Pakistan J. Zool., vol. 50(2), pp 559-566, 2018. DOI: http://dx.doi.org/10.17582/journal.pjz/2018.50.2.559.566

Field and Molecular Epidemiology of Peste des Petits Ruminants in Pakistan

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Article Information
Received 14 September 2017
Revised 12 December 2017
Accepted 02 January 2018
Available online 20 February 2018

Authors' Contribution
MA, ABZ and KN planned the study and support its execution. MAK, SQ and ABZ supervised the research work. MA and ABZ analysed the data.

Key words
Molecular epidemiology, PPR virus, Sheep and goats.

ABSTRACT

Peste des Petits Ruminants (PPR) is a highly fatal and economically devastating disease of sheep and goats. Present study was designed to have an insight into the epidemiology of PPR under field conditions in the country using molecular tools. A total of eighty-four (n = 84) PPR outbreaks were investigated during the study (2010 to 2013). The highest number of outbreaks was reported from Punjab province (n = 38) followed by Sindh (n = 21) and Khyber Pakhtunkhwa (KPK, n = 10). In 48 out of 84 outbreaks, disease occurred in goats only while 18 outbreaks affected sheep only and the remaining occurred in mixed herds. A total of 6221 animals were affected in these outbreaks. Sheep were less severely affected in comparison with goats. The morbidity, mortality and case fatality rate were 26.79%, 10.83% and 40.41% in sheep in comparison with 34.90%, 16.34% and 46.82% in goats, respectively. Overall, disease affected all three age groups of sheep and goats but the younger animals were more severely affected with a morbidity rate of 37.19%. The mortality and case fatality rates were also higher in young which were 46.86% and 17.39%, respectively. Yearly data of outbreaks was suggestive that a cyclic as well as seasonal pattern of disease occurred. The results of the phylogenetic tree indicated that all Pakistani PPRV strains, regardless of the gene used either F or N, clustered in lineage IV which is the most prominent and prevalent lineage of Asia. The distribution of Pakistani strains of PPRV was more dispersed as the isolate collected from Taxilla was clustered slightly distinct compared to rest of the isolates collected from Pakistan. Our findings are indicative of PPR endemic state of the country.

INTRODUCTION

Peste des Petits Ruminants (PPR) is a viral disease, highly contagious and infectious in nature, and affect mainly small ruminants such as goats and sheep but can also affect wild animals infrequently. The PPRV infection is characterized by elevated temperature, purulent ocular and nasal discharges, necrosis, pneumonia, mucous membrane ulceration and gastro-intestinal tract inflammation which ultimately lead to severe diarrhea (Gibbs *et al.*, 1979). It is a viral disease against sheep and goats herds and is considered as the strongest limiting factor to demanding sheep and goat farming in developing countries (Abubakar and Munir, 2014).

PPR has been recognized in Pakistan since 1991, earlier on the basis of clinical signs (Pervez *et al.*, 1993; Athar *et al.*, 1995; Amjad *et al.*, 1996) and later by using the advanced diagnostic techniques like Enzyme Linked Immuno-Sorbent Assay (ELISA) and Polymerase Chain Reaction (PCR) (Khan *et al*. 2007; Abubakar *et al.*, 2008; Munir *et al.*, 2012).

Under the existing production and marketing system in Pakistan, sanitation and prophylactic vaccination are two workable options for control of PPR in the country. Limited work has been documented regarding the occurrence of PPR in Pakistan. Present study was conducted to determine the field epidemiology as well as genetic analysis of PPRV in sheep and goats.

MATERIALS AND METHODS

Field epidemiology of PPRV

A total of eighty-four PPR outbreaks were investigated during the study period (2010 to 2013). A case definition was established as a sheep or goat having a combination of respiratory and alimentary symptoms with fever.

Swabs from the conjunctival discharges, nasal discharges and buccal mucosae were collected from

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diseased animals for the detection, identification and genetic characterization of PPRV by using conventional as well as molecular tools (Diallo *et al*., 1988; Libeau *et al*., 1995; Forsyth and Barrett, 1995).

The samples were tested by Immuno-capture ELISA which was performed as the method adopted by Anderson and McKay (1994) with the help of Kit produced by BDSL Company, UK with the collaboration of Flow Laboratories and CIRAD, EMVT, France.

Genetic characterization of PPRV

A RT-PCR technique based on the amplification of the N and F genes proteins was standardized for the specific diagnosis and confirmation of PPR (Forsyth and Barrett, 1995). Representative PPRV isolates (n= 12) from different outbreaks were processed for sequencing to provide phylogenetic analysis of nucleoprotein and fusion protein gene segments to get an insight genetic picture of PPRV as well as its cross border transmission. The PCR products were purified using Purification kit (Invitrogen, Pure link PCR purification kit, Cat # k3100-01). Then the purified samples were sequenced on DNA sequencer (GeXP, BackMann Coulter, USA).

Sequence data analysis

The consensus sequences were then included in a dataset containing either all the available sequences (N gene sequences) in the Gen-Bank or representative sequences for each lineage (N and F gene sequences). All sequences datasets were aligned in the BioEdit using ClustalW algorithm and were cut to equal length. Construction of phylogenetic trees was performed with the neighbor-joining method using Kimura two-parameter model in MEGA5 version 5 (CEMI, Tempe, AZ, USA) with 2000 replications. The bootstrap values lower than 50% were not shown in the tree. Horizontal distances are proportional to sequence distances. Sequencing was carried out in National Veterinary Laboratory, Islamabad, Pakistan on GeXP, BackMann Coulter, USA (Supplementary Figs. 1 and 2).

RESULTS

Area-wise occurrence of PPR

During the period of 2010 to 2013, a total of eighty-four (84) PPR outbreaks were attended and investigated. A total of 471 samples were taken out of which 288 samples were found positive for PPRV antigen giving a positive percentage of 61.15% (Table I).

The disease outbreaks occurred throughout the country. The highest number of outbreaks was reported from Punjab province followed by Sindh and KPK (Khyber Pakhtunkha). More number of positive samples was from ICT (Islamabad Capital Territory) as compared to other regions (Table I).

Table I.- Province/Region-wise summary of PPR outbreaks and samples tested.

Province/ Region	No. of outbreaks	Samples tested	Positive	% Positive
Punjab	38	216	125	57.87
KPK	10	59	35	59.32
Baluchistan	3	15	9	60.00
Sindh	21	120	81	67.50
AJK	5	24	13	54.17
Gilgit	2	9	4	44.44
Islamabad	5	28	21	75.00
Overall total	84	471	288	61.15

In the Punjab province, the highest number of outbreaks was confirmed from northern Punjab (65.79%) as compared to other regions. In Northern Punjab, the highest occurrence was in Attock (73.33%) followed by Chakwal and Rawalpindi. In central Punjab, the highest occurrence was in Jhang (64.29%) followed by Hafizabad and Faisalabad. In South Punjab, the highest occurrence was in Bahawalpur (77.78%) while Bahawalnagar and Layyah were at second and third place, respectively (Supplementary Table I).

In KPK, the highest occurrence was in Abbotabad (64.29%) followed by Charsadda and Peshawar. The overall occurrence of PPR positive samples was 59.32%. In Baluchistan, the percent positivity was 60%. In Sindh, the highest number of positive occurred in Mithi (75%) followed by TandoJam and Hyderabad.

In Azad-Jammu& Kashmir (AJK), the highest occurrence was in Kotli (80%) followed by Muzaffarabad and Mirpur. In Gilgit-Baltistan, it was 44.44% as compared to ICT (Islamabad Capital Territory) it was the highest as 75% (Supplementary Table I).

Seasonal occurrence of PPR

The disease outbreaks occurred throughout the study period which was highest in the year 2011 followed in 2013 while in comparison, these were less in 2010 and 2012 (Table II) which is suggestive that a cyclic pattern of disease occurred. Although the disease is not considered as seasonal but as we compared the data of four years, the disease outbreaks occurred in higher number from October to February.

Species-wise occurrence of PPR

PPR outbreaks affected both sheep and goats. In sheep, disease was less severe as compared to goats (Table III). In 48 out of 84 outbreaks, disease occurred

in goats only while 18 outbreaks affected sheep only while remaining occurred in mixed herds. A total of 6221 animals were affected in these outbreaks. Sheep were less severely affected in comparison with goats as morbidity, mortality as well as case fatality rates were higher in goats. The morbidity rate was 26.79% in sheep in comparison with 34.90% in goats. The mortality rate was 10.83% in sheep as compared with 16.34% in goats. Similarly case fatality rate was 46.82 in goats which was slightly higher than sheep that was 40.41% (Table III). Statistical analysis showed p-value of 0.099 which is non-significant and depict that both specie are equally susceptible to PPRV. As the mortality rates are quite less as reported by the literature so it shows the possible endemicity of disease.

Table II.- Month-wise summary of PPR outbreaks and samples tested.

Months	Years-wise No. of outbreaks			
	2010	2011	2012	2013
January	2	4	3	4
February	2	3	2	3
March	1	3	1	1
April	1	1	1	1
May	1	2	1	2
June	1	1	0	0
July	0	1	0	1
August	2	2	1	1
September	1	2	1	1
October	1	3	1	4
November	2	4	1	3
December	2	3	2	4
Overall total	16	29	14	25

Age-wise occurrence of PPR

Overall, disease affected all three age groups of sheep and goats but the younger animals were more severely affected with a morbidity rate of 37.19%. The mortality and case fatality rates were also higher in young which were 46.86% and 17.39%, respectively (Table IV).

In sheep, the disease caused high morbidity and mortality rates in animals with age group less than 6 months which were 35.71 and 15.25%, respectively. The case fatality rate was almost giving a similar trend in all age groups while mortality rate was higher in younger animals as compared to aged (Table IV).

Table III.- Species-wise distribution of PPR in sheep and goats.

Species	Morbidity	Mortality	Case fatality
Sheep	688/2568 (26.79%)	278/2568 (10.83%)	278/688 (40.41%)
Goat	1275/3653 (34.90%)	597/3653 (16.34%)	597/1275 (46.82%)

Chi-test (p-value) = 0.099.

In goats, the morbidity, mortality and case fatality rates were higher in first two age groups in comparison with animals with age more than 2 years (Table IV).

Statistical comparison of age groups between both species showed p-values of 0.384, 0.002 and 0.061 in age group 1, 2 and 3, respectively. This depicted that there is significant difference in second age group (6 months to 2 years) towards disease susceptibility and goats are more susceptible than sheep.

Genetic characterization of selected isolates

The sequenced samples of PPRV during this study (total 12 isolates which were selected based on geographical distinction) indicated similarity and were clustered in Lineage IV which is for Asian isolates (labeled in Phylogenetic trees as in Table V).

Detection and confirmation of PPRV by F and N gene based RT-PCR amplification with primers F1b/F2d and F1/F2, resulted an expected amplicon of 328 and 372bp, respectively. A total of 12 representative samples, were selected for sequencing. The viruses of the different locations (Islamabad, Taxilla, Khaniwal, Layyah, Attock, Faisalabad, Sargodha, Rawalpindi, TandoJam and Mithi) were analyzed for molecular epidemiology. The results of the phylogenetic tree indicated that all Pakistani PPRV strains, regardless of the gene used either F or N, were clustered in lineage IV which is the most prominent and prevalent lineage of Asia (Figs. 1, 2).

Table IV.- Age-wise distribution of PPR in sheep.

	Distribution of PPR								
	< 6 months			6 months- 2 years			> 2 Years		
	MB	MT	CF	MB	MT	CF	MB	MT	CF
Sheep	370/1036 (35.71%)	158/1036 (15.25%)	158/370 (42.70%)	202/932 (21.67%)	72/932 (7.73%)	72/202 (35.64%)	116/600 (19.33%)	48/600 (8.00%)	48/116 (41.38%)
Goats	537/1403 (38.28%)	267/1403 (19.03%)	267/537 (49.72%)	447/1296 (34.49%)	213/1296 (16.44%)	213/447 (47.65%)	291/954 (30.50%)	117/954 (12.26%)	117/291 (40.21%)

MB, morbidity; MT, mortality; CF, case fatality.

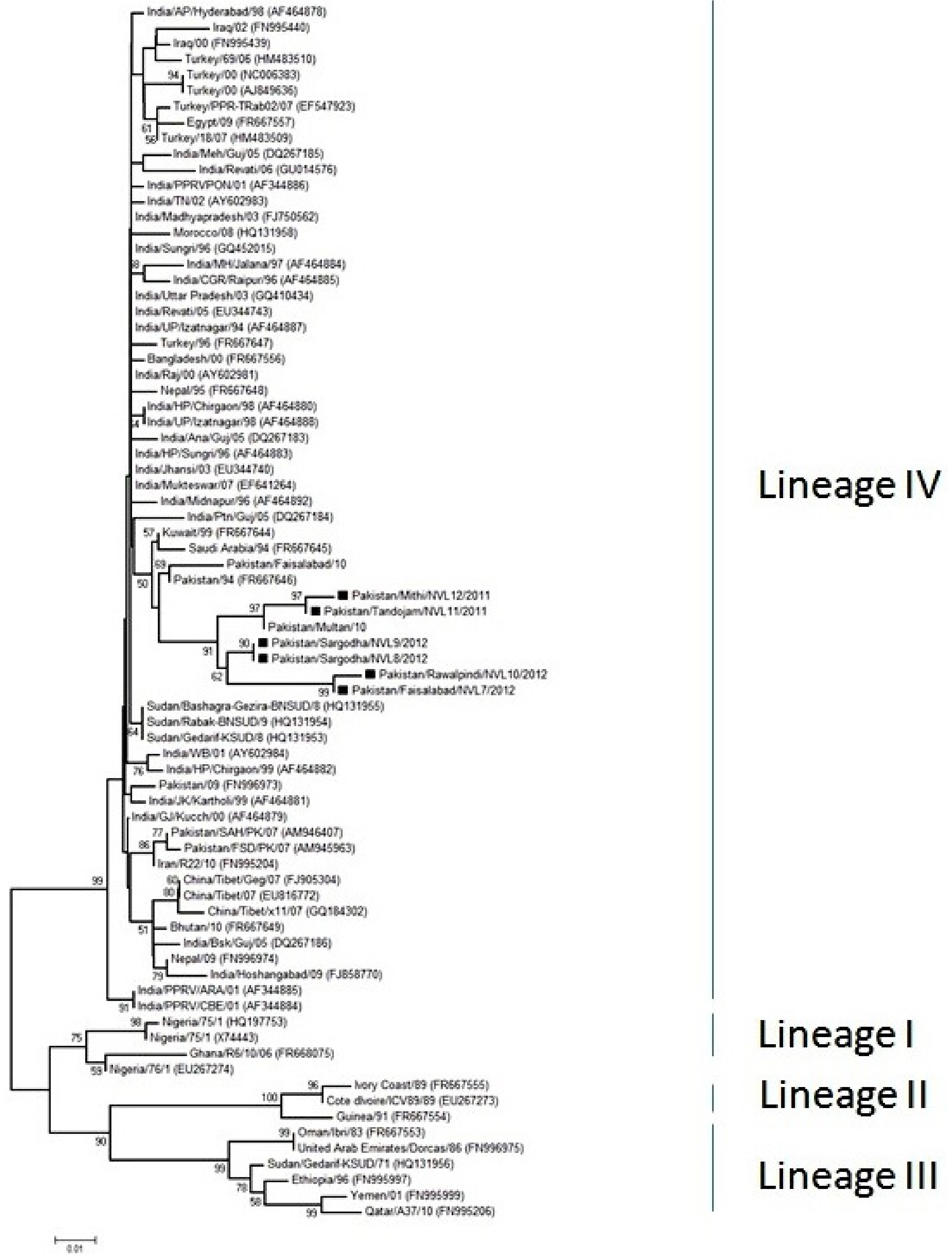

Fig. 1. Phylogenetic analysis of PPRV isolates with respect to F-gene.

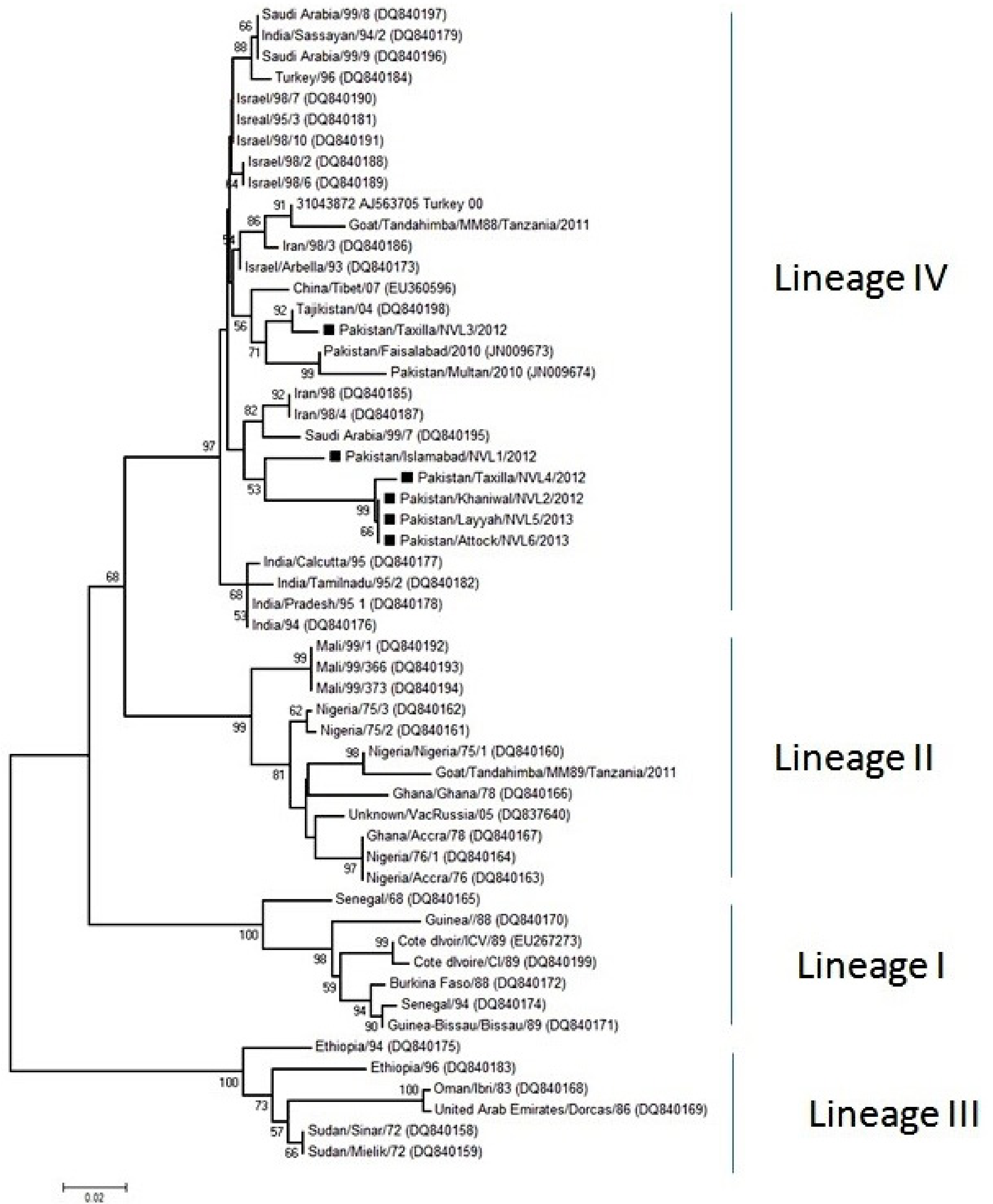

Fig. 2. Phylogenetic analysis of PPRV isolates with respect to N-gene.

Table V.- List of PPRV isolates used for the sequencing of F and N gene.

Description of isolate	Area of Outbreak	GenBank accession numbers
Used for N-gene		
Pakistan/Islamabad/NVL1/2012	Islamabad	KU991159
Pakistan/Khaniwal/NVL2/2012	Khanewal	KU991160
Pakistan/Taxilla/NVL3/2011	Taxilla	KU991161
Pakistan/Taxilla/NVL4/2012	Taxilla	KU991162
Pakistan/Layyah/NVL5/2013	Layyah	KU991163
Pakistan/Attock/NVL6/2013	Attock	KU991164
Used for F-gene		
Pakistan/Faisalabad/NVL7/2012	Faisalabad	KU991153
Pakistan/Sargodha/NVL8/2012	Sargodha	KU991154
Pakistan/Sargodha/NVL9/2011	Sargodha	KU991155
Pakistan/Rawalpindi/NVL10/2012	Rawalpindi	KU991156
Pakistan/TandoJam/NVL11/2011	TandoJam	KU991157
Pakistan/Mithi/NVL12/2011	Mithi	KU991158

Sequence identity

Using Clustal W alignment tools within BioEdit, the sequences for the both the F and N genes were aligned and their nucleotide and amino acid similarity was graphically presented. All the sequences reported are highly similar between each other and have at least 15 substitutions in the entire stretch of N gene whereas 11 substitutions in the F genes. Majority of these substitutions are synonymous and thus do not impact the final amino acid sequence where they have shown high level of amino acid identity in all isolates except the Faisalabad strain. This strain, due to shift in the open reading frame, has unique sequence compared to the rest of Pakistani strains reported. It would be worth considering the exact biological role of these substitutions and their impact on the pathobiology of PPRV in future.

Percent identity and divergence matrix

Direct comparison of the nucleotide sequences of all reported Pakistani strains of PPRV in this study, it was evident that these share high nucleotide identity from 93% to 99% based on the N gene whereas from 94% to 100% in case of the F gene. The corresponding divergences indicated the report of unique strains reported from individual outbreaks and the internal variations between these strains of PPRV prevalent in the small ruminant population of Pakistan.

DISCUSSION

The present study provided substantial evidences on the endemicity of PPR in about variation in the incidence rate of PPR in different areas, during different season in sheep and goats of different age groups. Results of study demonstrated that highest number of outbreaks occurred in Punjab during the study period as compared to other provinces of the country. This could be due to a better disease reporting system of the Punjab as compared with other provinces. Few reports were also documented outbreaks from Punjab previously (Khan *et al*., 2007; Abubakar *et al*., 2008). Field study of Punjab also demonstrated that more number of PPR cases was reported from Southern Punjab than any other Punjab region. Zahur *et al*. (2006) has also reported similar findings from Punjab but their data size was less as compared to present study.

Temporal evaluation of outbreaks gives us a wave like pattern of disease outbreaks *i.e.*, rise in one year and decline in the next year repetitively. This specific pattern can be linked to decrease in vaccination during the year when PPR cases are lower that result in more outbreaks in coming year. The present study also showed the seasonal variation of PPR outbreaks over a longer period of study. During the study period it was observed that PPR incidence is more during winter season. This can be linked to migration of susceptible, unvaccinated sheep and goat herds from mountains to plains of the country. These results are in contrast to other studies in Pakistan that reported few seasonal trends of PPR but for shorter periods of study (Zahur *et al*., 2009, 2011; Abubakar *et al*., 2011; Ullah *et al*., 2015). In contrast to other studies from the world, Opasina (1983) also proved in western Nigeria that PPR incidence is increased during cold season.

In our study, all the sequenced isolates were related to PPRV lineage IV which is the Asian lineage and has been found in most of the region. The sequence and Phylogenetic analysis revealed that two groups; one of them was clustering with other previous Pakistani isolate while the others were grouped with the regional strains like India and Iran. Anees *et al*. (2013) conducted a similar study and specified the grouping of the sequences in lineage IV along with PPRV strains from Asian and Middle East. In contrast to our findings, Kumar *et al*. (2014) studied a PPRV isolate from an outbreak that occurred in sheep and goats in Nanakpur village of Mathura District in Uttar Pradesh (India) and their findings were that the PPRV/Nkp1/2012 may not be closely related to lineage IV PPR viruses believed to be there in the Indian subcontinent. Munir *et al*. (2012) examined samples from two different outbreaks of PPR in goats and its phylogenetic analysis revealed that Pakistani samples clustered with Chinese,

Tajikistani and Iranian isolates, which is indicative of the true geographical pattern of PPRV. In a similar study by Munir *et al*. (2013) on the samples of PPR suspected outbreaks from different regions of Pakistan, fusion and nucleoprotein genes were sequenced.

The sequence identity pointed to the possibility of at least one group of PPRV from a diverse source and its passage in the small ruminants of Pakistan. In another study from the neighboring country India, Balamurugan *et al*. (2010) performed comparative sequence analyses of four genes (nucleocapsid (N), the matrix (M), the fusion (F) and the haemagglutinin H) of their isolates with already published sequences and found an identity of 97.7-100% and 97.7-99.8% among the Asian lineage IV and 89.6-98.7% and 89.8-98.9% with other lineages of PPRV at nucleotide and amino acid levels, respectively.

CONCLUSION

In conclusion, the rapid detection by suitable and appropriate methods of antigen and nucleic acid detection of PPRV in infected animals will assist in early judgment of infection and subsequently control of disease in Pakistan.

ACKNOWLEDGEMENTS

The study was supported by FAO Regional Project (GTFS/INT/907/ITA) and FAO PPR Project (GCP/PAK/127/USA). We would like to thank the field veterinarians and support staff for the sample collection and outbreak reporting.

Statement of conflict of interest

Authors declare that there is no conflict of interest.

Supplementary material

There is supplementary material associated with this article. Access the material online at: http://dx.doi.org/10.17582/journal.pjz/2018.50.2.559.566

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