CHAPTER 3.8.9.

PESTE DES PETITS RUMINANTS (INFECTION WITH SMALL RUMINANT MORBILLIVIRUS)

SUMMARY

Description of the disease: *Peste des petits ruminants (PPR), is an acute contagious disease caused by the small ruminant* Morbillivirus *in the family* Paramyxoviridae*. It affects mainly sheep and goats and occasionally wild small ruminants. Based on the fact that PPR has been reported on a few occasions in camels, cattle and buffaloes, those animal species are considered to be susceptible although their potential role in the circulation of PPR virus has not been formally established. PPR occurs in Africa except Southern Africa, in the Arabian Peninsula, throughout most of the Near East and Middle East, and in Central and South-East Asia.*

The clinical disease resembles rinderpest in cattle. It is usually acute and characterised by pyrexia, serous ocular and nasal discharges, diarrhoea and pneumonia, and erosive lesions on different mucous membranes particularly in the mouth. At necropsy, erosions may be noted in the gastrointestinal and urogenital tracts. The lungs may show interstitial bronchopneumonia and often secondary bacterial pneumonia. PPR can also occur in subclinical form.

PPR must be confirmed by laboratory methods, as bluetongue, foot and mouth disease and other erosive or vesicular conditions, as well as contagious caprine pleuropneumonia, can cause clinically similar disease.

Detection of the agent: *The collection of specimens at the correct time is important to achieve diagnosis by virus isolation and they should be obtained in the acute phase of the disease when clinical signs are apparent. The recommended specimens from live animals are swabs of conjunctival discharges, nasal secretions, buccal and rectal mucosae, and anticoagulant-treated blood.*

Laboratory diagnosis is done by immunocapture enzyme-linked immunosorbent assay (ELISA) or polymerase chain reaction. Counter immunoelectrophoresis and agar gel immunodiffusion may also be used. A penside test is available for field use.

Serological tests: *The serological tests that are routinely used are virus neutralisation and competitive ELISA.*

Requirements for vaccines: *Effective live attenuated PPR virus vaccines are widely available. Since the global eradication of rinderpest, the use of rinderpest vaccines to protect against PPR is forbidden.*

A. INTRODUCTION

Peste des petits ruminants (PPR) is an acute viral disease of small ruminants characterised by fever, oculo-nasal discharges, stomatitis, diarrhoea and pneumonia with foul offensive breath. Because of the respiratory signs, PPR can be confused with contagious caprine pleuropneumonia (CCPP) or pasteurellosis. In many cases, pasteurellosis is a secondary infection of PPR, a consequence of the immunosuppression that is induced by the virus. The virus is transmitted mainly by aerosols between animals living in close contact (Lefevre & Diallo, 1990). Infected animals present clinical signs similar to those historically seen with rinderpest in cattle, although the two diseases are caused by distinct virus species.

On the basis of its similarities to rinderpest, canine distemper and measles viruses, the virus causing PPR has been classified within the genus *Morbillivirus* in the family *Paramyxoviridae* (Gibbs *et al.*, 1979). It is now officially named *Small Ruminant Morbillivirus* (SRMV) (ICTV, 2019). Pending further revision of this chapter, it is also referred to below as PPR virus (PPRV). Virus members of this group have six structural proteins: the nucleocapsid protein (N), which encapsidates the viral genomic RNA, the phosphoprotein (P), which associates with the polymerase (L for large protein) protein, the matrix (M) protein, the fusion (F) protein and the haemagglutinin (H) protein. The matrix protein, intimately associated with the internal face of the viral envelope, makes a link between the nucleocapsid and the virus external glycoproteins: H and F, which are responsible, respectively, for the attachment and the penetration of the virus into the cell to be infected. The PPRV genome also encodes two nonstructural proteins, C and V.

PPR was first described in Côte d'Ivoire (Gargadennec & Lalanne, 1942). Since the late 1990s, it has expanded its range to cover large regions of Africa, from North Africa to Tanzania, and the Middle East, and is also widespread in countries from central Asia to South and East Asia (reviewed in Banyard et *al.,* 2010).

The natural disease affects mainly goats and sheep. It is generally considered that cattle are only naturally infected subclinically, although in the 1950s, disease and death were recorded in calves experimentally infected with PPRV-infected tissue and PPRV was isolated from an outbreak of rinderpest-like disease in buffaloes in India in 1995. Antibodies to PPRV as well as PPRV antigen and nucleic acid were detected in some samples from an epizootic disease that affected dromedaries in Ethiopia and Sudan. PPR affects a number of wild species within the order *Artiodactyla* some of which species are highly endangered, e.g. the Mongolian saiga antelope. The American white-tailed deer (*Odocoileus virginianus*) can be infected experimentally with PPRV. Dual infections can occur with other viruses such as pestivirus or goatpox virus.

The incubation period is typically 4–6 days, but may range between 3 and 10 days. The clinical disease is acute, with a pyrexia up to 41°C that can last for 3–5 days; the animals become depressed, anorexic and develop a dry muzzle. Serous oculonasal discharges become progressively mucopurulent and, if death does not ensue, persist for around 14 days. Within 4 days of the onset of fever, the gums become hyperaemic, and erosive lesions develop in the oral cavity with excessive salivation. These lesions may become necrotic. A watery blood-stained diarrhoea is common in the later stage. Pneumonia, coughing, pleural rales and abdominal breathing also occur. The morbidity rate can be up to 100% with very high case fatality in severe cases. However, morbidity and mortality may be much lower in milder outbreaks, and the disease may be overlooked. A tentative diagnosis of PPR can be made on clinical signs, but this diagnosis is considered provisional until laboratory confirmation is made for differential diagnosis with other diseases with similar signs.

At necropsy, the lesions are very similar to those observed in cattle affected with rinderpest, except that prominent crusty scabs along the outer lips and severe interstitial pneumonia frequently occur with PPR. Erosive lesions may extend from the mouth to the reticulo–rumen junction. Characteristic linear red areas of congestion or haemorrhage may occur along the longitudinal mucosal folds of the large intestine and rectum (zebra stripes), but they are not a consistent finding. Erosive or haemorrhagic enteritis is usually present and the ileo-caecal junction is commonly involved. Peyer's patches may be necrotic. Lymph nodes are enlarged, and the spleen and liver may show necrotic lesions.

There are no known health risks to humans working with PPRV as no report of human infection with the virus exists. Laboratory manipulations should be carried out at an appropriate containment level determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

B. DIAGNOSTIC TECHNIQUES

A list including all the types of tests available for PPR is given in Table 1. Assays applied to individuals or populations may have different purposes, such as confirming diagnosis of clinical cases, determining infection status for trade and/or movement, estimates of infection or exposure prevalence (surveillance) or checking post-vaccination immune status (monitoring).

Table 1. Test methods available for the diagnosis of peste des petits ruminants and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent[a]						
RT-PCR	–	++	++	+++	+	–
Real-time RT-PCR	–	++	+++	+++	+	–
Virus isolation in cell culture	–	–	–	++	–	–
Immunocapture ELISA		+	++	+++	+	–
Penside test (LFD)	–	–	++	++	–	–
AGID	–	–	+	+	–	–
Counter immune-electrophoresis	–	–	–	+	–	–
Detection of immune response						
Virus neutralisation	+++	–	–	++	++	++
Competitive ELISA	+++	–	+++	+	+++	+++
AGID	–	–	+	+	–	+
Counter immune-electrophoresis	–	–	–	+	–	–

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
RT-PCR = reverse-transcription polymerase chain reaction;
ELISA = enzyme-linked immunosorbent assay; LFD = lateral flow device; AGID = agar gel immunodiffusion.
[a]A combination of agent identification methods applied to the same clinical sample is recommended.

1. Collection of samples

Samples for virus isolation must be kept chilled in transit to the laboratory. In live animals, swabs are made of the conjunctival discharges or from the nasal, buccal or rectal mucosae. During the very early stage of the disease, whole blood is also collected in anticoagulant for virus isolation, polymerase chain reaction (PCR) and haematology (either ethylene diamine tetra-acetic acid or heparin can be used as anticoagulant, though the former is preferred for samples that will be tested using PCR). At necropsy, samples from two to three animals should be collected aseptically from lymph nodes, especially the mesenteric and bronchial nodes, lungs, spleen and intestinal mucosae, chilled on ice and transported under refrigeration. Samples of organs collected for histopathology are placed in 10% neutral buffered formalin. It is good practice to collect blood for serological diagnosis at all stages, but particularly later in the outbreak.

2. Detection of the agent

2.1. Immunocapture enzyme-linked immunosorbent assay

The immunocapture enzyme-linked immunosorbent assay (IC-ELISA) (Libeau *et al.,* 1994) using two monoclonal antibodies (MAb) raised to the N protein allows rapid identification of PPRV.

Advice on the use and applicability of the IC-ELISA is available from the WOAH Reference Laboratories for PPR[1]. The assay is available as a commercial kit. The instructions provided by the kit supplier should be followed. The general principle of the kit is:

i)   Wells are provided pre-coated with an anti-PPRV-N antibody.

ii)   Samples to be tested, and included controls, are added to the wells. PPRV, if present, forms an antibody–antigen complex.

iii)   After washing, an anti-PPRV-N MAb–horse radish peroxidase (HRP) conjugate is added, forming an antibody–antigen–MAb–HRP complex.

iv)   After further washing to eliminate the excess conjugate, the substrate solution (TMB: tetramethyl benzidine) is added. The resulting coloration depends on the quantity of PPRV present in the sample tested. In the presence of PPRV, a blue coloration appears that becomes yellow after addition of the stop solution. In the absence of PPRV, no coloration appears. The microplate is read at 450 nm.

The test is very specific and sensitive (it can detect $10^{0.6}$ TCID$_{50}$/well of PPRV). The results are obtained in less than 2 hours.

A sandwich form of the IC-ELISA is widely used in India (Singh *et al.*, 2004): the sample is first allowed to react with the detection MAb and the immunocomplex is then captured by an MAb or polyclonal antibody adsorbed onto the ELISA plate. The assay shows high correlation to the cell infectivity assay (TCID$_{50}$) with a minimum detection limit of 10^3 TCID$_{50}$/ml.

2.2. Nucleic acid recognition methods

Reverse transcription PCR (RT-PCR) techniques based on the amplification of parts of the N and F protein genes have been developed for the specific diagnosis of PPR (Couacy-Hymann *et al.*, 2002). This technique is 1000 times more sensitive than classical virus titration on Vero cells (Couacy-Hymann *et al.,* 2002) with the advantage that results are obtained in 5 hours, including the RNA extraction, instead of 10–12 days for virus isolation. The two most commonly used protocols are given in some detail below. A multiplex RT-PCR, based on the amplification of fragments of N and M protein genes, has been reported (George *et al.*, 2006). Another format of the N gene-based RT-PCR has also been described (Saravanan *et al.,* 2004). Instead of analysing the amplified product – the amplicon – by agarose gel electrophoresis, it is detected on a plate by ELISA through the use of a labelled probe. This RT-PCR-ELISA is ten times more sensitive than the classical RT-PCR.

In recent years, nucleic acid amplification methods for PPR diagnosis have been significantly improved with quantitative real-time RT-PCR (e.g. Bao *et al.,* 2008; Batten *et al.,* 2011; Kwiatek *et al.,* 2010). This method is also ten times more sensitive than conventional RT-PCR, as well as minimising the risk of contamination; a real-time RT-PCR assay specific for lineage IV strains has also been described (Li *et al.*, 2016). An alternative to RT-PCR is loop-mediated isothermal amplification (Li *et al.*, 2010). The sensitivity of this assay seems to be similar to that of the real-time RT-PCR, it is simple to implement, rapid and the result can be read by the naked eye.

In all cases RNA must first be purified from blood or tissue samples. Viral RNA can be purified from spleen (not ideal because of its high blood content), lymph node or tonsil, lung tissue, whole blood, buffy coat or purified peripheral blood lymphocytes (PBLs), or swabs from eyes or nose. Tissue samples should be extracted with acidified guanidinum thiocyante phenol using one of the commercial preparations available. Solid tissues (0.5–1.0 g) are minced and homogenised with 10 ml reagent. Conjunctival, oral or nasal swabs can be extracted, and whole blood, buffy coat or purified PBLs homogenised with, the same reagent; RNA is then purified according to the manufacturer's instructions. For tissues, blood, white cells or swabs, RNA extraction based on magnetic beads or spin columns are also suitable. The resulting RNA is stored at –70°C (or –20°C if –70°C not available) until required.

Although a real-time RT-PCR assay is the method of choice for laboratories that have the necessary equipment, none of the existing assays has proven satisfactory against all PPRV isolates tested. Because

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1   https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3

this is a rapidly developing field, users are advised to contact WOAH (see footnote 1) and FAO[2] Reference Laboratories for PPR for advice on the most appropriate techniques.

2.2.1. RT-PCR for the diagnosis of PPRV based on the amplification of part of the N gene

The tests described in this section and the next require: a one-step RT-PCR kit, distilled water, the relevant primers, a thermal cycler, plus reagents and equipment for agarose gel electrophoresis. In each case, the master mix recipe and thermal cycling conditions given are for a specific one-step RT-PCR kit, other reagents could also be used, but these should be optimised and validated for use in the relevant assay.

N gene amplification is based on the initial protocol described by Couacy-Hymann *et al.* (2002) in a one-step RT-PCR method. The conventional RT-PCR based on the primers described here is capable of detecting all four viral lineages. Other primers targeting the N gene could be used, but may not suitable for this aim.

i)   Primer sequences used:

Primer   Sequence

NP3:   5'-GTC-TCG-GAA-ATC-GCC-TCA-CAG-ACT-3';

NP4:   5'-CCT-CCT-CCT-GGT-CCT-CCA-GAA-TCT-3'.

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 10μM is obtained with a final volume of 50 μl.

iii) Add 5 μl of RNA template to 45 μl of PCR master mix containing:

Reagent	Mix (1 reaction)	Final concentration
Distilled water	15 μl	
5× RT-PCR Buffer	10 μl	1×
dNTP Mix	2 μl	
Q solution	10 μl	
Primer NP3 (10 μM)	3 μl	0.6 μM
Primer NP4 (10 μM)	3 μl	0.6 μM
Enzyme mix	2 μl	
Final volume	50 μl	

iv) Distilled water (5 μl) is used in place of RNA to provide a negative control which has to be included into each set of PCR tests.

v)   Thermal cycler conditions are set 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		
60°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)	–	–

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2   Food and Agriculture Organization of the United Nations.

vi) The RT-PCR gives an amplification product of 351 bp. 10 µl of these products are analysed by electrophoresis on a 1.5 % agarose gel. For all positive results, 40 µl of the final product may be directly used for sequencing.

2.2.2. RT-PCR for the diagnosis of PPRV based on the amplification of part of the F gene

This assay is based on that originally published in Forsyth *et al.* (2003). Like the N gene PCR, it detects virus from all of the four lineages.

i) Sequences of primers used in this protocol:

Primer	Sequence
F1b	5'-AGT-ACA-AAA-GAT-TGC-TGA-TCA-CAG-T-3'
F2d	5'-GGG-TCT-CGA-AGG-CTA-GGC-CCG-AAT-A-3'
F1	5'-ATC-ACA-GTG-TTA-AAG-CCT-GTA-GAG-G-3'
F2	5'-GAG-ACT-GAG-TTT-GTG-ACC-TAC-AAG-C-3'

ii) The first stage of this assay uses the consists of a one-tube RT-PCR reaction using PPRV primers F1b and F2d designed against the PPRV F gene. The protocol given has been validated using a commercial kit, but other reagents could be used after appropriate testing and validation.

iii) Combine 20 µl reaction mix with 5 µl RNA in a 0.5 ml PCR tube. Each assay requires, at a minimum, the sample, negative control, positive control and no-template control (RNase-free water instead of RNA sample).

iv) Transfer the reactions to a thermal cycler and start the following programme:

50°C for 30 minutes	1 cycle	Reverse transcription step
94°C for 2 minutes	1 cycle	Inactivates RT and activates polymerase
94°C for 1 minute		
55 °C for 1 minute	35 cycles	PCR amplification of the cDNA
72°C for 1 minute		
72°C for 7 minutes	1 cycle	Final extension
4°C (indefinite)	–	–

v) Analyse 10 µl of the reaction product by agarose gel electrophoresis using a 2% agarose gel in either TBE or TAE buffers. If present, PPRV RNA will be amplified to give a DNA fragment of 447 bp. If no DNA product, or a very weak DNA product, is seen, a second round of PCR can be completed to increase the amount of PCR product. This nested PCR can be carried out using primers F1 and F2 and any good quality PCR reagents and enzyme that have been suitably validated.

vi) Combine 24 µl reaction mix with 1 µl of the first stage PCR in a 0.5 ml PCR tube. Each assay requires, at a minimum, the sample, negative control, positive control and no-template control (RNase-free water instead of PCR product). Transfer the reactions to a thermal cycler and start the following programme:

94°C for 3 minutes	1 cycle	Activates polymerase
94°C for 1 minute		
55°C for 1 minute	35 cycles	PCR amplification of the cDNA
72°C for 1 minute		
72°C for 10 minutes	1 cycle	Final extension
4°C (indefinite)	–	–

vii) Analyse 10 µl of the reaction product by agarose gel electrophoresis using a 2% agarose gel in either TBE or TAE buffers. If present, PPRV PCR product will be amplified to give a DNA fragment of 371 bp.

viii) DNA product remaining from positive samples identified in Section 2.4.2 steps v or vii can be purified and subjected to DNA sequencing.

2.3. Culture and isolation methods

Even when diagnosis has been carried out by rapid techniques, the virus should always be isolated from field samples in tissue culture for further studies.

PPRV may be isolated in primary lamb kidney/lung cells and some cell lines (Vero, B95a). Unfortunately, PPRV isolation using such cells is not always successful on first passage and may require multiple blind passages. Recently, derivatives of cell lines (Vero, CV1) expressing the morbillivirus receptor, the signalling lymphocyte activation molecule (SLAM or CD150), have been developed that enable isolation of field viruses from pathological specimens in less than 1 week, without requirement for blind passages. These include a derivative of the monkey cell line CV1 expressing goat SLAM (Adombi *et al.*, 2011) and derivatives of Vero cells expressing dog SLAM. Monolayer cultures are inoculated with suspect material (swab material, buffy coat or 10% tissue suspensions) and examined daily for evidence of cytopathic effect (CPE). The CPE produced by PPRV can develop within 5 days and consists of cell rounding and aggregation culminating in syncytia formation in lamb kidney cells and cell lines expressing SLAM. In unmodified Vero cells, it is sometimes difficult to see the syncytia. If they exist, they are very small. However, small syncytia are always seen in infected Vero cells stained with haematoxylin and eosin. Syncytia are recognised by a circular arrangement of nuclei giving a 'clock face' appearance. Cover-slip cultures may show CPE earlier than day 5. Some cells may contain intracytoplasmic and intranuclear inclusions, others may be vacuolated. Similar cellular changes may be seen in stained histopathological sections of infected tissues. After 5–6 days, blind passages should always be carried out as CPE may take time to appear.

2.4. Penside (field) test for PPRV antigen

Commercial tests for PPRV antigen are available for use in the field. These tests are based on so-called lateral flow technology. Conjunctival, nasal or oral swabs are taken from suspect animals, the swabs are rinsed with buffer and this buffer is applied to one end of a chromatographic strip. The sample mixes with coloured beads coated with a specific MAb that recognises PPRV antigen. Buffer flow moves the beads along the chromatographic strip. If the sample contains PPRV antigen captured by the swab, this binds to the beads, and the antigen and beads complex is then captured by a line of anti-PPRV MAb part-way along the strip, making a coloured line to indicate a positive result. In the absence of PPRV antigen, no beads are bound by the test line. The tests take 20 minutes and require no additional equipment. Both tests have been validated against PPRV isolates from all four lineages, and show sensitivity similar to IC-ELISA, and 100% specificity in laboratory testing. One test has also been validated in field use in the Côte d'Ivoire, Ethiopia, Pakistan and Uganda (Baron *et al.,* 2014). The tests are designed for use with external swabs and are not suitable for use on blood or tissue samples.

2.5. Agar gel immunodiffusion

Agar gel immunodiffusion (AGID) is a very simple and inexpensive test that can be performed in any laboratory and even in the field. Standard PPRV antigen is prepared from infected mesenteric or bronchial lymph nodes, spleen or lung material and ground up as 1/3 suspensions (w/v) in buffered saline (Durojaiye *et al.*, 1983). These are centrifuged at 500 *g* for 10–20 minutes, and the supernatant fluids are stored in aliquots at –20°C. The cotton material from the cotton bud used to collect conjunctival or nasal swabs is removed using a scalpel and inserted into a 1 ml syringe. With 0.2 ml of phosphate buffered saline (PBS), the sample is extracted by repeatedly expelling and filling the 0.2 ml of PBS into an Eppendorf tube using the syringe plunger. The resulting conjunctival/nasal swab extracted sample, like the tissue ground material prepared above, may be stored at –20°C until used. They may be retained for 1–3 years. Negative control antigen is prepared similarly from normal tissues. Standard antiserum is made by hyperimmunising sheep with 1 ml of PPRV with a titre of 10^4 TCID$_{50}$ (50% tissue culture infective dose) per ml given at weekly intervals for 4 weeks. The animals are bled 5–7 days after the last injection (Durojaiye, 1982).

i) Dispense 1% agar in normal saline, containing thiomersal (0.4 g/litre) or sodium azide (1.25 g/litre) as a bacteriostatic agent, into Petri dishes (6 ml/5 cm dish).

ii) Six wells are punched in the agar following a hexagonal pattern with a central well. The wells are 5 mm in diameter and 5 mm apart.

iii) The central well is filled with positive antiserum, three peripheral wells with positive antigen, and one well with negative antigen. The two remaining peripheral wells are filled with test antigen, such that the test and negative control antigens alternate with the positive control antigens.

iv) Usually, 1–3 precipitin lines will develop between the serum and antigens within 18–24 hours at room temperature (Durojaiye *et al.*, 1983). These are intensified by washing the agar with 5% glacial acetic acid for 5 minutes (this procedure should be carried out with all apparently negative tests before recording a negative result). Positive reactions show lines of identity with the positive control antigen.

Results are obtained in one day, but the test is not sensitive enough to detect mild forms of PPR due to the low quantity of viral antigen that is excreted.

2.6. Counter immunoelectrophoresis

Counter immunoelectrophoresis (CIEP) is a more rapid version of the AGID test (Majiyagbe *et al.*, 1984). It is carried out on a horizontal surface using a suitable electrophoresis bath, which consists of two compartments connected through a bridge. The apparatus is connected to a high-voltage source. Agar or agarose (1–2%, [w/v]) dissolved in 0.025 M barbitone acetate buffer is dispensed on to microscope slides in 3-ml volumes. From six to nine pairs of wells are punched in the solidified agar. The reagents are the same as those used for the AGID test. The electrophoresis bath is filled with 0.1 M barbitone acetate buffer. The pairs of wells in the agar are filled with the reactants: sera in the anodal wells and antigen in the cathodal wells. The slide is placed on the connecting bridge and the ends are connected to the buffer in the troughs by wetted porous paper. The apparatus is covered, and a current of 10–12 milliamps per slide is applied for 30–60 minutes. The current is switched off and the slides are viewed by intense light: the presence of 1–3 precipitation lines between pairs of wells is a positive reaction. There should be no reactions between wells containing the negative controls.

3. Serological tests

The demonstration of antibodies in PPRV infected goats and sheep can be used to support a diagnosis based on clinical signs, but such antibodies may also arise from vaccination with any of the current PPRV vaccines. Tests that are routinely used are the virus neutralisation test (VNT) and the competitive ELISA.

3.1. Virus neutralisation

This test is sensitive and specific, but it is time-consuming. The standard neutralisation test is now usually carried out in 96-well microtitre plates although roller-tube cultures may also be used. Vero cells are preferred, but primary lamb kidney cells may also be used.

This test requires the following materials: cell suspensions at 600,000/ml; 96-well cell culture plates; sera to be titrated (inactivated by heating to 56°C for 30 minutes); complete cell culture medium; PPRV diluted to give 1000, 100, 10 and 1 $TCID_{50}$/ml.

i) Dilute the sera 1/5, and then make twofold serial dilutions in cell culture medium.

ii) Mix 100 µl of virus at 1000 $TCID_{50}$/ml (to give 100 $TCID_{50}$ in each well) and 100 µl of a given dilution of serum (using six wells per dilution) in the wells of the cell culture plate.

iii) Arrange a series of control wells for virus and uninfected cells as follows: six wells with 100 $TCID_{50}$ (100 µl) per well; six wells with 10 $TCID_{50}$ (100 µl) per well; six wells with 1 $TCID_{50}$ (100 µl) per well; six wells with 0.1 $TCID_{50}$ (100 µl) per well; and six wells with 200 µl of virus-free culture medium per well. Make the wells containing the virus dilutions up to 200 µl with complete culture medium, and incubate the plates for 1 hour at 37°C.

v) Add 50 µl of cell suspension to each well, pat the sides of the plate lightly to distribute the cells in the well and cover. Incubate the plates at 37°C in the presence of CO_2.

vi) Read the plates after 1 and 2 weeks of incubation. The results should be as follows:

If the virus dilution has been done correctly, all the virus control wells with 100 and 10 $TCID_{50}$/well will show CPE, 50% of the wells will show CPE for the 1 $TCID_{50}$/well dilution, and none should show CPE for the 0.1 $TCID_{50}$/well dilution. The test is only valid if the virus has been suitably diluted.

For the serum titration, there will be no CPE in wells where the virus had been neutralised by serum during the test; any level of CPE means that the virus had not been neutralised by serum. The neutralising titre is the dilution of serum that neutralises virus in half the wells. A neutralising titre of greater than 10 is positive.

3.2. Competitive enzyme-linked immunosorbent assay

Several competitive ELISAs (C-ELISA) have been described, based on the use of MAbs that recognise virus proteins. They are of two types: those where the MAb recognises the N protein and use recombinant N protein produced in baculovirus as the antigen (e.g. Libeau *et al.*, 1995); and those with a viral attachment protein (H) specific MAb and antigen consisting of purified or part-purified PPRV (vaccine strain) (e.g. Anderson & McKay, 1994; Saliki *et al.,* 1993). All the assays work on the principle that antibodies to PPRV in test sera can block the binding of the MAb to the antigen.

Advice on the use and applicability of ELISA methods is available from the WOAH Reference Laboratories for PPR. Some methods are available as commercial kits; these are the only practicable way to carry out this test. Before use, laboratories should seek assurance that the kit has been validated in accordance with the WOAH Validation Standard (see Chapter 1.1.6 *Validation of diagnostic tests for infectious diseases of terrestrial animals*). The only alternative would be for a laboratory to develop and validate all the reagents (monoclonals and antigens) in house.

C. REQUIREMENTS FOR VACCINES

1. Background

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given below and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

1.1. Rationale and intended use of the product

Sheep and goats vaccinated with an attenuated strain of PPR or that recover from PPR develop an active life-long immunity against the disease (Durojaiye, 1982). Several PPR vaccines are available, all of which are cell culture-attenuated strains of natural PPRV (Sen *et al.,* 2010). The two most commonly used vaccine strains (Nigeria/75/1 and Sungri/96) have both been shown in experimental trials to protect animals against PPRV isolates of all lineages (Hodgson *et al.*, 2018); the Nigeria/75/1 vaccine has also been proven to provide such complete cross-lineage protection in field use in a large number of countries.

2. Outline of production and minimum requirements for conventional vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

The history of the vaccine received and stored in the laboratory as master seed should be well known and registered: the origin of the vaccine, the number of passages in cell culture, and the range of the number of passages in cell culture shown to be effective in providing protection against PPR for at least 3 years when administered at the recommended dose. Such a PPR virus vaccine strain should not be excreted by inoculated animals in such a way that it can spread to in-contact animals. It should be proven that the vaccine strain has not reverted to virulence following at least three back passages in sheep and goats (Diallo *et al.,* 1989).

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

The seed should be controlled and tested free from bacterial, fungal and mycoplasma contamination. It should be tested free from pestivirus and other potentially contaminating viruses. Only live attenuated PPR virus should be present. The seed should either have passed an innocuity test in animals (rodents, sheep and goats) or have been shown to be free of harmful contaminants by other validated means; it should have demonstrated its efficacy to protect sheep and goats against PPR with the recommended dose.

2.2. Method of manufacture

2.2.1. Requirements for substrates and media

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

i)   Cells

All current PPRV vaccine strains are grown in Vero cells. Cells used for the production of PPR vaccine must be free from bacterial, fungal and viral contaminations. The source of these cells should be known and documented.

ii)   Serum

The serum used in the cell culture should be free from adventitious virus, in particular pestiviruses. It is recommended to use irradiated serum. The country of origin of the serum must be known (use of serum from countries with high risk of transmissible spongiform encephalopathy [TSE] infections should be avoided).

iii) Culture medium

The traditional culture medium is minimal essential medium (MEM); recent work has shown that Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose or 25 mM fructose gives significantly higher (tenfold) yields of virus (Silva *et al.*, 2011). The medium can be supplemented with antibiotics (e.g. penicillin + streptomycin at final concentrations of 100 IU [International Units]/ml and 100 μg/ml, respectively) and an antifungal agent (e.g. nystatin at a final concentration of 50 μg/ml) if required. The medium is enriched with 10% (v/v) fetal calf serum (complete medium) for cell growth. This proportion of serum is reduced to 2% (v/v) for maintenance medium when the cell monolayer is complete.

2.2.2. In-process controls

i)   Cells used in cultures must be checked for normal appearance and shown to be free from contaminating mycoplasma and viruses, especially bovine viral diarrhoea virus.

ii)   A virus titration must be undertaken on the working and production seed lots and on samples from the final product.

●   **Test procedure**

a)   Using serum-free medium, make a series of tenfold dilutions (0.5 ml virus + 4.5 ml diluent) down to 10^{-6} of the material to be titrated.

b)   Trypsinise Vero cells and suspend in complete culture medium at 300,000/ml. Distribute the cells on a 96-well plate (30,000 cells per well, equivalent to 100 μl of cell suspension).

c)   Add 100 μl of diluted virus to the cells (dilutions ranging from 10^{-2} to 10^{-6}). Use one row for each virus dilution; one row of wells serves as a negative control consisting of uninfected cells to which virus-free culture medium (100 μl) is added.

d)   Incubate the plate at 37°C in the presence of CO_2.

e)   Read the plates (by examining for CPE) 7–10 days after infection. Virus titre is calculated by the Spearman–Kärber method.

iii) The identity of the virus is confirmed at the final batch stage. For this purpose, anti-PPRV serum is used to neutralise the virus in cell culture.

   a) Mix the contents of two vaccine bottles with sterile double-distilled water to provide a volume equal to the volume before freeze-drying.

   b) Make tenfold dilutions of the reconstituted vaccine in serum-free culture medium (0.5 ml of viral suspension + 4.5 ml of medium).

   c) Make two series of mixtures for virus dilutions from each bottle on a 96-well plate as follows:

Series 1:	Dilutions of viral suspension:	−1	−2	−3	−4
	Viral suspension (in µl)	50	50	50	50
	Culture medium (in µl)	50	50	50	50
Series 2:	Dilutions of viral suspension:	−1	−2	−3	−4
	Viral suspension (in µl)	50	50	50	50
	PPR antiserum (in µl)	50	50	50	50

   (**Note:** PPR antiserum used for this purpose is prepared in goats and freeze-dried. It is reconstituted with 1 ml of sterile double-distilled water in a dilution of 1/10.)

   d) Incubate the mixtures at 37°C for 1 hour

   e) Add to each well 100 µl of cells suspended in complete culture medium (30,000 cells/well).

   f) Incubate the microplate at 37°C in the presence of CO_2.

   g) Read the plate after 7–10 days of incubation.

   Normally CPE is present only in the wells containing cells infected with the mixture of virus and culture medium. If it is detected in the wells of Series 2, it will be necessary to identify PPRV by immunofluorescence, using a PPR MAb, or by immunocapture ELISA (using the commercially available kit). If this identification confirms the presence of PPRV, the PPR antiserum used must have been too weak, or the batch must be changed. If immunofluorescence or immunocapture is negative, or if repeating the test with a new PPRV antiserum gives the same result, a viral contaminant must be present, and the material under test must be destroyed.

2.2.3 Manufacturing procedure

A protocol for the production and validation of vaccine from master seeds of the available attenuated PPRV strains is described here. Other protocols for the production of commercial stocks of the vaccine may be used as long as the essential criteria are observed and the final product is appropriately validated.

Essential criteria:

i) The master seed stock should have a known and documented history and be shown to be free of contaminating pathogens.

ii) The virus should be grown at all stages in the same cell line as that used to produce the master seed, since passage in a different cell line is likely to lead to the accumulation of sequence changes in the vaccine, changes which may have effects on the vaccine properties.

iii) The master seed must not be passed more than 10 times for the production of final vaccine batches.

iv) Sterility and potency tests must be carried out on the product and fully documented.

Once the manufacturer has received samples from an institution holding the PPRV vaccine seed – the master seed – it should prepare primary and secondary working seed batches. The production seed batch, from which the final vaccine is produced, is prepared from the secondary working seed. By preparing primary and secondary working seeds, the need to carry out a large

number of passages from the master seed is avoided. In this way, it is possible to comply with the WOAH recommendation of no more than 5–10 passages after the master seed (see chapter 1.1.8 *Principles of veterinary vaccine production*).

i) Primary and secondary working seed batches

When preparing primary and secondary working seed batches it is important to avoid infecting the cells with high doses of virus (high multiplicity of infection [m.o.i.]), as this will lead to the accumulation of defective particles in the viral suspension produced, which will diminish the titre of subsequent products. On the other hand, very low m.o.i. (e.g. 0.0001) will prolong the culture time. The manufacturer is responsible for optimising production work flow for their own cells and culture medium; as a guide, infections at each stage should be at a m.o.i. of 0.001–0.01.

The freeze-dried contents of a flask from the master seed are reconstituted with 2 ml of cell culture medium without serum. This liquid is mixed with Vero cells (if the vaccine is produced on Vero cells), suspended in complete culture medium to provide at least 0.001 $TCID_{50}$ per cell. Cell culture flasks are filled with this virus/cell mixture (around 2×10^7 Vero cells in a 175 cm^2 flask), and are incubated at 37°C. The cultured cells are examined regularly to detect CPE. The medium is renewed every 2 days, reducing the proportion of serum to 2% once the cell monolayer is complete. Virus (the cell culture medium over the cells) is first harvested when there is 40–50% CPE. This viral suspension is stored at –70°C. Successive harvesting is carried out every 2 days until the CPE reaches 70–80%, at which point the culture flasks are frozen at –70°C. In general, at least two further harvestings can be made before final freezing of the culture flasks. All suspensions of virus collected are submitted to two freeze–thaw cycles, then pooled to form a single batch, which serves as the working seed batch. The suspension of virus may be clarified by low speed centrifugation (e.g. 5 minutes at 1250 *g*) to remove cell debris. The working seed batch is divided into small volumes in bottles and stored at –70°C. The contents of five of these bottles are thawed and titrated (minimum titre required: 10^5 $TCID_{50}$/ml). It is best to freeze-dry this primary working seed in order to store it at –20°C. In this case it will be necessary to titrate the freeze-dried virus (five bottles). A batch made up in this way must pass all tests for sterility.

An aliquot of the primary working seed is then cultured in the same way to prepare the secondary working seed.

ii) Production seed batch

The production seed batch is prepared under the same conditions as for the working seed batches. A large stock of virus is formed, from which the final vaccine will be produced. This batch is distributed into receptacles and stored at –70°C. As previously, five samples are titrated (minimum titre required: 10^6 $TCID_{50}$/ml), and the production seed batch must satisfy tests for sterility.

iii) Vaccine production

This operation is performed on a larger scale, but is otherwise carried out as for the preparation of the production seed. Products of the various harvests, after two freeze–thaw cycles, are brought together to form the final product and stored at –70°C pending the results of titration and tests for sterility. If these results are satisfactory, the vaccine is freeze-dried.

v) Freeze-drying

Several freeze-drying media have been assessed for use with PPRV. Weybridge medium has been used most widely, and is composed of 2.5% (w/v) lactalbumin hydrolysate (LAH), 5% (w/v) sucrose and 1% (w/v) sodium glutamate, in Hank's balanced salt solution (HBSS) pH 7.2. Significantly improved stability at temperatures above 4°C has been reported using a freeze-drying medium containing 5%(w/v) LAH and 10% sucrose in HBSS (Mariner *et al*. 2017; Sarkar *et al*., 2003) or containing 20 mM Tris–HCl pH 7.4, 2 mM EDTA, 0.02% (w/v) Tween 80 and 1 M (34.2% (w/v)) trehalose (Silva *et al*., 2011). The freeze-drying medium is

added to an equal volume of viral suspension (which may have been diluted beforehand to provide the desired number of vaccine doses per bottle). The resulting mixture is kept cool, homogenised, then distributed into bottles and freeze-dried. At the end of a freeze–drying cycle, the probe is adjusted and kept at 35°C for 4 hours. Once this operation has been completed, the bottles are capped under vacuum. Randomly selected samples of this final batch are subjected to tests for innocuity, efficacy and sterility, and residual moisture is estimated by the Karl Fisher method (optimum ≤3.5%). If the tests give unsatisfactory results, the entire batch is destroyed.

2.2.4. Final product batch tests

Determination of the number of samples to be tested will depend on the batch size and the accuracy of the test involved; the principles of Appendix 1.1.2.1 *Epidemiological approaches to sampling: sample size calculations* of Chapter 1.1.2 *Collection, submission and storage of diagnostic specimens* apply. Manufacturers must sample sufficient vials to have >95% confidence of detecting contamination at the 0.1% level.

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 batch must be tested and proven not to be contaminated by bacteria, fungus, mycoplasma or other viruses. These tests are carried out on the vaccine batch before and after freeze-drying. Any product that fails these tests for sterility is destroyed.

The content of at least one container from each filling lot must be checked for identity by culture after neutralisation with specific antiserum.

ii) Safety and efficacy

As stated in Chapter 1.1.8 *Principles of veterinary vaccine production*, the target animal batch safety test (TABST) may be waived by the regulatory authority when a sufficient number of production batches have been produced under the control of a seed lot system and found to comply with the test, thus demonstrating consistency of the manufacturing process. Some regulatory authorities still require safety tests for the release of each batch. Where required, a safety test should be done on the host recipient of the vaccine, currently sheep and goats. Unless specifically required by a local regulatory authority, the safety test in rodents is not considered necessary for live attenuated PPRV vaccines.

Normally the minimum immunising dose is 100× the lowest dose of vaccine virus able to induce a 50% immunising response. For example, in the case of the attenuated Nigeria 75/1 vaccine the required minimum titre per dose has been shown to be $10^{2.5}$ $TCID_{50}$.

For the TABST, five bottles of the PPR vaccine are reconstituted in the normal diluent, mixed and diluted to give a solution of 100 doses/ml. Two goats and two sheep are used, all approximately 1-year old and free from PPR antibodies. The animals are vaccinated subcutaneously with 100 doses per animal. The animals are subjected to daily clinical examination for 3 weeks including recording of rectal temperatures. The vaccine is considered as safe if no abnormal clinical signs are observed in the vaccinated animals.

For the established live attenuated PPRV vaccines, efficacy is determined by the titre of live virus in the vaccine batch (batch potency); challenge with wild type virus is not required. Novel killed, subunit or other vaccines must establish appropriate correlates of protection, or must be batch tested by showing protection against challenge.

iii) Batch potency

For the established live attenuated PPRV vaccines, the potency of each batch can be determined by virus titration in cell culture coupled with confirmation that the virus has not undergone significant mutation during the production process. This confirmation can be provided (a) *in vitro* by carrying out full genome sequencing of the final batch material and

showing an absence of changes in the main immunogenic proteins (N and H) or (b) *in vivo* by inoculating at least six target animals with 1 dose per animal of vaccine and showing that all animals have a neutralising antibody titre of at least 1/10 at 3 weeks post-vaccination.

Potency for novel vaccines will have to be established by other means, depending on the type of vaccine.

2.3. Requirements for regulatory approval

2.3.1. Safety requirements

i) Target animal safety

The vaccine should be safe for use in all species of target animals, including young and pregnant recipients. Once manufacture has established a consistent safety record, they may apply to their regulatory authority for permission to omit this test.

ii) Reversion-to-virulence for attenuated/live vaccines

Information should be available to indicate that studies have been carried out and have demonstrated that the vaccine strain which has been used has not reverted to virulence after at least three back passages.

iii) Environmental consideration

Information should be available to indicate that studies have been carried out and have demonstrated that the vaccine strain which has been used is not excreted by the inoculated animals.

2.3.2. Efficacy requirements

i) For animal production

Field or other tests should have proven that the attenuated PPR vaccine is efficacious for use in all sheep and goats species, including young and pregnant animals.

ii) For control and eradication

Sheep and goats which have recovered from a PPR infection appear to be protected against a subsequent infection for the rest of their lives. Neutralising antibodies against PPRV were found in sheep and goats up to three years after vaccination with the attenuated PPRV vaccine strain Nigeria 75/1. Other attenuated PPR vaccines that have been developed in India (Sungri/96) have been shown to produce long-lasting immunity too (Saravanan *et al.,* 2010a; Sen et *al.,* 2010). All these data indicate that PPR is a disease that can be well controlled, even eradicated, following a large-scale and well planned vaccination campaign such as that done for rinderpest.

2.3.3. Stability

PPR vaccine freeze-dried in the Weybridge medium can be kept for at least 2 years at 2–8°C (although storage at –20°C is better), provided it is stored under vacuum and protected from light.

2.3.4. Vaccines permitting a DIVA strategy (detection of infection in vaccinated animals)

No DIVA vaccine and associated test is currently available for field use.

2.3.5. Duration of immunity

The duration of protective immunity should be determined for each vaccine strain in animal trials. For the Nigeria 75/1 and Sungri/96 vaccines, this has been shown to be at least 3 years (Saravanan *et al*., 2010b; Zahur *et al*., 2014). Commercial preparations of these vaccines should therefore offer at least 3 years protection.

3. Vaccines based on biotechnology

3.1. Vaccines available and their advantages

Preliminary results on recombinant capripox-based PPR vaccines indicate that they can protect against both capripox and PPR (Berhe *et al.*, 2003; Caufour *et al.,* 2014; Chen *et al.*, 2010; Diallo *et al.*, 2002). Novel vaccines based on recombinant adenoviruses have also been shown to be effective (Herbert *et al.,* 2014; Holzer *et al.,* 2016; Qin *et al.,* 2012; Rojas *et al.,* 2014; Wang *et al.,* 2013); such vaccines also offer the possibility of providing a DIVA capability. None of these vaccines has yet been validated for field use.

3.2. Special requirements for biotechnological vaccines, if any

None.

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

NB: FIRST ADOPTED IN 1989. MOST RECENT UPDATES ADOPTED IN 2021.