Original Research

Phylogenetic Analysis of Non Structural Protein in *Peste des petits ruminants* (PPR) Virus

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Abstract

Peste des petits ruminants (PPR) is an acute, highly contagious, and economically important transboundary viral disease of sheep and goats associated with high morbidity and mortality. The PPR virus virion exhibits 6 major proteins, on surface, nucleocapsid. The proteins play an important role in viral pathogenesis and its survivability in host. The protein inherits structure to function relationship and needs to be studied closely. The genetic diversity of non-structural V protein encoding region of P gene (894bp) of PPR virus was accomplished by using phylogenetic analysis of different strains of PPRV. Analysis of the sequence revealed that it could demonstrate the genetic diversity among various strains of PPRV. Based on the V protein tree, the PPRV was categorized into three different clades each representing different lineages. The data could be validated for the survey of viral dynamics, epidemiological studies as well as prevention and control of the disease.

Key words: PPRV, Non-Structural Protein, V Protein, Phylogeny

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Introduction

PPR is one of the most devastating, acute viral diseases of small ruminants caused by PPR virus (PPRV), a single stranded negative sense Morbillivirus that belongs to the Paramyxoviridae family (Gibbs *et al.*, 1979) with other members of the genus, which include rinderpest virus (RPV), measles virus (MV), canine distemper virus (CDV), phocine distemper virus (PDV) and dolphin and porpoise morbillivirus

(DMV) (Barrett *et al*., 1993). The virus is a pleomorphic particle with a lipoprotein membrane enveloping a ribo-nucleoprotein core, which contains RNA genome (Haffar *et al*., 1999). The genome is a negative sense single stranded-RNA, approximately 16 Kilo bases (kb) long with negative polarity (Balamurugan *et al*., 2014). The genes are arranged in the order of 3′ N–P/C/V–M–F–H–L 5′ (Bailey *et al*., 2005; Diallo, 1990). This disease is highly contagious in goats whereas sheep and other related wild animals seem to suffer a comparatively lesser insult. For a very long time this disease was thought to be caused by a variant of rinderpest virus, adapted to small ruminants as PPR clinically mimics cattle plague (Rinderpest), characterized by fever, erosive stomatitis, enteritis, pneumonia, and death (Gibbs *et al.*, 1979). This disease gained momentum when a severe case of RP like ailment was recorded in the herds of sheep and goat which failed to cause any clinical signs in the cattle that were reared in the close vicinity (Taylor and Abegunde, 1979; Taylor *et al*., 1990). The disease was reported in 1942 for the first time in the Côte d' Ivoire (Gargadennec and Lalanne, 1942; Kumar *et al*., 2014) and was designated as a separate member of the genus Morbillivirus in 1979 (Gibbs *et al.*, 1979). After its designation, the prevalence of PPRV has been reported from sub Saharan Africa, the Middle East, Turkey and the Indian subcontinent. Recently, outbreaks have been reported for the first time in China, Kenya, Uganda, Tanzania, Morocco and Tunisia (Anees *et al*., 2013)

India is home to 65.06 million sheep and 135.17 million goats (19th Livestock census, 2012). The goat population in our country has declined by 3.82% over the 18th census of livestock and one of the major reasons for this drastic decline in the goat population is PPR outbreaks (Balamurugan *et al*., 2012). In India, PPR is enzootic and the outbreaks are reported around the year (Dhar *et al.*, 2002). Although the disease was reported for the first time in 1989 in Tamilnadu (Shaila *et al*., 1989), during 1993-1994 various veterinary laboratories across the nation reported outbreaks of pasteurellosis which were typical PPR cases. The economic importance of PPR is mainly due to its contagious nature, with a case fatality rate as high as 100%. In India, PPR causes a net loss of $2500 millions/year (Balamurugan *et al.*, 2012). PPRV has caused numerous serious epidemics in small ruminant populations across sub-Saharan Africa, the Middle East as well. In the last decade, PPRV has shown to extend its range southward in Africa as far as southern Tanzania (2008) and the Democratic Republic of Congo and Angola (2012). Outbreaks have also been reported from North Africa, including within Tunisia (2006), Morocco (2008), and Algeria (2011). Cases of the disease have also been reported from Europe. During, 2011–2012 approximately 20 laboratory-confirmed PPR outbreaks in sheep and goats were reported from Turkey. In southwestern Asia, the virus spread to Tibet (2007) and has recently been reported throughout China (2013–2014). Although the factors that contributed to the emergence and the spread of the disease is still unclear, the health of millions of small ruminants across these regions is now at risk.

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The molecular epidemiology of PPRV, which is based on sequence comparison of a small region of the fusion (F) gene (322 nt) or the nucleoprotein (N) gene (255 nt), has identified 4 distinct lineages (I–IV) of PPRV (Banyard *et al*.,2010). However, this analysis has not generated much information on the evolution and dispersal of PPRV lineages. Lineage I PPRV had gone undetected for 19 years being detected in Senegal in 1994. Lineage IV PPRV, which was believed initially to be confined to India and the Middle East, now has a wider geographic presence and appears to be evolving rapidly. Many aspects of PPRV evolution, such as ancestral virus location, divergence and time of origin, and historical and geographic patterns of spread, are poorly understood (Muniraju *et al*., 2014). A better understanding of the evolution of PPRV would enable prediction of how these viruses will lead to further outbreaks and epidemics and provide data for control strategies. Advanced sequencing tools have enabled molecular epidemiologic studies of viruses in which whole gene and complete genome data can be used to improve and elucidate the evolutionary dynamics of viral infectious disease. Here, the V protein coding region of the P gene was analysed and the genetic diversity was evaluated. The phylogenetic tree was constructed to support the evidence of genetic diversity of the PPRV.

Materials and Methods

Propagation and Titration of PPRV

B95a cells procured from Division of Biological Products, IVRI, Izatnagar were propagated in RPMI-1640 medium containing 10% Fetal Bovine Serum (FBS) in a CO_2 atmosphere. The cells were maintained in the same medium containing 2% FBS. The vaccine strain of PPRV, Sungri/96 (Indian isolate belongs to genetic lineage IV) which was originally isolated in Vero cells adapted to B95a cells by subsequent passage (5^{th} passage level). This B95a adapted virus was used in further studies. The virus was propagated by infecting B95a cells, cultured in 75 cm^2 cell culture flasks in RPMI-1640 medium containing 5% FBS at multiplicity of infection (MOI) of 0.1 CCID50/ml. When viral cytopathic effect (CPE) is almost complete (~80%), the cells were freeze-thawed 3 times and aliquots were stored at -80°C. To study the infectivity titer of PPRV, virus titration was performed (Mariner *et al*., 1990). Briefly, B95a cells were subcultured and 100 µl of the cell suspension was added to each well (0.5×10^6 cells/ml) in 96 well tissue culture plates. Plates were incubated at 37°C in a 5% CO_2 incubator. The next day, virus was diluted 10-fold in RPMI-1640 containing 2% FBS in deep well plate. An aliquot of 100 µl of virus suspension was added to each well keeping four replicates per dilution. One well was kept as control (without virus) per replicate. The plates were incubated at 37°C in the presence of 5% CO2. Media were replaced each alternate day with RPMI-1640 containing 2% FBS. Reading was taken on the sixth day of titration. End points were calculated using Reed and Muench method (1938).

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Isolation and Quantification of Viral RNA

The viral RNA was extracted using Pure Link® Viral RNA/DNA Mini Kit (Thermo scientific, Lithuania, EU) as per the manufacturer's instructions. RNA concentration was determined using a biophotometer (Eppendrof, Hamburg, Germany). The quality of RNA was tested by evaluation on 1.8% TAE-agarose gel before being used for cDNA production. To exclude genomic DNA contamination, total RNA was treated with RNase free DNase.

First Strand cDNA Synthesis

Total RNA was reverse transcribed into cDNA in a 20 µl reaction mixture using revert aid first strand cDNA synthesis kit (Thermo scientific, Lithuania, EU) according to the manufacturer's instructions. cDNA was synthesized using 125 ng of sample RNA as template and oligo dT primers. A mixture without the reverse transcriptase enzyme was run in parallel to those with the enzyme included to ensure that there was no disturbance from genomic DNA.

Preparation of the Gene Construct

The nucleotide sequence of the PPRV-Vprotein (vaccine strain Sungri-96) was obtained from the GenBank (ID No. KJ956930.1). For amplifying target DNA, both forward and reverse primers were designed by adding the sequence identical to the end of the vector adjacent to the cloning site as per-Expresso Rhamnose SUMO Cloning and Expression System. The primer details for V protein have been presented in Table 1.

Table1: Primers for V protein

Forward	3'CGCGAACAGATTGGAGGTCAGAAGGGGCTGGAATGTA5'
Reverse	3'GTGGCGGCCGCTCTATTAGTACCAGATTCTTGTGTCGACT5'

PCR amplification of desired coding sequence for V protein encoding gene was performed using the viral cDNA as template by Taq Polymerase (GCC Biotech). The samples were run in 50 µl reaction mixture which consisted of 38 µl PCR-nuclease free water, 5 µl 10× Taq buffer, 1 µl dNTP mix (10 mM), 1.25 µl each forward and reverse primers (10 µM), 1 µl Taq DNA Polymerase and 2.5 µl DNA template. The PCR (Quanta biotech S-96 End PCR) was run with initial denaturation at 94 °C for 3 min, followed by 40 cycles of denaturation at 94 °C for 30s, annealing at 65 °C for 30 s and extension at 72 °C for 1 min, followed by final extension at 72 °C for 10 min. The confirmation of amplification of specific PCR amplicon was done by agarose gel electrophoresis. Briefly, the gel (1%) was run at a voltage of 5 V/cm using 100bp molecular weight marker along with the PCR amplicon. The bands were visualized under Safe light non UV documentation system (Chromus Biotech Pvt. Ltd). After confirming the PCR amplicon of desired product length, it was extracted from the agarose gel using Pure Link Quick Gel

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Extraction Kit (Invitrogen) as per manufacturer's instructions. The PCR product (DNA) was eluted and the concentration was measured in nanodrop spectrophotometer. The ligation free transformation of competent cells using the pRham vector was done by the Expresso Rhamnose SUMO cloning and expression system (Lucigen, USA). Briefly, the *E. coli* 10G competent cells and recovery medium were taken out from the −80 °C freezer and thawed completely over ice. Then 2 µl (25 ng) of pRham vector DNA and 1–3 µl (25–100 ng) of insert PCR product were added to the competent cells. The mixture was stirred briefly with pipette tip and incubated on ice for 30 min. Subsequently, the cells were given heat shock by exposing them to 42 °C in water bath for 45 s followed by returning the tube of cells over ice for 2 min. Recovery medium (960 µl) was added to the cells in culture tube and the tube was then placed in a shaking incubator at 250 rpm for 1 h at 37 °C. 100 µl of transformed cells were plated on LB agar plates containing 30 µg per ml Kanamycin antibiotic and plate was incubated overnight at 37 °C. The colonies which grew on agar LB agar plate overnight culture were picked for screening for the presence of inserts by colony PCR with the previously mentioned mixture of Taq DNA polymerase, reaction buffer, dNTPs, both forward and reverse primers and template DNA isolated from recombinant colonies. The amplicon length of desired insert was confirmed by agarose gel electrophoresis under UV light. Recombinant clones represented as white colonies as well as colony PCR positive were picked up and inoculated into 5ml of LB broth containing Kanamycin (30 µg per ml) and grown overnight at 37°C in a shaker incubator for plasmid isolation and characterization.

Isolation and Sequencing of Plasmid

Plasmid was extracted with AuPrep SPIN Miniprep Kit [Life Technologies India (Pvt.) Ltd, New Delhi, India] following manufacturer's protocol. Briefly, Five millilitres of overnight culture was taken in a 2.0 ml microcentrifuge tube and centrifuged at 8,000 x g for 2 min. Supernatant was discarded and the pellet was resuspended with 250 µl of MX1 (supplied by manufacturer) by vortexing. About 250 µl of MX2 (supplied by manufacturer) was added to it and was mixed gently by inverting until lysate becomes clear. The mixture was kept for 1- 5 min at RT. About 350 µl of MX3 (supplied by manufacturer) was added to it, inverted upside down to mix gently. The mix was then centrifuged at 13000 x g for 5 min and the supernatant was transferred to a SPIN^{m} Column onto a collection tube. The spin column was centrifuged for 60 sec at 8000 x g. The flow-through was discarded; the column was washed once with 0.5 ml WF buffer (supplied by manufacturer) by centrifuging for 60 sec. The flow-through was discarded. The column was washed once with 0.7 ml WS (supplied by manufacturer) buffer by centrifuging for 60 sec and the flow-through was discarded. The column was centrifuged at full speed for another 3 min to remove residual ethanol. The column was placed onto a fresh 1.5 ml microcentrifuge tube; 50 µl of NFW was added onto the column which was then left to stand for 1-2 min and centrifuged for 1-2 min to elute

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DNA. The eluted DNA was preserved at -20°C for future use. DNA sequencing was performed in both forward and reverse direction based on nucleotide sequencing using ABI 3730 (48 capillary) electrophoresis instruments (Sanger Bioserve, Hyderabad). The nucleotide sequence data were analyzed using software of ABI PRISM Model 3100 version 3.7 and BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) in comparison to V protein of the other PPRV that have been previously reported in GenBank(Supplementary Information).

Phylogenetic Analysis

The V protein coding region of P gene nucleotide sequence data of the PPRV available at GenBank were retrieved based on the BLAST results regarding the location and the year of isolation (Table 2).

Table 2: V protein coding region of P gene of *Peste des petits ruminants virus* isolates used for phylogenetic analysis

Virus Isolate	Specific Host/Lab Host	Lineage	Accession Number
Netherlands/Sungri/1996	Goat	IV	KJ867542.1
India/Izatnagar/1994	Goat	IV	KR140086.1
India/Sungri/1996	Goat	IV	AY560591.3
Synthetic construct	Synthetic construct	IV	KJ956930.1
Morocco/2008	Alpine goat/Vero cell line	IV	KC594074.1
China33/2007	Goat	IV	KX421388.1
Algeria/S15/2015	Goat	IV	KY885100.1
China/Tibet/2007	Goat	IV	JF939201.1
China/Tibet/Geg30/2007	Goat	IV	FJ905304.1
China/Tibet0701/2007	Small ruminant	IV	EU360596.1
China/CQ/2014	Goat	IV	MF443353.1
PPRV-FY/China/2015	Goat	IV	KX354359.1
Ethiopia/2010	Goat	IV	KJ867541.1
China/Tibet Bharal/2008	Wild bharal	IV	JX217850.1
Turkey/2000	Sheep/Vero cell line	IV	AJ849636.2
Mongolia/9/2016	Goat	IV	KY888168.1
India/Tamil Nadu/04/2015	Sheep	IV	KT860065.1
India/Tamil Nadu/02/2014	Goat	IV	KT860063.1
India/Tamil Nadu/01/2014	Goat	IV	KT270355.1
India/Tamil Nadu/Gingee/2014	Goat	IV	KR261605.1
India/Delhi/05/2016	Goat	IV	KX033350.1
India/ Izatnagar/Sungri96/2016	Goat	IV	KF727981.2
Nigeria/1975/1	Goat	II	HQ197753.1
Nigeria/Phosphoprotein/1975/1	Goat	II	AJ298897.2
Benin/10/2011	Sheep	II	KR781449.1
Ghana/NK1/2010	Sheep	II	KJ466104.1
Libya/2015	Goat	II	KU236379.1
UAE/1986	Dorcas gazelle	III	KJ867545.1
Oman/1983	Goat	III	KJ867544.1
Uganda/2012	Goat	III	KJ867543.1
Kenya/KN5/2011	Goat	III	KM463083.1
Ethiopia/1994	Goat	III	KJ867540.1
Canine distemper	Canine	---	M32418.1

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The retrieved data were aligned separately using the Clustal-W algorithm incorporated in DNAStar Lasergene Version 6.0 and MEGA 6.0.Phylogenetic analyses was carried out using the neighbour joining(NJ) method for all regions numbered according to the start codon in each gene) following the Tamura 3-parameter nucleotide substitution model. Sequences that did not fit these parameters or containing gaps were removed from the data set. NJ tree was then constructed using the distance matrices in MEGA 6.0 with 1000 bootstrap replicates to test the robustness of the tree topology using *Canine Distemper* P and C protein as the outgroup. Gamma parameter was set as 0. Gaps were treated by pair-wise deletion, *i.e*. ignoring only those gaps that are involved in the comparison of a pair of sequences. Sequence homology was determined using MEGAlign software(Supplementary Information).

Results

Upon PCR amplification of V protein coding region of P gene, the product of 894 bp in size was obtained. After ligation and transformation processes of this PCR product, positive white colonies were randomly selected. BLAST data of this V protein coding region of P gene sequence revealed that it was corresponding to that of Peste-des-petits-ruminants virus isolate Sungri 1996 MSD (The Netherlands) (KJ 867542.1) identity of 99%. The phylogenic tree constructed from the complete V protein coding region of P gene nucleotide sequence (894 bp) in comparison to those of PPRV strains from GenBank. The phylogenic tree inferred from the data clearly showed the genetic diversity of the virus. According to the tree, three main clades (Clade 1, Clade 2, and Clade 3) of V protein coding region of P gene of PPRV were clearly separated with a bootstrap value 98% (Fig.1). Clade 1 was further divided into 7 sub-clades (SC); SC 1a-1f.SC 1a contained our query sequence (Vprotein Sungri India) along with V protein coding region of P gene of PPRV/Sungri (AY560591.3; KJ867542.1; KR140086.1) and synthetic construct V protein gene (KJ956930.1) with bootstrap value 99%. Two virus sequences from china (MF443353.1, 2014 and KX354359.1, 2015) and a sequence from Mongolia (KY888168, 2016) were clustered within SC-2 with bootstrap value 99%. SC-3 has two virus sequences clustered together with bootstrap value 99% namely; Turkey (AJ849636.2, 2000) and Sungri/96(KF727981.2, 1996). The Ethiopian viral sequence (KJ867541.1) with bootstrap value 98% consists of the SC-4. The viral protein sequence from Morocco (KC594074.1, 2008) and Algeria (KY885100.1, 2015) with bootstrap value 97% were clustered together in SC-5. Five viral sequences from China/Tibet (JX217850.1, 2008; KX421388.1, 2007; JF939201.1, 2007; FJ905304.1, 2007 and EU360596.1, 2007) make up the SC-6 with bootstrap value 100%. The Indian isolates under SC-7 form three distinct groups based on geographical location. The first group comprises of virus from Tamilnadu isolated in 2015 (KT860065.1), the second group consists of virus sequence from Delhi isolated in 2016 (KX033350.1) and the third group comprises of viral sequences isolated in Tamilnadu during 2014 (KT860063.1; KT270355.1 and KT261605.1).

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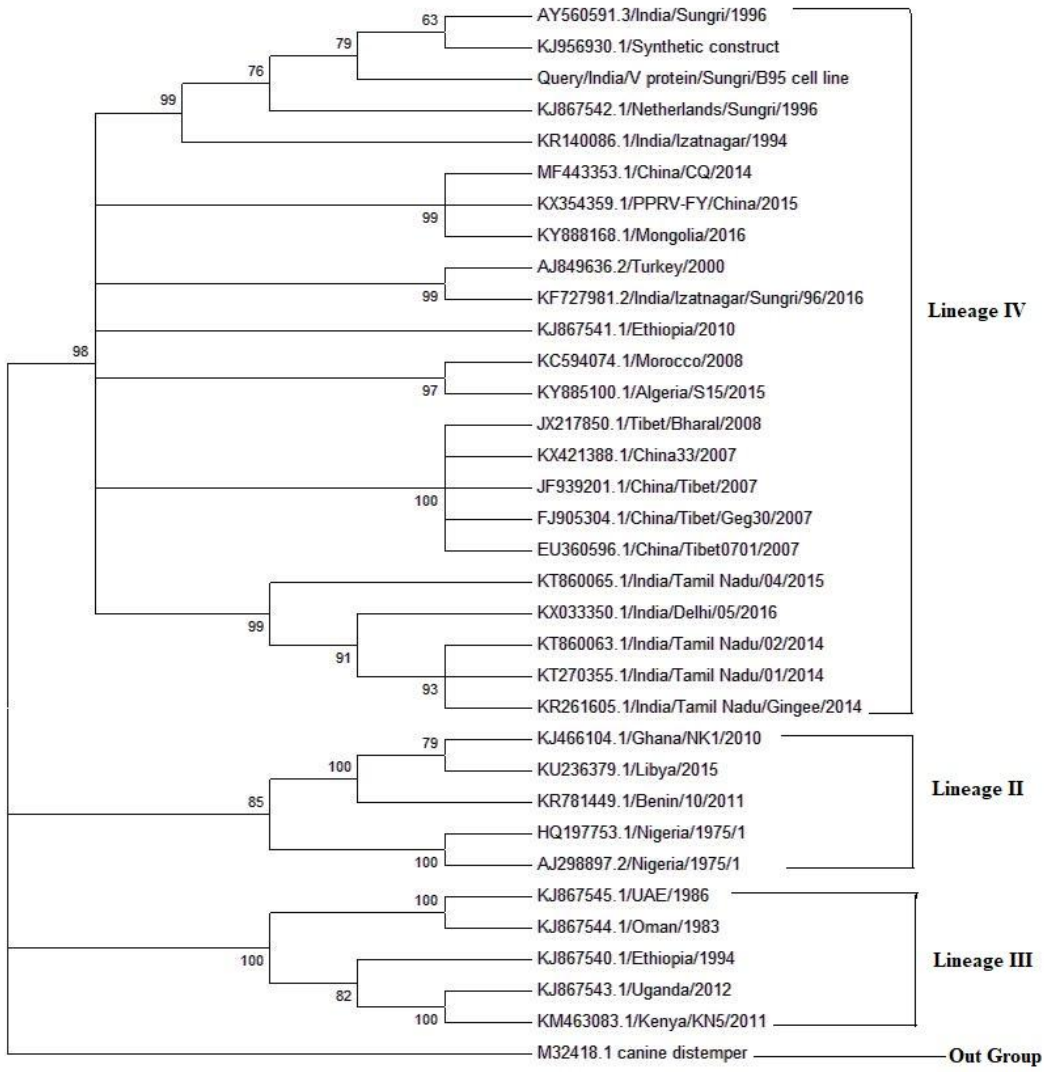

Fig. 1: Phylogenetic analyses was carried out using the neighbour joining (NJ) method for all regions numbered according to the start codon in each gene following the Tamura 3-parameter nucleotide substitution model. The numbers at the nodes represent the percentage of times the group occurred out of 1000 trees. Branches with less than 60% bootstrap support were collapsed. No significance is placed on the lengths of the branches connecting the species.

Within clade 2, there are two sub clusters SC-8 and SC-9 consists of virus sequences from West African countries. SC-8 consists of viruses from Ghana (KJ466140.1), Libya (KU236379.1), and Benin (KR781449.1) isolated between 2010 and 2015. Whereas SC-9 comprises of viral sequences isolated from Nigeria (HQ197753.1; AJ298897.2) in the year 1975. The clade 3 also comprises of two sub clusters SC-10 and SC-11.SC-10 comprises of isolates from Middle East (Oman and UAE) isolated during 1983 (KJ867544.1) and 1986 (KJ867545.1) respectively. Isolates from East African countries (Ethiopia, Uganda, and Kenya) that were isolated between 1994 and 2012 were clustered into SC-11. The nucleotide

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sequence homology of the query sequence was 89.2% - 99.6% with other sequences derived from Asian, African, and Middle Eastern Countries. Whereas the sequence homology with the sequences derived from Indian isolates was 96.2% - 99.4%.

Discussion

Being highly contagious and acute in nature, small ruminant borne *Peste des petits ruminants* (PPR) disease threatens the food security and livelihood of millions of farmers who depend on these animals for socio-economic stability. Being aware of its destructive nature, the Food and Agriculture Organization (FAO) and World Organization for Animal Health (OIE) have prioritized for complete eradication of peste des petits ruminants (PPR) disease that causes a very high mortality among small ruminants across Asia and Africa (Diallo, 2006; Oadhi and Ma, 2014). It is important to design effective control measures and know how these viral pathogens spread the disease as sudden outbreaks are rampant. One of the plausible methods to elucidate the genetic and evolutionary relationship between different viral isolates gathered from different geographical and agro-economical climatic zones is the incorporation of phylogenetic approaches to viral DNA. The phylogenetic analyses shall provide us with important insights about the transmission and the epidemiology of this viral disease. In the current study, we elucidate the evolutionary and epidemiological dynamics of PPRV through the analyses of the V protein coding region of P gene of Peste des petits ruminants virus isolates.

The phylogenetic tree was constructed using the available V protein coding region of P gene data in GenBank and V protein coding region of P gene data from the virus harvested in B95a cells. Three clades exist and each represent different lineage namely, clade 1 represents Lineage 1V, clade 2 represents Lineage II and clade 3 represents Lineage III. The clade 1 is further clustered into 7 subclades. All the viral sequences isolated from the Asian countries fall into these 7 subclades. Along with the Asian viral isolates, the viral sequences sampled from Eastern African country of Ethiopia and the North African counties of Algeria and Morocco. This is in accordance with works of Banyard *et al*., 2010. Although the presence of lineage IV PPRV in Africa came to light following the Moroccan outbreak in 2008, but its seems to have introduced in the 1990s that caused outbreaks in both small ruminants and camels (Kwiatek *et al.*, 2011). Studies from Sudan during 2000 and 2009 demonstrated how the virulent lineage IV PPRV has progressively replaced the pre-existing lineage III PPRV. This trans-boundary occurrence of disease illustrates a possibility of genetic drift following trans-boundary transmission. This also indicates the increase in the virulence of this lineage. The isolates sampled from Tamilnadu, India during 2014 and 2015 fall into the same subclades (SC-7) but different groups suggesting that these isolates are significantly distant to each other despite being related in the V protein coding region of P gene global tree. The detection of PPRV for the first time in 1996 and afterwards in Turkey has shown how illustrated

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the ability of the PPRV to spread to rest of Europe. SC-10 contains isolates from Oman and UAE suggest the transmission of virus the Asian countries to Middle Eastern countries mainly through animal trade.

Conclusion

In a nutshell, these findings demonstrate how PPRV is prevalent in Asian and African countries and posses threat to the ruminants population in rest of the World. Here, the isolates have been analysed based on available datasets and these datasets need enhancing to provide a better phylogenetic assessment. The presence of multiple subclades and groups within a country indicates the possibility of frequent mobility of the diseased individuals across the region. The sudden outbreaks in the recent years may also be attributed to rinderpest virus control and eradication that lowered the cross-immunity in small ruminants and increased their risk for PPRV.

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Conflict of Interest

The authors declare that they have no competing interests.

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