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Original Article

Epidemiology and genetic characterization of Peste des petits ruminants virus in Bangladesh

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Abstract

Peste des petits ruminants (PPR) is an acute, highly contagious disease responsible for high morbidity and mortality rates in susceptible sheep and goats. Adequate knowledge of the diversity of circulating strains of PPR virus will help livestock authorities choose appropriate vaccines. The objective of this study was to describe the epidemiology of PPR and characterize the strains circulating in Bangladesh. Veterinarians enrolled goats showing signs consistent with PPR, including diarrhoea, fever and respiratory distress, from three veterinary hospitals. Post-treatment follow up was carried out to ascertain health outcomes of the goats. Faecal and throat swab samples were collected from the goats and tested for PPRV RNA using real-time reverse transcription polymerase chain reaction (rRT-PCR). Nucleotide sequence-based phylogenetic analyses of two structural genes, the nucleocapsid (N gene), and the haemagglutinin (H gene) were studied to determine the genetic variations of PPRV strains. Of the 539 goats enrolled, 38% (203/539) had detectable RNA for PPRV. We were able to follow up with 91% (184/203) of the PPRV infected goats; 44 of them died (24%). PPRV was more frequently identified in the summer (45%) than in the rainy season (29%) (Odds ratio = 1.9, 95% confidence interval: 1.3–3.1). Bangladeshi strains were phylogenetically similar to the lineage IV PPRV strains; showing particularly strong affiliation with Tibetan and Indian strains. PPR is a common viral infection of the goats in Bangladesh, with a high case-fatality rate. This study confirms the circulation of lineage IV PPRV in the country with unique amino acid substitutions in N and H proteins and provides baseline data for vaccine development and implementation.

Keywords: Peste des petits ruminants virus, PPR, PPRV, case-fatality rate, goats, real time RT-PCR, Lineage IV, Bangladesh.

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Introduction

Peste des petits ruminants (PPR) is an acute, highly contagious animal disease and is responsible for high morbidity and mortality in sheep and goats. The disease is characterized by high fever, depression and loss of appetite, followed by ocular and nose discharge, erosive mouth lesions,

pneumonia and severe diarrhoea (Balamurugan *et al.* 2014). The morbidity rate in a susceptible population can reach up to 100% and the mortality rate can be 23–100% (Chowdhury *et al.* 2014). The high rates of morbidity and mortality make PPRV a significant threat in areas where the local economy depends on ruminant production. In Bangladesh, the rearing of goats is a profitable household enterprise for rural populations due to the animal's prolific breeding potential, survivability

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and consumption of locally collected feed (Islam *et al.* 2011). The country currently has approximately 25.7 million goats, representing 47% of all ruminants (<http://dls.portal.gov.bd>).

PPRV belongs to the genus *Morbillivirus* of the family *Paramyxoviridae* and has four genetically distinct lineages (lineage I, II, III & IV) (Maganga *et al.* 2013). Lineages I and II are commonly found in Western Africa, lineage III in Eastern Africa and the Middle East, and lineage IV is widely distributed in Asia and parts of the Middle East (El Arbi *et al.* 2014). Within the south Asian subcontinent, PPRV, belonging to lineage IV, was first reported in 1987 in the southern part of India (Shaila *et al.* 1989). In Bangladesh, PPR was first reported in goats in 1993 and since then it has become endemic in the country (Islam *et al.* 2001). Most of the previous PPR studies conducted in Bangladesh were based on either serology or clinical signs except a few recent publications with genetic characterization (Chowdhury *et al.* 2014; Rahman *et al.* 2016). The overall PPR sero-prevalence was 21% in 2008, ranging from 6% to 49% in different geographical locations/districts in Bangladesh (Bhuiyan 2012). A recent study conducted in 2009–2010 on laboratory confirmed outbreaks of PPR in Black Bengal goats resulted in 75% and 59% flock morbidity and mortality, respectively, with a case fatality rate of 74% (Chowdhury *et al.* 2014). Approximately 84 000 veterinary clinical cases of PPR were recorded in 2010 in Bangladesh, but it might not represent the actual burden of the disease as they all were not laboratory confirmed and presumptive diagnosis by veterinarians only has a moderate predictive value (Chowdhury *et al.* 2014; Haider *et al.* 2016). As the clinical signs of PPR are similar to other diseases such as foot-and-mouth disease (FMD), capripox, contagious pustular dermatitis, bluetongue and contagious caprine pleuropneumonia (Singh *et al.* 2009), differential diagnosis confirmed by appropriate laboratory tests will increase the diagnostic accuracy of the veterinarians. A limited number of studies described laboratory confirmed PPRV in goats and information about the genotypic diversity of

PPRV in Bangladesh (Chowdhury *et al.* 2014; Rahman *et al.* 2016). These studies identified Lineage IV PPRV among Bangladeshi goats that formed a sub-cluster along with recent isolates from Nepal, Bhutan and China (Rahman *et al.* 2016).

Investigating the diversity of circulating PPRV strains is important to assess the impact and usefulness of the available vaccines in Bangladesh. Various types of PPR vaccines, such as inactivated, vector based, protein based, recombinant and live-attenuated, have been developed. Among them live-attenuated PPR vaccines are considered to be the best choice for disease prevention in endemic regions (Balamurugan *et al.* 2014). The first attenuated PPR vaccine PPRV/Nigeria/75/1 (goat origin, lineage II) was developed in 1989 and was found to provide substantial protection against virulent viruses without any adverse effects (Diallo *et al.* 1989). In India, three PPR vaccines (lineage IV), namely PPRV/Sungri/96, PPRV/Coimbatore/97 (goat origin) and PPRV/Arasur/87 (sheep origin) were extensively tested and found to be safe and efficacious (Sen *et al.* 2010). Bangladesh Livestock Research Institute (BLRI) developed a tissue culture adapted vaccine against PPRV in 2001 and Department of Livestock Services has been using the vaccine to immunize the goats countrywide since November 2001 (Rahman *et al.* 2011). This vaccine required a good cold chain facility, which was difficult to maintain in field conditions in Bangladesh and led to BLRI to develop a thermostable vaccine, named Titu (lineage IV, goat origin) (EFSA-AHAW-Panel, 2015). However, questions are often raised by the practising veterinarians and goat farmers about its effectiveness as vaccinated goats are often observed with the PPR infection.

This study had three objectives: (1) estimate the prevalence of laboratory-confirmed PPR in goats presenting with clinical signs compatible with PPRV diseases from different areas of the country; (2) describe the clinical and demographic profile of infected animals and (3) characterize the circulating PPRV genotypes to understand the possible impact of currently available vaccines.

Materials and methods

Enrolment of goats and collection of specimens

From May 2009 to August 2010, we conducted surveillance for sick goats at three government veterinary hospitals in three sub-districts of Bangladesh located in the northwestern (Netrokona), northern (Dinajpur) and southeastern (Chittagong) areas of the country. Veterinarians at each of the three hospitals obtained informed consent from the owners/handlers of the goats and enrolled the animals in the study with any of the clinical signs: diarrhoea, breathing difficulties, and/or measured rectal temperature greater than or equal to 39.4°C (103 °F). (Encyclopedia, 1994; Radostits *et al.* 2000). After enrolment, veterinarians interviewed the owners of the animals to collect demographic data including age, breed, sex and clinical history. The veterinarians also asked whether the goats had received PPR vaccination during the last year and recorded the addresses of each owner to follow up on the goats' clinical outcomes. Veterinarians collected faecal and nasal swabs from goats meeting the screening criteria in cryovials containing lysis buffer (RLT buffer, Qiagen, USA). These samples were then stored in a liquid nitrogen dry shipper until transferred to the laboratory for further processing. We visited each household with a sampled animal between 14 and 60 days after the clinic visit to ask about the animals' health outcomes at 14 days of veterinary hospital visit. We classified season of patient presentation as winter (November to February), summer (March to June) and rainy season (July to October) (Buet 2008).

RNA extraction and polymerase chain reaction

Total RNA was extracted from 100 µL of the swab sample, which was collected in RLT buffer from the RNA easy mini kit (Qiagen, Germany) as per manufacturer instructions. Real time, one step RT-PCR was carried out with the BioRad CFX-96 Real-Time system (BioRad, USA) using the Superscript III/Platinum Taq One-step qRT-PCR kit (Invitrogen) for detection of PPRV according to procedures described elsewhere (Bao *et al.* 2008). Ten per cent of PCR positive samples were randomly selected for

genetic characterization by sequencing nucleocapsid (N) and haemagglutinin (H) genes fragments. These genes fragments were amplified using the primer sets Nad1/Pad1 for N gene and hrf3/hre4 for H gene followed by direct amplicon sequencing (Couacy-Hymann *et al.* 2002), (Balamurugan *et al.* 2006). The amplified products were analysed by electrophoresis through a 1% agarose gel and staining with ethidium bromide. The PCR products were purified by ExoSAP (Affymetrix, CA, USA) treatment for nucleotide sequencing using an automated Genetic Analyzer ABI 3500 XL (Applied Biosystem, Foster City, CA) and Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem). The nucleotide sequences were deposited in GenBank under the accession numbers KT253989–KT253999 for the N gene and accession numbers KT254000–KT254005 for the H gene.

Sequence analysis

The electropherogram files of the nucleotide sequences were examined and edited using Chromas 2.23 (Technelysium). Sequence similarity searches were performed using the Basic Local Alignment Search Tool (BLAST) server on the GenBank database. Sequences were aligned using BioEdit 7.0.5 (Hall 1999). Phylogenetic trees were constructed according to the maximum likelihood method using the MEGA (Molecular Evolutionary Genetics Analysis) version 6.0 (Tamura *et al.* 2011). The bootstrap probability at each branching point was calculated with 1000 pseudoreplicate data sets. Evolutionary distances in the phylogenetic tree were computed using the Kimura-2 parameter model (Kimura 1980; Tamura *et al.* 2011). The prediction of linear B cell epitopes based on virtual analysis of the amino acid sequences, derived from the respective H gene nucleotide sequences, were conducted using web-based antibody epitope prediction tool (<http://tools.immuneepitope.org/bcell/>).

Statistical analysis

We performed descriptive analysis and compared the frequency of each laboratory confirmed infection

associated with breed, location of sampling and season through Chi square or Fishers' exact test and report the *P* value. We calculated the odds ratio (OR) of laboratory confirmed infections among goats presenting with compatible clinical signs associated with breed, location and season with a 95% confidence interval (CI).

Results

We enrolled 539 goats from three government veterinary hospitals (Table 1). The mean age of the goats was 19 months (range: 1–120 months) and 60% ($n = 325$) of the goats were female. Only five goats were reported as being vaccinated against PPR. Thirty-eight per cent ($n = 203$) of the goats had detectable RNA for PPRV. The most common clinical signs in PPRV-positive goats were fever, respiratory distress and diarrhoea (Table 1).

Among the 203 goats that had detectable RNA for PPRV, 91% ($n = 184$) were followed up. The address of the remaining animals 9% ($n = 19$) could not be identified correctly or the owner could not be contacted. Among the 184 animals followed up, 24% ($n = 44$) goats died. Fifty per cent of the followed up animals ($n = 92$) recovered, 11% remained sick during the follow up ($n = 21$), 11% were sold ($n = 20$) and 4% were slaughtered ($n = 7$). The proportion of PPRV-positive samples varied according to their location, breed, and season. PPR was more common in cross-bred (51%; $P = 0.009$) and Jamunapari (46%; $P = 0.004$) breeds than it was in the Black Bengal (33%) breed. PPR was more common in the summer (45%, OR = 1.9, CI: 1.3–3.1) than in the rainy season (Table 2). Among the five goats in our study previously vaccinated against PPRV, three were infected with PPRV.

We retrieved 11 PPRV N gene fragments (542 bp) from a sub set of real time RT-PCR positive samples (10%, $n = 20$). The nucleotide sequence similarity of the N gene fragments revealed that the Bangladeshi PPRV strains were 99% ($\pm 0.5\%$) identical to each other and to the Tibetan strains found in China. They had 96–98% nucleotide conserved identities with those reported from India. In contrast, less than 90% identity with African strains (Nigeria, Guinea-Bissau,

Table 1. Characteristics of goats enrolled from three veterinary hospitals in Bangladesh between May 2009 and August 2010

Variables		Frequency	Per cent
Goats enrolled at three veterinary hospitals	Netrokona	93	17
	Dinajpur	179	33
	Chittagong	267	50
	Total	539	100
Sex	Female	325	60
Mean age in months (range)	Goats	539	19 (1–120)
Goat breed	Black Bengal (BB)	353	66
	Jamunapari (JP)	137	25
	Crossbred (BB × JP)	49	9
Fever		483	89
Respiratory distress		418	78
Diarrhea		286	53
Neurological signs		133	25
Interval in days between onset of illness and bringing the animals to veterinary hospitals for medication [mean (median) (range)]		539	4.5 (2) (0–30)
Animals treated before bringing them to veterinary hospitals* PPR vaccinated		121	23
		5	1

*By another practicing veterinarian or local non-certified veterinary practitioner.

Oman, Cote d'Ivoire, Ethiopia and Sudan). Phylogenetically, the Bangladeshi PPRV strains clustered with strains that belong to the PPRV lineage IV (Fig. 1). A number of amino acid substitutions ranging from single to three amino acids were found in the partial N gene fragments of Bangladeshi strains when compared with others (Fig. 2). Two of those substitutions, K423Q and E426G, were consistent with the previously reported Chinese isolates (Chowdhury *et al.* 2014). Two unique substitutions K439R and I471F were frequently observed but only in Bangladeshi strains. The study strains also varied from the previously reported Bangladeshi strains by a mutation A → T amino acid at position 401 (Fig. 2). In accordance to the reference sequence for PPRV (NC006383), three consecutive amino acid

Table 2. The odds ratios (OR) of PPR infections among the goats presenting with compatible clinical signs at three veterinary hospitals in Bangladesh between May 2009 and August 2010

Name of disease	No. of animals tested	No. positive (%)	OR [†] (95% CI [‡])
<i>Peste des petits ruminants</i> (PPR) in goats	539	203 (38)	–
By goat breed			
Black Bengal	353	115 (33)	1
Jamunapari	137	63 (46)	1.8 (1.2–2.7)*
Crossbred	49	25 (51)	2.1 (1.2–4.1)*
By location			
Netrokona	93	12 (8)	1
Dinajpur	179	62 (35)	3.6 (1.7–7.8)*
Chittagong	267	129 (48)	6.3 (3.2–13.3)*
By season			
Rainy (July–Oct)	164	48 (29)	1
Winter (Nov–Feb)	171	64 (37)	1.4 (0.9–2.3)
Summer (Mar–Jun)	204	91 (45)	1.9 (1.3–3.1)*

*Significant at $P < 0.05$. [†]Odds ratio, [‡]Confidence Interval.

substitutions, EIM to DFI, at the positions 471–473 were observed in KT253989, KT253990 and KT253997 (Fig. 2).

Six H gene nucleotide sequences (523 bp) were retrieved and phylogenetic analysis of the sequence data also indicated that the Bangladeshi PPRV strains were mostly related to the Tibetan strains found in China, showing 95.9–98.8% nucleotide sequence identity (Fig. 3). The study strains had 94.7–97.7% nucleotide sequence identity with Indian strains. Among the Bangladeshi strains, up to 2.9% divergence was observed in the H gene fragment. A number of amino acid substitutions were found when compared with the reference PPRV sequence (NC006383). The study strains had a unique amino acid substitution, G396D, which was consistently found among the study strains but not in the Tibetan strains (Fig. 4). The study strains varied from Indian strains by the substitutions, E → K at position 317, D → G at positions 396, 424 and L → S at position 419 (Fig. 4). In the predicted linear B cell epitope of SDPGVVI, an amino acid substitution G424D, was frequently observed among PPRV strains from Bangladesh and Tibet, China but not from India (Fig. 4).

Discussion

More than one-third of the goats were positive for PPRV and one quarter of the enrolled goats died from the disease. This case fatality rate is consistent with the previously reported rates from India (30%) and Pakistan (30%) (Sharma *et al.* 2007; Khan 2010), confirming the severity of PPR in goats in this region. In Bangladesh, not all goats are presented to veterinary clinics. The goat farmers bring their animals to veterinary hospitals on foot as they usually cannot bear the expense of renting vehicle. In addition, the animals in our study also benefitted from treatment with antibiotics and anti-histamines according to the standard practices for PPR clinical management (Haider *et al.* 2016; Radostits *et al.* 2000). Thus, it is possible that the case fatality rate of PPR among Bangladeshi goats reported elsewhere and in our study is underestimated.

Our results indicate that the local goats (Black Bengal) appear less susceptible to PPR than Jamunapari and the cross-bred goats, contrasting with the previous findings of Mondal *et al.* (1995) but in agreement with a recent study (Rony *et al.* 2017) and the Food and Agricultural Organization (FAO, 2010) and International Atomic Energy Agency who reported that the Black Bengal goats had innate resistance against common diseases and were well adapted to the local environment.

We found a higher frequency of PPR in goats during the summer season, which is in agreement with previous studies in India, Pakistan, and Bangladesh (Das *et al.* 2007; Singh *et al.* 2009; Khan 2010). During summer, the goats are more frequently allowed to graze in communal grazing field which might increase the chance of virus transmission from diseased goat to healthy ones.

The PPRV strains circulating in Bangladeshi goats were genetically similar although the samples were collected from different districts, Dinajpur, Netrokona and Chittagong bordering Indian districts, Dakshin Dinajpur, Meghalaya and Tripura which are very close to Tibet, China. Genetic analysis reveals that PPRV strains circulating in this region share a common phylogenetic lineage IV (Figs. 1 and 3) and possibly spread either from India and/or China to

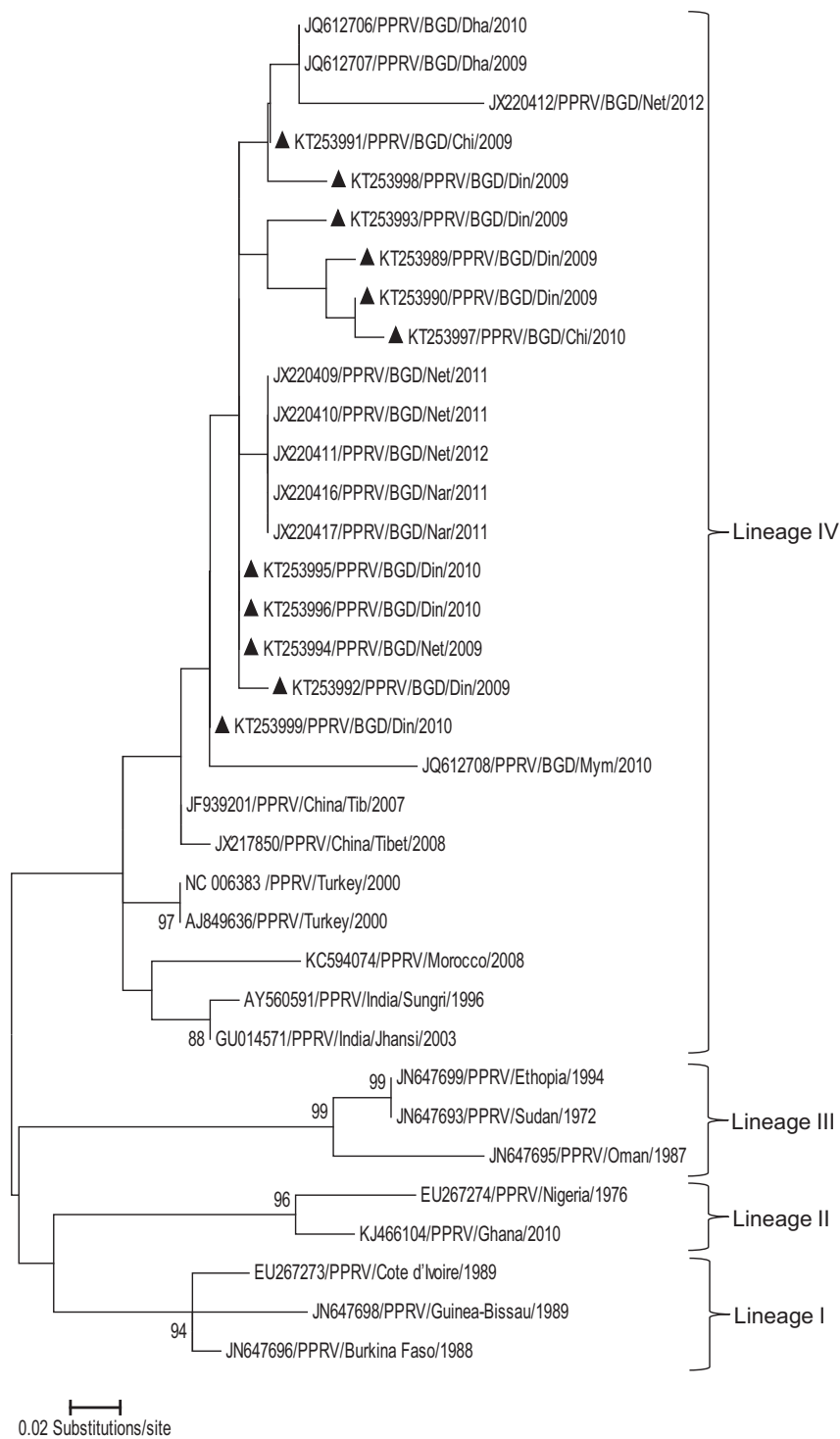


Fig. 1. The Phylogenetic analysis of N protein amino acid sequences (85 amino acid residues) of Bangladeshi strains and the representative strains of each lineage of PPRV using Maximum Likelihood method. The study Bangladeshi strains were denoted with black triangle (▲). Bootstrap values (1000 pseudo-replicates) above 80 are shown.

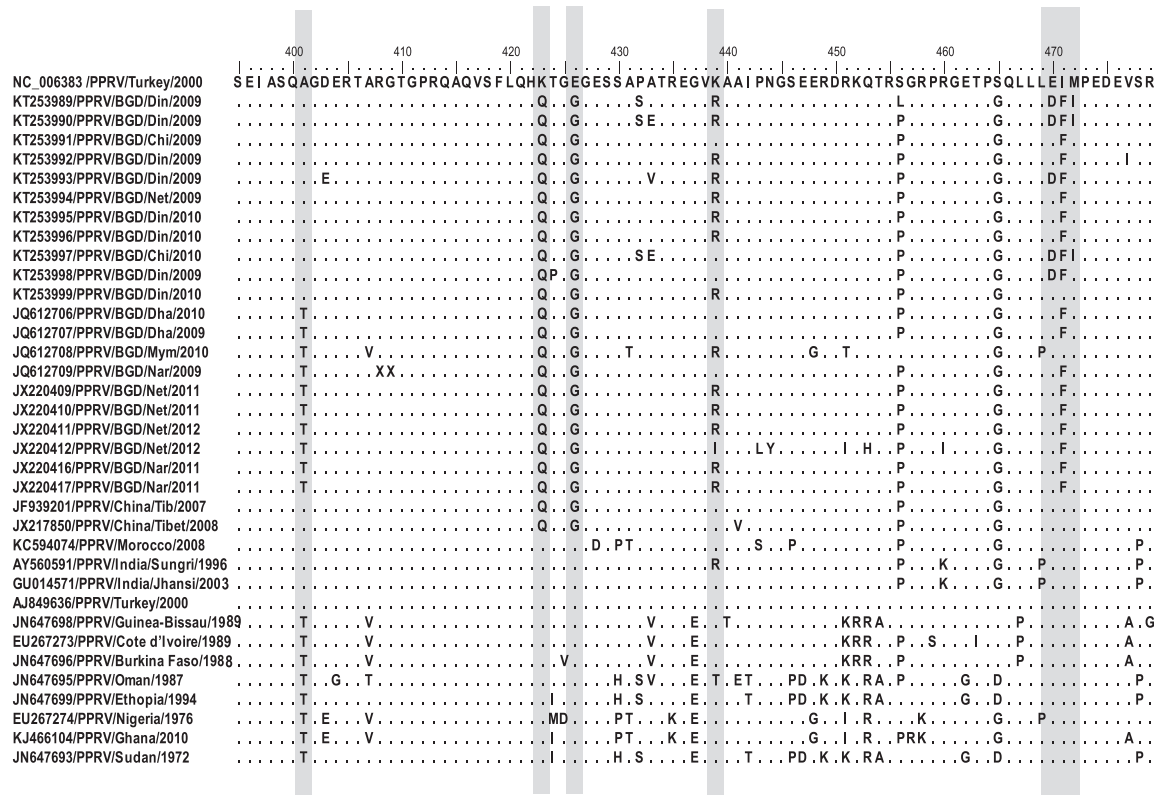


Fig. 2. Multiple sequence alignment of the partial amino acid N protein sequences (85 aa) of the PPRV strains ($n = 11$) detected in Bangladesh during 2009–2010 along with other Bangladeshi strains and representative PPRV strains of Lineages I to IV. The sequences were annotated according to PPRV Turkey strains, NC 006383. The gray-highlights indicate the significant amino acid indels among the strains.

Bangladesh or vice versa, through legal or illegal trans-boundary commerce. In contrast, they showed a number of substitutions in the amino acid sequences in both H and N gene fragments when compared with global strains. We detected amino acid substitutions in the predicted B cell epitopes of the H-proteins which regulates viral adsorption, host entry, its pathogenicity, release of newly produced viral particles (Liang *et al.* 2016) and is an important target for neutralizing antibodies against PPRV (Rota *et al.* 1992). Furthermore studies based on reverse genetics experiments will also be helpful to identify whether these changes have any impact on antigenicity and pathogenicity of PPRV.

The multiple sequence alignment of the N-gene amino acid sequences revealed that most of the amino acid substitutions were detected in a 59 amino acid window of the N-protein (420 and 479) (Fig. 2).

By using this window we were able to phylogenetically differentiate the lineages of PPRV strains with the similar topology in the phylogenetic tree (Supplementary Figure S1). Thus, the sequence variations in this 59 amino acid window in the N gene can be used as a culture-independent PPRV genotyping tool.

A very small proportion of the goats enrolled in our study were vaccinated against PPR and half of them were still infected with the disease. The government of Bangladesh delivers the PPRV vaccine at a very low subsidized cost (0.60 USD (50 BDT) per vial for 100 doses) but the effectiveness of the vaccine had been in question from the very beginning of its introduction (Sarkar *et al.* 2003). The present study revealed that the Bangladeshi PPRV strains are genetically diverse and may have broader spectrum of antigenic variations. Continuous PPRV

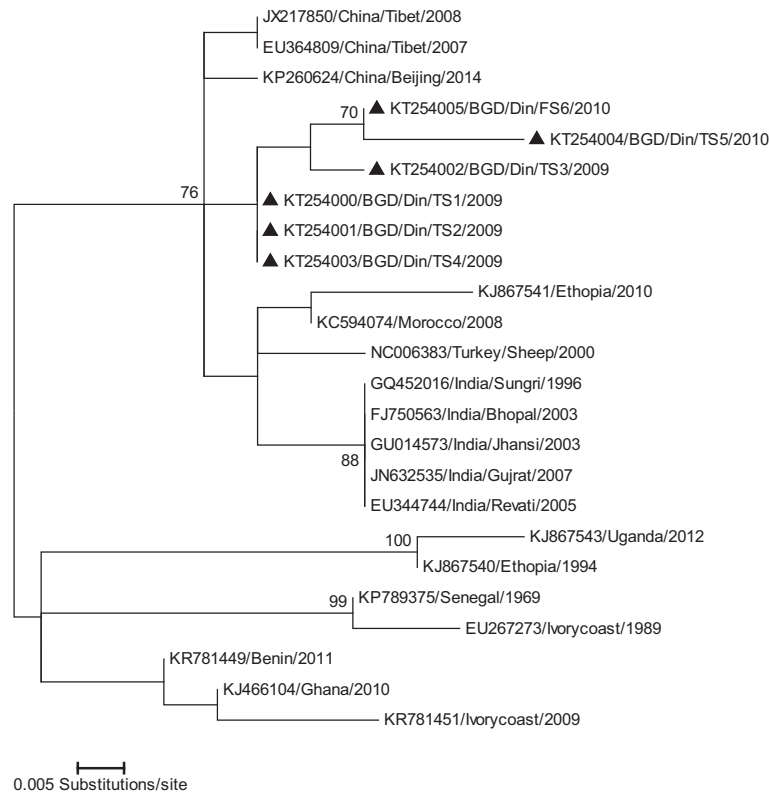


Fig. 3. Phylogenetic analysis of H gene amino acid sequences (174 aa) of the Bangladeshi study strains and representative strains of PPRV using Maximum Likelihood method. The study Bangladeshi strains were denoted with black triangle (▲). Bootstrap values (1000 pseudo-replicates) above 70 are shown.

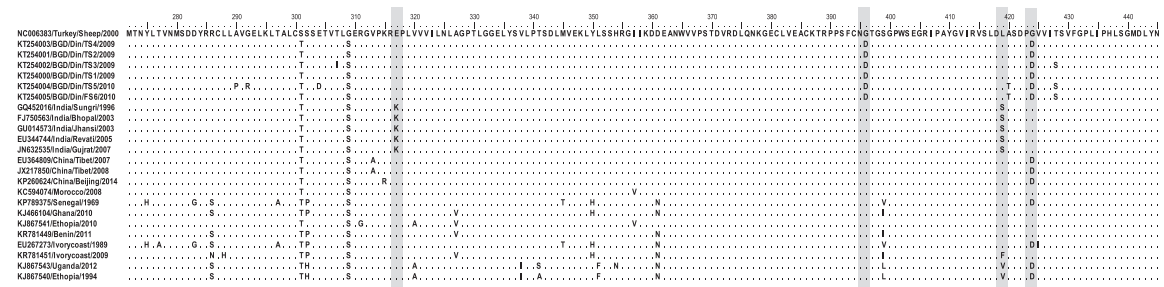


Fig. 4. Multiple sequence alignment showing H gene amino acid (174 aa) differences between Bangladeshi PPRV strains and other circulatory representative strains in Asia and Africa. The sequences were annotated according to PPRV Turkey strains, NC 006383. The gray-highlights indicate the significant amino acid indels among the strains.

surveillance is required to know more about the genotypic and antigenic variations of PPRV strains circulating in Bangladesh as well as selecting possible vaccine candidates for a successful immunization programme.

There are several limitations in our study. First, the study was carried out in veterinary hospitals only, which might exclude household sick animals. Second, all the sequences were retrieved directly from the clinical samples that might have low nucleic acid

content of PPRV and remained undetectable, causing false negatives. Sometimes, PCR produced multiple bands that hindered the quality sequence data. Third, our genotyping analysis was based on partial N and H genes sequences. Tissue culture-based PPRV isolation followed by its whole genome-based analysis is needed to reveal more about the PPRV strain diversity and predicted antigenic variations.

Conclusion

PPR is a common infectious disease of goats with a high case fatality in Bangladesh.

Bangladeshi PPRV strains clustered with strains that belong to the PPRV lineage IV with unique amino acid substitutions. Furthermore country-wide surveillance and monitoring are required to determine the socioeconomic impact of PPRV strains in Bangladesh as well as for their pathogenicity, virulence and selection of vaccine type.

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Conflicts of interest

Authors have no financial and other conflicts of interest to declare.

Ethical statement

Our trained veterinary officers obtained informed written consent from the owners of cattle and goats enrolled in the respective veterinary hospitals. The

team also took appropriate personal protection during the biological specimen collection. All the sampling procedures have been reviewed and approved by the animal experimentation and ethics committee of icddr,b.

Contributions

MZR, NH conceived, designed, and supervised the study; ESG, JHE, NZ protocol supervision and reviewed the manuscript; MGO, SAK supported field activities; SA involved in lab testing; MBH performed epidemiological data analysis; AI involved in sample and data collection; MZR and MEH supported lab data analysis and manuscript preparation; MR reviewed the manuscript.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. The Phylogenetic analysis of N protein amino acid sequences (59 amino acid residues) of Bangladeshi strains and the representative strains of each lineage of PPRV by using Maximum Likelihood method. The study Bangladeshi strains were denoted with black triangle (▲). Bootstrap values (1000 pseudo-replicates) above 80 are shown.