

## CHAPTER 2.7.11.

# PESTE DES PETITS RUMINANTS

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### SUMMARY

*Peste des petits ruminants (PPR), is an acute contagious disease caused by a 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 (PPRV) 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 rinderpest, bluetongue, foot and mouth disease and other erosive or vesicular conditions as well as contagious caprine pleuropneumonia, can cause clinically similar disease.*

**Identification 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.*

*Rapid diagnosis is done by immunocapture enzyme-linked immunosorbent assay (ELISA), counter immunoelectrophoresis and agar gel immunodiffusion. Polymerase chain reaction may also be used.*

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

**Requirements for vaccines:** *Effective live attenuated PPR virus vaccines are now widely available. Since the global eradication of rinderpest, heterologous vaccines should not be used to protect against PPR.*

### 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 PPR virus (PPRV). PPRV 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, PPRV has been classified within the genus *Morbillivirus* in the family *Paramyxoviridae* (Gibbs *et al.*, 1979). Virus members of this group have six structural proteins: the nucleocapsid protein (N), which encapsulates the virus 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 PPR genome also encodes two nonstructural proteins, C and V.

PPR was first described in Côte d'Ivoire (Gargadennec & Lalanne, 1942), but it occurs in most African countries from North Africa to Tanzania, in nearly all Middle Eastern countries to Turkey, and is also widespread in countries from central Asia to South and South-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. Cases of clinical disease have been reported in wildlife resulting in deaths of wild small ruminants. 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 1.1.3 *Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities*).

## B. DIAGNOSTIC TECHNIQUES

A list including all the types of tests available for peste des petits ruminants 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	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Competitive ELISA	++	++	–	+++	+++
Virus neutralisation	+++	+++	–	+++	+++

Method	Purpose				
	Population freedom from infection	Individual animal freedom from infection	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
RT-PCR	–	–	+++	–	–
Real-time RT-PCR (QRT-PCR)	–	–	+++	–	–
Virus isolation in cell culture	–	–	++	–	–
Immunocapture ELISA	–	–	+++	–	–
Agar gel immunodiffusion	–	–	+	–	+
Counter immunoelectrophoresis	–	–	+	–	–

Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose.

Although not all of the tests listed as category +++ or ++ have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

ELISA = enzyme-linked immunosorbent assay; RT-PCR = reverse transcription polymerase chain reaction.

## 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 and from the nasal and buccal 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. Identification of the agent

### a) 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 PPR viral 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 eye 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 eye/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<sup>4</sup> TCID<sub>50</sub> (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.

#### **b) Counter immunoelectrophoresis**

Counter immunoelectrophoresis (CIEP) is the most rapid test for viral antigen detection (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.

#### **c) Immunocapture enzyme-linked immunosorbent assay**

Advice on the use and applicability of the immunocapture enzyme-linked immunosorbent assay (icELISA) method is available from the OIE Reference Laboratories for PPR. The method described is available as a commercial kit.

The icELISA (Libeau *et al.*, 1994) using two monoclonal antibodies (MAB) raised to the N protein allows a rapid identification of PPRV. The instructions provided by kit supplier should be followed, but the following shows a typical procedure for the test.

- i) Microtitre ELISA plates are coated with 100 µl of a capture MAB solution (diluted according to the instructions of the kit supplier). Coating may be overnight at 4°C or for 1 hour at 37°C.
- ii) After washing, 50 µl of the sample suspension is added to each of two wells, and two block (control) wells are filled with buffer.
- iii) Immediately add 25 µl of a detection biotinylated MAB for PPR and 25 µl of streptavidin/peroxidase to two wells.
- iv) The plates are incubated at 37°C for 1 hour with constant agitation.
- v) After three vigorous washes, 100 µl of ortho-phenylenediamine (OPD) in 0.03% (v/v) hydrogen peroxide is added, and the plates are incubated for 10 minutes at room temperature.
- vi) The reaction is stopped by the addition of 100 µl of 1 N sulphuric acid, and the absorbance is measured at 492 nm on a spectrophotometer/ELISA reader.

The cut-off above which samples are considered to be positive is calculated from the blank control as three times the mean absorbance values of the control wells.

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

A sandwich form of the immunocapture 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 the MAB or polyclonal antibody adsorbed on to the ELISA plate. The assay shows high correlation to the cell infectivity assay (TCID<sub>50</sub>) with a minimum detection limit of  $10^3$  TCID<sub>50</sub>/ml.

#### **d) 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; Forsyth & Barrett, 1995). 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 the conventional RT-PCR, as well as minimising the risk of contamination. The application of nucleic acid isothermal amplification to PPR diagnosis has also been described (Li *et al.*, 2010). The sensitivity of this assay seems to be similar to that of the real-time RT-PCR. This assay is simple to implement, rapid and the result can be read by naked eye.

Because this is a rapidly developing field, users are advised to contact the OIE and FAO<sup>1</sup> Reference Laboratories for PPR (see Table given in Part 4 of this *Terrestrial Manual*) for advice on the most appropriate techniques.

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

N gene amplification is based on the initial protocol described by Couacy-Hymann *et al.* (2002) in a one-step RT-PCR method available as a commercial kit. The test described requires the following materials: Qiagen One-step RT-PCR kit, distilled water and primers.

i) Sequence of primer used:

Primer	Sequence
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NP3:	5'-GTC-TCG-GAA-ATC-GCC-TCA-CAG-ACT-3';
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NP4:	5'-CCT-CCT-CCT-GGT-CCT-CCA-GAA-TCT-3').
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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 Qiagen	2 µl	
Q solution	10 µl	
Primer NP3 (10 µM)	3 µl	0.6 µM
Primer NP4 (10 µM)	3 µl	0.6 µM
Qiagen 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
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94°C for 30 seconds		
60°C for 30 seconds	40 cycles	PCR amplification of the cDNA
72°C for 1 minute		
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72°C for 5 minutes	1 cycle	Final extension
4°C (indefinite)	–	–

1 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 1.5 % agarose gel. For all positive results, 40 µl of the final product may be directly used for sequencing.

• **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 & Barrett (1995).

- 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 Superscript III One-Step RT-PCR Platinum Taq HiFi kit (Life Technologies) and amplification is achieved by using PPRV primers F1b and F2d designed against the PPRV F gene. The following is required per reaction:

2× reaction mix	12.5 µl
Enzyme mix	0.5 µl
RNAse free distilled water	5 µl
Primer F1b (10µM)	1 µl
Primer F2d (10µM)	1 µl
Total Volume	20 µl

*Note 1: all reagents except primers and RNAse free distilled water are supplied in the Superscript III One-Step RT-PCR Platinum Taq HiFi kit*

*Note 2: A mastermix for the required number of reactions can be prepared.*

- 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 is carried out using the Taq PCR Master Mix (Life Technologies) and primers F1 and F2. The following is required per reaction:

Taq Mastermix	12.5 µl
Nuclease free distilled water	9.5 µl
Primer F1 (10µM)	1 µl
Primer F2 (10µM)	1 µl
Total Volume	24 µl

*Note: A mastermix for the required number of reactions can be prepared.*

- 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 RNA will be amplified to give a DNA fragment of 371 bp.
- viii) DNA product remaining from positive samples identified in (e) or (g) can be purified and subjected to DNA sequencing.

### e) 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 cultures 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 can 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.

## 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 (VN) test and the competitive ELISA.

### a) Virus neutralisation (the prescribed test for international trade)

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<sub>50</sub>/ml.

- Dilute the sera 1/5, and then make twofold serial dilutions in cell culture medium.
- Mix 100 µl of virus at 1000 TCID<sub>50</sub>/ml (to give 100 TCID<sub>50</sub> 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.
- Arrange a series of control wells for virus and uninfected cells as follows: six wells with 100 TCID<sub>50</sub> (100 µl) per well; six wells with 10 TCID<sub>50</sub> (100 µl) per well; six wells with 1 TCID<sub>50</sub> (100 µl) per well; six wells with 0.1 TCID<sub>50</sub> (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<sub>2</sub>.
- 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<sub>50</sub>/well will show CPE, 50% of the wells will show CPE for the 1 TCID<sub>50</sub>/well dilution, and none should show CPE for the 0.1 TCID<sub>50</sub>/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.

#### b) 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 OIE 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 OIE Validation Standard (see Chapter 1.1.5 *Principles and methods of validation of diagnostic tests for infectious diseases*). 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 1.1.6 *Principles of veterinary vaccine production*. The guidelines given below and in chapter 1.1.6 are intended to be general in nature and may be supplemented by national and regional requirements.

#### a) 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 homologous PPR vaccines are available, being cell culture-attenuated strains of natural PPRV (Sen *et al.*, 2010). In 1998, the OIE World Assembly (formally OIE International Committee) endorsed the use of such a vaccine in countries that have decided to follow the 'OIE pathway' for epidemiological surveillance for rinderpest in order to avoid confusion when serological surveys are performed. There have also been three published reports on the preliminary results from recombinant capripox-based PPR vaccines that are able to protect against both capripox and PPR (Berhe *et al.*, 2003; Chen *et al.*, 2010; Diallo *et al.*, 2002). The production and validation of vaccine from the commercially available attenuated PPRV is described here.

### 2. Outline of production and minimum requirements for conventional vaccines

#### a) Characteristics of the seed

##### i) Biological characteristics

The history of the vaccine received and stored in the laboratory as master seed should be well known and registered: origin, level of passages in cell culture, the range of the number of passages of the vaccine in cell culture that has been tested and shown to be effective in providing protection in animals against PPR with the recommended dose for vaccination for at least 3 years. This PPR virus vaccine strain should not be able to be excreted by inoculated animals and 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).

ii) *Quality criteria (sterility, purity, freedom from extraneous agents)*

The seed should be controlled and tested free from bacterial, fungus and mycoplasma contamination. It should be tested free from pestivirus and any other known extraneous virus. Only live attenuated PPR virus should be present. The seed should have passed an innocuity test in animals (rodents, sheep and goats) and have demonstrated its efficacy to protect sheep and goats against PPR with the recommended dose.

**b) Method of manufacture**

i) *Procedure*

Once the manufacturer has received samples from an institution holding the PPRV vaccine bank – the master seed – it must 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 over the master seed is avoided for the vaccine production. In this way, it is possible to comply with one of the OIE recommendations: 5–10 passages maximum after the master seed (see chapter 1.1.6).

- a) *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 weak m.o.i. (e.g. 0.0001) will prolong the culture time.

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<sub>50</sub> per cell. Cell culture flasks are filled with this virus/cell mixture (around  $2 \times 10^7$  Vero cells in a 175 cm<sup>2</sup> flask), and are incubated at 37°C. The cultured cells are examined regularly to detect a CPE. The medium is renewed every 2 days, reducing the proportion of serum to 2% once the cell monolayer is complete. Virus 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%, which is the time for final freezing of the culture flasks (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 added to form a single batch, which serves as the working seed batch. This is divided into small volumes in bottles and stored at –70°C. The contents of five flasks are thawed and titrated (minimum titre required: 10<sup>5</sup> TCID<sub>50</sub>/ml). It is best to freeze-dry this 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.

- b) *Preparation of the production seed batch:* The production seed batch is prepared under the same conditions as for the working seed batch apart from the fact that infection of the cells can be done with a higher m.o.i. compared with the previous cases and usually with at least m.o.i. 1–10. 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. It must satisfy tests for sterility. Five samples are titrated (minimum titre required: 10<sup>6</sup> TCID<sub>50</sub>/ml).

- c) *Validation as a vaccine:* It is necessary to confirm or rule out the presence of PPRV in the product under test. For this purpose, anti-PPR serum is used to neutralise the virus in cell culture.

• **Test procedure:**

- i) Mix the contents of two vaccine bottles with sterile double-distilled water to provide a volume equal to the volume before freeze-drying.
- ii) Make tenfold dilutions of the reconstituted vaccine in serum-free culture medium (0.5 ml of viral suspension + 4.5 ml of medium).
- iii) 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.)

- iv) Incubate the mixtures at 37°C for 1 hour
- v) Add to each well 100 µl of cells suspended in complete culture medium (30,000 cells/well).
- vi) Incubate the microplate at 37°C in the presence of CO<sub>2</sub>.
- vii) Read the plate after 7–10 days of incubation.

Normally a 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 (specific PPR MAb, and the immunocapture test kit are available from the OIE Reference Laboratory for PPR in France [see Table given in Part 4 of this *Terrestrial Manual*]). 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, a viral contaminant must be present, and the material under test must be destroyed.

- d) *Vaccine production*: This operation is performed on a larger scale. Cells can be infected with virus at a m.o.i. as before or with high doses, e.g. up to 0.01. 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.
  - e) *Freeze-drying*: The freeze-drying medium (Weybridge medium) is composed of 2.5% (w/v) lactalbumin, 5% (w/v) sucrose and 1% (w/v) sodium glutamate, pH 7.2. This medium is added to an equal volume of viral suspension for freeze-drying (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 (e.g. 5%) of this final batch are submitted 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.
- ii) *Requirements for substrates and media*
- a) *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.
  - b) *Serum*: The serum used in the cell culture should be free from adventive virus, in particular pestiviruses. It is recommended to use irradiated serum. The country origin of the serum should be known (it should be avoided to use the serum from countries with high risk of transmissible spongiform encephalopathies (TSE) infections).
  - c) *Culture medium*: The culture medium consists of minimal essential medium (MEM) supplemented with antibiotics (for example penicillin + streptomycin at final concentrations of 100 IU [International Units]/ml and 100 µg/ml, respectively), and an antifungal agent (nystatin [Mycostatin] at a final concentration of 50 µg/ml). The medium is enriched with 10% fetal calf serum (complete medium) for cell growth. This proportion of serum is reduced to 2% for maintenance medium when the cell monolayer is complete.

iii) *In-process controls*

Cells used in cultures must be checked for normal appearance and shown to be free from contaminating viruses, especially bovine viral diarrhoea virus. A virus titration must be undertaken on the seed lot: using MEM (serum-free) medium, a series of tenfold dilutions is made (0.5 ml virus + 4.5 ml diluent) down to 10<sup>-6</sup> of the product to be titrated. Vero cells from one flask are trypsinised and suspended in complete culture medium at 300,000/ml. They are distributed on a 96-well plate (30,000 cells per well, equivalent to 100 µl of cell suspension). Then, 100 µl of virus diluted tenfold is added to the cells (dilutions ranging from 10<sup>-2</sup> to 10<sup>-6</sup>). One row of wells serves as a control for uninfected cells to which virus-free culture medium (100 µl) is added. The plate is incubated at 37°C in the presence of CO<sub>2</sub>. The plates are read (by examining for CPE) 7–10 days after infection.

Virus titre is determined by the Spearman–Kärber method.

iv) *Final product batch tests*

*Sterility and purity*

Tests for sterility and freedom from contamination of biological materials may be found in chapter 1.1.7.

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

It should be tested and proven to be non-contaminated by bacteria, fungus or other viruses. The test carried out on cells and sera before their use in vaccine production, and on the seed stock and the vaccine before and after freeze-drying. Any product that fails this test for sterility is destroyed.

Tests for sterility and freedom from contamination of biological materials may be found in chapter 1.1.7.

#### *Safety and efficacy*

A safety test should be done in rodents in order to detect any nonspecific toxicity associated with the product and also on host recipient of the vaccine, currently sheep and goats.

For rodents, six guinea-pigs, each weighing 200–250 g; ten unweaned mice (17–22 g, Swiss line or similar). The vaccine contents of five bottles are mixed and used. The vaccine, 0.5 ml, is injected intramuscularly into a hind limb of two guinea-pigs, 0.5 ml into the peritoneal cavity of two guinea-pigs, and 0.1 ml into the peritoneal cavity of six mice. Two guinea-pigs and four mice are kept as uninoculated controls. The animals are observed for 3 weeks. If one guinea-pig or two mice die, the test must be repeated. Dead animals undergo post-mortem examination to ascertain the cause of death. At the end of 3 weeks of observation, all animals are killed for post-mortem examination. All the results are recorded. The vaccine is considered to be satisfactory if, during the first or second test, at least 80% of animals remain in good health during the period of observation, and no significant post-mortem lesion is found.

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<sub>50</sub>.

For the target host safety test, the PPR vaccine must be reconstituted with normal saline. The mixed contents of five bottles are reconstituted in the diluent to give solutions of 100 doses/ml and 0.1 dose/ml. Six goats and six sheep are used, all approximately 1-year old and free from PPR antibodies. Vaccinate two goats and two sheep subcutaneously with 100 doses per animal; vaccinate two goats and two sheep subcutaneously with 0.1 dose per animal; keep the remaining animals as in-contact controls. The animals are subjected to daily clinical examination for 3 weeks including recording of rectal temperatures. At the end of this period, blood is taken from all animals for the preparation of sera. All animals are challenged by subcutaneous injection of a 1 ml suspension of pathogenic PPRV previously tested for inducing PPR clinical signs in sheep and goats. The animals are observed and their body temperature measurements are recorded daily for 2 weeks.

The vaccine is considered as safe if no abnormal clinical signs are observed in the vaccinated animals, in particular those which have received the highest dose.

Its efficacy is proven if all vaccinated animals resist the challenge while the in-contact animals remain susceptible to the challenge virus.

#### *Batch potency*

The potency of each batch should be determined by virus titration in cell culture and shown to meet the minimum immunising dose requirements in the efficacy test.

The potency of the vaccine is proven if animals vaccinated with the dose used in the efficacy test (see above) develop antibody against PPRV when tested 3 weeks after vaccination by virus neutralisation at a serum dilution of > 1/10). The titration of neutralising antibody is carried out as described in section B.3.a above.

It might possible that no seroconversion is detected in some animals 3 weeks after vaccination but those animals will resist challenge if the vaccine is efficacious. Resistance in these cases is attributed to the cell-mediated immunity induced by PPRV (Saravanan *et al.*, 2010).

### **c) Requirements for authorisation**

#### *i) Safety requirements*

##### *Target and non-target animal safety*

The vaccine should be safe for use in all species of targeted animals, including young and pregnant recipients.

##### *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.

*Environmental consideration*

PPR vaccine should not be excreted by the inoculated animals.

ii) *Efficacy requirements**For animal production*

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

*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 anti 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 have been shown to produce strong immunity too (Saravanan *et al.*, 2010; Sen *et al.*, 2010). All these information indicate that PPR is a disease that can be well controlled, even eradicated following mass and well planned vaccination campaign as that was done for rinderpest. There is an example of a country which has succeeded in the implementation of this strategy.

iii) *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.

iv) *Vaccines permitting a DIVA strategy (detection of infection in vaccinated animals)*

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

v) *Duration of immunity*

Duration of immunity should be determined for each vaccine strain in animal trials. For the Nigeria 75/1 vaccine, this has been shown to be at least 3 years.

**3. Vaccines based on biotechnology****a) 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; Diallo *et al.*, 2002, Chen *et al.*, 2010). They are not yet validated for field use.

**b) Special requirements for biotechnological vaccines, if any**

None.

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**NB:** There are OIE Reference Laboratories for Peste des petits ruminants (see Table in Part 4 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: <http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/> ). Please contact the OIE Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for Peste des petits ruminants