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. 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 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:

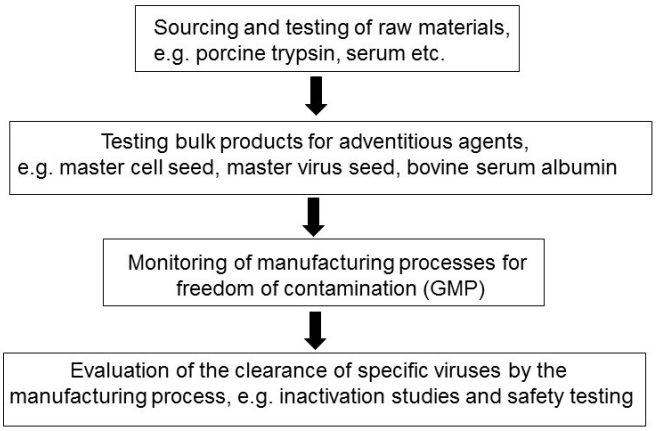
- Primary materials must be collected from sources shown to be free from contamination and handled in such a way as to minimise contamination and the opportunities for any contaminants to multiply (Figure 1).

- 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*.

Figure 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).
   - Department of Agriculture, Forest and Fisheries (Australia) (2013).
   - Department of Agriculture, Forest and Fisheries (Australia) (2021b) Live Veterinary Vaccines.
   - Regulation on Veterinary Drug Administration (China [People's Rep. of]) (2020).
   - European Medicines Agency Sciences Medicines Health (2016).
   - European Pharmacopoeia, 11th Edition (2023).
   - World Health Organization (WHO) (1998; 2012).

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. 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 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 seed has not already been tested and shown to be free from extraneous agents. An example of a simple inactivation study could include assessment of the titre of live vaccine before and after inactivation and assessing the $\log_{10}$ drop in titre during the inactivation process. 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 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 25 July 2023.

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 is recommended to ensure testing in 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. SERA, PLASMA AND DIAGNOSTIC AGENTS FOR ADMINISTRATION TO ANIMALS

1. Section B applies for sera/diagnostic agents that are not inactivated. Section C applies for inactivated sera/diagnostic agents.

2. 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. 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. 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.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 G.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 24 July 2023). 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).

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

1 American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia 20110-2209, USA.
2 PDA: potato dextrose agar

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 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 25 July 2023).

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 burnetti* (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 2.5.7 *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 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.

5.1. Example of general testing procedures for the exclusion of viruses from virus and cell seed stocks used in production of veterinary vaccines

5.1.1 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.

5.1.2 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.

5.1.3 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.

5.2. Example of specific agent exclusion testing of biologicals used in the production of veterinary vaccines

5.2.1. 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. 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.

- Outline of the Regulatory System of Veterinary Drugs in Japan (2015) Assurance of the Quality, Efficacy, and Safety Based on the Law for Ensuring the Quality, Efficacy, and Safety of Drugs and Medical Devices.

- 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) to allow the GMO to be dealt with.

I. 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. 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.