Occurrence of Peste Des Petits Ruminants in Five Districts of Punjab, Pakistan

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ABSTRACT

The present study was carried out to know the seroprevalence, molecular characterization and molecular epidemiology of peste des petitis ruminants (PPR) virus. The serum (n=440) and tissue (n=242) samples were collected from sheep and goat population reared in the central and peripheral districts of the Punjab province. The specimens were subjected to RT-PCR for the specific detection of fusion (F) protein genes of PPR virus. The cELISA test was applied to serum samples to know seroprevalence. Overall 51.5 and 46.5% seropositivity was found in sheep and goat, respectively. Seropositivity under different ages 1, 2 and >2 years was recorded as 46.5, 60.5 and 37.8%, respectively. Species and sex variation of seropositivity was recorded at 60.3 and 32.8% in buck and doe, respectively and 64.8 and 50.7% in ewes and ram, respectively. Sixty percent goat and 55.6% sheep population in Faisalabad district was seropositive for PPR virus, followed by the Bhakkar district where PPR virus seropositivity was 55.1% in sheep and 53.5% in goat. Specimen samples recovered from outbreak cases showed 372bp RT-PCR product indicating the presence of F specific protein region of PPR virus. Maximum predilection for PPR virus was lymph nodes (87.5%) in sheep as well as in goats followed by spleen (62.5%), nasal swabs (59.1%) and blood (51.4%). The phylogenetic analysis showed similar percentages and relationship with already reported data of this region. The present study reports that PPR virus is affecting the sheep and goat of less than two years of age with high frequency. The prevalent PPR virus is homologous with most of the reported PPR viruses from the Asian outbreaks.

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Key words: c-ELISA, Pakistan, Peste des petits ruminants, Punjab, RT-PCR

INTRODUCTION

Peste des petits ruminants (PPR) is an acute and highly contagious viral disease of small ruminants caused by a morbillivirus (Roeder et al., 1994), which is antigenically related to other morbilliviruses such as Canine distemper virus, Measles virus and Rinderpest virus. PPR is endemic in many parts of the world, including Africa, Asia, Middle East and sub Saharan Africa, India, Pakistan, Iran, Iraq, Bhutan and Bangladesh (Shailla et al., 1996; Kwiatek et al., 2007; Sande et al., 2011; Abubakar et al., 2011a). PPR was first recognized in Pakistan during 1991 by Amjad et al. (1996) and in Punjab province by Athar et al. (1995). Since then, the disease is reported in many parts of country (Hussain et al., 2003). The disease has serious economic and trade implications because of its high mortality (Asim et al., 2009). Naturally, the disease affects both sheep and goat but more severity with high case fatality recorded in goats than sheep (Atta-ur-Rehman et al., 2004).

PPR is characterized by high fever, mucopurulent discharge, ulceration of mucus membrane and inflammation of gastrointestinal tract with morbidity between 80-90% and mortality between 50-80% (Khan et al., 2008b). Abubakar et al. (2011b) reported the morbidity and mortality as high as 100 and 90%, respectively, and in certain cases these rates were recorded as low as 20%. The mortality rate varies between 70-80% in acute out break cases with death of affected animals in 10-12 days (Diallo et al., 2007). Due to its high mortality and morbidity rates, PPR has been rated as a notifiable viral disease by OIE.
PPR virus has been reported continuously from Pakistan with differing frequencies using a variety of techniques i.e., virus isolation, ELISA, and PCR (Anderson and Meckay, 1994; Singh et al., 2004; Khan et al., 2008b). PPR outbreaks were reported annually and in various areas and seasons in Pakistan (Tahir et al., 1998; Hussain et al., 1998; Khan et al., 2008a; Khan et al., 2009). The previous records were based upon small number of samples per district encompassing many districts and secondly RT-PCR was not the main techniques to conduct epidemiology of PPR on large scale. Moreover detailed analysis regarding age, sex and species were not reported solely from the area selected in the present study. The present study hypothesized to bridge the gap regarding seroprevalence of PPRV using cELISA and PCR to compile molecular epidemiology of PPRV with reference to location, age sex in sheep and goat samples collected from different districts of Punjab.

MATERIALS AND METHODS

Sample collection: Blood and specimen samples were collected from sheep n=134 and goats n=108 at different outbreaks in and around five districts (Faisalabad, Attock, DG Khan, Bhakkar, Kasur) of Punjab, Pakistan (January-December 2010). The districts heavily populated with sheep and goats (Anonymous, 2006) were selected for the present study. A total of 440 serum samples were collected from the randomly selected apparently healthy sheep and goat for specific antibody screening, while 242 specimen samples were collected from convalescent cases for antigen detection (Table 1). The samples preservation was done according to FAO Manual for Trans-boundary diseases. Ice pads were used for the transportation of lymph node, spleen and serum samples to the laboratory.

Detection of antibodies by cELISA: Sera were titered for antibodies by cELISA kit manufactured by Institute for Animal Health, Pirbright laboratory UK following instruction of the manufacturer. The negative and positive cut off values was used for the control of test procedure. The ELISA plates were read using (Humma Reader, USA) inference filter of 492 nm. EDI (ELISA data interchange) software was used to read and calculate the percentage of inhibition (PI). The optical density (OD) was converted to PI by using following formula (Libeau et al., 1992).

\[
PI = 100 - \left( \frac{OD \text{ control/test serum}}{OD \text{ monoclonal control}} \right) \times 100
\]

Samples having PI >50% were considered as positive.

Detection of PPRV using RT-PCR: The samples were subjected to RNA extraction using the protocol described by Nanda et al. (1996). Extracted RNA was subjected to cDNA synthesis by using iScript protocol (Bio-Rad Laboratories CA). The iScript reaction mix (4µl) was added into 1µl reverse transcriptase, nuclease free water (10µl) and RNA template (5µl) to make final volume of reaction mixture 20µl. This reaction mixture was incubated at 25°C for 5 minutes and 42°C for 30 min and 85°C for 5 minutes with final holding at 4°C.

PCR amplification: A pair of primers i.e., F1 (Forward) 5’ATCACAGTTAAAGCCTGTAGAGG3 and primer F2 (Reverse) 5’GAGACTGAGTTGTGACCTACAAGC3 were added to 5µl cDNA, 5 µl of 10x PCR buffer, 1.5 µl of 50 mM Mgcl2, Taq polymerase (5 µl) and 1µl dNTP (10mM each) and sterile distilled water to make final volume 50 µl. The cocktail was finally subjected to denaturation, annealing, synthesis and extension as reported by Nanda et al. (1996). PCR product was subjected to 1.5% agarose gel electrophoresis and was visualized by UV trans-illuminator Dolphin Doc, Wealtech USA. PCR product was sent to Eurofins MWG Operon U.S.A and phylogenetic analysis was performed using clustal W2 program of multiple sequence alignment. Chi-square analysis was performed to statistically analyze the differences found in sero-prevalence of PPR in sheep and goat.

RESULTS

Detection of PPR antibodies: A total of 440 serum samples from goat (n=131) and sheep (n=309) were randomly collected from five districts of Punjab. Of these 51.5% sheep and 46.5% Goat samples were positive. The percent positivity of the sheep samples is significantly different (P=0.042) and in goat samples highly significant (P=0.000) among different districts under study (Table 1). Age and gender based interaction is described in Table 2. It was found that the seroprevalence of PPR in ewes upto 1 year with buck 2 years and doe more than 2 years of age were significantly higher than other groups. While gender wise the values showed that the seroprevalence in ewe and buck, and ram and doe are significantly different.

Virus detection and F gene sequencing: Samples of sheep (n=134) and goat (n=108) were collected from various outbreak cases at five different districts of Punjab (Table 3). RT-PCR was applied on these samples targeting protein sequence of 372bp size (Fig. 1). Maximum PPRV was detected from lymph node (87.5%) followed by spleen, nasal swabs and blood. Whereas significant differences were observed in nasal swab and blood samples (P=0.000) followed by spleen samples from sheep, which were significantly different (P=0.001). The analysis of F gene sequence revealed that homology between previously reported and characterized Pakistani and surrounding areas isolates ranges between 98-99%. A phylogenetic tree was constructed for F gene based on sequence recovered from gene bank representing lineages. This tree revealed that the sample in this study describing the F gene sequence cluster together in the region of South East Asia and Middle East with the identification of new indigenous isolate of PPR virus found in the lineage 4 (Fig. 2) of isolate was obtained after submission of sequencing results to NCBI, USA.

DISCUSSION

PPR is an acute viral disease caused by a morbillivirus that mainly effect sheep and goats. The disease is having variable degree of morbidity and mortality. So far field cases in Pakistan are diagnosed on
Goats specificity and sensitivity in diagnosis (Abubakar technique is employed simultaneously for attaining more reaction between PPR and RPV that is why specific serological tests often gave confusing results due to cross location where this disease is epidemic. Conventional and detection of virus. Case recording helps to find out the three prong strategy i.e. recording, detection of antibodies and detection of virus. Case recording helps to find out the location where this disease is epidemic. Conventional serological tests often gave confusing results due to cross reaction between PPR and RPV that is why specific technique is employed simultaneously for attaining more specificity and sensitivity in diagnosis (Abubakar et al., 2011a).

In the present study, the overall seroprevalence in sheep and goat population in Punjab was 51.5 and 46.5%, respectively, which was higher than the already reported 51.5 and 46.5% as reported by Bahadar et al. (2007), but in the present study goat samples showed significantly high prevalence. It may be possible that Khan et al. (2007) could have conducted a study having more emphasis on goat only. The seroprevalence in sheep population was significantly higher (P=0.042) than the seroprevalence of the sheep samples collected from central Punjab.

Significantly higher seroprevalence of PPR in sheep and goat population in western Punjab has been reported by Khan et al. (2007), but in the present study goat samples showed significantly high prevalence. It may be possible that Khan et al. (2007) could have conducted a study having more emphasis on goat only. The seroprevalence in sheep population was significantly higher (P=0.042) than the seroprevalence of the sheep samples collected from central Punjab.

Table 1: Statistics of PPR Virus detected through RT-PCR in specimen samples of sheep and goat in five districts of Punjab

<table>
<thead>
<tr>
<th>Sampling Area</th>
<th>Total population</th>
<th>Total sample collected</th>
<th>Possible samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Central Punjab (Faisalabad district)</td>
<td>5475</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>Northern Punjab (Attock district)</td>
<td>180947</td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td>Southern Punjab (D.G Khan district)</td>
<td>645035</td>
<td>24</td>
<td>14</td>
</tr>
<tr>
<td>Western Punjab (Bhsksr district)</td>
<td>459540</td>
<td>12</td>
<td>4</td>
</tr>
<tr>
<td>Eastern Punjab (Kasur ditrcit)</td>
<td>98730</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>1439005</td>
<td>66</td>
<td>39</td>
</tr>
<tr>
<td>Prevalence percentage</td>
<td>59.100</td>
<td>51.400</td>
<td>53.200</td>
</tr>
<tr>
<td>Chi square values</td>
<td>33.636</td>
<td>17.579</td>
<td>17.14</td>
</tr>
<tr>
<td>P values</td>
<td>0.000</td>
<td>0.000</td>
<td>0.001</td>
</tr>
<tr>
<td>Goat</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Central Punjab (Faisalabad district)</td>
<td>904918</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>Northern Punjab (Attock district)</td>
<td>628642</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>Southern Punjab (D.G Khan district)</td>
<td>858437</td>
<td>27</td>
<td>2</td>
</tr>
<tr>
<td>Western Punjab (Bhsksr district)</td>
<td>655774</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>Eastern Punjab (Kasur ditrcit)</td>
<td>365792</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>3413564</td>
<td>67</td>
<td>36</td>
</tr>
<tr>
<td>Prevalence percentage</td>
<td>53.700</td>
<td>52</td>
<td>62.500</td>
</tr>
<tr>
<td>Chi square values</td>
<td>91.134</td>
<td>18.440</td>
<td>7.852</td>
</tr>
<tr>
<td>P values</td>
<td>0.000</td>
<td>0.002</td>
<td>0.049</td>
</tr>
</tbody>
</table>

Fig. 1: Ethidium bromide stained agarose gel electrophrosis of the product amplified with PCR using specific primers for PPR virus. M; 100bp DNA ladder Lane 1 showing 372 bp size lanes 2-5 showing –ve samples; +ve lane showing vaccine positive band.

three prong strategy i.e. recording, detection of antibodies and detection of virus. Case recording helps to find out the location where this disease is epidemic. Conventional serological tests often gave confusing results due to cross reaction between PPR and RPV that is why specific technique is employed simultaneously for attaining more specificity and sensitivity in diagnosis (Abubakar et al., 2011a).

Table 2: Seroprevalence of PPR virus in sheep and goat using c-ELISA

<table>
<thead>
<tr>
<th>Age group</th>
<th>+ve Sheep</th>
<th>+ve Goat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Positive/Total)</td>
<td>(Positive/Total)</td>
</tr>
<tr>
<td>Ram</td>
<td>48/82</td>
<td>12/25</td>
</tr>
<tr>
<td>Ewe</td>
<td>47/82</td>
<td>12/25</td>
</tr>
<tr>
<td>Total</td>
<td>93/164</td>
<td>24/50</td>
</tr>
<tr>
<td>Chi square values</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>P values</td>
<td>0.000</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Higher (P=0.000) in goats of central and western Punjab with 60 and 53.5%, respectively as compared to eastern and northern Punjab with 33.3 and 37.5%, respectively. The seroprevalence in sheep population was significantly higher (P=0.042) than the seroprevalence of the sheep samples collected from central Punjab.

Significantly higher seroprevalence of PPR in sheep and goat population in western Punjab has been reported by Khan et al. (2007), but in the present study goat samples showed significantly high prevalence. It may be possible that Khan et al. (2007) could have conducted a study having more emphasis on goat only. The seroprevalence in sheep population was significantly higher (P=0.042) than the seroprevalence of the sheep samples collected from central Punjab.
In F gene phylogenetic relationship the sequence in study clustered with sequence pattern from Pak 09 with accession no. (FN996973.1), Bhutan 10 (FR667649.1) and Bangladesh 2000 (FR667556.1), branching pattern following neighbor joining method of corresponding sequences indicating high mutation rate, which need to be investigated further (Fig. 3). Variation in F gene based on nucleotide variation for analysis of phylogenetic relationship. Similarity percentages in already reported and the sequence of F gene from the present study is in agreement with Munir et al. (2012).

**Conclusion:** It was concluded that young sheep and goat population is getting infection of PPR virus with higher frequency than the adults and the predilection site of PPR virus remained the lymph nodes.

**REFERENCES**


