

# Genetic Characterization of Peste des Petits Ruminants Virus from Recently Emerging Wave of Outbreaks in Pakistan

## Summary

Peste des petits ruminants (PPR) is endemic in Pakistan and despite exhaustive vaccination, outbreaks are on the rise annually across different parts of the country. Clinical outcome is largely employed to diagnose disease, while detailed investigations on the genomic features of prevailing PPRV usually remain elusive. Here, we present comparative sequence based phylogenomic of field strains from three districts representing different agro-livestock production systems during an emerging wave of outbreaks in 2015, together with complete genome sequencing of one of the selected strains for the first time from Pakistan. The analysis revealed clustering of under-study strains to lineage IV close to isolates from India and China. Investigation of inter- and intra-lineage genetic distances revealed a higher genetic distance between study strain to lineage III viruses than lineages I and II. The strain showed a high percentage of genetic distance from ancestral isolates originating from **Nigeria** indicating a possible evolution of PPRV. Based on these observations, an integrated cross-protection investigation is warranted in the future, not only to define the protective efficacy of currently applied vaccines, but also to continuously elucidate the genomic and evolutionary nature of circulating viruses in the country to achieve PPR eradication by 2030.

**KEYWORDS** Peste des petits ruminants, Complete genome, Phylogenomic analysis, Percent identity, Genetic distance, Pakistan

## 1 INTRODUCTION

Peste des petits ruminants (PPR), caused by peste des petits ruminant virus (PPRV), is a contagious transboundary disease of small domestic and/or wild ruminants (Parida et al., 2015; Baron et al., 2016). The virus belongs to the genus *Morbillivirus* within the family *Paramyxoviridae* and consists of a non-segmented, negative sense, single stranded RNA genome that encodes six structural [nucleocapsid (N), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin (H) and polymerase (L)] and two non-structural (C and V) proteins (Gibbs et al., 1979). The virus has been classified into four distinct lineages (I-IV) on the basis of partial sequence of N (225 bp) and F (322 bp) genes (Parida et al., 2015). All four lineages have been extensively isolated from different African countries whereas, in Asian countries, only lineage IV has been implicated for the epidemics (Parida et al., 2015; Baron et al., 2016).

33 Pakistan has an agriculture-based economy and livestock plays an integral role in the  
34 sustainability of national economy. However, it is challenged by various infectious and non-  
35 infectious factors resulting in severe annual losses. Among infectious threats, the occurrence  
36 of PPR is the leading infection since first reported in 1991 in Pakistan (Amjad et al., 1996).  
37 Despite available disease control measures, a number of outbreaks are being reported across  
38 the country with subsequent economic impacts. The economic loss for [three PPR outbreaks](#)  
39 was estimated to be US\$ 12,211 including direct and indirect financial loss; [the number of](#)  
40 [outbreaks throughout a year could result in](#) US\$ 240 million annual losses (Abubakar &  
41 Munir, 2014). Given the country-wide disease situation and global PPR eradication program  
42 by 2030, control of PPR is a benchmark of global food security and poverty alleviation. We  
43 have previously provided data on the nature of PPRV in Pakistani small ruminants based on  
44 the partial N and F gene sequencing (Munir et al., 2012; Anees et al., 2013). However, in  
45 order to offer higher resolution analysis of the virus currently circulating in the country,  
46 phylogenomic analysis of the entire genome is imperative. The goal of the current study was  
47 to perform a genome based comprehensive phylogenetic and genetic distance analysis of the  
48 complete genome of PPRV for the first time in Pakistan, one of the most PPRV-affected  
49 countries around the world.

## 50 **2 MATERIALS AND METHODS**

### 51 **2.1 Study area and sampling**

52 During 2015, an emerging wave of outbreaks were observed in small ruminants originating  
53 from different districts of Punjab province of Pakistan. These outbreaks generally [infect](#) the  
54 entire affected herd, irrespective of species and breed. Additionally, these outbreaks emerged  
55 in early winter of 2015 with an unusually high rate of infectivity in herds. Clinical symptoms  
56 such as high fever, nasal and lacrimal discharge, erosions in the oral mucosae, severe  
57 diarrhoea and abortion in pregnant animals were suggestive of PPR. We collected and  
58 processed the clinical samples (blood, mucosal erosion, nasal and lacrimal discharge) from  
59 herds of sheep (n = 7) and goat (n = 11) originating from three districts as per convenience in  
60 logistics and farmer consent: Lahore (n = 7, two sheep and five goat), Faisalabad (n = 8, three  
61 sheep and five goat) and Layyah (n = 3, two sheep and one goat). The selected districts  
62 represent traditional and mixed agro-livestock production systems in the country. The number  
63 of animals in the herds ranged from 10-150 heads with age ranging from 3 months to 4 years.  
64 The breeds of animals included Beetal, Daira Din Panah, Teddy and non-descript for goat,  
65 and Kajli, Thalli and Lohi for sheep. Each studied herd had no previous history of  
66 vaccination. A majority of animals died within the first five days of clinical disease, with

67 morbidity and case fatality ranging from 70-90% and 40-100%, respectively. All animals  
68 were found to be equally susceptible within infected herds, irrespective of age and breed.

## 69 **2.2 Genome amplification and sequencing**

70 All 92 samples representing 18 herds were confirmed to be PPRV positive by polymerase  
71 chain reaction (Couacy-Hymann et al., 2002). One sample per district was processed for  
72 partial sequencing of the N-gene using the primers applied for the detection, while one was  
73 processed for complete genome sequencing. Briefly, viral RNA was extracted as per  
74 manufacturer's guidelines (QIAamp Viral RNA extraction Mini Kit, Qiagen, Valencia city,  
75 CA, USA). The partial N-gene (Couacy-Hymann et al., 2002) and whole genome was  
76 amplified by one-step reverse transcriptase polymerase chain reaction (RT-PCR) using virus  
77 specific primers (List of primers can be provided upon request). The amplified PCR products  
78 were purified (Wizard<sup>®</sup> SV Gel and PCR Clean-Up System, Promega, Co., Madison, WI,  
79 USA) and sequenced in both directions through ABI PRISM Genetic Analyzer 3130x1  
80 version (Applied Biosystems, Foster City, CA, USA).

## 81 **2.3 Phylogenomic Analysis**

82 The consensus sequence for one complete genome and two partial N sequences were  
83 assembled by Geneios<sup>®</sup> version 8.1.6 and submitted in GenBank under accession numbers  
84 (KY967608, MH004284 and MH004285). Partial and complete nucleotide sequences were  
85 compared to corresponding GenBank database using BLAST tool at NCBI  
86 (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Sequences were aligned with strains reported earlier  
87 from different countries using ClustalW methods in BioEdit<sup>®</sup> version 5.0.6 (Hall, 1999).  
88 Comparative phylogeny using complete genome, partial N (255 bp) gene of under-study  
89 isolates and previously reported representative strains of each lineage was estimated by  
90 neighbour-joining (1000 replication bootstrap values) method in MEGA<sup>®</sup> version 6.0  
91 software (Tamura et al., 2013). The percent identity of nucleotide and amino acid of studied  
92 isolates was estimated by Pairwise Sequence Comparisons (PASC) analysis with  
93 representative strain using Kimura-2 model (1000 bootstrap) in MEGA software. For  
94 phylogenomic analysis of the complete genome, Splits Tree program v 4.95 was employed  
95 using Neighbor-Net graph method based on pairwise distance estimated by uncorrected *p*-  
96 distance and angle split transformation setting (Huson & Bryant, 2006). In order to estimate  
97 inter- and intra-lineage genetic distance, PASC analysis was performed using maximum  
98 composite likelihood method (d: Transitions + Transversions model) with in MEGA software  
99 (Tamura et al., 2013).

## 100 **3 RESULTS AND DISCUSSION**

101 All clinical samples were found positive, indicating a wide spectrum of susceptibility  
102 irrespective of animal breed, age and geographical area. Topology of partial N-gene revealed  
103 a wide geographical relationship of circulating PPR strains in the country (Figure 1A)  
104 consistent with observations made previously (Balamurugan et al., 2010; Munir et al., 2012;  
105 Muniraju et al., 2014). Since partial gene (either N or F gene)-based phylogeny represents an  
106 evolutionary pattern of a particular gene only, whole genome sequence analysis is  
107 recommended for more precise phylogenetic and evolutionary relationship of the circulating  
108 strains in a particular geographical setting (Muniraju et al., 2014), such as Pakistan where  
109 full-genomes have not yet been considered. Hence complete genome sequencing was  
110 performed using primers and protocols designed to represent local strains.

111 Genomes of PPRVs have been previously reported to range from 15942 to 15954 nucleotides  
112 (nt) in length, due to six-nucleotide indels in noncoding region between M and F gene  
113 (Balamurugan et al., 2010). The complete sequence of the under-study isolate was found to  
114 be 15948 nt in length, following the characteristic “rule-of-six” feature of paramyxoviruses  
115 required for efficient replication. Similar to previously reported genomes, the full genome of  
116 this isolate encoded six structural genes in order of 3'-N-P-M-F-H-L-'5 with a 52 nt long  
117 leader at 3' UTR and 73 nt long trailer at 5' UTR. Total length of coding genes varied in  
118 length across the whole genome: N with 1689 nt (55-1744), P with 1655 nt (1748-3402), M  
119 with 1484 nt (3406-4888), F with 2410 nt (4892-7302), H with 1957 nt (7306-9262) and L  
120 with 6643 nt (9266-1548). All genes were separated by similarly conserved non-coding  
121 intergenic trinucleotide (CTT) (Baron et al., 2016).

122 Based on complete genome sequences, phylogenetic analysis clustered the under-study  
123 isolate within lineage IV, close to previously reported isolates from China and India (Figure  
124 1B). A highest nucleotide identity (97.9%) was observed with an Indian strain (KR140086)  
125 isolated in 1994 followed by 97.5% with Chinese strain (KX421388) isolated in 2007 and  
126 97.3% with an Indian isolate (KX033350) reported recently in 2016. The reported isolate  
127 showed a higher nucleotide divergence (87.5%) with a strain in lineage III (KJ867543)  
128 followed by 88.4% with lineage I (EU267273) and 92.1% lineage II (HQ197753) (Table 1).  
129 Such a pattern of genetic divergence suggests an ongoing evolution among strains  
130 representing different lineages (Muniraju et al., 2014). Individual gene-based comparative  
131 analysis showed maximum homology for M-gene than other genes of representative strains in  
132 lineage IV (nucleotide: 96.7-98.7%, aa: 94.3-97.7%), whereas, it was found to be varying  
133 from 90.5-93.7% for nucleotides and 85.4-86.2% for residues within lineage I-III. The H  
134 gene was found to be the most divergent than other genes within lineage IV (nucleotide: 92.8-

135 95.9%, aa: 96.4-98.1%). Similarly, the nucleotide and residue divergence varied from 85.7 to  
136 91.1% and 79.1%-85.8%, respectively within lineage I-III (Table 1). These findings are  
137 consistent with observations made previously where higher divergence and/or more hyper-  
138 variability has been reported in H gene than other genes of the PPRV (Yu et al., 1998).

139 The under-study strain shared high percentage genetic distance with Moroccan and Ethiopian  
140 isolates as compared to Chinese and Indian isolates with 12.4% inter-lineage genetic distance  
141 between lineage III and II and/ I, 12.1% between lineage IV and III, 10.1% between lineage  
142 IV and I, and, 8.2% between lineage IV and II (Figure 2A). The current study presented the  
143 first intra-lineage genetic distance analysis of PPRV isolates originating from different  
144 countries (Figure 2B). Based on findings of genetic distance, we suggest that the under-study  
145 isolate is closer to Chinese and Indian isolates as compared to isolates from Nigeria,  
146 considered the ancestral origin of all circulating PPRV strains. This finding also indicates the  
147 continuous evolution of PPRV (Muniraju et al., 2014). Comparative residue analysis of the  
148 complete N and F gene showed several conserved motifs in our isolate similar to previously  
149 reported isolates (Balamurugan et al., 2010). These include three motifs in N-gene: a nuclear  
150 export signal motif (<sup>4</sup>LLKSLALF<sup>11</sup>), a nuclear localization signal motif (<sup>70</sup>TGVMISML<sup>77</sup>)  
151 and the RNA binding motif (<sup>324</sup>FSAGAYPLLWSYAMG<sup>338</sup>) involved in interaction of N  
152 with N monomers of RNA during genomic RNA binding and thought to be required for N-N  
153 self-interaction (Yu et al., 1998). Three conserved motifs were also noticed in the F gene: a  
154 signal peptide (<sup>1</sup>MTRVAILAFLFLNAVAC<sup>19</sup>), cleavage site motif (<sup>103</sup>GRRTRR<sup>108</sup>)  
155 responsible for virulence and adaptation in the environment and a leucine zipper domain  
156 (<sup>459</sup>LGNAVTRLENKELLDASDQIL<sup>380</sup>) involved in maintenance of protein tertiary  
157 structure (Lamb & Parks, 2007).

158 Taken together, assessing the complete genetic nature of field-circulating PPRV strains  
159 highlights the level of divergence and genetic differences compared to vaccine or circulating  
160 isolates from neighbouring countries. A possible link of intra-lineage genetic diversity of  
161 isolates from different countries would be helpful not only in understanding the genetic basis  
162 of circulating viruses but also to facilitate the establishment of foundations to exploit such  
163 information in designing future vaccine viruses that confer better protection in a given  
164 country or situation. Additionally, continuous monitoring of the disease emergence to  
165 determine the nature of the virus and to assess the potential of viral evolution, would be a  
166 benchmark of success in disease eradication from the globe.

## 167 **ACKNOWLEDGMENT**

168 None

169 **CONFLICT OF INTEREST**

170 All authors declare no competing of interest

171 **FIGURE LEGENDS**

172 **FIGURE 1** Phylogenetic tree of partial N-gene (**A**) and whole genome (**B**) of under-study  
173 PPRV sequences compared to representative strains from different geography was  
174 constructed using MEGA version 6.0. The study PPRV isolates are marked with black-square  
175 (■).

176 **FIGURE 2** Inter and intra-lineage genetic distance for complete genome of PPRV strains are  
177 determined using evolutionary network in Splits Tree software. The isolates originated from  
178 different countries are highlighted with different colours accordingly.

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