Diagnosis and Control Strategies for Peste Des Petits Ruminants Virus: Global and Pakistan Perspectives

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ABSTRACT
Peste des petits ruminants virus (PPRV) causes an acute and highly contagious disease and belongs to the family Paramyxoviridae and genus Morbillivirus. The control of animal diseases is one of the primary objectives of government livestock departments in Pakistan. Control of any viral disease requires rapid, specific and sensitive diagnostic tests and efficacious vaccines. Small ruminants infected with PPRV are routinely diagnosed on the basis of clinical examination, gross pathology, histological findings and laboratory confirmation. A number of serological and molecular diagnostic tests are used for the detection of PPRV. Control of PPRV may be attained using measures including movement control of sheep and goats from affected areas, quarantine of infected animals, removal of potentially infected fomites and a restriction on the importation of sheep and goats from infected areas. The effective way to control PPR in Pakistan is by mass immunization of small ruminants as strict sanitary control measures, including the stamping out policy are not economically viable. Therefore, the control of PPR requires an effective vaccine and for this purpose several vaccines such as homologous and recombinant vaccines have been developed.

INTRODUCTION
Worldwide, the livestock sector accounts for 40% of the agricultural gross domestic product (GDP) (Steinfeld et al., 2006). Within Pakistan, the livestock sector plays a vital role in the economy as it contributes more than 50% to the value addition in the agriculture sector and almost 11% to the Pakistani GDP. In the rural community of Pakistan, about 30-35 million people raise livestock such as sheep and goats as a supplementary occupation, with 5-6 sheep/goats per family. In Pakistan, the total population of sheep and goats is approximately 25.5 and 61.9 million, respectively. Collectively, they produce 31 million tons of milk, 782.1 thousand tons mutton, 45.2 thousand tons of wool, 21.5 thousand tons of hair and 51.2 million skins per year (Anonymous, 2005–2006).

Control of animal diseases is one of the primary objectives of government livestock departments in Pakistan. Priority pathogens for intervention measures in Pakistan include several transboundary animal diseases (TADs) including rinderpest, foot and mouth disease (FMD), Peste des Petits Ruminants (PPR) and avian influenza (AI). It is estimated that PPR causes annual losses of more than US$ 342.15 million through high levels of morbidity and mortality and the resulting depletion of genetic stock (Hussain et al., 2008).

PPR is an acute and highly contagious viral disease of mainly domestic small ruminants such as goats and sheep but the virus can also affect wild animals. The morbidity and mortality rates can be as high as 100 and 90%, respectively (LeFebre and Diallo, 1990), and in a severe outbreak, mortality can reach to 100% (Radostitis et al., 2000) although these rates can vary enormously with mortality sometimes being as low as 20% (Roeder and Obi, 1999). Due to the high mortality, PPRV was classified as an A-list viral pathogen by the OIE (Office International des Epizooties) but according to the new classification it is included as an OIE ‘notifiable’ disease. PPRV has been reported to be endemic in various parts of Asia and Africa (Roeder and Obi, 1999; Dhar et al., 2002;
The etiological agent, PPRV virus belongs to the Order Mononegavirales, family Paramyxoviridae, genus Morbillivirus (Gibbs et al., 1979). Virions are pleomorphic in nature, varying between 130-390 nm in diameter. The virus envelope is 8-15 nm thick with glycoprotein spikes of 8.5-14.5 nm length being present throughout the membrane. PPRV has a non-segmented, single-strand negative sense RNA genome of 15,948 nucleotides that encodes eight proteins including six structural proteins namely: the nucleoprotein (N), the phosphoprotein (P), the matrix protein (M), the fusion protein (F), the haemagglutinin protein (H) and the large polymerase protein (L), and two nonstructural proteins V and C; in the order 3’-N-P(C/V)-M-F-H-L-5’ (Bailey et al., 2005). The N protein totally encapsidates the viral RNA and encapsidated genomes can be observed under the electron microscope as ‘herringbone-like’ structures of approximately 14-23 nm in length (Durojaiye et al., 1985; Barrett et al., 1993). These structures containing the genomic RNA, completely encapsidated in N protein, and in association with the P and L proteins, form the minimal replicative unit of these viruses, the ribonucleocapsid protein (RNP) complex. The P protein acts as a molecular intermediate that is proposed to bridge components of the RNP during both the replicase and transcriptase activities of the viral life cycle. Three viral proteins: M, F and H are associated with the viral envelope which is derived from the host cell membrane. The M protein is located inside the envelope and serves as a link between the RNP and the two external viral proteins, F and H, and is believed to be important for virus particle assembly. The virus binds to the host cell receptor through H during the first step of the infection process. Following attachment, F mediates the fusion of the viral envelope with the host cell membrane, introducing the viral RNP complex into the cell cytoplasm. F and in particular, H are considered to be very important for inducing protective host immune response against the virus (Barrett et al., 2005).

The acute form of PPR is characterized by pyrexia (up to 41°C), anorexia, necrosis and ulceration of mucous membranes, sores in the mouth, mucopurulent nasal and ocular discharge, necrotizing and erosive stomatitis, pneumonia and inflammation of gastrointestinal tract, leading to severe diarrhea (Lefevre and Diallo, 1990; Roeder and Obi, 1999). The sub-acute form of the disease is seen as a mild form, characterized by moderate hyperthermia, 39-40°C over a 1 to 2 day period; diarrhea is slight and may last for 2-3 days and mucosal discharges are less abundant. There is no mortality in the sub-acute form of PPR and animals go on to convalesce (Diallo, 2006). The main routes of PPRV transmission are via oral and respiratory secretions and transmission generally occurs following close contact between infected and susceptible animals (Lefevre and Diallo, 1990). It has been estimated that PPRV has half life of 2.2 minutes at 56 C and 3.3 hours at 37 C (Rossiter and Taylor, 1994). It has a long survival time in chilled and frozen tissues and is stable at pH 4-10 (Anonymous, 2002).

PPRV is lymphotropic and epitheliotropic and produces severe lesions in organ systems rich in lymphoid and epithelial tissues. After entry through the respiratory route, PPRV multiplies in the pharyngeal and mandibular lymph nodes and tonsils. Two to three days post-infection and one to two days before the appearance of first clinical signs, viremia may develop, which then results in dissemination of the virus to the spleen, bone marrow and mucosa of the gastro-intestinal tract and the respiratory system (Scott, 1981). Acute disease is usually accompanied by lymphopenia and immuno-suppression, leading to secondary opportunistic infections (Appel and Summers, 1995; Murphy and Parks, 1999).

Studies have shown that the apoptosis of infected cells seems to play an important role in the pathogenesis of PPRV in goats and sheep. In vitro infection of goat peripheral blood mononuclear cells with PPRV causes nuclear condensation and fragmentation, DNA fragmentation, blebbing of the plasma membrane and an increase in apoptotic index; supporting the fact that PPRV induces peripheral blood mononuclear cells to undergo apoptosis (Mondal et al., 2001; Guvenc and Gurcan, 2009).

PPRV is sometimes reported as being more severe in goats than in sheep. However, there have been reports of both an increased susceptibility of sheep populations, goat populations and outbreaks that appear to have affected sheep and goat populations equally (Taylor and Abegunde, 1979; Roeder et al., 1994; Taylor et al., 2002; Singh et al., 2004; Yesilbag et al., 2005; Chauhan et al., 2009; Wang et al., 2009). PPRV is known to infect cattle asymptomatically (Diallo et al., 1989; Anderson and McKay, 1994) and can also affect camels (Roger et al., 2001).

ADVANCES IN DIAGNOSIS

Effective implementation of control measures for PPR require rapid, specific and sensitive methods for diagnosis. Small ruminants infected with PPRV are routinely diagnosed on the basis of clinical examination, gross pathology, histological findings, and laboratory confirmation (Diallo et al., 1995). A number of serological and molecular diagnostic tests are currently used for the detection of PPRV.

Conventional Methods

Conventional techniques such as the Agar Gel Immuno Diffusion (AGID) test are not routinely used for standard diagnosis as they lack sensitivity when compared to other assays (Nussieba et al., 2008; Aslam et al., 2009). However, Haemagglutination tests (HA) and Haemagglutination Inhibition tests (HI) tests can be used for routine screening purposes in control programmes as they display comparative sensitivity alongside being simple to perform and cheap to produce (Manoharan et al., 2005).
Virus isolation in cell culture can be attempted with several different cell lines where samples permit. Although Vero 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 support better growth of PPRV lineage IV as compared to Vero cells (Sreenivasa et al., 2006). More recently, Vero cells expressing the SLAM receptor have been used as an effective alternative for isolation in cell culture (Ono et al., 2001; Seki et al., 2003). The fragility of morbillivirus virions generally renders techniques such as virus isolation redundant for routine diagnostic use, especially where sample quality is poor. Such techniques are also considered to be time-consuming and cumbersome (Brindha et al., 2001). Virus isolation does, however, play an important role from a research perspective.

ELISA tests using monoclonal antibodies are often used for serological diagnosis and antigen detection for diagnostic and screening purposes. For PPR antibody detection, the competitive ELISA is the most suitable choice as it is sensitive, specific, reliable, and has a high diagnostic specificity (99.8%) and sensitivity (90.5%) (Anderson and McKay, 1994; Choi et al., 2005; Khan et al., 2007; Khan et al., 2008; Abubakar et al., 2009). Immunocapture ELISA (ICE) is a rapid, sensitive and virus specific test for PPRV antigen detection and it can differentiate between RPV and PPRV (Diop et al., 2005; Abubakar et al., 2008) and has been reported to be more sensitive than the AGID test (Abraham and Berhan, 2001). For rapid diagnosis to enable a swift implementation of control measures, further development and validation of pen-side tests such as the chromatographic strip test (Hussain et al., 2003) and the dot ELISA that can be performed without the need for equipments or technical expertise are highly desirable (Anonymous, 2008). Conventional techniques used for PPRV detection are detailed in table 1.

**Molecular Methods**

Molecular techniques such as reverse transcription polymerase chain reaction (RT-PCR) (Forstyth and Barrett, 1995; Brindha et al., 2001; Couacy-Hymann et al., 2007; Farooq et al., 2008; Albayrak and Alkan, 2009) and nucleic acid hybridization (Shaila et al., 1989; Diallo et al., 1995) are generally used. These genome based techniques are largely used because of their high specificity and sensitivity. However, modern one step real-time RT-PCR assays specific for PPRV (Bao et al., 2008; Kwiatek et al., 2010; Batten et al., 2011) and loop-mediated isothermal amplification techniques (Wei et al., 2009) are more sensitive techniques for PPRV detection but do not allow genetic typing of positive samples.

RT-PCR coupled with ELISA have also been used to increase the analytical sensitivity of visualization of RT-PCR products and to overcome the drawbacks of electrophoresis-based detection such as use of ethidium bromide, exposure to UV light etc (Kumar et al., 2007). The assay is reported to detect viral RNA in infected tissue culture fluid with a virus titre as low as 0.01 TCID50 /100 µl and has been reported as being 100 and 10,000 times more sensitive than the sandwich ELISA and RT-PCR, respectively (Saravanan et al., 2004).

**CLASSICAL CONTROL MEASURES**

Control of PPR may be achieved using classical measures. These measures include restricted movement of sheep and goats from affected areas, quarantine of affected animals, elimination of contact fomites and restriction on the importation of sheep and goats from infected areas. Infected animals should be slaughtered and the carcasses should be burned within deep burial grounds (Saliki, 1998). Affected premises should be cleaned and disinfected with lipid solvent solutions of high or low pH, disinfectants (phenol, sodium hydroxide, alcohol, ether) and detergents (Anonymous, 2002). PPR outbreaks should be carefully monitored. Farmers and other livestock handlers need to receive education on reactionary procedures following disease identification and control measures such as movement restriction of animals with clinical signs, basic hygienic practices, and biosecurity levels within and between flocks. This is important to limit the spread and severity of PPR outbreaks (Balmarurugan et al., 2010). Control of PPR outbreaks may also rely on movement control (quarantine) combined with the use of focused (“ring”) vaccination and prophylactic immunization in high-risk populations.

**Vaccines**

The most effective way to control PPR is mass immunization of small ruminants as often, farmers in areas where the virus is endemic are unable to afford and implement the strict sanitary control measures, including the stamping out policy, required to contain the virus. Therefore the control of PPR requires an effective vaccine and for this purpose several vaccines including both homologous and recombinant vaccines have been developed.

**Conventional vaccines**

Early attempts to control PPR involved the use of the live attenuated tissue culture RPV vaccine (TCRV) and adequate protection was afforded to small ruminants using this vaccination policy (Plowright and Ferris, 1962). Sera from vaccinated animals showed the presence of neutralising antibodies against RPV but not against PPRV and after PPRV challenge, the neutralizing antibody response to PPRV increased and all animals were protected (Taylor, 1979). However, the RPV eradication campaign required a halt in the use of the TCRV as serological testing was not able to differentiate between naturally infected and vaccinated animals. This meant that vaccine derived RPV serological positivity complicated surveillance initiatives set up to try and detect the presence of naturally circulating forms of rinderpest of low pathogenicity. A homologous PPRV vaccine was introduced after the successful attenuation of the Nigeria 75/1 strain through serial passage on Vero cells (Diallo et al., 1989). A similar vaccine was produced in Pakistan using the PPRV Nigeria 75/1 (PPR 75-1 LK 6 Vero 75) from France and it was found that it could be safely used for immunization of small ruminants against PPR (Asim et al., 2009). In India, several vaccines have been
developed including the live attenuated PPRV Sungri/96 vaccine strain that has been standardized for use (Hegde et al., 2008).

Thermostable vaccines
As PPRV is thermolabile, it is necessary to maintain a cold chain to preserve vaccine potency. The thermostability of the TCRV vaccine was improved through the application of freeze drying technology (Mariner et al., 1990). Similarly, Worrall et al. (2001) developed a thermo-tolerant vaccine freeze dried in the presence of a cryo-protectant containing trehalose. This vaccine is stable at 45°C for 14 days with minimal loss of potency. Two thermostable vaccines were developed and their thermo-stability when checked at both 37°C and 40°C, demonstrated a shelf life of 7.62 and 3.68 days, respectively (Sen et al., 2010). Heavy water has been reported to enhance the thermo-stability of PPRV vaccines. When compared to conventional virus, deuterated virus reconstituted in heavy water-based diluents resulted in the maintenance of higher titers (Sen et al., 2010).

Recombinant vaccines
Following the successful eradication of RPV, attention has now been focused on the potential to eradicate PPRV. Currently, a live attenuated vaccine is available, however, as seen with the RPV eradication campaign, application of the current vaccines and companion serological diagnostic ELISA test does not allow serological differentiation of naturally infected from vaccinated animals, the so called ‘DIVA’ concept. The development of recombinant vaccines that fulfill DIVA requirements is very important for the eradication of the disease from the country.

Historically, the F and H proteins of several morbilliviruses have been expressed in heterologous live virus vector systems which have proven to be effective as vaccines (Romero et al., 1994; Stephensen et al., 1997). One such recombinant dual vaccine was developed using attenuated capripoxvirus strain KS-1 that expressed the PPRV F protein which proved to be effective at a low dose (0.1 PFU) (Berhe et al., 2003). Similarly two recombinant capripoxviruses, expressing PPRV glycoprotein H or F, were constructed. These DIVA vaccines proved to induce long-lasting immunity against PPRV (Chen et al., 2010). Other studies used attenuated modified vaccinia virus Ankara strain (MVA) as a vector for PPRV immunogens. Two recombinant viruses were constructed namely MVA-F and MVA-H expressing the full length PPRV F and H glycoproteins, respectively. Goats vaccinated with these vaccines were completely protected from the disease which gave an indication that mass vaccination with both or one of the two recombinant virus vaccines could help in PPRV eradication (Chandran et al., 2010). One caution when considering use of such pox-vectored vaccines is the possibility of preexisting immunological responses to naturally circulating poxviruses that may interfere with this vaccination strategy. Furthermore, when relying solely on the PPRV competitive ELISA, use of these vectored vaccines does not enable DIVA.

To reduce the cost of vaccination, bi or trivalent vaccines should be developed that can provide protection against PPRV as well as other important diseases of sheep and goats. This would enhance poverty alleviation in areas where multiple viral pathogens of small ruminants exist (Diallo, 2006; Banyard et al., 2010; Abubakar et al., 2011).

Edible Vaccines
Recent work has provided data to support the prospect of generating vaccines in plants and has shown that transgenic plants can synthesize and assemble immunogenic proteins. Furthermore, oral immunization with transgenic plants expressing vaccine antigens has been shown to produce specific immune responses (Mason et al., 1992). Transgenic plants could be an

Table 1: Detail of conventional methods for the detection and confirmation of PPR

<table>
<thead>
<tr>
<th>Sr. #</th>
<th>Test Name</th>
<th>Acronym</th>
<th>Application (Lab or Field)</th>
<th>Feature Detected (Antigen or Antibody)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.</td>
<td>Dot enzyme immunoassay</td>
<td>--</td>
<td>Lab</td>
<td>Antigen</td>
<td>Obei and Ojeh (1989)</td>
</tr>
<tr>
<td>4.</td>
<td>Differential immuno-histo-chemical staining of tissue sections</td>
<td>IH staining</td>
<td>Lab</td>
<td>Antigen</td>
<td>Saliki et al. (1994)</td>
</tr>
<tr>
<td>5.</td>
<td>Haemagglutination and Haemagglutination inhibition tests</td>
<td>HA and HI</td>
<td>Both</td>
<td>Both</td>
<td>Raj et al. (2000)</td>
</tr>
<tr>
<td>6.</td>
<td>Immuno-filtration</td>
<td>IF</td>
<td>Lab</td>
<td>Antigen</td>
<td>Raj et al. (2008)</td>
</tr>
<tr>
<td>7.</td>
<td>Latex agglutination tests</td>
<td>LA</td>
<td>Field</td>
<td>Antigen</td>
<td>Keerti et al. (2009)</td>
</tr>
<tr>
<td>8.</td>
<td>Virus isolation</td>
<td>VI</td>
<td>Lab</td>
<td>Antigen</td>
<td>Brindha et al. (2001)</td>
</tr>
<tr>
<td>9.</td>
<td>Competitive enzyme-linked immunosorbent assay (c-ELISA)</td>
<td>cELISA</td>
<td>Lab</td>
<td>Antibody</td>
<td>Anderson et al. (1991)</td>
</tr>
<tr>
<td>10.</td>
<td>Novel sandwich ELISA</td>
<td>sELISA</td>
<td>Lab</td>
<td>Antigen</td>
<td>Saravanan et al. (2008)</td>
</tr>
<tr>
<td>11.</td>
<td>Immuno-capture enzyme-linked immunosorbent assay</td>
<td>ic-ELISA</td>
<td>Lab</td>
<td>Antigen</td>
<td>Singh et al. (2004)</td>
</tr>
</tbody>
</table>

effective way of vaccination against viral diseases endemic across the developing world as production costs are greatly reduced when compared to vaccines produced using expensive cell culture-based expression system. Compared with parenteral antigen delivery, oral vaccine delivery is a more convenient way of immunization and by the induction of mucosal immunity, offers more effective protection against pathogens interacting with host mucosal surfaces (Prasad et al., 2004; Streatfield 2005).

Expression of the H gene of PPRV in pigeonpea (Cajanus cajan (L.) Millsp.) for the development of an edible vaccine against PPR has been reported (Prasad et al., 2004). A 2 kb fragment containing the coding region of the H gene from an Indian PPRV isolate was cloned into the binary vector pBI121 and mobilized into Agrobacterium tumefaciens strain GV3 101. However, the immunogenicity and extent of protection offered by oral delivery of the H protein expressed in pigeonpea remains to be tested. The production of vaccine antigens in plants in their immunogenic forms creates the possibility of economically favourable “bio-farming” for the development of PPRV vaccine.

Whilst the generation and ingestion of transgenic plants could be an attractive mechanism of immunization, one drawback is the potential inclusion of pesticide residues as possible contaminants within plants. This may increase production costs through the necessity to remove these substances during the purification process (Floss et al., 2007).

ANTIVIRAL THERAPY

Whilst extensive research into vaccination strategies to protect small ruminants against PPRV is ongoing, ultimately vaccines can only serve as preventive tools to induce protective immune responses prior to infection. Research into antiviral therapies may develop effective therapeutics that could help virus control when animals are newly infected or are at high risk of exposure to virus within the herd. However, questions related to the use of antiviral compounds such as efficacy, selectivity, safety, route of administration, uptake and clearance of the compound in the animal, development of drug-resistant mutants, possible risk to humans of residual compound in food producing animals, etc. need to be addressed and further in vivo research will be required for this purpose (De Almeida et al., 2007; Goris et al., 2008).

To control PPRV following the onset of clinical disease, one antiviral strategy that could be pursued is the silencing of gene expression using RNA interference (RNAi). In vitro inhibition of PPRV and RPV replication by using synthetic short interfering RNAs (siRNAs) has been demonstrated (De Almeida et al., 2007). siRNAs targeting the N gene of PPRV and RPV resulted in a >80% reduction in virus replication in vitro. siRNAsNPPR V6 and NPPR V7, targeting two conserved regions of PPRV, showed clear inhibition of PPRV replication, reported as a marked decrease in cytopathic effect, detection of virus antigen by immunofluorescence staining, decreased viral titres and decreased quantities of viral RNA being detected. In vivo therapy of RNAi requires the efficient delivery of siRNA molecules to the appropriate tissues. Both plasmids and viral vectors expressing short hairpin RNA sequences specific for virus genes may be used for this purpose. Another antiviral therapy was investigated by Sujatha et al. (2009) who reported the in vitro antiviral activity of 4, 4-(arylmethylene)bis(3-methyl-1-phenyl-pyrazol-5-ols) against PPRV. The synthesized compounds showed excellent antiviral activity against PPRV and were found to be more potent than the standard drug ribavirin used.

Conclusions

The ongoing scientific research into the detection of, protection against and molecular characteristics exhibited by PPRV and related morbilliviruses across the globe will hopefully lead to the development of further safe and effective mechanisms by which to control these viruses. PPRV continues to constitute a huge economic burden on the agricultural sustainability both within Pakistan and across the developing world. The lessons learnt from the success of the RPV eradication campaign must now be translated to PPRV. The development of novel vaccines and companion diagnostic assays that enable DIVA requirements to be met may make a PPRV eradication campaign economically viable. With the appropriate tools in place, attempts to globally eradicate PPR in the same way as has been achieved for RPV may be a realistic goal.

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