

Serodiagnosis of *Peste des Petits Ruminants Virus*

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1. Introduction

The Peste des Petits Ruminants (PPR) is one of the epizootic diseases of small ruminants, which is highly infectious and causes high mortality (Kitching, 1988). The clinical outcomes of the disease give it all the hallmarks of an economic and social disaster, especially in the small industries in developing nations. Given the impact on animal health and the economic relevance, PPR is regarded as an Office International des Epizooties (OIE) list A (A050) disease. The OIE and FAO are keen to control and subsequently eradicate PPR from the globe, as has been practiced for Rinderpest (RP). The first step in the eradication of a disease is accurate and reliable diagnosis. It is impossible to ascertain the magnitude and variability of a disease within susceptible populations without efficient diagnosis, which may lead to failure of the eradication program (Banyard *et al.*, 2010). In this chapter, we will review the background of PPRV research focusing on its contribution to basic virology and technological development. We will also highlight some old discoveries and will provide crucial momentum to the development of the current concepts and technologies in the serodiagnosis of this deadly disease.

1.1 An overview of *Peste des Petits Ruminants*

The PPR, a contagious viral infection of both wild and domestic cloven-hoofed small ruminants, is characterized by fever, pneumonia, profuse diarrhoea, and inflammation of the mucous membrane of the respiratory and digestive tracts (Ismail & House, 1990). Although all wild ruminants are susceptible to infection, PPR has only been diagnosed in

Gazellinae (Dorcas gazelle), Caprinae (Nubian ibex and Laristan sheep), Hippotraginae (gemsbok) and *Capra aegagrus blythi* (Sindh Ibex) (Abubakar *et al.*, 2011; Furley *et al.*, 1987).

In contrast to rinderpest (RP), which is one the best-known diseases historically, PPR was identified not long ago. The PPR virus was first detected in 1942 when Gargadennec and Lalanne realized that a RP like disease caused symptoms in sheep and goats but was not transmittable to cattle (Gargadennec & Lalanne, 1942). After demonstration in 1956 that PPRV is antigenically distinct from RP and its isolation in cell culture in 1962, Gibbs and coworkers categorized PPRV as another member of genus *Morbilliviruses* (Gibbs *et al.*, 1979). Initially, susceptible populations of sheep and goats were immunized using already available live attenuated RP vaccine, due to antigenic similarity of PPRV with the RP virus. Later, in 1989, a live attenuated PPRV vaccine was applied for disease control after its first successful attenuation [reviewed in (Sen *et al.*, 2010)]. Currently, comparison of various characteristics between PPR and RP, and rescuing the chimeric viruses for differentiation of infected and vaccinated animals (DIVA) and full genome sequencing of PPRV, have made substantial advances in understanding of the disease.

Phylogenetically, based on the fusion (F) and nucleocapsid (N) genes, PPRV can be classified into four distinct lineages (Figure 1) (Shaila *et al.*, 1996). PPRV belonging to lineages I and II are exclusively isolated from the countries of PPRV origin in West Africa. Lineage III is restricted to Middle East (Yemen, Qatar and Oman) and East Africa, although some of the viruses that belong to lineage III have also been isolated from southern India. Lineage IV is considered a new lineage comprising newly emerging viruses, and is most prevalent in Asian countries (Munir *et al.*, 2011; Shaila *et al.*, 1996). The proper understanding of lineage distribution in a specified region is essential when choosing the appropriate homologous prototype to ensure efficient immunization. The continued application of heterologous vaccine candidates hitherto not prevalent may lead to generation of novel lineages, or allow the existing population to evade protection, especially in RNA viruses. Therefore, identification of the lineage is a pre-requisite for fruitful diagnosis, epidemiology and control.

1.2 Peste des Petits Ruminants virus

PPR virus belong to the family *Paramyxoviridae*, order *Mononegavirales* and is a member of the genus *Morbillivirus* along with the rinderpest, canine distemper and measles viruses (Gibbs *et al.*, 1979). PPR viruses are pleomorphic in shape and are enveloped. The genome is single stranded RNA, and is enclosed in a ribonucleoprotein core together with nucleocapsid protein (Figure 2 A, B). The genome is composed of 15,948 nucleotides, which is the longest of all the *Morbillivirus* members (Bailey *et al.*, 2005). The PPRV genome encodes six genes, each responsible for transcription of a single protein in the order N, phosphoprotein (P), matrix (M), F, hemagglutinin (H) and the large RNA polymerase (L) (Figure 2, C). The P gene encodes for two additional non-structural proteins, C and V. The three viral proteins (M, F and H) are associated with the host-derived envelope. The matrix (M) protein is linked to the nucleocapsid and surface proteins (F and H) (Mahapatra *et al.*, 2006). The L protein acts as RNA dependent RNA polymerase. The association of P protein to N and L is linked to viral cycle control, transcription and translation regulation.

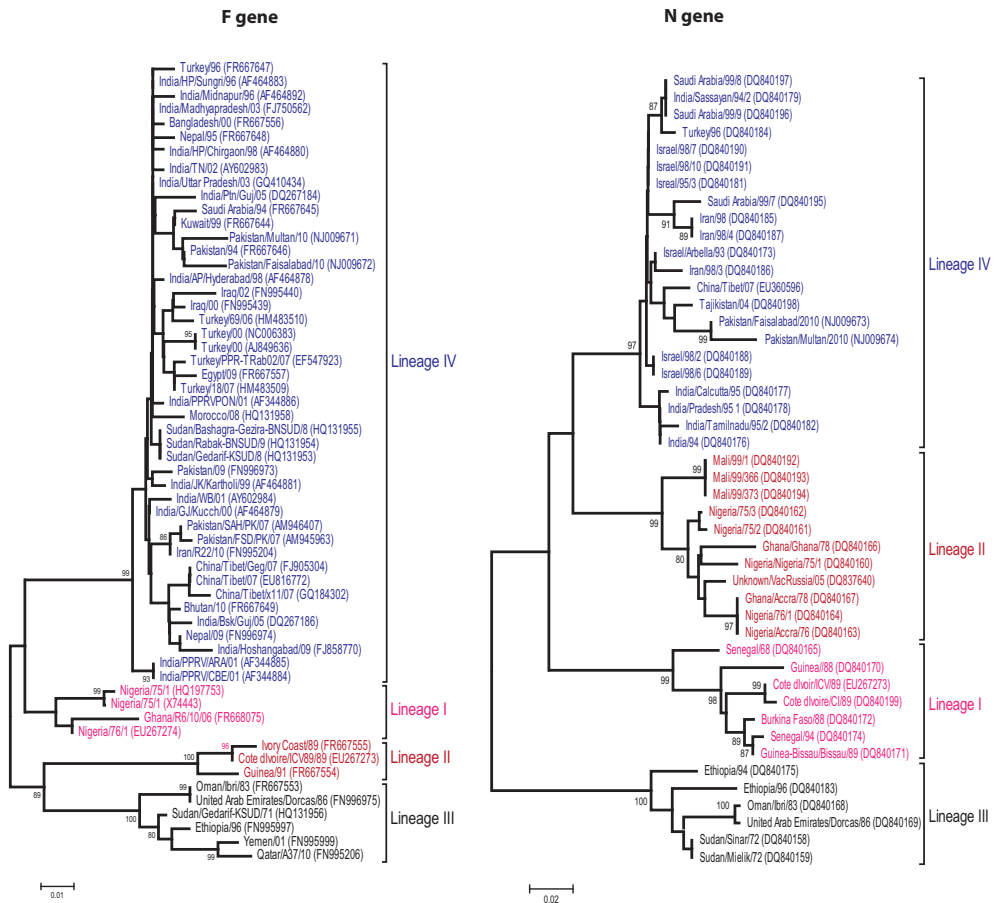


Fig. 1. Phylogenetic analysis of the F gene (left panel) and N gene (right panel) of representative PPRV isolates. Trees were generated by the neighbor-joining method in the MEGA 5.0 program. Numbers above the branches indicate neighbor-joining bootstrap values (values above 80% are shown).

2. Host response to PPRV and potential for serodiagnosis

The protective immune response is usually elicited against the surface F and H proteins of PPRV. However, among the viral proteins most of the neutralizing antibodies are directed against the H protein during PPRV infection (Diallo *et al.*, 2007). In all members of the genus *Morbillivirus* including PPRV, the N protein is the most abundant viral protein due to its presence at the extreme 3'-end of the viral genome. Owing to its high quantity during infection, the N protein is considered the most immunogenic, but the immunity produced against N protein does not protect the animals from the disease. By virtue of the nature of the H and N proteins, these remain the most acceptable targets for the design of PPRV diagnostic tools (Munir, 2011).

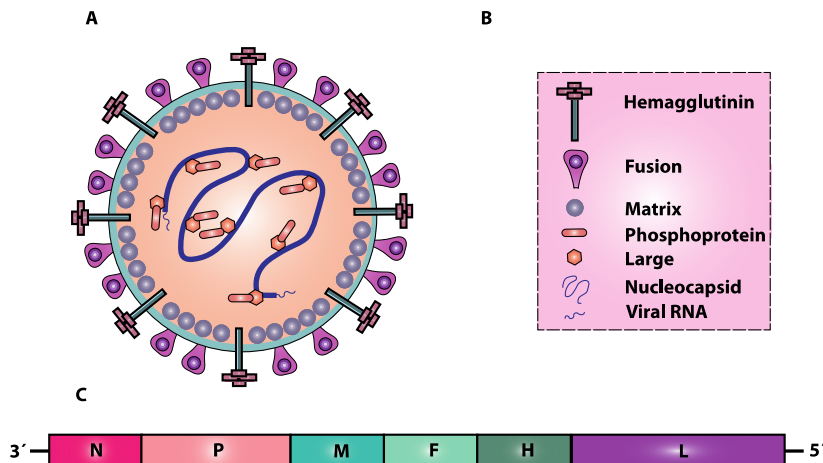


Fig. 2. A schematic illustration of the PPR virus structure. The arrangement of the viral proteins is shown in the structure (A). The names of the viral proteins present in the virus structure are shown in the box (B). The viral genome organization and arrangement of the different viral proteins in the genome are shown (C).

Based on putative amino acid sequences, hypothetically the N protein can be divided into four regions (I-IV) (Figure 3 A). Region I includes amino acids 1-120, region II includes 121-145, region III comprises amino acids 146-398, and region IV finishes with amino acids 421-525. Recently, it has been demonstrated that regions I and II are comparatively more immunogenic than regions III and IV (Choi *et al.*, 2005b). Another study demonstrated that the amino acids from 452-472 are the most immunogenic part within the N protein. It has further been summarized that there is development of earlier immune response to region I and II than region III and IV (Bodjo *et al.*, 2007). Most of the diagnostic assays for PPRV have been developed based on monoclonal antibodies (mAb) raised against the N protein (Libeau *et al.*, 1995).

The H protein of PPRV, on the other hand, is the most diverse among all the members of *Morbilliviruses*. This can be seen from the fact that the two most similar member of the genus share only 50% similarity in their H proteins. The most variable nature of H protein probably reflects the role of this protein is species specificity. If this is the case, H proteins of RP and PPR virus may have significant potential for differentiation of infected from vaccinated animals (DIVA) strategies. Since the H protein determines the cell tropism, most of the protective host immune response is raised against the H protein (Renukaradhya *et al.*, 2002). For this reason, and the preponderance of the neutralizing antibodies again the H protein, it has remained under continuous immunological pressure. The H protein is not only involved in cell-tropism but studies indicate that it may have a role as a neuraminidase. PPRV is unique among *Morbilliviruses*, which carry this function. Mapping of the functional domain, using monoclonal antibodies, has demonstrated that two regions, one at amino acids 263-368 and other at 539-609, are the most immuno-dominant epitopes (Seth & Shaila, 2001) (Figure 3 B). There is an increasing tendency to design DIVA strategies targeting the H protein of PPRV.

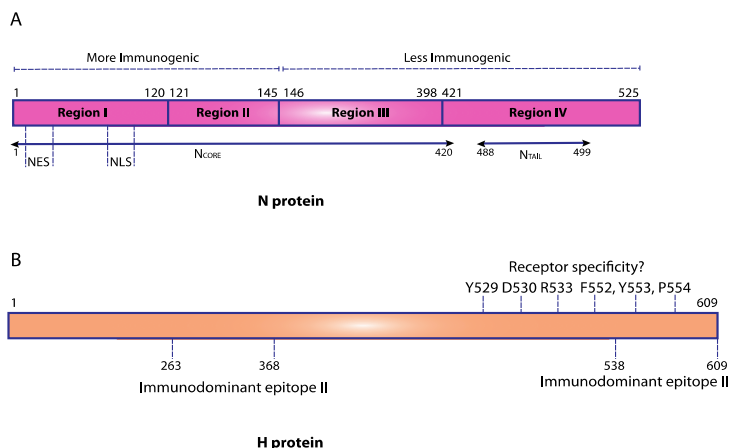


Fig. 3. Schematic illustration of the structural arrangement of the N and H proteins. The N protein is divided into four regions (I, II, III, IV) or two domains (Ncore and Ntail). The first two regions are reported to be more immunogenic than the other two. The nuclear localization and nuclear export signals are located in region I (A). Residues 263-368 and 538-609 in the H protein are considered to be immunodominant epitopes. Several amino acids in and around the second immunodominant epitope are identical to the receptor binding site for SLAM recognized for measles virus (B).

3. Specimen collection, processing and shipment

Efficient documentation and processing is the key to successful laboratory diagnosis of any pathogen. Therefore, most care must be taken to address these elements.

3.1 Documenting sample collection

Before collecting or sending any sample from animals with suspected PPR disease, the appropriate authorities should be contacted. Samples should be sent only under secure conditions and to authorized laboratories, to prevent the spread of the disease.

In the animals that survive the disease, swabs are taken from the conjunctival discharges and the nasal and buccal mucosae, and debris from oral lesions should be collected; a spatula can be rubbed across the gum and inside the lips to collect samples from oral lesions. During the very early phase of the disease, whole blood is also collected in anticoagulant for virus isolation, polymerase chain reaction (PCR) and haematology. At necropsy, lymph nodes, especially the mesenteric and bronchial nodes, lungs, spleen and intestinal mucosae should also be collected aseptically, chilled on ice and transported under refrigeration. Fragments of organs collected for histopathology are placed in 10% formalin.

3.2 Serological samples for antibody detection

At the end of the outbreak, blood can be collected for serological diagnosis. Depending upon the distance from the diagnostic laboratory and the time required for shipment, choose the best source for maintaining cold such as ice, ice pads, ice cooler or dry ice. In either case:

1. Place the glass tubes containing serum samples into mailing canisters, and put these into sealable plastic bags and seal them.
2. Every shipping icebox should contain a plastic liner. Place all the contents, such as dry ice and samples, inside this box liner.
3. Place the sealed plastic bag with canister in the dry ice box.
4. An adequate amount of dry ice is essential. Calculate according to the need as 3-4 kg of dry ice/day of shipment or 8 kg for overnight shipment. Label this amount on the box.
5. Label these boxes with the clinical specimens, not the biological specimen, and include the forms recommended by the concerned lab.
6. Inform the lab about this shipment and instruct to store these samples at -20°C.

3.3 Samples for virus isolation

Even when diagnosis has been carried out by rapid techniques, the virus should always be isolated from field samples in tissue cultures for further confirmation and research studies. Whole blood (in EDTA) should be taken for virus isolation. Samples for virus isolation should be collected during the acute stage of the disease, when clinical signs are present, and these samples should be taken from animals with high fever and before the onset of diarrhea.

3.4 Samples for antigen detection

At necropsy, samples can be collected from lymph nodes (particularly the mesenteric and mediastinal nodes), lungs, spleen, tonsils and affected sections of the intestinal tract (e.g. ileum and large intestine). These samples should be taken from euthanized or freshly dead animals. Samples for virus isolation should be transported chilled on ice. Similar samples should be collected in formalin for histopathology. Whenever possible, paired sera should be taken rather than single samples. However, in countries that are PPR-free, a single serum sample (taken at least a week after the onset of clinical signs) may be diagnostic.

4. Laboratory diagnosis of PPR

Successful implementation of control measures for PPR requires rapid, specific and sensitive methods for diagnosis. Small ruminants infected with PPR are routinely diagnosed on the basis of clinical examination, gross pathology, histological findings and laboratory confirmation (Bruning-Richardson *et al.*, 2011; Atta-ur-Rahman *et al.*, 2004). A number of serological and molecular diagnostic tests are used for the detection of PPR virus.

Conventional techniques used for PPRV detection are: agar gel immunodiffusion (AGID) (Munir *et al.*, 2009b), counter immunoelectrophoresis (CIEP) (Diallo *et al.*, 1995; Obi & Ojeh, 1989), dot enzyme immunoassay (Perl *et al.*, 1995; Obi & Patrick, 1984), differential immunohistochemical staining of tissue sections (Saliki *et al.*, 1994), haemagglutination (HA) and haemagglutination inhibition (HI) tests (Raj *et al.*, 2008; Saravanan *et al.*, 2006; Manoharan, 2005), virus isolation (Manoharan, 2005; Brindha *et al.*, 2001), competitive enzyme-linked immunosorbent assay (c-ELISA) (Ezeibe *et al.*, 2008; Anderson *et al.*, 1991), novel sandwich ELISA (Munir *et al.*, 2009b; Anderson & McKay, 1994), immuno-capture enzyme-linked immunosorbent assay (IC-ELISA) (Abubakar *et al.*, 2008; Saravanan *et al.*, 2008; Khan *et al.*, 2007; Singh *et al.*, 2004; Libeau *et al.*, 1994), immunofiltration (Diop *et al.*, 2005), and latex agglutination tests (Keerti *et al.*, 2009).

Conventional techniques such as AGID cannot be used for routine diagnosis, as these are less sensitive and not reliable (Keerti *et al.*, 2009; Osman *et al.*, 2008). However, HA and HI tests, being simple, cheaper and comparatively sensitive, can be used for routine screening purposes in control programmes (Munir *et al.*, 2009b; Osman *et al.*, 2008). For quick diagnosis and control measures, pen-side tests such as chromatographic strip test (Aslam *et al.*, 2009; Hussain *et al.*, 2003), dot ELISA etc. that can be performed without the need for equipment or technical expertise, are highly desirable (Hussain *et al.*, 2003).

Virus isolation in cell culture can be attempted using several different cell lines. Although Vero (African green monkey) cells have been the choice for isolation and propagation of PPRV, it is reported that B95a, an adherent cell line derived from Epstein-Barr virus-transformed marmoset B-lymphoblastoid cells, is more sensitive and supports better growth of PPRV lineage IV as compared to Vero cells (Bruning-Richardson *et al.*, 2011). Techniques for virus isolation cannot be used as routine diagnostic tests as they are time-consuming and cumbersome (OIE, 2008).

Serological tests include virus neutralization and competitive ELISA assays. Both tests can distinguish peste des petits ruminants from rinderpest; this is not always possible with older serological tests such as complement fixation. ELISAs using monoclonal antibodies have been used for serological diagnosis and antigen detection for diagnostic and screening purposes. For PPR antibody detection, competitive ELISA is a better choice as it is sensitive, specific, reliable, and has a high diagnostic specificity (99.8%) and sensitivity (90.5%) (Sreenivasa *et al.*, 2006; Choi *et al.*, 2005b; Brindha *et al.*, 2001). Immunocapture ELISA is a rapid, sensitive and virus specific test for PPRV antigen detection, and it can differentiate between RP and PPR viruses. Moreover, it is more sensitive than AGID (Abraham & Berhan, 2001).

5. Serodiagnosis of PPRV

5.1 Serum neutralization tests

This test requires the following: 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 TCID₅₀/ml. Dilute the sera at 1/5, then make a twofold dilution in cell culture medium. Mix 100 µl of virus at 1000 TCID₅₀/ml (to give 100 TCID₅₀ 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 TCID₅₀ (100 µl) per well; six wells with 10 TCID₅₀ (100 µl) per well; six wells with 1 TCID₅₀ (100 µl) per well; six wells with 0.1 TCID₅₀ (100 µl) per well; and six wells with 200 µl of virus-free culture (control cells) per well. Make the wells containing the virus controls up to 100 µl with complete culture medium, and incubate the plates for 1 hour at 37°C. Add 50 µl of cell suspension to each well. Incubate the plates at 37°C in the presence of CO₂. Read the plates after 1 and 2 weeks of incubation. The results should be as follows: 100% CPE in virus control wells of 100 and 10 TCID₅₀, 50% CPE for the 1 TCID₅₀ dilution, no CPE for the 0.1 TCID₅₀ dilution, no CPE in wells where the virus had been neutralised by serum during the test, and CPE in wells where the virus had not been neutralised by serum during the test (OIE, 2008).

5.2 Haemagglutination tests

The haemagglutination test (HA) is a straightforward and rapid tool for the serological and confirmatory demonstration of antibodies against PPRV (Munir, 2011). It has been demonstrated that PPRV and MV are unique among morbilliviruses in carrying haemagglutination abilities (Wosu, 1985). The two domains within the H protein of PPRV have been demonstrated to act as immune dominant epitopes. It is now well accepted that the H protein of PPRV is not only responsible for viral attachment to cells and agglutinating erythrocytes but also cleaves sialic acid residues to help budding out the virus. Using this haemagglutinating character of PPRV, HA and HAI tests have successfully been employed for the confirmatory diagnosis of PPRV.

5.3 Haemagglutination inhibition test

This test is widely used for the quantitative measurement of PPRV antibodies usually in a suspension. In this regard, a two fold serial dilution of serum is practiced in a microwell plate. The dilution of antibodies still able to inhibit agglutination is regarded as the titer of the serum sample in the suspension. However, it is also possible to titrate the PPRV antigen using HA and HAI tests (Osman *et al.*, 2008).

5.4 Competitive ELISA

Competitive ELISA (cELISA) is one of the most extensively used tests for serological screening and diagnosis of PPRV infected animals. The detailed procedure is described below and outlined in Figure 4.

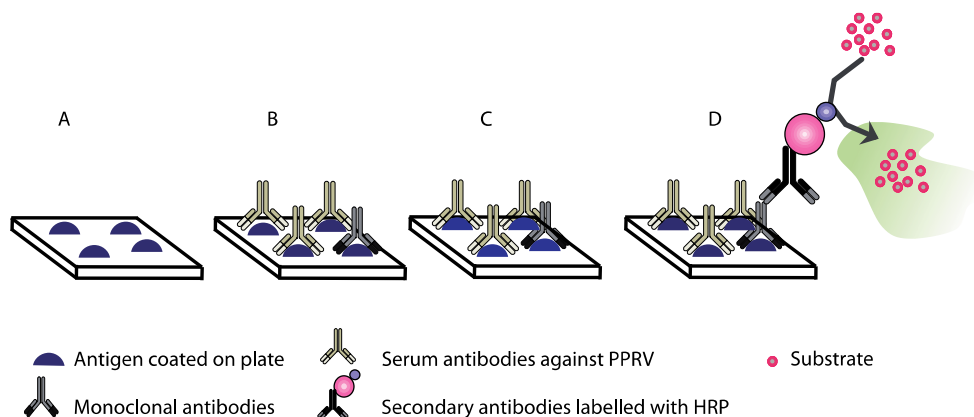


Fig. 4. Principle of competitive ELISA assays. (A) Coating the antigen on a polystyrene plate. (B) Addition of serum sample and monoclonal antibodies raised against the N protein of PPRV. (C) Addition of secondary antibodies labeled with enzyme. (D) Addition of substrate and reading the plate.

A competitive ELISA based on the use of MAb anti-nucleocapsid protein and a recombinant nucleocapsid protein produced in the baculovirus has been described (Libeau *et al.*, 1995; Libeau *et al.*, 1994).

1. Coat microtitre plates (e.g. high adsorption capacity NuncMaxisorb) with 50 μ l of a predetermined dilution of N-PPR protein (produced by a recombinant baculovirus) for 1 hour at 37°C with constant agitation.
2. Wash the plates with washing solution (available in the kit) three times and blot dry.
3. Distribute 45 μ l of blocking buffer (PBS + 0.5% Tween 20 + 0.5 fetal calf serum) to all wells, and then add 5 μ l of test sera to test wells (at a final dilution of 1/20) and 5 μ l of the different control sera (strong positive, weak positive and negative serum) to the control wells.
4. Add 50 μ l of MAb diluted 1/100 in blocking buffer, and incubate at 37°C for 1 hour.
5. Wash the plates three times and blot dry.
6. Add 50 μ l of anti-mouse conjugate diluted 1/1000, and incubate at 37°C for 1 hour.
7. Wash the plates three times.
8. Prepare OPD in hydrogen peroxide solution. Add 50 μ l of substrate/conjugate mixture to each well.
9. Stop the reaction after 10 minutes with 50 μ l of 1 M sulphuric acid.
10. Read on an ELISA reader at 492 nm.
The absorbance is converted to percentage inhibition (PI) using the formula:
$$PI = 100 - \frac{\text{Absorbance of the test wells}}{\text{Absorbance of the MAb control wells}} \times 100$$
11. Sera showing PI greater than 50% are positive.

Another competitive ELISA technique, based on the use of monoclonal anti-haemagglutinin (H), has also been described (Choi *et al.*, 2005a).

5.5 Indirect immunofluorescence

The immunofluorescent antibody technique (IFA) has been practiced for rapid diagnosis of several pathogens including viruses and bacteria (Munir, 2011; Sumption *et al.*, 1998). However, if IFA is to play a reliable part in diagnostic virology then it is necessary to show that the results obtained are confirmed by the established procedures. This technique has been used to detect the PPRV antigen in conjunctival smears.

Principally, IFA is a technique allowing the visualization of a specific protein or antigen in cells or tissue sections by binding a specific antibody chemically conjugated with a fluorescent dye such as fluorescein isothiocyanate (FITC).

There are two major types of immunofluorescence staining methods:

1. Direct immunofluorescence staining in which the primary antibody is labeled with fluorescence dye, and
2. Indirect immunofluorescence staining in which a secondary antibody labeled with fluorochrome is used to recognize a primary antibody. Immunofluorescence staining can be performed on cells fixed on slides and tissue sections. Immunofluorescence stained samples are examined under a fluorescence microscope or confocal microscope. Due to this requirement of microscopy it is hard to apply this technique under field conditions.

5.6 Immunofiltration test

Immunofiltration (IF) assay is based on the surface adsorption of the antigen on the nitrocellulose membrane, and subsequent detection. Mainly, IF has been applied for the

semiquantitative and/or qualitative detection of a wide range of pathogens. Since its development, IF has replaced several of the other immunoassays. The main advantage of the IF principle is its application in field conditions, and it can be taken as pen side test. Recently, IF has been employed for the detection of PPRV and compared with antigen-competition ELISA (AC-ELISA) (Raj *et al.*, 2008). It was proposed that IF is the best in screening larger samples in the field, and AC-ELISA can be used to confirm the important samples (Munir, 2011)

5.7 *In situ* immuno-peroxidase immunohistochemistry

A commercial streptavidin/biotin immunoperoxidase kit (LSAB 2 system, HRP, DacoCytomation, Denmark) can be used for this purpose. Tissue sections are digested with Proteinase K (0.1%) and incubated with a rabbit antirinderpest antibody (Institute for Animal Health, Pirbright, UK) at a dilution of 1/500. An aminoethylcarbazolechromogen substrate system (Labvision Corp., Fremont, CA) is applied for color reaction. Peste des petits ruminants virus positive tissues, previously confirmed with RT-PCR, are used as positive controls. Immunoperoxidase scoring is done on the basis of positively stained cells observed in 3 different areas at 403 microscope objective. The scores should be as follows: 0 (none): absent, 1+ (mild): a few immunopositive cells, 2+ (moderate): focal prominent Immuno-positivity, and 3+ (intense): strong immunopositivity in more than 50% of the cells.

5.8 Agar Gel Immunodiffusion test

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 mesenteric or bronchial lymph nodes, spleen or lung material and ground up as 1/3 suspensions in buffered saline. 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 104 TCID₅₀ (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 (Munir *et al.*, 2009b; Osman *et al.*, 2008).

1. 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).
2. 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.
3. 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.
4. Usually, 1–3 precipitin lines will develop between the serum and antigens within 18–24 hours (10).

5. 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.
6. 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.

5.9 Counter Immunoelectrophoresis

Counter immunoelectrophoresis (CIEP) is the most rapid test for viral antigen detection. However, it has been applied for the serological detection of PPRV with a certain level of satisfaction (Munir *et al.*, 2009a). 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 onto 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 (Munir, 2011).

5.10 Nucleic acid detection of PPRV

There has been a substantial improvement in the detection of nucleic acid of PPRV in recent years. Demonstration of several real-time PCR assays have provided powerful and novel means of not only detection but also quantification of PPRV nucleic acids in several kinds of clinical samples. However, on the other side, these diagnostic tools are not readily available in all diagnostic laboratories especially in developing nations. There is a need to establish reliable, sensitive and affordable diagnostic tools that will be promptly accessible at low cost, independent of laboratory type. Therefore, there has been a strong tendency to increase the number of diagnostic tools based on diverse principles. While doing so, it is extremely important to design these assays in a way that these will be requiring less time, should be readily available, affordable for developing nations, not require high-tech facilities in laboratories, and must not be complex while performing under field conditions.

6. DIVA for PPRV and RP

The currently used live attenuated vaccine, using lineage I African isolate Nigeria 75/1, provides strong protection for at least three years. This vaccine, however, has certain limitations, including requirement for cold-chain maintenance, and inability to differentiate vaccinated from infected animals (DIVA). Development of such a technique would provide a practical and useful means to control the disease, especially in tropical countries. Research in several laboratories is in progress to produce a recombinant marker vaccine carrying

either positive marker (addition of irrelevant B epitope), or negative marker (suppression of an epitope), with primary focus on serologically differentiating PPRV from RP.

7. Potential and need for future advances in serodiagnosis of PPR

Some of the conventional diagnostic serological methods that are currently available are rather time consuming, labour intensive and expensive. As the ultimate goal is to eradicate PPRV, as has been successfully done with RPV, the diagnostic methods that aid this goal have to be reliable, simple and quite cheap. They also need to work reliably during field conditions. As a vaccination strategy will be part of the eradication efforts, some kind of DIVA approach will be necessary. Also, the methods need to work independent of the genetic variants of PPRV that may exist. There is no doubt that the technology development in the field of diagnostic methods will expand fast. We have seen this especially in the nucleic acid detection field. Many methods can detect extremely small quantities of viral nucleic acid, down to one copy per PCR reaction. At this point it is difficult to judge what kind of technology to detect antibodies against PPRV will be the one that will be developed and accepted globally, if any. Probably, several types will be developed, tested and used in parallel, since many academic and corporate laboratories are working on this intensively. Usually, most technologies that are developed are too high tech to be accepted in routine diagnostic laboratories, and it takes some time to replace the already existing ones.

It is likely that we will have a combination of a simple field test device, such as a “dip-stick” that detects antibodies against PPRV, and possibly also some kind of vaccine marker. This can be used in combination with a simple field PCR machine, also with possibilities to differentiate between vaccinated and infected animals. In reference laboratories more advanced serological methods can be used, probably in combination with some kind of nucleic acid detection method.

In the more advanced reference laboratories flow cytometric bead-based technology has been successfully introduced, especially in human medicine. This technology will add new approaches that open up possibilities for simultaneous measurement of multiple molecules in samples. This relatively new technology allows for: (1) evaluation of multiple molecules in a single sample; (2) extremely small sample volumes; (3) high reproducibility; (4) direct comparison with existing assays; and finally (5) a more rapid evaluation of multiple samples in a single platform. For example, one can measure both IgG and IgM to be able to judge recent versus early infection, in combination with some key cytokines to determine the status of the infection, or even if this is a vaccinated animal, or vaccinated and infected in spite of the vaccination. Similarly, other pathogens can be tested simultaneously. However, at present the method needs rather expensive equipment and reagents; but we envision that this will become cheaper in the future.

8. Quality assurance in diagnostic laboratories

The veterinary diagnostic laboratories provide strategic support to field veterinarian, veterinary clinical services and public decision makers in controlling the important animal diseases. However, these activities can benefit veterinary services and public health only if the results produced by the laboratories are reliable, reproducible and rapid enough to be useful.

In many countries, veterinary diagnostic laboratories have not been able to contribute optimally to the community development because of: lack of clearly defined national policies for the laboratory services; shortage of qualified manpower; inappropriate laboratory equipment; poor development of internal quality control methods; and insufficient external quality assessment.

Today, providing funding for quality assurance should not be viewed only as a cost for the laboratories, but as an investment in building a permanent infrastructure and the competitive advantage for the laboratory, not only nationally but also internationally, in order to be able to respond to new animal health threats.

Setting up a quality assurance system in a veterinary diagnostic laboratory includes defining the structure, responsibilities, standard operating procedures and resources necessary to prevent the risks, to correct the errors and improve the efficiency of the laboratory, to ensure the quality of the results. One of the first steps to assure quality in laboratory practice is to conduct an assessment of laboratory systems. The quality assurance system comprises two types of activities: internal quality control that includes appropriate measures taken during day-to-day activities to control all possible variables that can influence the outcome of laboratory results: and external quality assessment to ensure comparability of results among laboratories.

A veterinary diagnostic laboratory working with diagnosis of PPRV should have the necessary staff with appropriate education and experience to carry out all the functions and responsibilities required from the personnel in a safe and accurate manner. This process should include not only management and scientific personnel but also administrative support, maintenance, cleaning and service team. All the staff should have a job description including: functions and responsibilities, academic training required and experience necessary.

The head of the laboratory must play the role of supervisor and motivator in ensuring quality of the results of the laboratory, and strive for continuous quality improvement throughout the organization.

9. Personal preparedness

The fundamental objective of the human resources policy should be to have competent staff with the scientific and/or appropriate technical training to apply appropriate laboratory procedures correctly. Staffing levels should be adequate to enable all the functions expected of the laboratory to be carried out without compromising safety the personal or the integrity of the processes performed in the laboratory.

PPRV is not known to infect humans in either laboratory or field settings. However, all of the personnel working with the potentially PPRV infected materials should wear protective clothing at all times for work in the laboratory; it is prohibited to wear laboratory clothing in any places outside the laboratory. Protective laboratory clothing that has been used in the laboratory must not be stored in the same lockers or cupboards as street clothing. Appropriate gloves must be worn for all procedures that may involve direct or accidental contact with specimens. After use, gloves should be removed aseptically and hands must

then be washed. Personnel must wash their hands before they leave the laboratory working areas. Eating, drinking, smoking and applying cosmetics are prohibited in the laboratory working areas.

The greatest risk of working with PPRV is the escape of the organism into a susceptible animal population. Personnel who are working directly with potentially infected material should therefore strictly follow the measures necessary to minimize the risk of spreading the virus to the susceptible animals. Due to the highly contagious nature of the agent and the severe economic consequences of disease, laboratory workers should have no contact with susceptible hosts for five days after working with the agent.

There are specialized activities within the laboratory that require staff with considerable experience, such as ELISA techniques, cell culture production and virus isolation in cell culture, and RT-PCR and sequencing techniques. The laboratory must develop a continuing education program and regularly arrange training courses to update the skills of both technical and scientific staff according to needs identified by the management. This training is offered as a means of contributing to the success of the quality assurance process. The staff-training program should be documented to allow the correction of errors or weaknesses, and can also be used as a tool for promotion and follow-up of the performance of each staff member based on the job description.

10. Role and function of the laboratory in PPRV control and prevention

Although a tentative diagnosis of PPR can be proffered based on clinical signs, laboratory confirmation is required for differential diagnosis from other diseases with similar signs. Disease severity and the clinical signs depend on various factors: PPRV lineage, species, breed, immune status of animals. So a definitive diagnosis of PPRV infection cannot be based on clinical impressions alone, but must rely on laboratory confirmation. Detection of PPRV specific antibodies in serum is the standard test for the rapid laboratory diagnosis of PPRV. Antibody testing is most commonly performed using commercial enzyme linked immunoassay (ELISA) kits. Systems for the direct detection of PPRV through RT-PCR are becoming more common and, although standard methods are becoming established, no single standard method has yet been developed. Although not recommended for routine laboratory diagnosis, culture of PPR virus from clinical specimens is an important component of disease control strategies.

11. Structure and activities of the laboratory

The veterinary diagnostic laboratory dealing with PPRV should have adequate space to safely perform all activities, store all necessary equipment, and allow for easy cleaning and maintenance. Laboratory manipulation of clinical specimens for PPRV detection and identification, and serologic testing, are all associated with potential risks. These risks must be recognized and appropriate safety measures taken to prevent spread of the disease from the laboratory. Ideally all activities involving handling of infectious and potentially infectious diagnostic materials should take place in a limited access area under biosafety level 2 (BSL-2) conditions, and all activities involving the amplification of viruses, either in vitro or in vivo, should take place under BSL-3 conditions.

Staff working with the live viruses must undergo appropriate training, in order to minimize risk to laboratory staff and spread of the virus outside the laboratory. All laboratory procedures using infectious or potentially infectious materials must be carried out in a fully functioning Biological Safety Cabinet (a ventilated laboratory workspace for safely working with materials contaminated with pathogens requiring a defined biosafety level). Cabinets must be adequately maintained and periodically tested for correct operation. All pipetting should be done using an automatic or manual safety pipetting device; mouth pipetting must not be permitted. Improper handling of the cell cultures must be avoided. Any laboratory accident should be reported to a supervisor *immediately* for the further action to minimize the effect of the accident.

There should be enough rooms to enable separation of infectious from non-infectious activities, to minimize the chances of contamination of clean areas. Lighting and ventilation should correspond to the needs of each working area, according to the specific requirements of the activity carried out. The surfaces of the workbenches should be smooth, easy to clean and made of material resistant to chemicals. Cell culture and media-making facilities should be separated from the laboratory where viral or other microbiological activities are being carried out. If space allows, specific areas and preferably specific rooms should be allocated for:

1. Reagents and consumables storage;
2. Instruments and equipment;
3. Specimen receipt and recording;
4. Specimen processing;
5. Serology;
6. Cell culture;
7. Virus handling;
8. Specialized activities;
9. Documentation and archiving;
10. The administrative area; and
11. Disposal of contaminated wastes.

12. Equipment and instruments

The laboratory should have the necessary equipment and instruments for the accurate performance of all tests to be performed. Before installation of equipment and organization of the laboratories biosafety and other safety standards should be taken into account. New instruments and equipment should be installed and calibrated if possible by the qualified person. All manuals and operating instructions should be stored in an area accessible to all users, and a regular maintenance and calibration schedule must be established. All users must be undergoing an introduction in order to be fully familiar with the operation, maintenance and validation procedures to ensure correct functioning of the equipment. Documentation of all malfunctions, maintenance and validation activities should be recorded. The laboratory should have a list of equipment and instruments that include:

1. The name;
2. Brand;
3. Supplier;
4. Maintenance company;
5. Maintenance schedule;

6. Inventory number;
7. Serial number;
8. Model and year;
9. Location;
10. Date of purchase; and
11. Copy of manufacturer's handbook.

13. Supplies

13.1 Reference materials

These include materials such as reference positive and negative control sera, used for calibration of the test procedures. Reference materials are also used to guarantee uniformity in determining activity. These materials should be purchased from certified suppliers, and purchasing, reception and distribution must be the responsibility of a qualified professional. A central record should be kept with the name of the reference material, supplier, and origin and lot number.

This registry should contain all the information relating to the properties of the reference material. The quality of the reference material should be verified when the conditions have been altered, and routinely once a year.

13.2 Reagents (including diagnostic kits)

These can be defined as materials of chemical or biological origin used in laboratory assays. Because of difficulty of transport to some regions, reagents should be ordered some months ahead of need. The reagents should be of appropriate quality and be obtained from recommended suppliers. A record should be kept of purchasing, reception and distribution to guarantee continuity, particularly with substances that need to be acquired in advance.

Reagents prepared in the laboratory should be prepared in conformity with written procedures and, where applicable, according to OIE/FAO standard recommendations, validated and labeled appropriately, stating the following:

1. Identification of the reagent;
2. Concentration;
3. Preparation and expiry date;
4. Storage conditions;
5. Initials of the technician responsible.

13.3 Laboratory safety

Each laboratory should have available a safety policy document that describes the essential biosafety, chemical, fire and electrical safety requirements to protect staff, the community and the environment. The content of this document should be well implemented in the daily work of the staff, and all the personnel should be familiar with the contents of the safety policy document and should proceed accordingly. All new staff should be made aware of the risks involved in working in a laboratory before starting work in the laboratory. The head of the laboratory is responsible for implementation of and compliance with the provisions of the safety policy document.

The major risk to staff in the laboratory is in handling the clinical samples. The clinical samples should always be considered as a potentially infectious material, and personnel should wear gloves when opening packages containing the clinical samples, aliquoting or transferring samples and when performing assays. Personnel who receive and unpack specimens should be aware of the potential health hazards involved, and should be trained to adopt standard precautions, particularly when dealing with broken or leaking containers. Primary specimen containers should be opened in a biological safety cabinet where possible. Disinfectants should be available in case of spills.

14. Annexes

14.1 Example for PPRV laboratory request form

Sample submission form

Basic Information

Sender name:.....	Name of sender lab:.....
Date of sample collection:.....	Sample ID:.....

Demographic Information

Name, address, location of the owner:.....	
Total animals in a herd:.....	goats are:.....sheep are:.....
Species: Goat:.....	Sheep:.....
Breed:.....	Age:.....
Sex:.....	

Disease Information

Diseased animals are:.....	Dead animals are:.....	Aborted animals are:.....
Health Status: Very good.....Good.....Poor.....Very poor.....		
Previous treatment to any disease:.....		
PPRV vaccination: Yes.....No.....		

Specimen Information

Whole Blood :.....	Oral Mucosa	Lungs.....	Brain.....
Serum	Tonsils.....	Liver	Kidney.....
Swab	Spleen	Intestine.....	

Clinical signs of the disease

Abortion.....
Diarrhea.....
Pneumonia.....
Oral mucosa lesions
Parasitism
Other (please specify)

Management of the herd

Housing.....
Nutrition.....
Feeding.....
Other (please specify)

Signature.....

14.2 Composition of media and reagents

14.2.1 Phosphate Buffered Saline (PBS)

To prepare 1 liter (L) of PBS, the following reagents will be required:

Reagents	Quantity
Sodium chloride NaCl	8g
Potassium chloride KCl	0.2g
Potassium dihydrogen phosphate anhydrous KH_2PO_4	0.2g
Disodium hydrogen phosphate anhydrous Na_2HPO_4 OR	0.92g
Disodium hydrogen phosphate dihydrous $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ OR	1.15g
Disodium hydrogen phosphate dodecahydrous $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$	2.32g
Distilled water to make up to 1 L	QS

1. Weigh all the reagents and add to a 5 L conical flask.
2. Add distilled water to make 1 L and mix well.
3. Adjust the pH to 7.3 to 7.4.
4. Pour into storage bottles.
5. Autoclave at 121°C and 15 pound per square inch (psi) for 15 minutes. Use a slow exhaust.
6. Allow cooling, then tighten the lids and label the bottles.

Similarly, a greater concentration or volume of PBS can be prepared.

14.2.2 Preparation of 1% nobal agar

To prepare 1% nobal agar, the following reagents will be required.

Reagents	Quantity
Nobal agar	1g
Tris-Borate EDTA (TBE) buffer	1L

1. Weigh 1 gram of nobal agar and add to 1 L of Tris-Borate EDTA (TBE) buffer in a 2 L Erlenmeyer flask.
2. Mix the agar until it dissolve and autoclave the mixture for 10 minutes. Continuously stir the contents by swirling to ensure a homogeneous mixture of ingredients after removing from the autoclave. After autoclaving, allow the agar to cool at room temperature (approximately 25°C) and 15 psi for 10 to 15 minutes before dispensing into Petri plates.
3. Dispence the agar into small quantities (daily working volumes) and store in airtight containers at 4°C for several weeks. These working flasks can be melted and dispensed into plates as needed.

NOTE: Agar or agar plates cannot be used if microbial contamination or precipitate are observed.

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