Research Article

Genetic Characterization of *Peste des Petits Ruminants* Virus Circulating in Bangladesh

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Abstract | *Peste des petits ruminants* (PPR) is an acute and highly contagious viral disease of small ruminants. The disease is endemic in Bangladesh and causes considerable economic losses due to its high morbidity and mortality. The study was undertaken to detect and characterize the PPR virus (PPRV) circulating in Bangladesh. Twelve local isolates of Bangladeshi PPRV were successfully detected from post mortem samples (lymph node, spleen and trachea) of goats and a sheep during the year 2008-2012. The partial N gene of ten isolates and partial F gene of eight isolates were amplified by RT-PCR, and were sequenced. The phylogenetic analysis revealed that PPRVs circulating in Bangladesh belonged to Lineage IV and they formed a separate sub-cluster along with recent isolates from Nepal, Bhutan and China. On genetic analyses, it appeared that the N gene of PPRV is less conserved as compared to the F gene. Two unique amino acid substitutions (K423Q and E426G) were observed in Bangladeshi and Chinese isolates in the partial sequence of N protein. The recent Bangladeshi isolates are somewhat divergent from the earlier Bangladeshi isolate, indicating that the PPRV strains in Bangladesh are continuously changing their genetic character.

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Introduction

Peste des petits ruminants (PPR) is an acute and highly contagious viral disease of small ruminants and camel. The disease is characterized by fever, erosive stomatitis, conjunctivitis, gastroenteritis and pneumonia. The morbidity and mortality of the disease can be as high as 100% and 90%, respectively (Abu-Elzein et al., 1990). PPR virus (PPRV) is classified as a member of the genus *Morbillivirus* under the family *Paramyxoviridae* and order *Mononegavirales* (Gibbs et al., 1979). PPRV when viewed under an electron microscope displays the typical structure of paramyxoviruses: a pleomorphic particle with a lipid envelope which encloses a helical nucleocapsid that contains the single-stranded negative-sense RNA genome of 15,948 nucleotides (Diallo et al., 2007). The genome of PPRV codes for six structural (N, P, M, F, H and L) and two non-structural (C and V) proteins in the order of 3´-N-P(C/V)-M-F-H-L-5´ (Diallo et al., 1994; Bailey et al., 2005; Mahapatra et al., 2006). Among them, the nucleoprotein and fusion protein genes have been the target for PCR based specific diagnosis of PPR (Forsyth and Barret, 1995;

Couacy-Hymann et al., 2002; Saravanan et al., 2004) and it is suggested that the N gene is more divergent and therefore more suitable for phylogenetic distinction between closely related circulating viruses (Couacy-Hymann et al., 2002; Saravanan et al., 2004; Kwiatek et al., 2007; Kerur et al., 2008). Currently PPRV virus isolates aredivided into four genetically distinct lineages (Shaila et al., 1996; Dhar et al., 2002) lineage I is being represented mainly by Western African isolates and Central African isolates; lineage II by West African isolates from the Ivory Coast, Guinea and Burkina Faso; lineage III by isolates from Eastern Africa, Sudan, Yemen and Oman; lineage IV includes all viruses isolated from recent outbreaks across the Arabian Peninsula, the Middle East, southern Asia and recently across several African territories (Ozkul et al., 2002; Banyard et al., 2010). Lineage differentiation is mainly determined by the sequence comparison of a small region of the F gene (Forsyth and Barrett, 1995) or N gene (Couacy-Hymann et al., 2002).This classification of PPRV helps to understand the molecular epidemiology and the worldwide movement of PPR viruses. In Bangladesh, the PPR virus was first identified by The Pirbright Institute (was then known as Institute for Animal Health), UK, during a severe outbreak of the disease in the year 1993. Since then, the disease has become endemic in Bangladesh and presently considered as one of the major threats to the small ruminants population of the country. Although PPR has been persisting for more than a decade in this country, studies on the virus are rather limited, especially at the molecular level. Previously, few research works were performed on PPR in Bangladesh mostly on sero-prevalence by C-ELISA(Razzaque et al., 2004), pathological investigation and diagnosis (Islam et al., 2001; Khan et al., 2005; Rahman et al., 2011a; Bhuiyan et al., 2014; Chowdhury et al., 2014), immunological evaluation of the vaccine (Rahman et al., 2011b) and an evaluation of antibiotic combined with hyper-immune serum therapy (Islam et al., 2003). The present study focused on the genetic characterization of PPRV circulating in Bangladesh.

Material and methods

Virus Samples

Detailed necropsy was conducted on eleven goats and a sheep from twelve outbreaks occurred at different places of Bangladesh during 2008 – 2012. Details of the outbreaks and samples can be seen in Table 1.

Table 1: *History of samples used in this study*

SL	Name of isolates	Date of collection	Species			History of clinical signs
			Species	Breed	Age	
1	BD/PPR/ Narayangong/09	16/02/2009	Goat	Black Bengal goat	2 years	Fever (104°F), oculo-nasal discharge, diarrhoea and death
2	BD/PPR/ BAU/Sheep/09	12/09/2009	Sheep	Local	12 month.	Sub normal temperature (100°F), diarrhoea and death.
3	BD/PPR/ Dhaka/09	26/09/2009	Goat	Black Bengal goat	12 month	High fever (106°F), oculo-nasal discharge, dyspnia, stomatitis, diarrhoea and death
4	BD/PPR/ Dhaka-2/09	27/12/2009	Goat	Black Bengal goat	1 years	Fever (104°F), oculo-nasal discharge, dyspnia, stomatitis, diarrhoea and death
5	BD/PPR/ Dhaka-1/10	10/03/2010	Goat	Jumunapari	18 month	High fever (106°F), oculo-nasal discharge, stomatitis, diarrhoea and death
6	BD/PPR/ Mymensingh/10	25/03/2010	Goat	Black Bengal goat	12 month.	Sub normal temperature (100°F), diarrhoea and death
7	BD/PPR/ Narayangonj-1/11	05/10/2011	Goat	Jumunapari	one month	Fever (104°F), oculo-nasal discharge, diarrhoea and death.
8	BD/PPR/ Narayangonj-2/11	12/11/2011	Goat	Jumunapari	one month	High fever (106°F), oculo-nasal discharge, diarrhoea and death.
9	BD/PPR/ Netrokona-1/12	15/01/2012	Goat	Black Bengal goat	18 month	Fever (103°F), oculo-nasal discharge, dyspnia, stomatitis, diarrhoea and death
10	BD/PPR/ Netrokona-2/12	15/01/2012	Goat	Jumunapari	18 month	Fever (104°F), stomatitis, diarrhoea and death
11	BD/PPR/ Netrokona-3/12	16/01/2012	Goat	Black Bengal goat	18 month	High fever (106°F), oculo-nasal discharge, dyspnia, diarrhoea and death
12	BD/PPR/ Netrokona-4/12	16/01/2012	Goat	Jumunapari	18 month	Fever (103°F), oculo-nasal discharge, diarrhoea and death

Mesenteric and bronchial lymph node, trachea and lung tissue samples were collected in aseptic condition in sterile Falcon tubes. Collected tissues from each animal were pooled and homogenized to make a 20% tissue suspension in PBS (pH7.2). Tissue suspension was centrifuged at 3000 rpm for 10 minutes; the supernatant was collected in a fresh sterile Falcon tube and stored at -80^0 C for molecular detection of the virus and subsequent virus isolation. Lyophilized freeze-dried live PPR vaccine, kindly provided by Livestock Research Institute, Mohakhali, Dhaka (Batch no. 26) was used as the reference virus.

RT-PCR for PPRV Detection and Gene Sequencing

RNA was extracted from the prepared tissue suspension and reference vaccine virus using the RNeasy Mini Kit (Qiagen, Germany). RT-PCR was done using the Qiagen One Step RT–PCR kit (Qiagen, Germany) with the previously described primers forF (Ozkul et al., 2002) and N genes (Couacy-Hymann et al., 2002). The PCR reaction conditions described earlier for both F (Forsyth and Barrett, 1995) and N genes (Couacy-Hymann et al., 2002)were followed with little modifications. The reaction conditions for N gene specific primers comprised initial denaturation at 95^0 C for 15 min followed by 35 cycles of denaturation at 94^0 C for 1 min, annealing at 50^0 C for 1 min and extension at 72^0 C for 30 sec, and a final extension at 72^0 C for 10 min. Similarly for F gene specific primers the reaction condition consisted of initial denaturation at 95^0 C for 15 min followed by 35 cycles of denaturation at 94^0 C for 30 sec, annealing at 55^0 C for 30 sec and extension at 72^0 C for 2 min, and a final extension at 72^0 C for 7 min. The RT-PCR products were visualized by electrophoresis on 1.5% agarose gel stained with ethidium bromide. RT-PCR products for both F and N genes were cleaned using the EZ-10 Spin Column DNA Gel Extraction kit (Bio Basic Inc., USA). The procedure described by the manufacturer was strictly followed. Quantification of purified DNA and sequencing of the products was done from 1st BASE Laboratories, Malaysia.

Sequence Analyses and Phylogenetic Tree Construction

The sequenced data were first confirmed to be the gene fragments of PPRV by NCBI Basic Local Alignment Search Tool (BLAST) search. Sequence editing, alignment, and homology study were carried out with the software package “Lasergene” (Modules - EditSeq and MegAlign; DNASTAR Inc., USA). In addition, sequenced data of Bangladeshi isolates were compared with other related sequences retrieved from the GenBank. Homology and divergence among the Bangladeshi isolates compared to other contemporary isolates were studied. Phylogenetic trees were constructed with the partial sequence of both F and N gene with the MEGA (Version 5.10) software using the Maximum Likelihood method based on the Tamura-Nei model with 1000 bootstraps (Tamura et al., 2011). All the sequence data have been submitted to the GenBank and are available under the GenBank accession no. JQ612706, JQ612707, JQ612708, JQ612709, JX220409, JX220410, JX220411, JX220412, JX220416, JX220417, JX094437, JX094440, JX094438, JX094439, JX094436, JX220413, JX220414 and JX220415.

Results

PRRV Detection by RT-PCR and Sequence Homology Analysis

A 448 bp fragment of F gene and 351 bp fragment of N gene of all the eleven PPRV suspected samples of Bangladesh and the reference vaccine virus were successfully amplified by RT-PCR. Out of 12 samples, amplified RT-PCR products of N gene of 10 isolates and F gene of 8 isolates were sequenced.

Multiple alignment of F gene fragment indicated that all isolates from Bangladesh were closely related to the isolates of Bhutan/2010 and China/Tibet-0702 with 99.7-99.4 % nucleotide identity, respectively. It was found that Bangladeshi isolates were 96.3-99.7% homologous to the lineage IV isolates, 88.2-90.1% to Lineage III, 92.5-94.4% to Lineage II and 87.0-88.5% to Lineage I isolates (Table 2). On the other hand multiple alignment of N gene fragment revealed that Bangladeshi isolates were highly similar to the China/ Tibet 0702 isolates with 94.5-98.8% homology. The N gene fragment of Bangladeshi isolates were 89.4-98.8% homologous to the lineage IV, 79.6-82.4% to Lineage III, 82.4-91.4% to Lineage II and 81.2-85.1% to Lineage I isolates (Table 2). It was also observed that the PPRVs from distant geographical regions vary to a greater extent in their N gene than in F gene sequences (Table 2). Among the Bangladeshi isolates of PPRVs maximum 2.2% divergence was observed in the F gene, while up to 8.8% divergence was noticed in the N gene fragment.

Table 2: *Percent nucleotide identity/divergence among Bangladeshi field isolates and between Bangladeshi field isolates and other representative isolates of different lineages*

Isolates	Country	Lineage	Identity		Divergence	
			N gene	F gene	N gene	F gene
Among Bangladeshi isolates*	Bangladesh	IV	91.4-95.7	97.5-100	4.4-8.8	0-2.2
Bangladeshi isolates vs.						
Bhutan /10	Bhutan	IV	N/A	97.8-99.7	N/A	0.3-1.9
China/Tibet-0702	China	IV	94.5-98.8	97.2-99.1	1.2-5.7	0.9-2.1
Sungri/96	India	IV	89.4-93.3	96.9-98.8	7.1-11.0	.03-2.9
Ana /Guj/05	India	IV	89.4-93.3	96.3-98.8	7.1-11.0	1.3-3.5
Nigeria/75.1	Nigeria	II	82.4-91.4	92.5-94.4	8.4-17.9	5.9-7.6
Ivory-cost/89	Cot-d-ivory	I	81.2-85.1	87.0-88.5	15.5-20.0	12.0-13.6
Ethiopia/96	Ethiopia	III	79.6-82.4	88.2-90.1	19.2-21.7	10.9-12.8

Among all the Bangladeshi field isolates described in this study;* **N/A: *sequence not available*

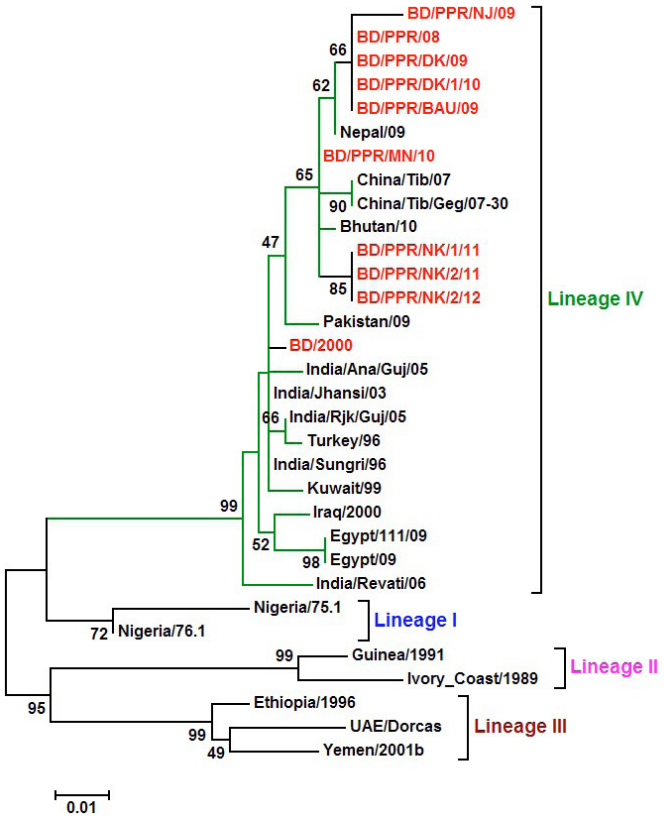

Figure 1A: *The Maximum Likelihood (ML) phylogenetic tree constructed based on the partial F gene sequences (320bp) of Bangladeshi and South Asian isolates and representative strains of each lineage of PPRV. The scale indicates the number of substitution per site. The sequences established in this study are labelled with red colour*

Phylogenetic Analysis

Partial nucleotide sequences of F and N genes of Bangladeshi isolates of PPRV and other Asian isolates available in the GenBank were aligned and phylogenetic trees were constructed. Bangladeshi isolates clustered under Lineage IV in both the F and N genes phylogeny and have a close relationship with other recent Asian isolates of China, India, Nepal, Bhutan and also with some Middle-eastern isolates (Figure 1A, 1B, 2A and 2B). On phylogenetic analysis based on F gene all recent Bangladeshi isolates clustered very closely with the recent isolates from Nepal, China and Bhutan, but slightly away from other Lineage IV isolates including an older Bangladeshi isolate BD/2000 (Figure 1A and 1B). In case of N gene, genetic diversity among PPRV was quite evident in the phylogenetic tree (Figure 2A). Within the Lineage IV several sub-clusters can be noticed. Even Bangladeshi isolates are also segregated into four apparent sub-clusters (Figure 2B). The isolate BD/ PPR / Mymensingh / 2010 is quite distinct from other Bangladeshi isolates and clustered with a strain from Tajikistan. Two other Bangladeshi isolates, Bangladesh/09 and

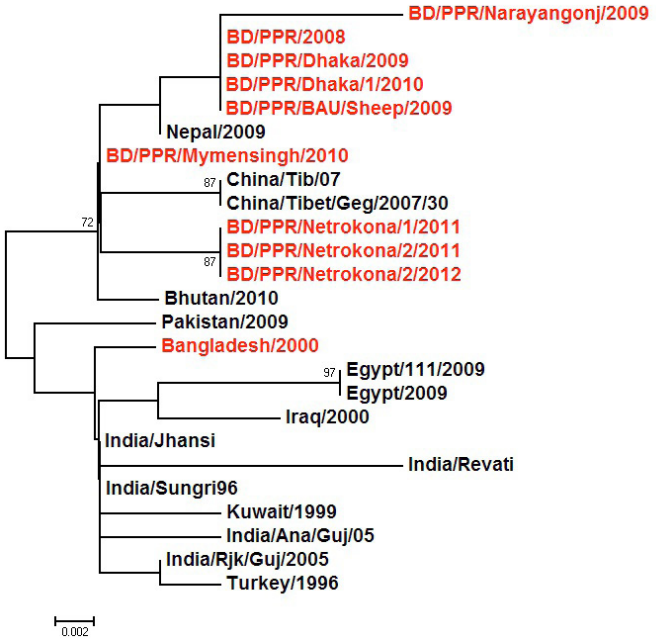

Figure 1B: *The Maximum Likelihood (ML) phylogenetic tree constructed based on the partial F gene sequences (320bp) of Bangladeshi isolates representing only lineage IV*

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Table 3: *Common nucleotide mutations and deduced amino acid substitutions in the F and N genes that are unique to Bangladeshi and Chinese isolates of PPR virus*

Sequence		F gene(5779-6088)			N gene (1360-1613)			
	Position	5789	5846	5921	1374	1384	1397	1547
Nucleotide	Consensus	A	C	A	A	A	T	G
	Substituted by	G	T	T	C	G	C	A
		F protein (91-180)			N protein (421-500)			
	Position				423		426	
Amino acid	Consensus				K		E	
	Substituted by	None			Q		G	

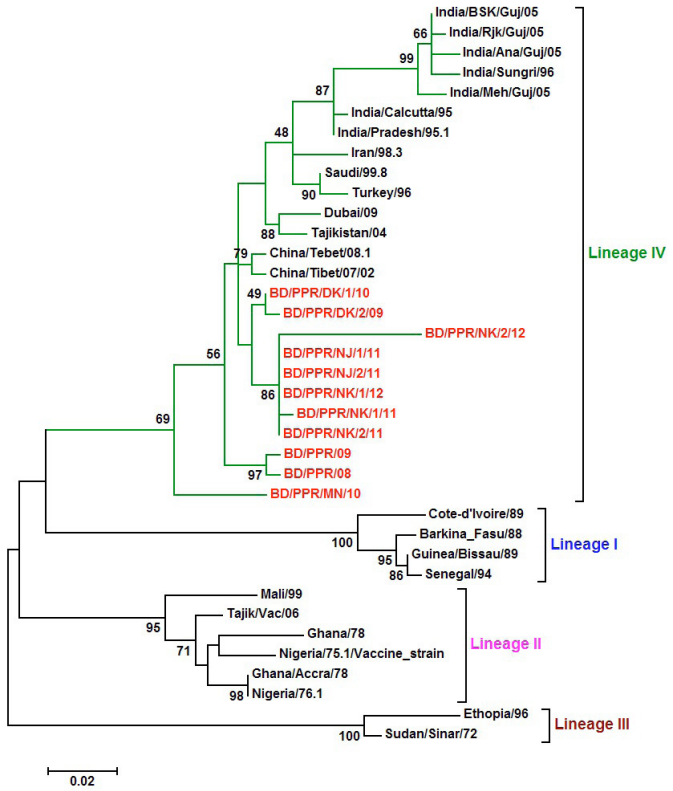

Figure 2A: *The Maximum Likelihood (ML) phylogenetic tree constructed based on the partial N gene sequences (255bp) of Bangladeshi and South Asian isolates and representative strains of each lineage of PPRV. The scale indicates the number of substitution per site. The sequences established in this study are labelled with red colour*

BD/PPR/08, made a separate sub-cluster. Other Bangladeshi isolates formed two more closely related sub-clusters (Figure 2B). These findings would indicate that the N gene of Bangladeshi PPRV is in somewhat evolution.

Nucleotide and Amino Acid Substitution Analysis

Alignment of nucleotide sequences of the partial F gene (322bp) revealed that there were three common nucleotide substitutions, A5789G, C5846T and A5921T, which were unique to all the recent Bangladeshi isolates and Tibet/08-1.B/China, Tibet/0702/China and Bhutan/2010 isolates (Table 3). Similarly, nucleotide analysis based on partial sequence of N gene (255bp) showed that Bangladeshi isolates and both the Tibet/China isolates shared four common nucleotide substitutions, A1374C, A1384G, T1397C and G1547A (Table 3). Furthermore, there were some scattered nucleotide substitutions in some strain for N gene (data not shown) but they were not common for all Bangladeshi isolates.

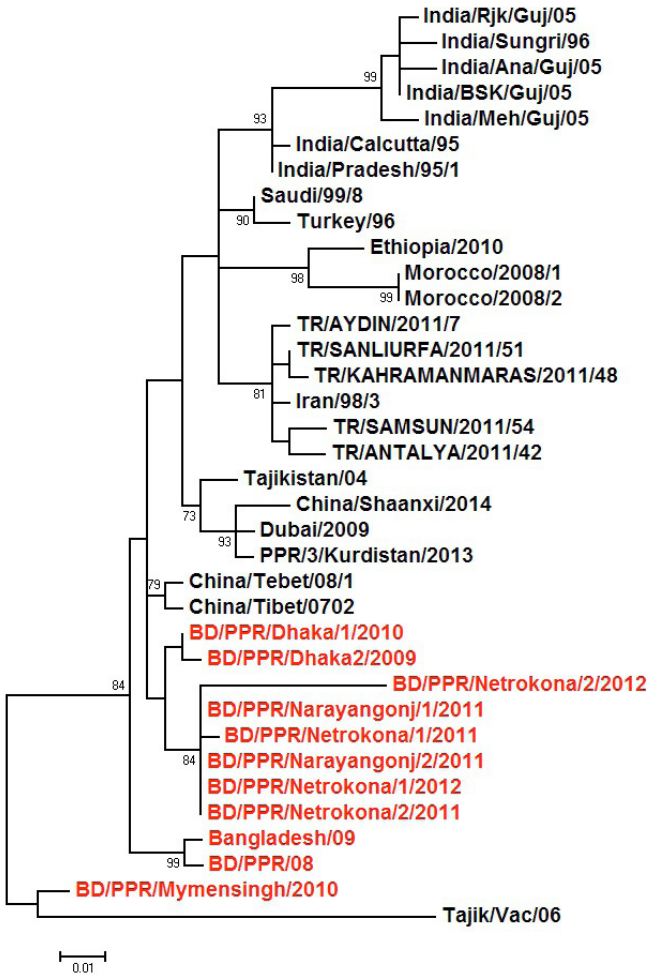

Figure 2B: *The Maximum Likelihood (ML) phylogenetic tree constructed based on the partial N gene sequences (320bp) of Bangladeshi isolates representing only lineage IV*

Alignment of deduced amino acid sequences revealed

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that all the three common nucleotide substitutions in the F gene of recent Bangladeshi, Chinese and Bhutan isolates were silent mutations and did not result into any amino acid substitutions. However, two of the four common nucleotide mutations in the N gene of recent Bangladeshi and Chinese isolates resulted into two amino acid substitutions, K423Q and E426G (Table 3), which were not evident in earlier lineage IV Asian isolates. Although there were amino acid substitutions in N protein of some isolates (data not shown), they were not common for all Bangladeshi isolates.

Discussion and Conclusions

Since 1993 PPR has been prevailing in Bangladesh with devastating effects on the goat husbandry (Sil et al., 1995; Chowdhury et al., 2014). Bangladeshi isolates of PPRV obtained during the period from 2008 to 2012 were characterized and all belonged to the lineage IV and remained close to other isolates from South and Southeast Asia (China, India, Pakistan, Nepal and Bhutan).There are four distinct genetic lineages (Lineages I, II, III, and IV) of PPRV circulating in the world (Shaila et al., 1996; Dhar et al., 2002). In Asia only Lineage IV virus is present (Banyard et al., 2010). Phylogenetic analysis based on partial F gene and N gene sequences of ten and eight Bangladeshi PPRV isolates, respectively, shows that within lineage IV, Bangladeshi isolates formed separate well defined sub-group along with isolates from China, Nepal and Bhutan. In previous studies, it has been urged that Asian isolates including the isolates of turkey formed a separate branch under lineage IV (Shaila et al., 1996; Nanda et al., 1996; Ozkul et al., 2002; Kwiatek et al., 2007; Wang et al., 2009; Banyard et al., 2010).

On partial sequence alignment analysis, the PPRV isolates of Bangladesh, Nepal, Bhutan and China (BNBC) sub-group differed from other Asian viruses by 3 common nucleotide substitutions for F gene and 4 for N gene. Two mutations in the N gene resulted into two amino acid substitutions (K423Q and E426G). Analysis of full genome sequence could reveal further unique amino acid substitutions. However, the significance of these mutations cannot be ascertained without antigenicity and pathogenicity study coupled with reverse genetics experiments. The evolutionary dynamics of RNA viruses are complex and their high mutation rates, rapid replication kinetics complicates the control of emerging infectious agents (Adam et al., 2010).

Some degree of divergence also existed within the circulating Bangladeshi isolates. The recent Bangladeshi isolates are somewhat divergent from the earlier Bangladeshi isolate, indicating that the PPRV are in continuous evolution. The findings of the present study warrant further detailed molecular epidemiological study, preferably with full-length gene sequences.

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Conflict of Interest

There is no conflict of interest.

Authors' Contribution

MMR, RP and ARB, designed and performed the experiments and drafted the manuscript. MG and SMZHC contributed to planning and coordination of the study and provided technical and logistic supports. EHC and MRI conceived the study, coordinated and supervised the whole work and edited the manuscript.

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