

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 might also be affected. 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, FAO¹ and WOAAH are collaborating in establishing the principle of international oversight and regulation of facilities holding rinderpest virus (RPV). All diagnostic testing, vaccine production and research activities that use live RPV or RPV-containing materials should be performed in an FAO-WOAAH approved Rinderpest Holding Facility.

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. WOAAH (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.

Identification of the agent: Laboratory confirmation is required, and is based on demonstrating the presence of the virus, viral RNA or 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 be done using estimation of neutralising antibody from the results of a competition enzyme-linked immunosorbent assay (C-ELISA). Any test used must be highly specific for RPV. Such tests can only be carried out in FAO-WOAAH approved Rinderpest Holding Facilities, as the 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. Under the terms of the Guidelines for Rinderpest Virus Sequestration, of Resolution No. 21 (adopted by the WOAAH Assembly, May 2017) governing the post-eradication era, it is not permitted to inoculate an animal with a rinderpest vaccine without prior permission from WOAAH and FAO.

1 FAO: Food and Agriculture Organization of the United Nations

In order to prepare for the possibility of a RPV re-emergence or release, FAO and WOA, 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 WOA.

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 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 WOA 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 research and approved vaccine manufacturing laboratories. To guard against the accidental release of virus from laboratory sources, FAO and WOA 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 RPV-containing materials² should be performed in an FAO-WOA approved Rinderpest Holding Facility and after approval of the activity by WOA 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. WOA 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 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 WOA 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 WOA *Terrestrial Code* for definition of Rinderpest virus containing materials.

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 eye 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 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	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
Identification of the agent^(a)						
Virus isolation	–	–	+	+++	–	–
Antigen detection (AGID)	–	–	+	+	+	–
(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)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; 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 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.

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. 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 WOA 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 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 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. A similar broad-spectrum cover can be obtained using neomycin at 50 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. 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.

1.3. 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 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. Laboratories, other than the WOA 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 a negative control using sterile distilled water instead of RNA, must be included. Positive reactions with a 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 or tonsil (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 guanidinium 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 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 3.8.9, 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 WOA 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 Laboratory³ in the UK, which is also a WOA Reference Laboratory for rinderpest, and the WOA Reference Laboratory in France⁴, 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 electrophoresis are also required.

i) Sequences of primers used:

Gene	Product size	Primer	Sequence (5' → 3')
RPV N	297 bp	B2	ATC-CTT-GTC-GTT-RTA-TGC-TCT-YRG
		B12	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 PCR master mix containing 0.6 μM final concentration of primers.
- 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.

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>

- v) 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	40 cycles	PCR amplification of the cDNA
72°C for 1 minute		
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.

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 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 contact the WOAHP Reference Laboratories.

2. Serological tests

2.1. The competitive enzyme-linked immunosorbent assay

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 μ l volumes of substrate/chromogen mixture (1 part 3% H₂O₂ to 250 parts OPD), and incubate at room temperature for 10 minutes without shaking. Then add 50 μ l 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.

2.3. 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} TCID₅₀ 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 × 10⁵ 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 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} TCID₅₀/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 μ l volumes of serum are incubated with 50 μ l volumes of virus diluted to contain between 10^{1.8} and 10^{2.8} TCID₅₀ (Taylor & Rowe, 1984). Following a 45-minute to overnight incubation, 50 μ l RPV-susceptible cells (between 1 and 2 × 10⁵ primary calf or lamb kidney cells, 5 × 10³ Vero or Vero-SLAM cells, or 5 × 10⁴ 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 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.

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, 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 to assess safety and efficacy. It is recommended to sequence 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.

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 bovine embryos or calves, or cells derived by up to ten serial subcultures from either of these sources. In addition, the vaccine may be manufactured in approved 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. 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

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), 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% polyvinylpyrrolidone 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 established virus seed stocks, animal-based testing of safety and efficacy may be deemed unnecessary.

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 carcasses 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 TCID₅₀). 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 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

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 spreads 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% polyvinylpyrrolidone/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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NB: There are WOA Reference Laboratories for rinderpest (please consult the WOA Web site: <https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>).

Please contact the WOA Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for rinderpest

NB: FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2018.