus* (*ASFV*). *The clinical syndromes vary from peracute, acute, subacute to chronic, depending on the virulence of the virus. Acute disease is characterised by high fever, haemorrhages in the reticuloendothelial system, and a high mortality rate. Soft ticks of the* Ornithodoros *genus, especially* O. moubata *and* O. erraticus, *have been shown to be both reservoirs and transmission vectors of ASFV. The virus is present in tick salivary glands and passed to new hosts* (*domestic or wild suids*) *when feeding. It can be transmitted sexually between ticks, transovarially to the eggs, or transtadially throughout the tick's life.*

ASFV is the only member of the Asfarviridae *family, genus* Asfivirus.

Laboratory diagnostic procedures for ASF fall into two groups: detection of the virus and serology. The selection of the tests to be carried out depends on the disease situation and laboratory diagnostic capacity in the area or country.

Identification of the agent: *Laboratory diagnosis must be directed towards isolation of the virus by inoculation of pig leukocyte or bone marrow cultures, the detection of antigen in smears or cryostat sections of tissues by fluorescent antibody test and/or the detection of genomic DNA by the polymerase chain reaction* (*PCR*) *or real-time PCR. The PCRs are excellent, highly sensitive, specific and rapid techniques for ASFV detection and are very useful under a wide range of circumstances. They are especially useful if the tissues are unsuitable for virus isolation and antigen detection. In doubtful cases, the material is passaged in leukocyte cell cultures and the procedures described above are repeated.*

Serological tests: *Pigs that survive natural infection usually develop antibodies against ASFV from 7–10 days post-infection and these antibodies persist for long periods of time. Where the disease is endemic, or where a primary outbreak is caused by a strain of low or moderate virulence, the investigation of new outbreaks should include the detection of specific antibodies in serum or extracts of the tissues submitted. A variety of methods such as the enzyme-linked immunosorbent assay* (*ELISA*), *the indirect fluorescent antibody test* (*IFAT*), *the indirect immunoperoxidase test* (*IPT*), *and the immunoblotting test* (*IBT*) *is available for antibody detection.*

Requirements for vaccines*: ~~At present, there is no vaccine for ASF.~~ Commercially produced modified live virus vaccines are ~~available and licenced~~ ~~under field evaluation~~ being evaluated and have received regulatory approval for field use in some countries.*

A. INTRODUCTION

The current distribution of African swine fever (ASF) extends across more than 50 countries in three continents (Africa, Asia and Europe). Several incursions of ASF out of Africa were reported between the 1960s and 1970s. In 2007, ASF was introduced into Georgia, from where it spread to neighbouring countries including the Russian Federation. From there ASF spread to eastern European countries extending westwards and reaching the European Union in 2014. Further westward and southern spread in Europe has occurred since that time. In all these countries, both hosts – domestic pig and wild boar – were affected by the disease. In August 2018, the People's Republic of China reported its first outbreak of ASF and further spread in Asia has occurred. ASF was identified on the island of Hispaniola (Haiti and the Dominican Republic) in 2021. See WAHIS (https://wahis.woah.org/#/home) for recent information on distribution at the country level.

ASF virus (ASFV) is a complex large, enveloped DNA virus with icosahedral morphology. It is currently classified as the only member of the *Asfaviridae* family, genus *Asfivirus* (Dixon *et al.*, 2005). More than 60 structural proteins have been identified in intracellular virus particles (200 nm) (Alejo *et al.,* 2018). More than a hundred infection-associated proteins have been identified in infected porcine macrophages, and at least 50 of them react with sera from infected or recovered pigs (Sánchez-Vizcaíno & Arias, 2012). The ASFV double-stranded linear DNA genome comprises between 170 and 193 kilobases (kb) and contains between 150 and 167 open reading frames with a conserved central region of about 125 kb and variable ends. These variable regions encode five multigene families that contribute to the variability of the virus genome. The complete genomes of several ASFV strains have been sequenced (Bishop *et al.,* 2015; Chapman *et al.,* 2011; de Villiers *et al.,* 2010; Portugal *et al.,* 2015). Different strains of ASFV vary in their ability to cause disease, but at present there is only one recognised serotype of the virus detectable by antibody tests.

The molecular epidemiology of the disease is investigated by sequencing of the 3' terminal end of the B646L open reading frame encoding the p72 protein major capsid protein, which differentiates up to 24 distinct genotypes (Achenbach *et al.*, 2017; Boshoff *et al.,* 2007; Quembo *et al.* 2018). To distinguish subgroups among closely related ASFV, sequence analysis of the tandem repeat sequences (TRS), located in the central variable region (CVR) within the B602L gene (Gallardo *et al.,* 2009; Lubisi *et al.,* 2005; Nix *et al.,* 2006) and in the intergenic region between the I73R and I329L genes, at the right end of the genome (Gallardo *et al.,* 2014), is undertaken. Several other gene regions such as the E183L encoding p54 protein, the CP204L encoding p30 protein, and the protein encoded by the EP402R gene (CD2v), have been proved as useful tools to analyse ASFVs from different locations and hence track virus spread.

ASF viruses produce a range of syndromes varying from peracute, acute to chronic disease and subclinical infections. Pigs are the only domestic animal species that is naturally infected by ASFV. European wild boar and feral pigs are also susceptible to the disease, exhibiting clinical signs and mortality rates similar to those observed in domestic pigs. In contrast African wild pigs such as warthogs (*Phacochoerus aethiopicus*), bush pigs (*Potamochoerus porcus*) and giant forest hogs (*Hylochoerus meinertzhageni*) are resistant to the disease and show few or no clinical signs. These species of wild pig act as reservoir hosts of ASFV in Africa (Costard *et al.,* 2013; Sánchez-Vizcaíno *et al.,* 2015).

The incubation period is usually 4–19 days. The more virulent strains produce peracute or acute haemorrhagic disease characterised by high fever, loss of appetite, haemorrhages in the skin and internal organs, and death in 4–10 days, sometimes even before the first clinical signs are observed. Case fatality rates may be as high as 100%. Less virulent strains produce mild clinical signs – slight fever, reduced appetite and depression – which can be readily confused with many other conditions in pigs and may not lead to suspicion of ASF. Moderately virulent strains are recognised that induce variable disease forms, ranging from acute to subacute. Low virulence, non-haemadsorbing strains can produce subclinical non-haemorrhagic infection and seroconversion, but some animals may develop discrete lesions in the lungs or on the skin in areas over bony protrusions and other areas subject to trauma. Animals that have recovered from either acute, subacute or chronic infections may potentially become persistently infected, acting as virus carriers. The biological basis for the persistence of ASFV is still not well understood, nor it is clear what role persistence plays in the epidemiology of the disease.

ASF cannot be differentiated from classical swine fever (CSF) by either clinical or post-mortem examination, and both diseases should be considered in the differential diagnosis of any acute febrile haemorrhagic syndrome of pigs. Bacterial septicaemias may also be confused with ASF and CSF. Laboratory tests are essential to distinguish between these diseases.

In countries free from ASF but suspecting its presence, the laboratory diagnosis must be directed towards isolation of the virus by the inoculation of pig leukocyte or bone marrow cultures, the detection of genomic DNA by polymerase chain reaction (PCR) or the detection of antigen in smears or cryostat sections of tissues by direct fluorescent antibody test (FAT). Currently the PCR is the most sensitive technique and can detect ASFV DNA from a very early stage of infection in tissues, ethylene diamine tetra-acetic acid (EDTA)-blood and serum samples. The PCR is particularly useful if samples submitted are unsuitable for virus isolation and antigen detection because they have undergone putrefaction. Pigs that have recovered from acute, subacute or chronic infections usually exhibit a viraemia for several weeks making the PCR test a very useful tool for the detection of ASFV DNA in pigs infected with low or moderately virulent strains. Virus isolation by the inoculation of pig leukocyte or bone marrow cultures and identification by haemadsorption tests (HAD) are recommended as a confirmatory test when ASF is positive by other methods, particularly in the event of a primary outbreak or a case of ASF.

~~As no vaccine is available, the presence of ASFV antibodies is indicative of previous infection and, as antibodies are produced from the first week of infection and persist for long periods, they are a good marker for the diagnosis of the disease, particularly in subacute and chronic forms.~~

Vaccines should be prepared in accordance with Chapter 1.1.8 *Principles of veterinary vaccine production*. Current ASF modified live virus (MLVs) vaccines are based on the live virus that have been naturally attenuated or attenuated by targeted genetic recombination through cell cultures (Gladue & Borca, 2022). MLV production is based on a seed-lot system consistent with the *European Pharmacopoeia* (11th edition) and that has been validated with respect to virus identity, sterility, purity, stability potency, ~~stability, safety~~ and immunogenicity ~~(including spread), non-transmissibility, stability and immunogenicity~~. ASF MLV first generation vaccines ~~– defined as those for which peer-reviewed publications are in the public domain –~~ should meet or exceed the minimum standards as described below. ~~Paramount~~ Demonstration of acceptable safety and efficacy against the epidemiologically relevant ASFV field strain(s) circulating in areas where the vaccine is intended for use ~~are~~ is required. At the present time, a variety of mutants (Forth *et al.*, 2023) and recombinants (Zhao *et al.*, 2023) have emerged globally, and the prevalence of these strains is increasing. In addition, there is a risk that vaccine strains ~~will~~ might revert to virulence and/or recombine with circulating strains. These conditions should be taken into account in vaccine development. ~~acceptable efficacy should be shown against the B646L (p72) genotype II pandemic virus lineage currently circulating widely in domestic pigs and wild boar.~~

ASF MLV first generation vaccines allowing the differentiation of infected animals from vaccinated animals (DIVA) by suitable methods (e.g. serology-based tests) are preferred. Minimum standards set out in this chapter include safety and efficacy testing in young pigs (4–10 weeks old) and safety testing in pregnant sows. Demonstration of MLV safety and efficacy in pigs at different growth stages, including ~~(suckling piglets, nursery pigs, fattening pigs), the safety in~~ breeding-age boars, ~~and gilts and pregnant sows,~~ and onset and duration of protective immunity, are also preferred but are not required to meet the minimum standard. Additional data will likely be required by Regulatory Authorities if ~~these~~ specific categories are included in the indications for the vaccine. Details of the onset of immunity (the interval of time elapsed between vaccination and challenge if protection is confirmed) and the duration of immunity (last time-point at which vaccine-induced immunity has been demonstrated) ~~(the time point at which vaccine induced immunity begins to decline and provides less protection)~~ are also required to meet minimum standards.

ASF epidemiology is complex with different epidemiological patterns of infection occurring in Africa and Europe. ASF occurs through transmission cycles involving domestic pigs, wild boar, wild African suids, and soft ticks (Sánchez-Vizcaíno *et al.*, 2015). In regions where *Ornithodoros* ~~soft bodied~~ ticks are present, the detection of ASFV in these reservoirs of infection contributes to a better understanding of the epidemiology of the disease. This is of major importance in establishing effective control and eradication programmes (Costard *et al.*, 2013).

ASF is not a zoonotic disease and does not affect public health (Sánchez-Vizcaíno *et al.*, 2009).

ASFV should be handled with an appropriate level of bio-containment, determined by risk analysis in accordance with Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*.

. . .

C. REQUIREMENTS FOR VACCINES [UNDER REVIEW]

~~At present there is no commercially available vaccine for ASF.~~ Commercially produced modified live virus vaccines are being evaluated and have ~~licensed~~ received regulatory approval for field use in some countries.

1. Background

The ASF p72 genotype II strains (ASFV Georgia 2007/1 lineage) (NCBI, 2020) are recognised to be the current highest global threat for domestic pig production worldwide (Penrith *et al.*, 2022). However, genotype I ~~attenuated~~ low virulent strains and genotype I/II recombinant strains have been reported to be circulating. In Africa, multiple genotypes are circulating.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of Veterinary Vaccine Production*. Varying additional requirements relating to quality (including purity and potency), safety, and efficacy will apply in particular countries or regions for manufacturers to comply with local regulatory requirements.

Wherever live, virulent ASFV or ASF MLVs are stored, handled and disposed, the appropriate biosecurity level, procedures and practices should be used. The ASF MLV vaccine production facility should meet the requirements for containment

outlined in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*.

An optimal ASF MLV first generation vaccine for the target host should have the following general characteristics (minimum standards):

- Safe: demonstrate absence of persistent fever as defined below (see Section 2.3.2) and clinical signs of acute or chronic ASF in vaccinated and in-contact animals, minimal and ideally no vaccine virus transmission, and absence of an increase in virulence (genetic and phenotypic stability);
- Efficacious: protects against mortality, reduces acute and other forms of disease (fever accompanied by the appearance of clinical signs caused by ASF) and reduces ~~vertical (boar semen and placental) and~~ levels of challenge virus viraemia and shedding ~~horizontal disease transmission~~;
- Quality – purity: free from wild-type ASFV and extraneous microorganisms that could adversely affect the safety~~, potency~~ or efficacy of the product;
- Quality – ~~potent~~ stability: the ~~log$_{10}$~~ virus titre maintained throughout the vaccine shelf life that guarantees the efficacy demonstrated by the established minimum immunising (protective) dose;
- ~~Identity~~ Vaccine matching: based on the capacity to protect against the ASFV B646L (p72) genotype II pandemic strain or other p72 genotypes of recognised epidemiological importance.

Vaccine production should be carried out using a validated, controlled and consistent manufacturing process.

ASF MLV first generation vaccines must be safe (i.e. an acceptable safety profile) for non-target species and the environment in general.

Ideally, ASF MLV first generation vaccines that meet the minimum standards should also fulfil the following additional general characteristics: i) prevents acute and persistent ~~(carrier state)~~ disease; ii) prevents horizontal and vertical disease transmission; iii) induces rapid protective immunity (e.g. < ~~2~~ 4 weeks); and iv) confers stable, life-long immunity.

Furthermore, ASF MLV second and future generation vaccines should meet the minimum safety and efficacy standards as ASF MLV first generation vaccines, and ideally provide additional product profile benefits, including but not limited to: i) contain a negative marker allowing the differentiation of infected from vaccinated animals (DIVA) by reliable discriminatory tests such as serology-based tests; and ii) confer cross protection against circulating related strains and some ~~broad range of protection against~~ other p72 genotype field strains of varying virulence (low, moderate, and high).

The majority of ASF global vaccine research groups and companies are currently focused on ASF MLV first generation vaccine candidate~~s that are safe and efficacious~~ against ASF viruses belonging to the ASFV p72 genotype II pandemic strain (ASFV Georgia 2007/1 lineage) (NCBI, 2020). More research is needed to determine whether these genotype II-specific MLVs can effectively protect against newly circulating variants of genotype II and recombinant strains.

Currently, two recombinant gene deleted MLV ~~recombinant~~ vaccines (ASFV-G-ΔI177L and ASFV-G-ΔMGF) have ~~been licensed~~ received regulatory approval ~~for field use~~ in Vietnam for use in domestic pigs ~~following supervised field testing to evaluate the safety and effectiveness of several vaccine batches~~.

There are numerous, promising ASF MLV vaccine candidates targeting the p72 genotype II pandemic strain under development, including:

- A naturally attenuated field strain (Lv17/WB/Rei1) (Barasona *et al.*, 2019) being developed as an oral bait vaccine for wild boar~~s~~;
- A laboratory thermo-attenuated field strain (ASFV-989) (Bourry *et al.*, 2022);
- Single gene-deleted, recombinant viruses (e.g. SY18ΔI226R, ASFV-G-ΔA137R) (Gladue *et al.*, 2021; Zhang *et al.*, 2021);
- Double gene-deleted, recombinant viruses (e.g. ASFV-G-Δ9GL/ΔUK; ASFV-SY18-ΔCD2v/UK; Arm-ΔCD2v-ΔA238L) (O'Donnell *et al.*, 2016; Pérez-Núñez *et al.*, 2022; Teklue *et al.*, 2020);
- Multiple gene-deleted, recombinant viruses (ASFV-G-ΔI177L/ΔLVR; ASFV-G-ΔMGF; ~~BA71ΔCD2;~~ HLJ/18-7GD; ASFVGZΔI177LΔCD2vΔMGF, Arm07ΔMGF) (Borca *et al.*, 2021; Chen *et al.*, 2020; Kitamura *et al.*, 2023; Liu *et al.*, 2023; Monteagudo *et al.*, 2017; O'Donnell *et al.*, 2015).

Information regarding many of these MLV vaccine candidates can be found in a recent review publication (Brake, 2022).

Different DIVA strategies using serological methods (e.g. ELISA) or genome detection methods (e.g. differential real-time PCR) have been published for some of the ~~are not widely available for these~~ ASF MLV first generation vaccine candidates (Borca *et al.*, 2024; Velazquez-Salinas *et al.*, 2021). ~~Therefore~~ However, there is still room for improvement with respect to marker vaccines and their companion diagnostic tests. The field use of genetically modified ASFV strains with marker genes should be compliant with the Cartagena Protocol's regulations for conserving biodiversity as set out in Chapter 1.1.8 Principles of Veterinary Vaccine Production, Section 7.2.3.2 Additional requirements for live rDNA products.

Inactivated (non-replicating) whole virus vaccines are not presently available and may be difficult to develop to meet minimum efficacy standards. Recombinant vectored, subunit vaccine candidates that can be produced in scalable vaccine platform expression systems and mRNA-based ASF vaccines are being evaluated in ongoing laboratory research, testing and evaluation in experimental challenge models. The publicly available *Center of Excellence for African Swine Fever Genomics* (ASFV Genomics, 2022[1]) that provides the structural protein predictions for all 193 ASFV proteins may help accelerate ASF first and second generation vaccine research and development.

Any future use of vaccine candidates should be based on a thorough risk–benefit assessment considering all safety and efficacy features, as well as the potential vaccination scenario. Fit-for-purpose vaccine use scenarios matched to the intended use in a domestic pig-specific type of production system may require different vaccine product profiles or may influence the focus of essential versus ideal vaccine requirements. Prudent use of ASF MLVs as part of strict, controlled vaccination programmes, especially in the areas where ASF is not prevalent, should be implemented. Transmission of vaccine virus to non-vaccinates (domestic or wildlife) could be particularly problematic in areas where ASF is not known to be present.

It is important to know what genotypes of ASFV are circulating in a population before vaccination is introduced. Due to the potential risk of recombination events between circulating low and high virulent field strains with future ~~licensed~~ vaccine strains with regulatory approval, and the possibility of reversion to virulence of vaccine strains, strict pharmacovigilance post-vaccination using stringent reporting criteria (e.g. any fever is reportable – in contrast to the safety testing criteria below of 2 days of fever) is essential. Field pharmacovigilance data should be collected and analysed during vaccination campaigns using ASF MLV first generation vaccines post-~~licensing~~ regulatory approval. Active post-vaccination surveillance programmes for the detection of new ASF viruses that may arise from MLV vaccine strains and naturally circulating wild-type virus recombination, as well as revertant vaccine strains, should be implemented. It is also recommended that vaccine manufacturers carry out laboratory experiments to further evaluate the risk of vaccine virus recombination with field and vaccine strains.

As with any ~~MLV~~ vaccine, all ASF ~~MLV~~ vaccines should be used according to the label instructions, under the strict control of the country's Regulatory Authority.

The minimum standards given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national, regional, and veterinary international medicinal product harmonised requirements. Minimum data requirements for ~~an authorisation~~ regulatory approval in exceptional circumstances (e.g. unexpected introduction of the virus, sudden outbreaks of the disease) should be considered where applicable.

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed virus

2.1.1. Biological characteristics of the master seed virus

ASF MLVs are generally produced from ASFV field strains derived from naturally attenuated field isolates or using DNA homologous (genetically targeted) recombination techniques in cell cultures to delete one or more ASFV genes or gene families. These molecular techniques typically involve replacement of the targeted ASFV gene(s) with one or more positive, marker fluorescent (e.g. BFP, eGFP, mCherry) or enzyme-based (e.g. β-glucuronidase) ASFV promoter-reporter gene systems that allow the use of imaging microscopy or flow cytometry to visualise, select, and clone gene-deleted, recombinant, ASF MLVs. MLV production is carried out in cell cultures based on a seed-lot system.

Master seed viruses (MSVs) for MLVs should be selected and produced based on their ease of growth in cell culture, virus yield (log_{10} infectious titre) and genetic stability over multiple cell passages. Preferably, a continuous well-characterised cell line (e.g. ZMAC-4; PIPEC; IPKM) (Borca *et al.*, 2021; Masujin *et al.*, 2021; Portugal *et al.*, 2020) is used to produce a master cell bank (MCB) on which the MSV and MSV-derived working seed virus (WSV) can be produced. The exact source of the underlying ASFV isolate, the whole genome sequence, and the passage history must be recorded.

[1] http://asfvgenomics.com. Accessed 4/4/2023.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

Only MSVs that have been established as sterile, pure (free of wild-type parental virus and free of extraneous agents as described in Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*, and those listed by the appropriate ~~licensing~~ regulatory approval authorities) and immunogenic, should be used as the vaccine virus (WSV and vaccine batch production). ~~Live vaccines must be shown not to cause disease or other adverse effects in target animals in accordance with chapter 1.1.8, Section 7.1 *Safety tests* (for live attenuated MSVs), that includes target animal safety tests, increase in virulence tests, assessing the risk to the environment) and if possible, no transmission to other animals.~~

Identity of the MSV must be confirmed using appropriate methods (e.g. through the use of vaccine strain-specific whole genome detection methods such as next generation sequencing).

Demonstration of MSV stability over several cell passages is necessary, typically through at least five passages (e.g. MSV+5). For those MLV vaccines for which attenuation is linked to specific characteristics (gene deletion, gene mutations, etc.), genetic stability of attenuation throughout the production process should be confirmed by full genome sequencing and confirmation of the vaccine phenotype, for example, by confirming the virus titre obtained by growth in the cell line used for production ~~using suitable methods. Suitable techniques to demonstrate genetic stability may include but are not limited to: genome sequencing, biochemical, proteomic, genotypic (e.g. detection of genetic markers) and phenotypic strain characterisation~~. If final product yields (infectious titres) are relatively low, as is typically the case with ASFV, demonstration of stability is required for the maximum passage for use in the final product manufacturing as defined by the producer ~~genetic stability at a minimum of MSV+10 should be demonstrated to allow more flexibility in the outline of production. For example, if MSV+8 is the maximum passage for use in final product manufacturing, demonstration of genetic stability to at least MSV+10 is warranted~~.

2.1.3. Validation as a vaccine strain

The vaccine derived from the MSV must be shown to be satisfactory with respect to safety and efficacy. Live vaccines must be shown to not cause disease or other adverse effects in target animals in accordance with chapter 1.1.8, Section 7.1 *Safety tests* (for live attenuated MSVs), that includes target animal safety tests, increase in virulence tests, assessing the risk to the environment) and if possible, no transmission to other animals.

Even if pigs are not known for susceptibility to transmissible spongiform encephalopathy (TSE) agents, consideration should also be given to minimising the risk of TSE transmission by ensuring that animal origin materials from TSE-relevant species, if no alternatives exist for vaccine virus propagation, comply with the measures on minimising the risk of transmission of TSE.

Ideally, the vaccine virus in the final product should generally not differ by more than five passages from the master seed lot.

ASF vaccines should be presented in a suitable pharmaceutical form (e.g. lyophilisate or liquid form).

2.2. Method of manufacture

2.2.1. Procedure

The MLV ~~virus~~ is used to infect swine primary cell cultures obtained from specific-pathogen free pigs, the requirements for which are defined in specific monographs (Chapter 2.3.3 *Minimum requirements for the organisation and management of a vaccine manufacturing facility*, Section 2.4.2). It should be noted that each donor pig should be considered a different "master cell stock" and be tested for purity and extraneous agents to account for the risk of contamination during cell collection and processing. Similar considerations should apply to collections over time, and the herd health of the donor pigs should be closely monitored. Compared with primary cell cultures, use of a continuous cell line generally allows for more consistency, higher serial volumes in manufacturing and aligns better with a seed lot system. Thus, preferably a master cell bank based on an established, continuous cell line shown to support genetically stable ASFV replication and acceptable titres over several passages should be used.

Cell cultures shall comply with the requirements for cell cultures for production of veterinary vaccines in chapter 1.1.8. Regardless of the production method, the substrate should be harvested under aseptic conditions and may be subjected to appropriate methods to release cell-associated virus (e.g. freeze–thaw cycles, detergent lysis). The harvest can be further processed by filtration and other purification methods.

A stabiliser or other excipients may be added as appropriate. The vaccine is homogenised to ensure a uniform batch/serial.

2.2.2. Requirements for ingredients

All ingredients used for vaccine production should be in line with requirements in chapter 1.1.8.

2.2.3. In-process controls

In-process controls will depend on the protocol of production; they include virus titration of bulk antigen and sterility tests. Biosafety precautions should align with the outcomes of a biosafety risk assessment and conform to local and national guidelines.

2.2.4. Final product batch tests

i) Sterility

Tests for sterility and freedom from contamination of biological materials intended for veterinary use may be found in chapter 1.1.9.

ii) Identity

Appropriate methods such as specific genome detection methods (e.g. specific differential real-time PCR and/or full genome sequencing) should be used for confirmation of the identity of the vaccine virus and differentiation from the parent strain of the virus as a potential contaminant.

iii) Purity

Appropriate methods should be used to ensure that the final product batch does not contain any residual wild-type ASFV.

iv) Safety

Batch safety testing is to be carried out unless consistent safety of the product is demonstrated and approved in the registration dossier and the production process is approved for consistency in accordance with the standard requirements referred to in chapter 1.1.8.

v) Batch/serial potency

Virus titration is a reliable indicator of vaccine potency once a relationship has been established between the vaccine minimum immunising dose (MID) (minimum protective dose) and titre of the modified live vaccine in vitro. In the absence of a demonstrated correlation between the virus titre and protection, an efficacy test will be necessary (Section C.2.3.3 *Efficacy requirements*, below).

vi) Residual humidity/residual moisture

The test should be carried out consistent with VICH[2] GL26 (*Biologicals: Testing of Residual Moisture*, 2003[3]). Required for MLV vaccines presented as lyophilisates ~~for suspension for injection~~.

2.3. Requirements for ~~authorisation/registration/licensing~~ regulatory approval

2.3.1. Manufacturing process

~~For regulatory approval of a vaccine,~~ All relevant details concerning history of the pre-MSV, preparation of MSV, manufacture of the vaccine and quality control testing (Sections C.2.1 *Characteristics of the seed* and C.2.2 *Method of manufacture*) should be submitted to the regulatory approval authorities.

Information shall be provided from preferably three or more ~~preferably~~ consecutive vaccine batches originating from the same MSV and representative of routine production~~, with a volume not less than 1/10, and more preferably with a volume not less than 1/3 of the typical industrial batch volume~~. The in-process controls are part of the manufacturing process.

[2] VICH: International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medical Products

[3] https://www.ema.europa.eu/en/documents/scientific-guideline/vich-gl26-biologicals-testing-residual-moisture-step-7_en.pdf

Genetic stability of attenuation throughout the production process (i.e. to the maximum passage level to be used for vaccine production) should be confirmed by full genome sequencing and confirmation of virus phenotype (e.g. virus yield in cell line used for production).

2.3.2. Safety requirements

For the purpose of gaining regulatory approval, the following safety tests should be performed ~~satisfactorily~~.

As a minimum standard, vaccines should be tested for any pathogenic effects on healthy domestic pigs of the target age intended for use. In addition, due to risks from horizontal spread of the vaccine virus, vaccine safety testing should be carried out in pregnant sows as the population that is likely to be the most sensitive indicator of any adverse effects. Additional demonstration of MLV safety in pigs at different growth stages, including breeding-age boars and gilts ~~in breeding-age gilts and pregnant sows~~ is preferred ~~but not required as a minimum standard~~. When the vaccine is recommended for use or may be used ~~If in the future a vaccine intended for use~~ in breeding animals ~~is developed~~, an evaluation of the impact of the vaccine on reproductive performance will be a standard safety requirement.

i) Safety in young animals

Carry out the test by each recommended route of administration using, in each case, piglets a minimum of ~~6~~ 4-weeks old and not older than 10-weeks old.

The test is conducted using no fewer than eight healthy piglets, and preferably no fewer than ten healthy piglets.

To obtain individual mean baseline temperatures, the body temperature of each piglet should be measured on at least the 3 consecutive days preceding administration of the vaccine. Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine.

Administer to each piglet a quantity of the vaccine virus ~~equivalent to~~ not less than ten times the maximum virus titre ~~(e.g. 50% haemadsorption dose [HAD_{50}], 50% tissue culture infective dose [$TCID_{50}$], quantitative PCR, etc.) (maximum release dose)~~ likely to be contained in one dose of the vaccine.

~~To obtain individual and group mean baseline temperatures, the body temperature of each vaccinated piglet is measured on at least the 3 consecutive days preceding administration of the vaccine.~~

To confirm the presence or absence of fever accompanied by acute and chronic disease, ~~observe~~ measure body temperature and clinical signs in the piglets 4 hours after vaccination and then at least once daily for at least 45 days, preferably 60 days post-vaccination. On each day during the observation period the maximum increase in body temperature above the baseline observed for each pig will be recorded. Carry out the daily observations for signs of acute and chronic disease using a quantitative clinical scoring system adding the values for multiple clinical signs (e.g. Gallardo *et al.*, 2015a). These clinical signs should include fever, anorexia, recumbency, skin haemorrhage or cyanosis, joint swelling and necrotic lesions around the joints, respiratory distress and digestive findings).

At a minimum of 45 days post-vaccination, humanely euthanise all vaccinated piglets. Conduct gross pathology on spleen, lung, tonsil, and kidney tissue samples and at least three different lymph nodes (which should include lymph node closest to site of inoculation, gastrohepatic and submandibular nodes).

The vaccine is compliant ~~complies with the test~~ if:

- No piglet shows abnormal (local or systemic) reactions or notable signs of disease, or reaches the pre-determined humane endpoint defined in the clinical scoring system or dies from causes attributable to the vaccine;
- ~~The average body temperature increase for all vaccinated piglets (group mean) for the observation period does not exceed 1.5°C above baseline; and no individual piglet shows a temperature rise above baseline greater than 2.5°C for a period exceeding 3 days.~~

- ~~On each day during the observation period, the maximum increase in body temperature above the baseline observed for each pig will be used to calculate the daily group mean temperature rise. This mean value should not exceed 1.5°C and~~ No individual pig should show a rise in temperature above baseline greater than 1.5°C for a period exceeding ~~3~~ 2 consecutive days that is attributable to ASFV infection. In cases where pigs exceed the temperature standard but show no behavioural changes or other clinical signs, regulators may determine vaccine safety without solely relying on temperature for non-compliance;

- No vaccinated pigs show notable signs of disease by gross pathology.

ii) Safety test in pregnant sows and test for transplacental transmission

There is limited ~~currently an absence of~~ published information on ASFV pathogenesis in ~~breeding-age gilts and in~~ pregnant sows associated with ASFV transplacental infection and fetus abortion/stillbirth. ~~If a label claim is pursued for use in breeding age gilts and sows, then~~ Due to risks from horizontal spread and contact transmission of the vaccine virus, a safety study in line with VICH GL44 (*Guidelines on Target Animal Safety for Veterinary Live and Inactivated Vaccines, Section 2.2. Reproductive Safety Test*, 2009[4]) should be completed.

Carry out the test with vaccination by a recommended route using no fewer than eight healthy sows of similar age, between the 55th and 70th day of gestation, that do not have antibodies against ASFV and blood samples are negative on PCR. Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine.

Administer to each sow a quantity of the vaccine virus equivalent to not less than the maximum virus titre likely to be contained in 1 dose of the vaccine. Clinical observation of animals is carried out daily until farrowing. Blood samples should be taken from newborn piglets before ingestion of colostrum.

The test is invalid if the vaccinated sows do not seroconvert before farrowing.

The vaccine is compliant if:

- No pregnant sows show abnormalities in their gestation or in their piglets;

- No pregnant sows show notable signs of disease or dies from causes attributable to the vaccine;

- No vaccine virus or antibodies against ASFV are present in blood samples from newborn piglets.

iii) Horizontal transmission

The test is conducted using no fewer than 12 healthy piglets~~,~~ a minimum of ~~6~~ 4-weeks old and not older than 10-weeks ~~old, and of the same origin, that~~ Piglets do not have antibodies against ASFV~~,~~ and blood samples are negative on real-time PCR. ~~All piglets are housed together from day 0 and the number of vaccinated animals is the same as the number of naïve, contact animals.~~

Co-mingle the piglets in the same pen or room so that equal numbers (at least six) can be ~~of~~ vaccinated and the same number can remain unvaccinated (naïve contact piglets) ~~and naïve, contact piglets from day 0 in the same pen or room.~~

To obtain individual mean baseline temperatures, the body temperature of each naive contact piglet should be measured on at least the 3 consecutive days preceding administration of the vaccine. Use vaccine virus at the least attenuated passage level that will be present between the master seed lot and a batch of the vaccine. Administer by ~~each~~ the recommended route of administration to no fewer than six piglets a quantity of the vaccine virus equivalent to not less than the maximum virus titre (maximum release dose) likely to be contained in 1 dose of the vaccine.

~~To obtain individual and group mean baseline temperatures, the body temperature of each naïve, contact piglet is measured on at least the 3 consecutive days preceding co-mingling with vaccinated piglets.~~ The body temperature of each naïve, contact piglet is then measured daily for at least 45 days, preferably 60 days. ~~To confirm the presence or absence of fever accompanied by disease, observe the naïve, contact piglets daily for at least 45 days, preferably 60 days.~~ On each day during the observation period the maximum increase in body temperature above the baseline observed for

[4] https://www.ema.europa.eu/en/documents/scientific-guideline/vich-gl44-target-animal-safety-veterinary-live-inactived-vaccines-step-7_en.pdf.

each pig will be recorded. ~~used to calculate the daily group mean temperature rise. This mean value should not exceed 1.5°C and no individual pig should show a rise in temperature above baseline greater than 1.5°C for a period exceeding 3 consecutive days.~~ Carry out ~~the~~ daily observations for signs of acute and chronic clinical disease using a quantitative clinical scoring system adding the values for multiple clinical signs (e.g. Gallardo *et al.*, 2015a). These clinical signs should include fever, anorexia, recumbency, skin haemorrhage or cyanosis, joint swelling and necrotic lesions around the joints, respiratory distress and digestive findings.

~~In addition,~~ Blood should be taken from the naïve contact piglets at least twice a week for the first 21 days post-vaccination and then on a weekly basis. From the blood samples, determine vaccine virus titres by quantitative virus isolation (HAD_{50}/ml, $TCID_{50}$/ml or other methods, e.g. titration using IPT or FAT detection). Real-time PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration as described above ~~infectious virus titres by quantitative virus isolation (e.g. HAD_{50}/ml or $TCID_{50}$/ml) and using a real-time PCR test~~.

~~If the vaccine virus is non haemadsorbing or does not cause cytopathic effects, a real-time PCR test only may be used.~~

Collect blood (serum) samples from the naïve contact pigs at least at day 21 and day 28 days and carry out an appropriate test to detect vaccine virus induced antibodies.

At a minimum of 45 days, humanely euthanise all naïve, contact piglets. Conduct gross pathology on spleen, lung, tonsil, and kidney tissue samples and at least three different lymph nodes. Determine virus titres in all collected samples as described above. ~~by quantitative virus isolation (HAD_{50}/mg or $TCID_{50}$/mg) or other appropriate methods (e.g. IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration as described above and real-time(RT)-PCR (see Section B.1. Identification of the agent). If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test or other appropriate method (e.g. titration using IPT or FAT detection) may be used.~~

The vaccine ~~complies with the test~~ is compliant if:

- No vaccinated or naïve contact piglet shows abnormal (local or systemic) reactions or notable signs of disease, reaches the predetermined humane endpoint defined in the clinical scoring system or dies from causes attributable to the vaccine;
- ~~On each day during the observation period the maximum increase in body temperature above the baseline observed for each pig will be used to calculate the daily group mean temperature rise. This mean value should not exceed 1.5°C and~~ No individual naïve contact pig ~~should~~ shows a rise in temperature above baseline greater than 1.5°C for a period exceeding ~~3~~ 2 consecutive days that is attributable to ASFV infection. In cases where pigs exceed the temperature standard but show no behavioural changes or other clinical signs, regulators may determine vaccine safety without solely relying on temperature for non-compliance;

~~The average body temperature increase for all naïve, contact piglets (group mean) for the observation period does not exceed 1.5°C above baseline; and no individual piglet shows a temperature rise above baseline greater than 2.5°C for a period exceeding 3 days~~;

- No naïve, contact pig~~let~~ shows notable signs of disease by gross pathology ~~and no virus is detected in their blood or tissue samples~~;
- ~~No or a low percentage of contact piglets test both real-time PCR positive and seropositive No naïve contact pigs test positive for antibodies to the vaccine virus~~ No or a low percentage of naïve, contact pigs test positive to the vaccine virus and/or to antibodies against the vaccine virus.

iv) ~~**Post-vaccination kinetics**~~ **Dissemination** ~~**of viral replication**~~ **of the vaccine strain in the vaccinated animals** ~~**(MLV blood and tissue dissemination) study**~~

Prior to the reversion to virulence study (Section C2.3.2.v. below), a minimum of one study should be performed to determine the dissemination of the vaccine strain ~~the post-vaccination kinetics of virus replication~~ in the blood (viremia), tissues and viral shedding.

The test consists of the administration of the vaccine virus from the master seed lot to no fewer than eight ~~healthy piglets,~~ and preferably ten healthy ~~piglets~~ pigs. Pigs should be a minimum of ~~6~~ 4-weeks old and not older than 10-weeks. They should test negative for ~~old and of the same origin, that do not have~~ antibodies against ASFV~~,~~ and ~~blood samples are negative on real-time~~ by PCR.

Administer to each piglet, using the recommended route of administration most likely to result in spread (such as the intramuscular route or intranasal route), a quantity of the master seed vaccine virus equivalent to not less than the maximum virus titre (maximum release dose) likely to be contained in 1 dose of the final product of the vaccine.

Record daily body temperatures and observe inoculated animals daily for clinical disease for at least 45 days, preferably 60 days.

Carry out the daily observations for signs of acute and chronic clinical disease using a quantitative clinical scoring system adding the values for multiple clinical signs (e.g. Gallardo *et al.* (2015a). These clinical signs should include fever, anorexia, recumbency, skin haemorrhage or cyanosis, joint swelling and necrotic lesions around the joints, respiratory distress and digestive findings.

Collect blood samples from all the piglets at least two times per week from 3 days post-vaccination for the first 2 weeks, then weekly for the duration of the test. Determine vaccine virus titres in the samples by quantitative virus isolation (HAD_{50}/ml or $TCID_{50}$/ml) or other appropriate methods (e.g. titration using IPT or FAT detection). Real-time PCR may be used to detect positive samples but results should be confirmed by infectious virus titration as described above ~~and using a real-time PCR test. If the vaccine virus is non haemadsorbing or does not cause cytopathic effects, a real-time PCR test only may be used~~.

Determine which blood timepoint(s) should be used in the design of the reversion to virulence study (Section C.2.3.2.v. below), for example, specific blood sample(s) at specific timepoints that show the highest titres should be considered for selection and use in the reversion to virulence study.

Collect oral, nasal and faecal swab samples (preferably devoid of blood to minimise assay interference) at least two times per week from 3-days post-vaccination for the first 2 weeks, then weekly for the duration of the test. ~~Test the swabs for the presence of vaccine virus.~~ Determine virus titres in all collected samples by quantitative virus isolation ~~(HAD_{50}/ml or $TCID_{50}$/ml) or other appropriate methods (e.g. titration using IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration~~ as described above ~~and using a real-time PCR test. If the vaccine virus is non haemadsorbing or does not cause cytopathic effects, a real-time PCR test or other appropriate method (e.g. titration using IPT or FAT detection) may be used~~.

Euthanise at least two piglets on days 5, 7, 14, 21, and preferably on day 28 (±2 days at each timepoint) and collect spleen, lung, tonsil, kidney tissue samples and at least three different lymph nodes (which should include lymph node closest to site of inoculation, gastrohepatic and submandibular nodes). Determine virus titres in all collected samples by quantitative virus isolation ~~(HAD_{50}/mg or $TCID_{50}$/mg) or other appropriate methods (e.g. titration using IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration~~ as described above ~~and using real-time PCR test. If the vaccine virus is non haemadsorbing or does not cause cytopathic effects, a real-time PCR test or other appropriate method (e.g. titration using IPT or FAT detection) may be used~~.

Determine which tissue(s) and timepoint(s) should be used to aid in the design of the reversion to virulence study (Section C.2.3.2.v), for example, specific tissues at specific timepoints which show the highest titres should be considered for selection and use in the reversion to virulence study.

v) Reversion to virulence

The test carried out should be consistent with VICH GL41 (Examination of live veterinary vaccines in target animals for absence of reversion to virulence, 2008[5]).

The test for increase in virulence consists of the administration of the vaccine master seed virus to healthy piglets of an age (e.g. between ~~6~~ 4 weeks and 10 weeks old) suitable for recovery of the strain. ~~and of the same origin, that do not have~~ Piglets should test negative for antibodies against ASFV, and ~~blood samples that are negative on real-time~~ by PCR. This protocol is typically repeated five times.

[5] https://www.ema.europa.eu/en/documents/scientific-guideline/vich-gl41-target-animal-safety-examination-live-veterinary-vaccines-target-animals-absence-reversion_en.pdf.

To obtain individual mean baseline temperatures, the body temperature of each piglet should be measured on at least the 3 consecutive days preceding administration of the vaccine or passaged material.

First passage (p1)

Administer to no fewer than two piglets, and preferably no fewer than four piglets using the intended route of administration for the final product, a quantity of the master seed vaccine virus equivalent to not less than the maximum virus titre (maximum release dose) likely to be contained in 1 dose of the final product of the vaccine. Observe inoculated animals daily for the appearance of ~~at least two and preferably at least three~~ clinical signs ~~and record daily body temperatures~~ using a quantitative clinical scoring system adding the values for multiple clinical signs (e.g. Gallardo *et al.*, 2015a) and record daily body temperatures.

Based on results from at least one completed post-vaccination kinetics of viral replication (MLV ~~vaccine shed and spread (virus~~ blood and tissue dissemination study (Section C.2.3.2.iv above), collect an appropriate quantity of blood from each piglet on the predetermined ~~single~~ timepoint(s) (i.e. day ~~5~~ 3–13). Determine virus titres in individual blood–samples by quantitative virus isolation (HAD_{50}/ml or $TCID_{50}$/ml) or other appropriate methods (e.g. titration using IPT or FAT detection). Real-time PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration as described above ~~and by real-time PCR. If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test or other appropriate method (e.g. titration using IPT or FAT detection) may be used~~. Identify the individual blood sample(s) with the highest infectious titre and reserve for the subsequent *in-vivo* passage (second pass, p2). If appropriate, blood samples with the highest infectious titres from different pigs should be pooled to prepare inoculum for further passages.

Based on results from at least one completed ~~vaccine virus~~ MLV blood and tissue ~~distribution~~ dissemination study (Section C.2.3.2.iv above), euthanise piglets on the predetermined timepoint (i.e. day 5, 7, 14, 21, or 28). Determine infectious virus titres in individual tissue samples by quantitative virus isolation ~~(HAD_{50}/ml or $TCID_{50}$/ml) or other appropriate methods (e.g. titration using IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration~~ as described above. ~~If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test or other appropriate method (e.g. titration using IPT or FAT detection) may be used.~~ Identify individual tissue–sample type(s) with the highest infectious titre~~. Pool the Use tissues with the highest titres~~ from different organs from ~~all~~ each animal~~s with the highest titres~~ and prepare ~~at least~~ a ~~10%~~ virus suspension ~~to obtain a virus titre within the range used for inoculation~~ in PBS, pH 7.2 kept at 4°C or at –70°C for longer storage. Identify the individual tissue samples with the highest infectious titres from each animal and reserve for subsequent *in-vivo* passage (second pass, p2). If appropriate, tissue samples with the highest infectious titres from different pigs should be pooled to prepare inoculum for further passages.

~~Test each blood and tissue sample pool used for inoculation by PCR to confirm the absence of potential viral agent contaminants (i.e. CSFV, FMDV, PRRSV, PCV2). Blood and pooled tissue (p1) are used to inoculate 2 ml of positive material diluted to the maximum release dose likely to be contained in 1 dose of the vaccine using the intended route of administration for the final product to each of at least two and ideally at least four further pigs of the same age and origin.~~

~~*Second pass (p2)*~~

If no virus is found at passage 1 (p1), repeat the administration by the intended route ~~once~~ again with the same master seed vaccine virus used in p1 (see above) ~~pooled material (blood and pooled tissue, p1)~~ in another ten healthy piglets of the same age and origin. If no virus is found at this point ~~during this second passage (p2) at this point~~, end the process here.

Second passage (p2)

If ~~however~~ virus is found ~~in~~ at p1, carry out a second series of passages by administering an appropriate volume (e.g. 2 ml) of positive material from p1 ~~diluted to the maximum release dose likely to be contained in 1 dose of the vaccine~~ using the intended route of administration for the final product to each of no fewer than two piglets, and preferably no fewer than four piglets of the same age and origin. Observe inoculated animals daily for the appearance of ~~at least two and preferably at least three~~ clinical signs using a quantitative clinical scoring system ~~adding the values for multiple clinical signs (e.g. Gallardo *et al.*, 2015a)~~, ~~and~~ record daily body temperatures, collect blood and tissue samples at the predetermined time points in the blood and tissue dissemination study (above) and determine infectious virus titres in individual blood and tissue samples as described for p1 above.

~~*Third and fourth pass (p3 and p4)*~~

If no virus is found at ~~in (~~p2~~)~~, repeat the ~~intramuscular~~ administration by the intended route ~~once again~~ with the same ~~pooled~~ material (blood and/or ~~pooled~~ tissue) from p~~2~~ 1) in another eight healthy piglets of the same age and origin. If no virus is found at this point, end the process here.

Third and fourth passage (p3 and p4)

If~~, however,~~ virus is found on p2, carry out further ~~this~~ passages ~~operation~~ no fewer than two additional times (p3 and p4) (to each of no fewer than two piglets, and preferably no fewer than four piglets of the same age and origin). Record clinical signs and body temperature daily, collect blood and tissue samples at the predetermined time points (see above) and determine infectious virus titres ~~verifying the presence of the virus at each passage~~ in individual blood and tissue samples as described for p1 above. ~~Observe inoculated animals daily for the appearance of at least two and preferably at least three clinical signs using a quantitative clinical scoring system adding the values for multiple clinical signs (e.g. Gallardo *et al.*, 2015a) and record daily body temperatures.~~

Fifth passage (p5)

Administer an appropriate volume (e.g. 2 ml) of the blood and ~~pooled~~ tissue (p4) to each of at least eight healthy piglets of the same age and origin. Observe inoculated animals daily for at least 28 days post-inoculation for the appearance of ~~at least two and preferably at least three~~ clinical signs using a quantitative clinical scoring system ~~adding the values for multiple clinical signs (e.g. Gallardo *et al.*, 2015a),~~ ~~and~~ record daily body temperature and determine infectious virus titres in individual blood and tissue samples as described above.

If the fifth group of animals shows no evidence of an increase in virulence indicative of reversion to virulence during the observation period, further testing is not required. Otherwise, materials used for the first passage and the final passage should be used in a separate experiment using at least 8 animals per group to directly compare the clinical signs and other relevant parameters. This study should be done by the route of administration that was used for previous passages.

The vaccine is compliant ~~complies with the test~~ if:

- No piglet shows abnormal (local or systemic) reactions, or notable signs of disease, or ~~reaches~~ the pre-determined humane endpoint defined in the clinical scoring system or dies from causes attributable to the vaccine; and
- There is no indication of increasing virulence (as monitored by daily body temperature increases above the baseline accompanied by clinical sign observations) of the maximally passaged virus compared with the master seed virus.

At a minimum, a safe MLV vaccine shall demonstrate ALL the following features (minimal standards):

- Absence of fever (~~on each day during the observation period, the maximum increase in body temperature above the baseline observed for each pig will be used to will be recorded calculate the daily group mean temperature rise. This mean value should not exceed 1.5°C and~~ no individual pig should show a rise in temperature above baseline greater than 1.5°C ~~(defined as average body temperature increase for all vaccinated piglets (group mean) for the observation period does not exceed 1.5°C above baseline; and no individual piglet shows a temperature rise above baseline greater than 2.5°C~~ for a period exceeding ~~3~~ 2 days that is attributable to ASFV infection). In cases where pigs exceed the temperature standard but show no behavioural changes or other clinical signs, regulators may determine vaccine safety without solely relying on temperature for non-compliance;
- Absence of chronic and acute clinical signs and gross pathology over the entire test period ~~or minimal chronic mild clinical signs (defined as e.g. mild swollen joints with a low clinical score that resolve within 1 week)~~;
- Absence of abnormal (local or systemic) reactions;
- ~~Minimal (defined as no naïve, contact piglet shows notable signs of disease by clinical signs and gross pathology and no or a low percentage of contact piglets test both real-time PCR positive and seropositive) or no vaccine virus transmission (defined as no naïve, contact piglet shows notable signs of disease by clinical signs and gross pathology and no contact piglets test both real-time PCR positive and seropositive) over the entire test period~~ No or a low percentage of naïve, contact pigs test positive to the vaccine virus and/or to antibodies against the vaccine virus;

- Absence of an increase in virulence (genetic and phenotypic stability) (complies with the reversion to virulence test).

In addition, for regulatory approval, ASF MLV ~~the~~ vaccines ~~in their commercial presentation before being authorised for general use~~ should be tested for safety ~~in the~~ under field conditions (see chapter 1.1.8 Section 7.2.3). ~~Additional~~ Field safety studies generally ~~evaluation studies~~ may include measurement of body temperatures, observation of local or systemic reactions and, where appropriate, performance measurements ~~such as but are not limited to~~: environmental persistence (e.g. determination of virus recovery from bedding or other surfaces), assessment of immunosuppression, and negative impacts on performance.

2.3.3. Efficacy requirements

i) Protective dose

Vaccine efficacy is estimated in immunised animals directly, by evaluating protection against ~~their resistance to~~ live virus challenge. The test consists of a vaccination/challenge trial in piglets a minimum of ~~6~~ 4-weeks old and not more than 10-weeks old, free of antibodies to ASFV, and negative ~~blood samples~~ by real-time PCR. The test is conducted using no fewer than 15 and preferably no fewer than 24 vaccinated pigs, and no fewer than five non-vaccinated control piglets.

The test is conducted to determine the minimal immunising dose (MID) (also referred to as the minimal protective dose [MPD] or protective fraction); using at least three groups of no fewer than five and preferably not fewer than eight vaccinated piglets per group, and one additional group of no fewer than five non-vaccinated piglets of the same age and origin as controls. Use vaccine containing virus at the highest passage level that will be present in a batch of vaccine. Each group of piglets, except the control group, is immunised with a different vaccine virus content in the same vaccine volume. In at least one vaccinated group, piglets are immunised with a vaccine dose containing not more than the minimum virus titre ~~(minimum release dose)~~ likely to be contained in one dose of the vaccine as stated on the label.

The MID of the vaccine is calculated from the number of animals protected in each group using an appropriate statistical test, such as the Spearman–Kärber method.

A suitable challenge model should be developed based on anticipated field usage of the vaccine. As a baseline protocol, ~~Twenty-eight~~ 28 days (±2 days) after the single ~~injection~~ dose of vaccine (or if using two ~~injections~~ doses of the vaccine then 28 days [±2 days] following the second ~~injection~~ dose), challenge all the piglets by the intramuscular route. If previous studies have demonstrated acceptable efficacy using IM challenge, then a different challenge route (e.g. direct contact, oral or oronasal) may be used. Challenged, vaccinated piglets may be housed in one or more separate pens in the same room or in different rooms. Challenged, naïve controls can be housed in one or more rooms that are separate from challenged, vaccinated piglets.

Carry out the test using an ASFV representative strain of the epidemiologically relevant field strain(s) where the vaccine is intended for use (e.g. ASFV B646L [p72] genotype II pandemic strain and other p72 virulent genotype of recognised epidemiologic importance). For gene deleted, recombinant MLV viruses, if neither challenge virus type is available, then carry out the test with the parental, virulent virus used to generate the MLV recombinant virus. Use a 10e3–10e4 HAD_{50} ~~(~~or $TCID_{50}$ ~~for non-HAD viruses)~~ challenge dose sufficient to cause death or meet the humane endpoint in 100% of the nonvaccinated piglets in less than 21 days. Higher or lower challenge doses can be considered if appropriately justified.

To obtain individual mean baseline temperatures, the body ~~rectal~~ temperature of each vaccinated piglet is measured on at least the 3 days preceding administration of the challenge virus. Body temperature is then measured, at the time of challenge, 4 hours after challenge, and then daily for the observation period of at least ~~28~~ 45 days, preferably ~~35~~ 60 days. ~~Observe the piglets at least daily for at least 28 days, preferably 35 days.~~ Carry out the daily observations for signs of acute and chronic clinical disease using a quantitative clinical scoring system adding the values for multiple clinical signs (e.g. Gallardo *et al.*, 2015). These clinical signs should include fever, anorexia, recumbency, skin haemorrhage or cyanosis, joint swelling and necrotic lesions around the joints, respiratory distress and digestive findings.

Collect oral, nasal, faecal swabs ~~anal~~ and blood samples from the vaccinated challenged piglets at least two times ~~once~~ per week from 3 days post-challenge for at least ~~28~~ 14 days, then weekly up to 35 days post-challenge and then every 14 days up to the end of the observation period ~~preferably 35 days~~. From the blood samples, determine infectious virus titres by quantitative virus isolation (HAD_{50}/ml or $TCID_{50}$/ml) or other appropriate methods (e.g. titration using IPT or FAT detection).

Real-time PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration as described above ~~and using a real-time PCR test. If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test only may be used~~.

At the end of the test period, humanely euthanise all vaccinated challenged piglets. Conduct gross pathology (and histopathology if considered necessary) on spleen, lung, tonsil, and kidney tissue samples and at least three different lymph nodes (which should include lymph node closest to site of inoculation, gastrohepatic and submandibular nodes). Determine virus titres in all collected samples by quantitative virus isolation ~~(HAD_{50}/mg or $TCID_{50}$/mg) or other appropriate methods (e.g. titration using IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration~~ as described above ~~and real-time PCR (see Section B.1. Identification of the agent). If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test or other appropriate method (e.g. titration using IPT or FAT detection) may be used~~.

If using a highly virulent challenge virus, as described above, the test is invalid if fewer than 100% ~~the difference between in the number~~ of unvaccinated control piglets infected with the live challenge virus ~~and the number of vaccinated / challenged piglets vaccinated with the minimum release dose that~~ die or reach a humane endpoint ~~is not statistically significant~~.

The vaccine (or a specific vaccine virus dose if conducting a vaccine dose titration study) is compliant ~~complies with the test~~ if:

- No vaccinated challenged piglet dies or shows abnormal (local or systemic) reactions, or reaches the humane endpoint ~~or dies~~ from causes attributable to ASF;
- ~~On each day during the observation period the maximum increase in body temperature above the baseline observed for each pig will be used to calculate the daily group mean. This mean value should not exceed 1.5°C and no individual pig should show a rise in temperature above baseline greater than 2.0°C for a period exceeding 2 consecutive days. The average body temperature increase for all vaccinated challenged piglets (group mean) for the observation period does not exceed 2.0°C above baseline; and no individual piglet shows a temperature rise above baseline greater than 2.0°C~~;
- The vaccinated challenged piglets display a reduction or absence of pyrexia, typical acute clinical signs or other forms of disease and gross pathology, and a reduction or absence of challenge virus levels in blood, swabs and tissues.

~~**ii) Assessment for horizontal transmission (challenge virus shed and spread study)**~~

~~The ASF basic reproduction number, R0, can be defined as the average number of secondary ASF disease cases caused by a single ASFV infectious pig during its entire infectious period in a fully susceptible population (Hayes *et al.*, 2021). In general, if the ASFV effective reproduction number Re=R0 × (S/N) (S= susceptible pigs; N= total number of pigs in a given population) is greater than 1.0, disease is predicted to spread. Ideally, ASF vaccination should reduce Re to less than 1.0 by reducing the number of susceptible, naïve, contact pigs exposed to vaccinated, infected pigs.~~

~~To evaluate ASF vaccine impact on ASF disease transmission, the test consists of a vaccination/challenge trial in piglets a minimum of 6 4-weeks old and not older than 10 weeks old, free of antibodies to ASFV, and negative blood samples by real-time PCR.~~

~~The test is conducted using no fewer than 15 healthy piglets at a ratio comprising twice the number of vaccinated piglets to naïve piglets (e.g. ten vaccinated and five naïve). Use vaccine containing virus at the highest passage level that will be present in a batch of the vaccine.~~

~~The quantity of vaccine virus administered to each pig is equivalent to be not less than the minimum virus titre (minimum dose) likely to be contained in one dose of the vaccine as stated on the label. Following immunisation, vaccinated and naïve piglets should continue to be co-mingled.~~

~~Twenty eight days (±2 days) after the single injection dose of vaccine (or if using two injections doses of the vaccine then 28 days [±2 days] following the second injection dose), temporarily separate [into different pen(s) or room(s)] all vaccinated piglets from naïve piglets. Challenge all vaccinated piglets by the intramuscular or other previously verified route. Carry out the challenge using an ASFV representative strain of the epidemiologically relevant field strain(s) where the vaccine is intended for use (e.g. ASFV B646L [p72] genotype II pandemic strain and other p72 virulent genotype of recognised epidemiological importance). For gene deleted, recombinant MLV viruses, if neither challenge virus type is available, then carry out the test with the parental, virulent virus used to~~

generate the MLV recombinant virus. Use a 10e3–10e4 HAD_{50} (or $TCID_{50}$ for non-HAD viruses challenge dose sufficient to cause death or met the humane endpoint in 100% of the nonvaccinated piglets in less than 21 days. Higher or lower challenge doses can be considered if appropriately justified.

Approximately 18–24 hours later, re-introduce naïve piglets to vaccinated, challenged piglets and allow for direct nose to nose contact exposure with vaccinated, challenged piglets. Allow for continuous contact exposure by co-mingling both groups through the end of the study. If more than one pen or room is used for co-housing, following reintroduction initially maintain a ratio of 2:1 of challenged, vaccinated piglets to contact exposed, naïve piglets.

The rectal temperature of each contact piglet is measured on at least the 3 days preceding administration of the challenge virus to vaccinated pigs, immediately prior to direct contact exposure, 4 hours post-contact exposure, and then daily for at least 28, preferably 35 days and twice a week for at least 60 days. Observe all contact exposed piglets at least daily for at least 28 days, and then twice a week for at least 60 days preferably for at least 35 days.

Carry out the daily observations in each contact piglet for signs of acute and chronic clinical disease using a quantitative clinical scoring system adding the values for multiple clinical signs (e.g. Gallardo *et al.*, 2015a). These clinical signs should include fever, anorexia, recumbency, skin haemorrhage or cyanosis, joint swelling and necrotic lesions around the joints, respiratory distress and digestive findings.

In addition, blood should be taken from the naïve contact piglets at least twice a week from 3 days post-contact exposure for the duration collect blood samples from the contact piglets at least two times per week from 3 days post-contact for at least 14 days, then weekly up to 35 days post-contact exposure and then every 14 days up to the end of the test period. Determine virus titres in all collected samples by quantitative virus isolation (HAD_{50}/mg or $TCID_{50}$/mg) or other appropriate methods (e.g. titration using IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration as described above From the blood samples, determine infectious challenge virus titres by quantitative virus isolation (HAD_{50}/ml or $TCID_{50}$/ml) and using a real-time PCR test. If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test only may be used.

Collect blood (serum) samples from the naïve contact pigs at least at day 21 and day 28 (±2 days), and at the end of the test period, and carry out an appropriate test to detect vaccine virus antibodies.

Collect oral, nasal and faecal swab samples (preferably devoid of blood to minimise assay interference) from all contact exposed naïve piglets at least two times per week from 3 days post-contact exposure for the first 2 weeks, then weekly for the duration of the test and test swabs for the presence of challenge virus. Determine virus titres in all collected samples by quantitative virus isolation (HAD_{50}/mg or $TCID_{50}$/mg) or other appropriate methods (e.g. titration using IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration as described above Determine virus titres in all collected samples by quantitative virus isolation (HAD_{50}/ml or $TCID_{50}$/ml) and using a real-time PCR test. If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test or other appropriate method (e.g. titration using IPT or FAT detection) may be used.

At the end of the test period, humanely euthanise all contact piglets. Conduct gross pathology on spleen, lung, tonsil, and kidney tissue samples and at least three different lymph nodes. (which should include lymph node closest to site of inoculation, gastrohepatic and submandibular nodes). Determine virus titres in all collected samples by quantitative virus isolation (HAD_{50}/mg or $TCID_{50}$/mg) or other appropriate methods (e.g. titration using IPT or FAT detection). Quantitative PCR may be used to detect positive samples, but results should be confirmed by infectious virus titration as described above Determine virus titres in all collected samples by quantitative virus isolation (HAD_{50}/mg or $TCID_{50}$/mg) and real-time PCR (see Section B.1. Identification of the agent). If the vaccine virus is non-haemadsorbing or does not cause cytopathic effects, a real-time PCR test or other appropriate method (e.g. titration using IPT or FAT detection) may be used.

The test is invalid if the vaccine fails to comply with the compliance criteria described for the protected dose test in vaccinated pigs (Section C.2.3.3.i above).

If the manufacturer claims that the vaccine induces sterilising immunity, the vaccine complies with the test for a reduction in horizontal disease transmission if all the following conditions are satisfied:

- ~~No naïve, contact exposed piglet shows abnormal (local or systemic) reactions, reaches the defined humane endpoint or dies from causes attributable to ASF;~~
- ~~No naïve, contact exposed piglet displays fever accompanied by typical signs of disease, including gross pathology.~~
- ~~Naïve contact pigs show an absence of challenge virus in blood and tissues.~~
- ~~No naïve contact pigs test positive for antibodies to the challenge virus.~~

~~Otherwise, the vaccine complies with the test for a reduction in horizontal disease transmission if:~~

- ~~Naïve contact pigs show a reduction or absence of challenge virus levels in blood and tissues.~~
- ~~None of or a reduced number of naïve contact exposed pigs test positive for antibodies to the challenge virus.~~

At a minimum, an efficacious MLV vaccine shall demonstrate ALL the following features (minimal standards):

- Protects against mortality;
- Reduces acute or other forms of disease (fever accompanied by a reduction of ~~typical~~ clinical and pathological signs of ~~acute~~ disease);
- ~~Reduces levels of viral shedding and viraemia.~~
- ~~Reduces horizontal disease transmission (no none of or a reduced number of naïve, contact exposed piglets shows abnormal [local or systemic] reactions, reaches the humane endpoint or dies from causes attributable to ASF, and displays fever accompanied by typical acute disease signs caused by ASF) and test positive for antibodies to the challenge virus.~~
- Reduces levels of viral shedding and viraemia.

In general, for regulatory approval, ASF MLV ~~addition, the~~ vaccines ~~in their commercial presentation before being authorised for general use~~ should be tested for efficacy ~~in the~~ under field conditions (see chapter 1.1.8 Section 7.2.3). ~~Additional~~ Field efficacy ~~evaluation~~ studies ~~may~~ generally include ~~but are not limited to: onset of immunity, duration of immunity, and impact on disease transmission~~ measurement of relevant efficacy parameters including but limited to mortality, clinical signs, impact on disease transmission, performance parameters.

2.3.4. Duration of immunity

Although not included in the guidance for ASF MLV first generation vaccines, manufacturers are ~~encouraged~~ required, as part of the ~~authorisation~~ regulatory approval procedure, to define and demonstrate the duration of immunity of a given vaccine ~~by evaluation of potency at the end of the claimed period of protection~~.

2.3.5. Stability

Stability of the vaccine should be demonstrated over the shelf life recommended for the product. Although not included in the standards for first generation MLV ASF vaccines, manufacturers are ~~encouraged~~ in general required, as part of the ~~authorisation~~ regulatory approval procedure, to generate data supporting the retention of immunogenicity over a defined period of ~~validity~~ time of a lyophilised or other pharmaceutical form of the ASF vaccine as part of the ~~authorisation~~ regulatory approval procedure.

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NB: There are WOAH Reference Laboratories for African swine fever
(please consult the WOAH Web site:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on
diagnostic tests and reagents for African swine fever

NB: FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2021.