

Recent advances in the laboratory diagnosis of peste des petits ruminants

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Summary

Peste des petits ruminants (PPR) is a highly contagious and economically important, viral disease of small ruminants caused by the peste des petits ruminants virus (PPRV), which belongs to the genus *Morbilivirus* in the family *Paramyxoviridae*. PPR control is achieved mostly through vaccination and/or slaughter of susceptible animals coupled with clinical or laboratory-based diagnosis. Since clinical signs of PPR are not disease-specific and clinical diagnostics is not reliable, it should be confirmed by laboratory testing. Laboratory confirmation of clinical suspicions is made by detection of PPRV in blood, swabs or post-mortem tissues through classical virus isolation (VI), agar gel immunodiffusion (AGID)/agar gel precipitation test (AGPT), counter-immunoelectrophoresis (CIE), immunoperoxidase test (IPT) or enzyme-linked immunosorbent (ELISA) assays. However, these conventional methods have been superseded by more rapid, sensitive and accurate molecular diagnostic techniques based on the amplification of parts of either nucleocapsid (N) or fusion (F) protein gene, such as RT-PCR, real-time RT-PCR, reverse transcription loop-mediated isothermal amplification (RT-LAMP), reverse transcription recombinase polymerase amplification (RT-RPA) and Oxford nanopore MinION technology. Although these molecular diagnostic assays are accurate, rapid and sensitive, they have to be performed in laboratory settings, and samples must be transported under appropriate conditions from the field to the laboratory, which can delay the confirmation of PPRV infection. The recently developed immunochromatographic lateral flow device (IC-LFD) assay can be used in the field ("pen-side") without the need for expensive equipment, so a well-established laboratory is not required. The control and eventual eradication of PPR is now one of the top priorities for the Food and Agriculture Organization (FAO) and the World Organization for Animal Health (OIE). In 2015, the international community agreed on a global strategy for PPR eradication, setting 2030 as a target date for elimination of the disease

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Peste des petits ruminants (PPR) is a highly contagious and economically important viral disease of small ruminants that affects primarily goats and sheep, but can also infect and cause disease in wild species, such as springbuck, gazelles and impala (35). The impact of PPR on the productivity of small ruminants includes loss of meat, milk, fibers and hides, weight loss, impaired growth and abortion. With morbidity and mortality rates as high as 90%, PPR has a devastating impact on the livelihood of poor farmers in endemic countries. The causative agent, peste des petits ruminants virus (PPRV), is a representative member of the genus *Morbilivirus* of the family *Paramyxoviridae* (12). PPRV contains a single-stranded negative-sense RNA genome of about 16 kb, organised into six transcription units encoding six structural proteins, nucleo-

protein (N), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin (H) and the large polymerase proteins (L), in the order 3'-N-P-M-F-H-L-5'. Among these, nucleocapsid (N) protein gene is the most abundantly transcribed gene in the host cells, and it is therefore the preferred target site for genomic detection of PPRV. In addition, two non-structural proteins C and V are translated from the P-gene open reading frame (ORF) (35). PPRV exists serologically as a single serotype, but the molecular epidemiology of PPRV, based on the sequence comparison of a small region of either the N or the F gene, has revealed the existence of four distinct lineages (I-IV) of the virus (29). Historically, the four lineages follow a geographic distribution, where lineages I and II are found in western and central Africa, lineage II in eastern Africa and the southern part of the

Middle East, and lineage IV is distributed in the Middle East and southern Asia (2). The classification of PPRV into lineages has broadened the understanding of the molecular epidemiology and worldwide movement of PPR viruses.

The first reports of the presence of a disease bearing a strong resemblance to rinderpest (RP) that caused epidemics in small ruminants appeared in 1871 and 1927 in Senegal and French Guinea (17). The disease was certainly widespread in West Africa by the late 19th and early 20th centuries, long before its first description by French veterinarians in early 1942 in Ivory Coast (25). In the following years, the disease extended its distribution in other parts of the world, in Africa, Middle East and Asia (8), and is still spreading globally, with emergence notably reported in China, Mongolia, the Indian subcontinent, Pakistan, Afghanistan, Iran, Iraq, Saudi Arabia, Turkey (3, https://www.oie.int/wahis_2/public/wahid.php/Wahidhome/Home), and most recently within the European Union in Bulgaria, where a PPR outbreak was reported on 24th June 2018 in the village of Voden, Bolyarovo municipality of the Yambol region, on the border with the Thrace region of Turkey (https://ec.europa.eu/food/sites/food/files/animals/docs/reg-com_ahw_20181211_pdpf_bul.pdf).

PPR control is achieved mostly through vaccination and/or slaughter of susceptible animals, coupled with clinical and laboratory-based diagnosis. Rapid and sensitive detection of PPRV in the field is crucial for effective disease control. PPR is tentatively diagnosed on the basis of clinical observation, characteristic symptoms, epidemiology, post-mortem lesions and laboratory confirmation by various recognition techniques. Common clinical signs of PPR in sheep and goats are pyrexia, oculo-nasal discharges, erosive stomatitis, anorexia, diarrhea, bronchopneumonia, gastro enteritis and dehydration, followed by either recovery or death (2). Further, secondary infections result in a change in the clinical picture of the original disease. However, confirmatory diagnosis of PPR under field conditions is not always possible due to the prevalence of PPR-

like diseases and the frequent occurrence of PPR as a mixed infection in small ruminants. This complicates differential diagnosis based on clinical signs typical of PPR because other diseases producing PPR-like signs in small ruminants, such as bluetongue (BT), contagious caprine pleuropneumonia (CCPP), contagious ecthyma (Orf), capripox and foot-and-mouth disease (FMD), can cause problems in clinical diagnosis (35).

Since clinical signs of PPR are not disease-specific and clinical diagnostics is not reliable, it should be confirmed by laboratory testing. The characteristics of an ideal diagnostic test include accuracy, wherever used, heat-stable reagents with an extended shelf life, portability, minimal technical skills for operation, rapid, sensitive, and specific results, minimal batch sizes, cost effective tests and suitability for a broad range of clinical samples. Most of PPR diagnostic tests meet these standards. A classification of diagnostic methods for PPR based on the target component of detection is presented in Fig. 1. Laboratory confirmation of clinical suspicion is obtained by detection of PPRV in blood, swabs (nasal/ocular/oral) or post mortem tissues through classical virus isolation (VI). PPRV can be isolated by this assay using primary goat/sheep cells, African green monkey kidney cells (Vero cells) or marmoset B-lymphoblastoid (B95a) cell lines (40). Despite the high sensitivity of primary cell cultures for VI, they are not commonly used, because of the risks of contamination, batch-to-batch variation and animal ethic issues. Although B95a cells are more sensitive to PPRV infection and support virus replication to a high titre, the Vero cells line is commonly used for virus isolation from clinical samples and for infectivity assays. Normally, during VI in Vero cells, several sequential blind passages are required before an appreciable cytopathic effect (CPE) can be observed. The virus manifests specific CPE after 3-5 days of infection, which include initial rounding of the infected cells in grape-bunch-like clusters, followed by vacuolation, granulation of the cell cytoplasm, fusion of the monolayer cells and formation of syncytia,

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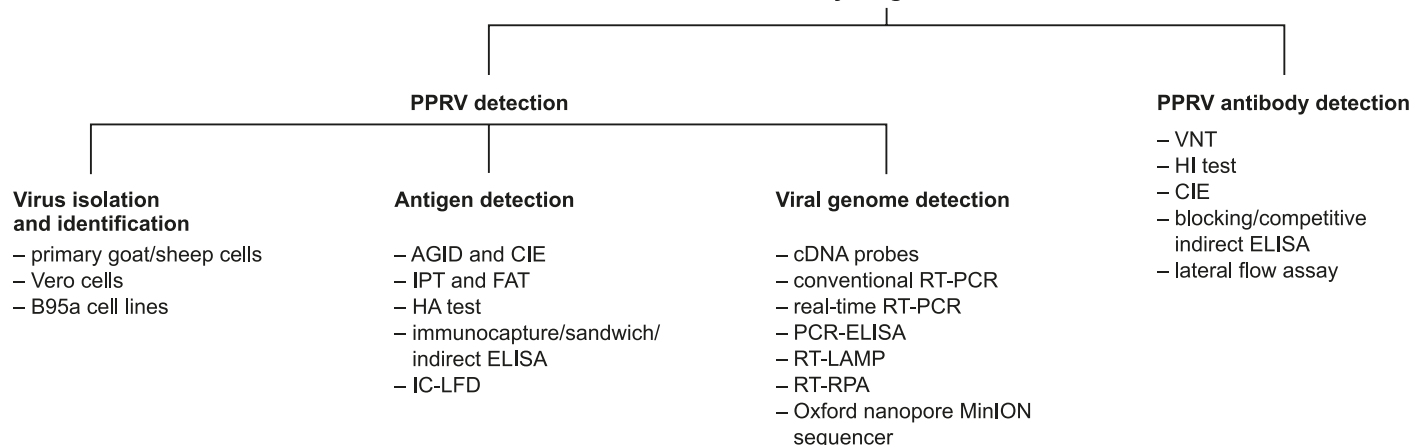


Fig. 1. Laboratory methods available for the diagnosis of PPR

which are characteristics of PPRV. However, the VI technique is time consuming, labour intensive, requires cell culture facilities and is relatively cumbersome to perform as a routine technique. Moreover, the preservation of samples collected under field conditions is not always adequate for successful laboratory results. Apart from VI, the conventional immunological methods, such as agar gel immunodiffusion (AGID)/agar gel precipitation test (AGPT), counter immunoelectrophoresis (CIE), haemagglutination test (HA) and immunocapture enzyme linked immunosorbent assay (ELISA), can be applied for PPRV detection. In addition, immunochemistry techniques were used to detect PPRV antigen in tissues. The monoclonal antibody (MAb)-based fluorescent antibody test (FAT) was developed to detect PPRV antigen from conjunctival smears (41). Although FAT is simple and quick to perform, it requires a fluorescent microscope and technical expertise. Therefore, a similar assay, the immunoperoxidase test (IPT), which requires only a light microscope and is available even to resource-poor laboratories, has been developed (1). Besides, the haemagglutination (HA) test using human blood group "O" and chicken red blood cells (RBCs) has been applied for detection of PPRV antigen from clinical specimens (42). These tests, even though they are not sensitive enough to detect early stages of disease, can be used to confirm CPE during PPRV infectivity titration, similar to the cell-ELISA techniques. There are different formats of ELISA for PPRV antigen detection based on sensitivity and specificity requirements. An immunocapture ELISA using MAbs raised against the nucleoprotein of PPRV is available as a commercial kit for antigen detection (28). Later, a sandwich ELISA was developed using polyclonal sera for antigen capture and MAb (4G6) raised against the N protein of PPRV as a detection antibody (38). In addition, an indirect ELISA, namely, cell-ELISA, for the detection of antigen in infected cells was developed using the anti-nucleoprotein MAb 4G6 and it can be used as a vaccine quality control tool, as both infectivity assays and specific detection of PPRV can be performed simultaneously using this assay (36). However, most of the conventional PPR diagnostic assays are time-consuming, labour-intensive, less sensitive, not rapid, and therefore, not suitable for primary diagnosis, but useful in secondary confirmatory testing and retrospective epidemiological studies. To overcome the drawbacks of these tests, the recent molecular biology assays have been used for rapid and sensitive detection of PPRV genome from clinical samples.

Over the past 30 years, many molecular diagnostic techniques for specific diagnosis of PPRV have been developed (13, 15, 18, 27, 31). Nucleic acid hybridization is suitable for making diagnosis based on field materials that are either collected from putrefied carcasses or become putrefied during the transit period (18).

Radioisotope (^{32}P)-based cDNA probes derived from the N gene of RP and PPR viruses were developed for differentiation of these viral infections without virus isolation, but despite high sensitivity, radiolabelled probes were not widely used, because of the short half-life of ^{32}P and the need for fresh specimens and an isotope handling facility. This led to the development of non-radioactive probes using biotinylated DNA or digoxigenin- (DIG) labelled oligonucleotides (19, 34). Subsequently, RT-PCR techniques based on amplification of parts of either PPRV nucleocapsid (N) or fusion (F) protein gene were developed to detect viral RNA in clinical specimens (4, 15). Moreover, the RT-PCR targeting M gene and the H gene of PPRV were described to detect and differentiate between RP and PPR (20). This was an improvement over the earlier PCR assays for the simultaneous detection and differentiation of PPRV. A two-step RT-PCR has been shown to be useful for the rapid detection of PPRV RNA in samples submitted for laboratory diagnosis (22). However, conventional RT-PCR methods (laborious and expensive) may not necessarily be applicable for routine clinical diagnosis in resource-poor laboratories when the sample size is very large. Furthermore, these techniques are sensitive to cross-contamination that often results from previous collection, processing and testing. The diagnostic sensitivity of these assays can also be affected by unforeseen variation in the primer binding site, RNA degradation and PCR inhibitors in the sample, which results in a false negative. As an improvement over conventional PCR assays, the real-time RT-PCR (rRT-PCR) techniques targeting either the N or the M gene using a *TaqMan* hydrolysis probe and SYBR Green with melting curve analysis have been used for rapid, highly sensitive and specific detection and quantification of PPRV (5, 6, 13, 24). Earlier, an N gene-based one-step *TaqMan* rRT-PCR was developed for detection of the PPRV genome in clinical samples with high sensitivity (9). The rRT-PCR assays offer certain advantages over conventional RT-PCR. They do not use agarose gel electrophoresis, thus decreasing the risk of contamination, and are suitable for large-scale testing and automation (13, 24). In addition, the use of a thermal cycler with 96-well plate formats further increases the capacity and speed of the analysis.

PCR-ELISA uses a combination of nucleic acid amplification and ELISA techniques for amplification and detection, respectively. In this assay, digoxigenin-labelled primers are used for amplification of viral RNA. Subsequently, biotinylated N-gene-specific probes are hybridized to these PCR amplicons and detected by ELISA (37). This PCR-ELISA is prone to the cross-contamination of samples, like PCR assays, and is time-consuming, but it would be worth using with samples that yield doubtful results in sandwich ELISA or other laboratory tests. The high sensitivity of this assay might be useful during the later phases of

PPR eradication, since even a single case of PPR needs confirmation by multiple tests. RT-PCR and simple and aqueous phase ELISA (SNAP-ELISA) techniques for differentiation of RPV from PPRV were developed by targeting the F gene of PPRV (21). In line with PCR-ELISA, this test also uses ELISA for detection of PCR amplicons, so it is easy to perform on a large scale. Another molecular technique for detection of PPRV in clinical samples is the reverse transcription loop-mediated isothermal amplification (RT-LAMP). An RT-LAMP assay based on the M-protein gene (26) and another based on the N-protein gene (31) have been developed. This sensitive, inexpensive and simple method can be more readily used in developing countries that do not have access to high technology laboratories. However, in each RT-LAMP assay, primers must be specifically designed to be compatible with the target nucleic acid sequences. In addition, the RT-LAMP assay requires six primers and has unsatisfactory reliability in detection of highly variable viruses. Nevertheless, this assay provides a solution for pen-side, rapid and inexpensive PPR diagnostic testing in the field in nascent PPR eradication programmes. Recently, a reverse transcription recombinase polymerase amplification (RT-RPA) assay has been developed for rapid detection of PPRV from clinical samples (27). This technique is highly specific for PPRV, as there is no cross reaction with FMD, BT and Orf virus, which may cause clinical signs similar to PPR in small ruminants. Thus it shows potential as a novel testing tool for differential diagnosis (43). The RT-RPA assay is more rapid than RT-LAMP, does not require expensive equipment, and the results can be read with the naked eye in less than 25 min. Sensitivity analysis revealed that the conventional RT-RPA assay could detect 852 copies of standard PPRV RNA per reaction at 95% probability within 20 min. at 41°C, whereas the real-time RT-RPA (rRT-RPA) assay could detect 103 copies of viral RNA molecules per reaction at 95% probability. Specificity analysis showed that neither of these assays had cross-reactivity with nucleic acid templates prepared from other viruses or common pathogens. The clinical evaluation of both RT-RPA assays showed that their performance was comparable to that of rRT-PCR (44). The recently developed, innovative Oxford nanopore MinION technology is capable of rapid comprehensive detection, diagnosis and bio-surveillance of emerging infectious diseases (32). This technology is a non-PCR-based tool for detection of viral RNA from clinical samples with the Oxford nanopore MinION sequencer. This diagnostic platform may provide the capacity for genomic surveillance of PPRV as well as other infectious diseases in resource-limited settings in real time.

Advances in the application of nanotechnology for disease diagnosis have paved the way for the development of immunochromatographic tests for

rapid diagnosis of PPR. These assays are simple, quick, cost effective and easy to perform. They give results within a matter of minutes and can be used as a “pen-side” diagnostic test. This test detects antigen-antibody interactions on a nitrocellulose membrane (NCM) platform by trapping the unknown antigen/antibody with a known antibody/antigen pre-spotted on the NCM. The immunochromatographic lateral flow device (IC-LFD) assay, based on the specificity and affinity of anti-H-protein MAb C77 both as a capture antibody on the test line and as a detection antibody, has been developed for PPRV antigen detection (11, 14). The performance of this diagnostic assay was evaluated in the laboratory and under field conditions on superficial samples (ocular or nasal swabs). The test showed a sensitivity and specificity of 84% and 95%, respectively, and detected as little as 10^3 TCID₅₀ of cell culture-grown PPRV. The test could detect PPRV in swabs from animals as early as 4 days post-infection at the time when clinical signs were minimal. It could therefore significantly reduce the negative impact of PPR. Furthermore, this technique can be used in the field without the need for expensive equipment, so it does not require a well-established laboratory. Without these tests, transportation of samples to diagnostic laboratories and diagnosis can take a few days, by which time the virus may already spread throughout the surrounding area. Although these “pen-side” tests are less sensitive than ELISA methods for PPRV antigen detection and nucleic-acid-based techniques, their direct application in herds in the field effectively offsets this disadvantage (11).

The virus neutralization test (VNT) is considered the gold standard for PPRV antibody detection, and it is an OIE-accepted diagnostic tool for international trade. This assay is the oldest reliable test for detection of morbillivirus antibodies. It is sensitive and specific, but expensive and time-consuming. Serum against either PPRV or RPV may neutralize both viruses, but it will neutralize the heterologous virus at a lower titer compared to the homologous virus. It was found that VNT offered a more rapid and higher throughput assessment of PPRV neutralization antibody titer for the recombinant virus compared with the traditional method (23). Due to the need for cell culture facilities and sterile serum, VNT is difficult to perform for routine serological surveillance or monitoring, especially where a large number of samples need to be screened. However, VNT is sometimes a good choice, e.g., when screening animals for experimental challenge studies, as the presence of a low antibody titre in the serum (less than 1 : 16) may not always be reliably detected by ELISA (39). A haemagglutination inhibition (HI) test can also be used to quantify virus-neutralizing antibodies as an alternative to VNT. HI is cheaper and simple, yet reliable. However, it is necessary to standardize the commonly available PPRV antigen.

Counter immunoelectrophoresis (CIE) can also be used for sero-epidemiological and experimental surveys to diagnose PPR. It was confirmed that its rapidity, simplicity and sensitivity made it a suitable technique in serological studies of PPR (35). The agar gel immunodiffusion (AGID) test can also be useful for diagnosis of PPR in the field. This method is based on the passive diffusion of soluble antigens and/or antibodies towards each other leading to their precipitation in a gel matrix (33). The most reliable and rapid test for PPRV antibody detection is a competition ELISA (cELISA) based on a monoclonal antibody directed against virus nucleoproteins N and H (30, 39). The cELISA based on an anti-N assay relies on the competition between the anti-PPR monoclonal antibody, which is directed against the haemagglutinin protein of PPRV, and the antibody in the serum sample. The sensitivity of cELISA is 99.4%, and its specificity amounts to 94.5% (30). The presence of antibodies to PPRV in the serum blocks the reactivity of MABs, which causes reduction in the expected colour following the addition of conjugate and chromogen solution. Similarly, other researchers developed cELISA based on an anti-H protein MAB using partially purified PPRV (39). The diagnostic sensitivity (92.4%) and diagnostic specificity (98.4%) of this test were comparable to those for VNT. These ELISAs are, in general, simple and convenient and are both time- and cost-effective when large-scale sample screening is needed, which makes them useful for the routine diagnosis of PPR. Both anti-N and anti-H protein MABs-based competitive ELISAs are available as commercial kits for detection of antibodies against PPRV (35). An indirect ELISA is a valuable alternative to competitive ELISA. It was developed for PPR antibody detection (7) with a relative diagnostic specificity of 95.09% and diagnostic sensitivity of 90.81% when compared to cELISA (39). Although a species-specific conjugated secondary antibody is required, this indirect ELISA could be used if the MAB clone used in competitive ELISA is lost due to some unavoidable situation or in laboratories where cELISA is not available. Recently, a fast and ultrasensitive quantum dots lateral flow immunoassay strip has been developed to detect anti-PPRV antibodies. In this assay, the N protein of PPRV is immobilised in the detection zone of the test strip, and luminescent water-soluble carboxyl-functionalised quantum dots are used as signal output and conjugated to streptococcal protein G. The performance of the test is extraordinary compared to that of cELISA and the IC-LFD assay (16). It is rapid, sensitive and suitable for on-site diagnosis and post-vaccination evaluation of PPRV. This test cannot be used for early detection of active infection where only IgM and viral particles are present in circulation. Alternatively, nucleic acid-based tests or PPRV antigen detection methods could be used during disease outbreaks. The recent develop-

ment of a helper cell-dependent recombinant PPRV has also yielded a promising, yet biologically safe, source of viral antigen for future diagnostics, since this system produces replication-incompetent virus (10). A helper cell-dependent form of PPRV has been created by removing the entire RNA polymerase gene and complementing it with polymerase made constitutively in a cell line. The resultant L-deleted virus grows efficiently in the L-expressing cell line, but not in other cells. Virus made with this system is indistinguishable from normal virus when used in diagnostic assays and can be grown in normal facilities without the need for high-level biocontainment. The L-deleted virus will thus make a positive contribution to the control and study of PPR (10).

In conclusion, it can be stated that if the global PPR eradication strategy recommends combining PPR control with control of other diseases of small ruminants, a cost-effective, multi-disease diagnostic test would be very useful for simultaneous surveillance of all target diseases. The availability and distribution of sensitive and specific field-deployable diagnostic tools in developing countries will improve the diagnostic capacity and early containment of PPR. Field-deployable and laboratory-based diagnostic tools have inherent strengths and weakness, so an optimal combination of both is essential for rapid and accurate diagnosis of PPR. Rapid tests, such as IC-FLD, can be useful for the field-level diagnosis of PPR, whereas molecular diagnostic techniques, such as RT-PCR, RT-LAMP and RT-RPA, as well as the combination of an immunological test and a molecular assay (PCR-ELISA), are especially recommended for laboratory diagnosis. For detection of PPR during early phases of the disease and for clinical samples that gave equivocal results in other tests and require re-confirmation, highly sensitive nucleic-acid-based diagnostic tests could be used. A good number of these tests are available commercially, including rRT-PCR, RT-LAMP and the Oxford nanopore MinION sequencer.

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