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RESEARCH ARTICLE

Elucidating the Genetic Diversity of Prevalent Strains of *Peste des Petits* Ruminants Virus in Gilgit-Baltistan Province, Pakistan

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Peste des petits ruminants caused by small ruminant morbillivirus (SRMV), is a highly contagious disease of small ruminant. It is endemic in Gligit-Baltistan (GB) territory of Pakistan where a number of clinical cases are being reported frequently. However, so far, the phylogenetic relationship of prevailing strains in this particular geography has remained elusive. We carried out a study to characterize partial N gene of PPR viruses from outbreaks during years 2017-18 from GB. Out of 30 clinical samples analyzed, 27 percent (n=8) were positive for N gene (351). However, owing to close relationship expected among study sequences, only two samples from sheep and one from goat were sequenced by phylogenomic analysis using a range of bioinformatics tools. Phylogeny analysis revealed a close relatedness with the previously reported viruses with lineage IV. Sequence composition showed high level of homology with circulating viruses suggesting that that these viruses do not undergo rapid genetic changes in N gene. However, a number of genomic and residue substitutions were noted within the prevalent viruses as compared to those reported previously. This study provides the first genetic evidence of SRMV strains sequence analysis of partial N gene involved in recent outbreaks in the GB region. Future studies are necessary to further ascertain the study outcomes and elucidate the molecular epidemiology of prevalent strains in the said geographical area for better disease control and management interventions.

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INTRODUCTION

The control of Peste des petits ruminants (PPR) virus has become important due to its disastrous impact on small ruminants population and other unusual hosts such as cattle and camels worldwide (Balamurugan *et al*., 2014; Omani *et al*., 2019). In fact, the poverty alleviation and global food security is at risk due to this particular deadly disease of small ruminants (Baron *et al*., 2016). This may be the reason that the malady has been targeted for eradication till 2030 by some authors and organizations (Albina *et al*., 2013; Banyard *et al*., 2016; FAO and WHO, 2016; Baron *et al*., 2017).

The causative agent, small ruminant morbillivirus (SRMV), belongs to family: *Paramyxoviridae* and genus: *Morbillivirus*. It is described to be pleomorphic, non-segmented, and has negative sense genome that encodes six proteins i.e. Nucleocapsid (N), haemagglutinin (H), Fusion(F), Matrix (M), Phosphoprotein(P) and Large RNA polymerase (L), also two non-basic proteins (V and C). Among structural proteins of virus, the F and N gene has been demonstrated to be hypervariable and consequently most appropriate for the molecular characterization of the virus (Kumar *et al*., 2014; Khan *et al*., 2018; Alemu *et al*., 2019). The virus was confined to West Africa first and then spread throughout African countries as well as Asia, Russia, Southern Tanzania, China, Middle East, Europeon region of Thrace and many other parts of the world (Parida *et al*., 2015; Zhu *et al*., 2016; Abubakar *et al*., 2017; Baazizi *et al*., 2017). There are four lineages circulating worldwide with varying distribution of viruses of different lineages in different geographical areas (Dundon *et al*., 2018).

Using different genetic markers, particularly the N gene, many studies showed that virus is spreading and evolving worldwide (Su *et al*., 2015). For instance, partial amplification of fusion gene was developed specifically for PPR diagnosis (Forsyth & Barrett, 1995). A cross border PPR virus transmission was diagnosed by fusion gene based reverse transcriptase PCR in Turkey (Ozkul *et al*., 2002). Multiplex RT-PCR by N and M gene amplification was reported well for PPR virus detection (Muthuchelvan *et al*., 2014; Donduashvili *et al*., 2018). The N gene based detection of PPR has been conducted previously using secretion of nasal swab, a cheapest source for sample collection for subsequent diagnosis (Senthil *et al*., 2014). Similarly, a set of primer NP3 and NP4 was designed to amplify 350 base pair nucleocapsid fragment through RT-PCR which has been found to be 1000 times more sensitive for PPR detection (Couacy-Hymann *et al*., 2002). This is simply because the N gene is much replicative of all the genes of PPR virus. This may be the reason that N gene based assay is most frequently used now a day for identification and subsequent phylogenomic studies to provide more comprehensive clustering pattern for prevalent viruses throughout the world.

The serological and molecular studies have been done from various outbreaks in many parts of country like Punjab, Sindh, Khyber Pakhtunkhaw and Azad Kashmir (Anees *et al*., 2013; Zahur *et al*., 2014; Munir *et al*., 2015; Aziz ul Rehman *et al*., 2016; Abubakar *et al*., 2017; Abubakar *et al.*, 2018), however, there is paucity of research data elucidating phylogenomic characteristics of prevalent strains of PPR from GB. With this brief background, we conducted genome based identification and phylogenetic analysis of prevalent strains for better elucidation of the molecular nature of circulating strains and subsequent decision for better disease control and management.

MATERIALS AND METHODS

This research was performed to evaluate the rate of occurrence of PPR virus in small ruminants from randomly selected herds of different areas in Gilgit Baltistan zone including Tattovat, Fairy-Maidow, Naltar lake and Bangle Naltar, where an emerging wave of clinical disease of PPR virus was reported (Fig. 2). Gilgit Baltistan shares border with China, Afghanistan and Tajikstan through Wakhan. Farmers and nomads mostly depend on sheep and goat population for their livelihood due to extensive pastures in the area. However, during winter season, they move to other areas due to snow and reduced grazing facilities. These movements may increase the potential for disease transmission across the livestock/wildlife interface, at upper elevations, where there occurs a competition for limited and depleted rangelands between domestic and wildlife. This means that wildlife species may be exposed to disease agents carried by domestic species, but also serve as a reservoir to reintroduce virus to domesticated animals.

Using sterile cotton and buccal swabs (n=30) were collected from each of those animals which exhibited flue like symptoms such as cough, nasal discharge, high temperature and pustules and were clinically suspected of PPR. The parameter like age of animal, breed, gender and geographical location along with locality was also noted during sampling. The number of animals in the herd varied from 27-50 animals and their age ranged from three months to five years. The breeds of animals were either Beetal or non-descript, without any history of vaccination in the past. For the buccal swab, the mouth was opened with consideration to keep away from nibbles (clench hand of hand among incisors and premolars) trailed by precise perception of tongue, cheeks and gums were for indications of PPR. Utilizing the cotton fleece swab, the tongue, cheeks and the gums were swabbed and put in the cryogenic vials. The swab stick is then cut discharging the example end of the swab into the cryogenic vial. The vial with the example was then all around anchored with the top, named, surface cleaned and put in a cool box/dry shipper or fluid nitrogen and, after achieving the research facility, they were protected at -20˚C before being prepared for sub-atomic portrayal. The samples were processed through reverse transcriptase polymerase chain reaction (RT-PCR) for the amplification of target N gene using OIE published primers named as NP3 and NP4 (Couacy-Hymann *et al*., 2002) giving a product of 255 bp. Briefly, the virus RNA was extracted from the samples as per instructions described (Kwiatek *et al*., 2010) (plasma and swab samples) using QI Aamp® DSP Virus Kit (LOT: 154029744) as per manufacturer's instructions.

The c-DNA was synthesized using Thermo-Scientific® Revert Aid First Strand cDNA (Cat: K1822) according to the instructions provided by the kit's manufacturer. Following cDNA synthesis, a set of primers were used for the amplification of NP-gene (Table 1). This amplification was performed in conventional thermal cycler (BIORAD, USA) in the final volume of 50 µL of reaction mixture. A brief detail of each of reagent used in reaction mixture is listed in Table 2. Similarly, the optimized PCR protocol or varying thermal condition for NP gene of PPR is given in Table 3.

Finally, the amplified products were electrophoresed (1.2%) using a 100 bp ladder and observed under UV light for demonstration of band of desired length in the gel showing at Fig. 1.

Table 1: Details of primer used for RT- PCR

Primer	Primer sequence position	Position	Product size
Forward	5′-ATC ACA GTG TTA AAG CCT GTA GAG G-3′	1232-1255	351 bp
Reverse	5′-GAG ACT GAG TTT GTG ACC TAC AAG C-3′	1583-1560	

Table 2: Recipe of PCR mixture for NP gene of PPRV

Reagents	Quantity (µL)
cDNA (10ng/µL)	2.0
Reverse Primer-NPR (10pmol)	1.0
Forward Primer-NPF (10pmol)	1.0
Dream Taq® Green Taq	20
Deionized Water	26
Total volume	50

Table 3: Thermal conditions of PCR for amplification of NP gene corresponding to PPRV

PCR Steps	Temperature	Time	Cycles
Initial denaturation	95°C	5 minutes	1
Denaturation	94°C	30 seconds	
Annealing	52°C	45 seconds	35 cycles
Extension	72°C	1 minute	
Final Extension	72°C	10 minutes	1

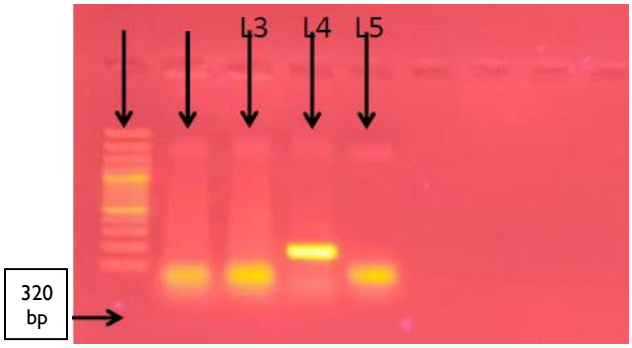

Fig. 1: An image showing PCR product on gel electrophoresis. L1: Ladder; L4: Positive sample; L2, L3 and L5: Negative sample.

Fig. 2: A map showing geographical location of different areas from Gilgit district of Gilgit-Baltistan and sampling sites are indicated by red-circles.

RESULTS

Out of 30 clinical samples, only 08 were positive for PPRV by NP3 and NP4 primer amplification with a positivity rate of =27%. The remaining 22 samples were negative. Samples collected from herds originating from Naltar lake (n=8), Tatovat (n=7), Fairy Meadows (n=7) and Bangle Naltar (n=8). Among these, only 06 were positive for sheep and 02 were positive for goat. However, owing to similar genetic pattern expected, only 02 were enough for sequencing and got band size of 351bp for both sequences which were submitted to the gene bank (Accession number: MN094749-MN094750). Identity of Nucleotide sequence among two sequences reported in this study and with previously characterized Pakistani strains of PPRV was found to be 99.98 and 99.98-99.93% similar respectively.

The Phylogenetic analysis of resulted sequences revealed that all the Pakistani strains of SRMV clustered into lineage IV, which is prevalent in Asian and Middle East countries. However, it is interesting to note that all the Gilgit-Baltistan originating strains of PPRV made a separate cluster closer to the isolates from Multan in 2011 (KC191632) and from Okara in 2012 (KC207867). In addition to these, all of these sequences reported here and the sequences from Multan and Okara were closer to the isolates from Nigerian isolate in 2013 (KR828813). Phylogenetic tree, based upon partial N gene, is shown (Fig. 2).

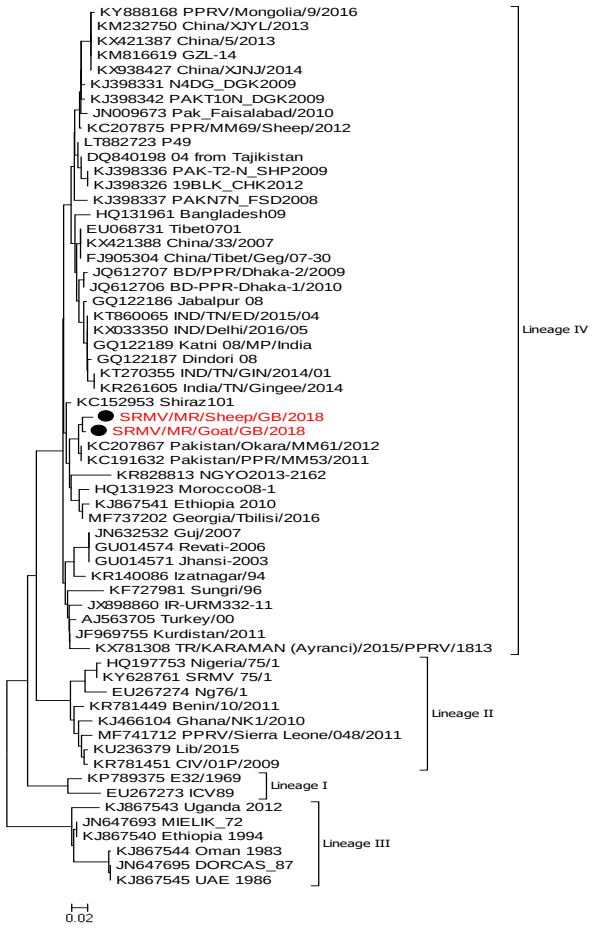

Fig. 2: Partial phylogenetic analysis of the study isolates along with representative isolates from.

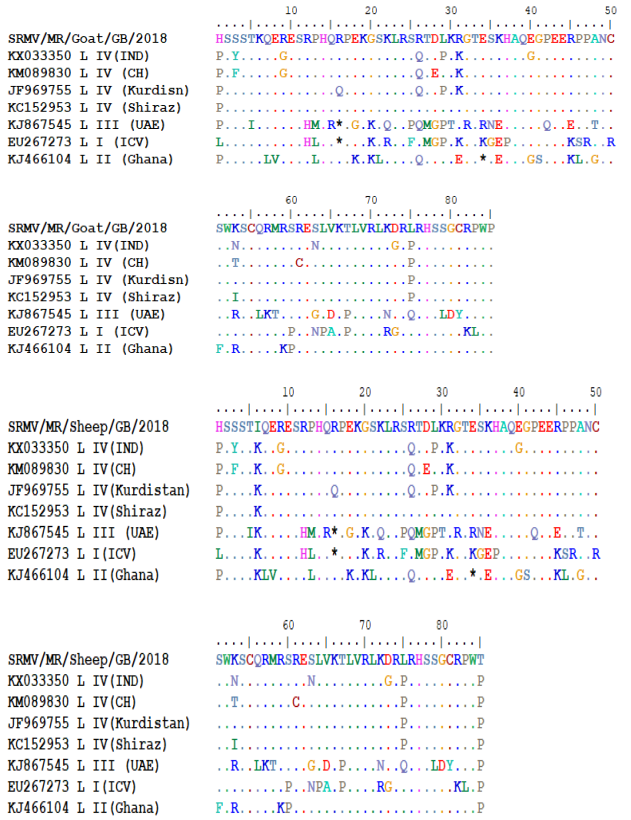

Fig. 3: Comparative residue analysis of the study isolates along with Asian countries isolates (India and China).

Comparative genomic analysis of current study isolates with reference strains from neighboring countries revealed significant mutations in our study isolates as compared to Lineage IV isolates shown in Fig. 3. For goat-originating strains, several substitutions located at position1 (P→H), 3(Y/F→S), 9(G→R), 26(Q→R), 28(E→D), 29(P→L), 31(K→R), 40(G→E), 53(N/T→K), 61(C→R), 63(N→S), 73(G→D) and 75(P→L) were observed. Similarly, for the sheep isolate, mutations were observed at residue number 1(P→H), 3(Y/F→S), 6(K→I), 9(G→R), 21(Q→R), 28(E→D), 29(P→L), 31(K→R), 40(G→E), 53(N/T→K), 61(C→R), 63(N→S), 73(G→D), 75(P→L), and 85(P→T).

DISCUSSION

Previously, the divergence and similarity among SRMV strains is characterized well on the basis of partial N gene (Kgotlele *et al*., 2014). Though this fact has been well established that PPRV has great propensity for genetic change and information about the molecular evidence of currently prevailing SRMV strains is lacking from GB region of Pakistan. Therefore, the current study was designed to characterize the currently prevailing SRMV strains in GB and Phylogenetic linkages were established. Close-clustering of studied isolates to Nigerian isolate was observed. This possible link of Pakistani strains to Nigerian strains could be due to the fact that Pakistan is currently using the vaccine originated from Nigerian isolates (Zahur *et al*., 2014). In addition, nomads of Gilgit Baltistan Province travel around the country to sell their animals, on or before religious events such as Eid-ul-Adha; a common practice not only in Pakistan but also in other neighboring Islamic countries, representing about 60% of total population. The clustering of studied strains with isolates from Multan and Okara revealed that PPRV isolates have undergone an evolutionary pathway, which might be due to several inductions of viruses from various sources. However, the outcome of current study emphasize on routine surveillance of currently prevailing strains to elucidate the molecular epidemiology of PPRV strains in GB.

Conclusions: The topography of N gene based Phylo-genetic tree revealed that the study SRMV strains were closely related to lineage IV viruses with several nucleotide and residue substitutions. Future studies are very much essential at relatively a higher resolution better intervene and devise appropriate disease control interventions.

Authors contribution: MR has done work for her research purpose and prepared this manuscript. TA, MF and NR helped in preparing the manuscript. MR, MY and NA helped in execution of research and writing of manuscript.

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