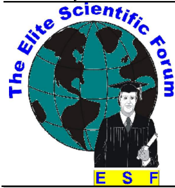

Pakistan Journal of Life and Social Sciences
www.pjlss.edu.pk

RESEARCH ARTICLE

Detection and Sequencing of Field Isolates of Peste Des Petits Ruminants Virus from Punjab Province, Pakistan

Haider Ali Khan[1,*], Sajjad-ur-Rahman[1], Ahrar Khan[2] and Qaiser Mahmood Khan[3]
[1]Institute of Microbiology, University of Agriculture, Faisalabad, Pakistan
[2]Department of Pathology, University of Agriculture, Faisalabad, Pakistan
[3]National Institute of Biotechnology and Genetic Engineering, Faisalabad, Pakistan

ARTICLE INFO
Received: Oct 25, 2013
Accepted: Oct 26, 2013
Online: Nov 20, 2013

Keywords
F gene
Peste des petits ruminants virus
Phylogeny
Sequencing

***Corresponding Author:**
imhkhan2002@gmail.com

ABSTRACT
Peste des petits ruminants (PPR), is a viral contagious and important transboundary animal disease (TAD), affecting domestic and wild small ruminants like sheep and goats, endemic in Indo-Pak subcontinent. The study was undertaken to characterize the indigenous PPRV by characterizing the fusion protein (F) gene segments by sequencing and their phylogenetic analysis for determination of genetic variation in the field viruses. Selected F gene segments of PPRV genome were amplified using RT-PCR from 110 tissues (lungs, lymph nodes and spleen) samples from dead animals and 41 swabs (nasal, occular and oral) from PPR suspected animals. The resulting amplicons were sequenced for phylogenetic analysis. The phylogenetic tree based on the F gene sequences of PPRV from different locations clustered them into lineage 4 along with the other isolates from Pakistan. Thus, genetic diversity of PPRV into the same lineages based on the F gene sequences showed the presence of lineage 4 emerged to give up the thought of molecular epidemiology for PPRV.

INTRODUCTION

Peste des petits ruminants (PPR) is an important viral disease of sheep and goats, endemic in Pakistan, affecting sheep and goats and wild small ruminants (Munir et al., 2008). PPRV is in the morbilliviruses genus of Paramyxoviridae family (Gibbs et al., 1979), share the same genome organization (Barrett et al., 1998). In most of the developing countries, the disease has considerable socioeconomic significance due to subsisting nature of farming and risk to livestock trade. It is endemic in countries located between the Sahara in Africa, the Middle East and the Indian sub continent (Diallo et al., 1995; Amjad et al., 1996). The virus was assumed for a long time to be a variant of rinderpest adopted in small ruminants causes PPR. Later on, these viruses were perceived with different genetically, antigenically and serologically, physicochemical traits. It was also revealed to be an immunologically diverse virus with a separate epizootology in areas where both viruses were enzootic.

PPR is a highly contagious disease with very high morbidity and mortality (Abu-Elzein et al., 1990). It was first reported in sheep and goats (Gargadennec and Lalanne, 1942) and in Sindh Ibex (Abubakar et al., 2011b), it was thought to have limited to the African countries. However, during the recent years, the disease has been also been reported in other parts of the world, including Pakistan, Bangladesh, India, Nepal, Saudi Arabia, Iran, Iraq, Israel, Kuwait, Oman Lebanon, Jordan, Yemen and the United Arab Emirates. In Pakistan, it was first detected by Amjad et al. (1996) and than by Khan et al. (2008) and Abubakar et al. (2011), resulting heavy economic losses and is currently defined as the major concern the small ruminants in the country.

The diagnostic techniques like PCR, improve the rapid and specific diagnosis of PPR (Forsyth and Barrett, 1995; Couacy et al., 2002). PPRV is grouped genetically into four different distinct lineages on the basis of fusion protein (F) gene sequence analysis. However, only a single serotype of PPRV has been previously reported (Shaila et al., 1996; Dhar et al., 2002; Ozkul et al., 2002). This characterization of PPRV into different lineages has been considered applicable for comprehensive molecular epidemiology of PPR worldwide. However, increase the awareness of PPR in Pakistan, the need was felt to probe PPRV into the molecular details of the field virus.

The PPRV was detected from suspected outbreaks among sheep and goats of Punjab Province, by using specific F gene (Forsyth and Barrett, 1995). Furthermore, the genetic diversity between these viruses were explored by evaluating the nucleotide sequences F and N genes PCR amplified products from other PPRV sequences available in GenBank of National Center for Biotechnology Information (NCBI).

MATERIALS AND METHODS

Sample collection for PPR antigen detection

Twenty one outbreaks of PPR were investigated during the course of study (2005-06) in various districts of Punjab, Pakistan. History of all the outbreaks, clinical signs and post mortem findings were recorded. A total of 110 tissues (lungs, lymph nodes and spleen) samples from dead animals and 41 swabs (nasal, occular and oral) from PPR suspected animals were collected and transported to the laboratory and kept at -20°C for further analysis.

Identification of PPR virus

Immunocapture Enzyme-Linked Immunosorbent Assay (IcELISA)

The immunocapture enzyme-linked immunosorbent assay (IcELISA), was performed as the method adopted by Anderson and McKay, (1994) in National Veterinary Laboratory, Islamabad, for rapid differential identification of PPR or rinderpest viruses using anti-N monoclonal antibodies (MAb) by using a commercial kit (BDSL, Flow Laboratories & CIRAD, EMVT, France).

Reverse transcription-polymerase Chain reaction

A Polymerase Chain Reaction (PCR) technique based on the amplification of the F protein gene was optimized (Forsyth and Barrett, 1995) for the specific diagnosis of PPR in National Institute of Biotechnology and Genetic Engineering (NIBGE), Faisalabad. PCR amplification was carried out from specific F protein gene for PPRV specific primer set as follows:

Specific Primers:

Two sets of PPR-specific set based on the equivalent region of Fusion protein (F) gene sequence of PPRV were used.

Set 1:

Forward: PPRVF1: 5/
ATCACAGTGTTAAAGCCTGTAGAGG 3/
Reverse: PPRVF2: 5/
GAGACTGAGTTTGTGACCTACAAGC 3/

Set 2:

Forward: PPRVF1b: 5/
ATGCTCTGTCAGTGATAACC 3/
Reverse: PPRVF2d: 5/
CTATGAACAGAGGGGACAAG 3/

These primers were selected from the F protein gene sequence, which amplify 308bp and 372bp DNA products.

RNA Extraction using Commercial kit

RNA extraction was conducted with the help of GM-1, Universal RNA purification Kit (catalog No. GM-RN-025 Vivantis) and amplification was conducted as follows:

Thermal cycling Program;

1 cycle: 50°C for 30min.
1 cycle: 94°C for 4min.
35 cycles: 95°C for 1min, 50°C for 1min, and 72°C for 45sec.
1cycle: 72°C for 10min.
Hold at 4 °C

After amplification, amplicons were stored overnight at 2-8°C for further use.

Analysis of RT-PCR products

The size of the PCR products was determined by observing the distance between the two primers in the primary sequence of the nucleic acid being analysed. The amplified product was visualized under UV light and observed by gel documentation system.

Sequencing and Phylogenetic Analysis

The sequencing was performed by First BASE Laboratories, Malaysia. Only twenty nine PCR products having sharp bands were purified and then sequenced in Gen-Bank using Clustal W (Thompson et al., 1994).

RESULTS

It was evident that the PPRV was circulating in Punjab and detected from suspected outbreaks among sheep and goats of Punjab Province.

Disease pattern and clinical findings

The most common clinical findings observed in all the twenty one PPR outbreaks were elevated body temperature (up to 107°F), muco-purulent ocular and nasal discharge, severe mucosal erosion, coughing, and respiratory distress. Diarrhoea was also observed in most of the affected small ruminants. The carcasses of the affected animals were emaciated due to loss of fluid and other lesions include; necrotic enteritis, pneumonia, enlargement of the lymph nodes (mesenteric and mediastinal) and spleen. All the outbreaks were associated with either the entry of newly purchased animals from a commercial market and inclusion of migratory animals with local animals. A complete history of the movement (forward and backward tracing) of animals was determined for all of the outbreaks investigated. All the confirmed outbreaks of PPR involved both sheep and goats except one outbreak which occurred in female of sheep in Southern districts of the Punjab province.

Table 1: Results of IcELISA for the Detection of PPRV Antigen

Sample Type	Number Tested	Number Positive	Percentage Positive	PP values
Spleen	64	42	65.62	34-275
Lungs	24	19	79.17	22-56
Lymph nodes	22	17	86.36	28-313
Swabs	41	18	43.90	15-44
Total	151	96	63.58	15-313

Detection of PPR virus antigen

All the samples, which were tested with AGID, were also subjected to Immunocapture ELISA (Table 2). The overall percentage of positive samples observed through IcELISA was 63.58%. It was observed that out of 64 spleen samples tested, 42 (65.62%) were positive, from 24 lungs samples, 19 (79.17%) were positive, from 22 lymph nodes 17 (86.36%) were positive and from 41 swabs, 18 (43.90%) were positive.

The samples having Percent Positive values greater than 50 in IcELISA were subjected to RT-PCR for the confirmation of PPRV. It was observed that all the samples, which were positive through IcELISA, were also found positive with RT-PCR. The F protein gene of PPR and RP were amplified by using specific (PPR specific) primers with the help of RT-PCR. PPR specific F protein gene specific primer was used for the synthesis of cDNA from suspected tissues and control RNA (PPR Vaccine Nig. 75/1). The figure shows bands of 308bp and 372bp (Fig.1 and 2) with the specific PPR (F protein) gene primers.

Molecular characterization of PPR virus

Detection of PPRV by F gene based RT-PCR amplification with primers F1b/F2d and F1/F2 yielded the amplicon of 308 and 372bp, respectively. Out of 110 representative samples, 96 were positive and the others were negative for F protein gene based RT-PCR. The virus of the four different locations (Faisalabad, Chakwal, Cholistan, T. T. Singh districts) was analyzed for molecular epidemiology. The analysis of F gene sequences (Fig.3) having accession Nos. EU784661, EU784662 and EU870808 revealed in phylogenetic tree as Chak/pk/06, Chol/pk/06, Fsd/pk/06 respectively. The homology among the field isolates of present study was 93% (for Pk/04, SAH/PK07, Jhansi 03, Uri99, Laxm98, Hyd98, Kacch200, Sung96, Revati 05, Izat94), 92% (for Mdn96, Trab01, Rai96, Chirg98, Ana/Guj/05, China/Tibet/07 and Rjk/Guj/05) and 91% (for Ptn/Guj/05, Jala97). None of the field isolates had homology less than 91% with any other isolates of the Pakistani and Indian origin.

The Phylogenetic tree based on the F gene sequence was constructed with the help of Geneious 3.7.0. software. It was evident that all the isolates were clustered in the lineage 4 with analogous of the isolates of turkey, isolate of India and Iran. The F gene sequences based network analysis resulted in two identical isolate Chak/pk/06, Iran 05 whereas Fsd/pk/08 resulted in three identical Nigeria 75/1, ICV89 and Nig/75/1. All the field isolates were differentiated into different clusters but in the same lineage. The isolate Chak/Pk/06 was closest to the Iran05 and the isolate of Cholistan desert (Chol/Pk/06) was the most distant from the other isolates.

DISCUSSION

Peste des petits ruminants was demonstrated with the presence of the disease by observing clinical manifestation and by molecular characterization of virus. This wide-ranging survey is the first to be carried out on this disease in Punjab, Pakistan. Previously several PPR outbreaks were unrecorded in the Punjab, Province of Pakistan due to insufficient animal disease reporting and surveillance system. In the past, most of the PPR outbreaks have been diagnosed just on the basis of clinical signs. An extensive clinical review of PPRV infection has been difficult because the specific diagnostic tests for the detection of PPRV, were not frequently employed. The development of a MAb-based Immunocapture ELISA (IcELISA) kit CIRAD/EMVT, France has greatly facilitated the diagnosis of PPRV. The IcELISA help for the specific diagnosis and will greatly assist in tracing PPR infection in different geographical regions.

The RT-PCR of F protein gene has gained much popularity (Forsyth and Barrett, 1995), and aid in the specific diagnosis of PPRV. The present study has achieved another milestone in the submission of different strains of PPR belonging to same lineage.

Detection of PPR virus antigen

As RP disease in cattle, PPR is economically significant for the sheep and goats. The global eradication programme has also been engaged to get rid of this disease (Rweyemamu & Cheneau, 1995). The sensitive and specific tests like IcELISA and RT-PCR are required to assist the eradication programme strategies across the world. The comparative detection of PPRV in same field samples by IcELISA and RT-PCR. Thus, in the present study, the PPRV was detected with IcELISA and RT-PCR.

The immunocapture ELISA (IcELISA) was found appropriate for specific diagnosis of PPRV in field samples (Abubakar et al., 2008) such as swabs (ocular and nasal) and tissues, (Diallo et al., 1995). However, this is not an economical as AGID. The monoclonal antibody-based (MAb) IcELISA was reported to be highly sensitive test for the detection of PPRV in secretions and tissues of infected animals (Saliki et al., 1994, Abubakar et al., 2011a).

A total of 151 clinical samples (tissues and swabs) from diseased animals and dead animals (sheep and goats), showing clinical signs and lesions indicative of PPR,

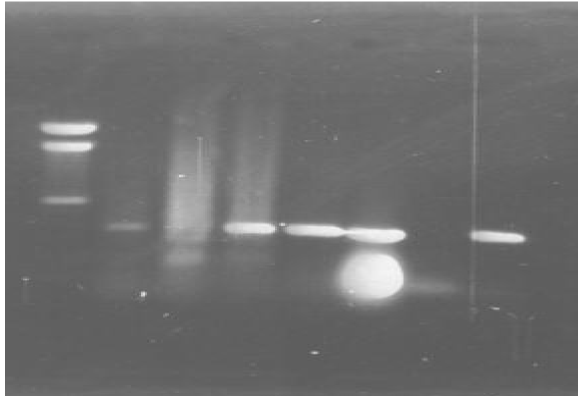

Fig. 1: Agarose Gel Electrophoresis of different PCR products amplified through Specific Gene primers obtained (308bp)

Lane 1: DNA Ladder (100 bp)
Lane 2: Positive Field sample (Faisalabad)
Lane 3: Positive field sample (Sargodha)
Lane 4: Positive Field sample (Cholistan)
Lane 5: Positive field sample (Toba Tek Singh)
Lane 6: Positive field sample (Multan)
Lane 7: Negative Control for RP
Lane 8: Positive control for PPRV

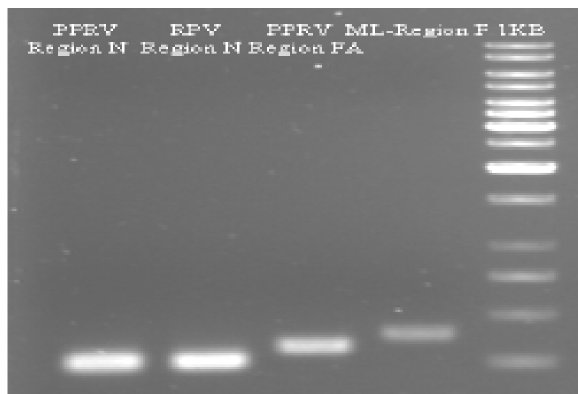

Fig. 2: Agarose Gel Electrophoresis of different PCR products amplified through Universal and Specific Gene primers obtained 238bp, 308bp, 372bp

PPRV N: Positive control of PPR
RPV N: Positive control of RP
PPRV FA: Positive Field sample (Chakwal)
ML F: Positive field sample (Multan)
Lane 5: 1KB DNA Ladder (bp): 250, 500, … 10000

were collected from different outbreaks all over the Punjab and tested using MAb-based IcELISA kit (CIRAD/EMVT France) for PPRV antigen detection. Higher percentage of positive samples was observed in lymph nodes and least was observed in swab samples. This might be attributed to the less concentration of virus in swabs when compared with lymph nodes as the concentrations of virus in swabs (nasal, oral and ocular) from diseased animals were found relatively low in virus as compared to various affected tissues for PPRV diagnosis using the IcELISA.

Currently, RT-PCR has appeared as highly specific and sensitive tests, which are also helpful in molecular characterization PPRV. Forsyth and Barrett (1995) developed highly sensitive F-gene primers based RT-PCR for the detection of PPRV. It has become the most conventional tool for the diagnosis and molecular charecterization (Shaila et al*.,* 1996), The sequencing of 308 and 372 bp F gene based RT-PCR product offers the benefit of identifying variation in PPRV from different geographical positions.

Apart from the diagnostic application of these tests, it could also be used for epidemiological studies and molecular characterization of the virus of PPR infection among different geographical locations. During the present investigations it was also observed that IcELISA performed equally well with RT-PCR and all the samples found positive for IcELISA, were also positive by RT-PCR. Thus it is logically apparent that performing RT-PCR on tissue and swab samples emerges successful for molecular characterization of PPR.

Molecular Characterization of PPR Virus

The PPR specific primers F1 and F2 developed by aforesaid workers (Forsyth and Barrett, 1995) give an amplification of a 372bp of PPRV F gene. In the present study, PPRV was detected from clinical samples tested, which confirmed PPRV in all the outbreaks of different locations in Southern (Multan, Cholistan), Central (Faisalabad, T.T Singh) and Northern (Chakwal) districts of Punjab, Pakistan. The complete history of the PPR outbreaks in Pakistan is not available, however, the present study reveal that the most of the outbreaks happened in Punjab during 2006-2007 with variable pattern of mortality.

In the present study, genetic relationship between PPR virus of different geographical origin was determined. It was found that both primers for F gene amplified the PCR products 308 and 372 bp comparable with the results of Shaila et al. (1996). By sequencing these fragments of F-gene, generated lineages 4 and lineage 1 of PPR viruses of distinct geographical origin (Barrett et al., 1998). It was demonstrated that all the virus isolates from subcontinent over the last few years were found to be of lineage 4 (Shaila et al*.,* 1996). However, Govindarajan et al*.* (1997) identified a lineage 3 of virus from a unique outbreak of PPR in buffaloes from southern part of India. Moreover, PPRV is not as recognized as other RNA viruses with respects to genetic variation and mutation because it is important for molecular epidemiology of the field virus as well as for expressing the probable appearance of any variants. The virus detected in this study belongs to the different lineage PPRV, which resembles with the viruses whose origins are in the Middle East, Saudi Arabia, and South

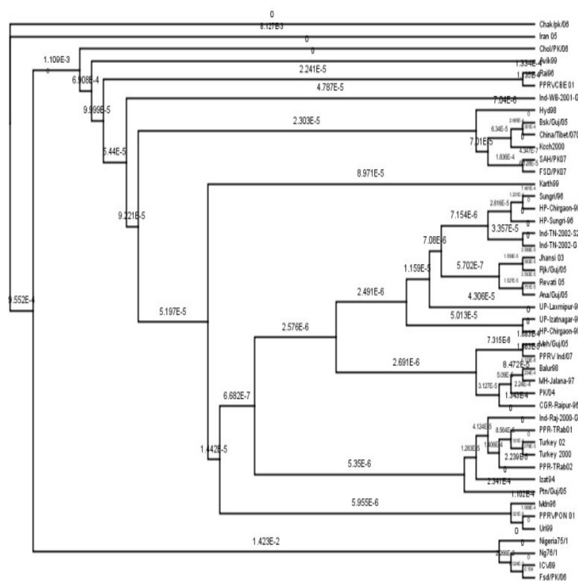

Fig. 3: Phylogenetic tree of PPRV of various districts of Punjab, Province based on F Genes through Geneious 3.7.0 software after alignment by Clustal W 1.83

Asia (Shaila et al., 1996). Therefore, the significance of PPRV as a threat to livestock should be measured, along with other diseases having economic importance.

The analysis of F gene sequences revealed that the greater homology observed between the field isolates (Chak/pk/06, Chol/pk/06, Fsd/pk/07) and other field isolates present in the Genbank (Pk/04, SAH/PK07, Jhansi 03, Uri99, Laxm98, Hyd98, Kacch200, Sung96, Revati 05, Izat94, Mdn96, Trab01, Rai96, Chirg98, Ana/Guj/05, China/Tibet/07) was suggested to be related with the lineage 4. The entire field isolates and vaccinal virus shared comparatively lesser homology while the isolate (Fsd/06 Accession No. EU870809) shared greater similarity with the Nigeria/75/1.

The phylogenetic network analysis based on the 308bp F gene segments by Geneious 3.7.0. Software allowed identification of the variation in the nucleotide sequences by depicting the position in each sequence. A consensus Phylogenetic tree based on the lineage specific 308bp F gene sequence was constructed, all the field isolates clustered together into a separate branch from the Nigerian (Nig/75/1) isolate. Whereas, only one isolate (Fsd/Pk/06) make close similarity with Nig/75/1. Thus, from the Phylogenetic tree it was evident that all the isolates of Punjab, Province clustered at separate positions with the other Asian isolates of lineage 4. Software analysis for the 308bp F gene sequences and similarly network analysis revealed that the isolates of Turkey, India and Nigeria resemble greatly from the isolates of this study. Thus, the Network analysis was in concordance with the branching pattern showed by the consensus tree. To assess the genetic similarity and divergence among the field PPRVs of Cholistan, Chakwal, T. T. Singh, Faisalabad as well as their relatedness to some of the previously described isolates. The partial F gene sequence based classification of PPRV into lineages placed the local isolates into lineage 4. Classification of PPRV into different lineages based on F gene sequence appeared to group the viruses in a better way, thus, giving better epidemiological picture about PPRV.

Conclusion

Genetic diversity of PPRV into the same lineages based on the F gene sequences showed the presence of lineage 4 emerged to give up the thought of molecular epidemiology for PPRV.

Acknowledgements

The authors greatly acknowledge the technical support provided by National Veterinary Laboratories (NVL), Islamabad and National Institute of Biotechnology and Genetic Engineering (NIBGE), Faislabad and financial support by Higher Education Commission, Islamabad.

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