Molecular Epidemiology Of Peste Des Petits Ruminants (PPR) In Goat

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ABSTRACT: The study was planned to carry out the molecular epidemiology of PPR. The data (n=143) were collected from two village goat and a market of Shibgonj, Bogra, Bangladesh and nasal swabs (n=72) were collected from clinically suspected goats from twelve Veterinary Hospitals of seven districts during July-October 2012. The virus was detected by RT-PCR technique in which PPRV: F1b & F2d; NP3 & NP4 primers were used to detect and F gene of three isolates (Named PPRV/BD/Rajshahi-1/2012, PPRV/BD/Rajshahi-2/2012 and PPRV/BD/Bogra/2012) were sequenced and phylogenetic analyses were performed. The survey resulted that the overall morbidity and mortality were 19.79 and 11.51 % respectively in the post outbreak period as well as the molecular study confirmed that 33.33 % samples were diagnosed as positive and this can be interpreted as, the goat were suffering from high fever with or without diarrhea still incubating the disease, the highest morbidity and mortality were found in male goats and in goats of age between 4-12 months. The N gene was revealed 100% confirmatory diagnosis in comparison with F. The findings of the nucleotide sequences of F gene fragments revealed that each of the isolates belonged to Lineage IV and most closely related to the others obtained from Gene Bank. PPRV/BD/Bogra/2012 has 100% homology in terms of partial sequence, 289bp of F gene with the viruses- Narayangonj/2009(Ac.No.JX094440), Dhaka/2009(Ac.No.JX094438) and BAU/Mymensingh/2010 (Ac.No.JX094436).

KEY WORDS: Peste des petits ruminants (PPR); Molecular Epidemiology; RT-PCR; F gene sequencing.

1. Introduction

Peste des petits ruminants (PPR) is an endemic disease of goat, caused by RNA virus of the genus Morbillivirus that was first described by [1] from Ivory Coast. RT-PCR is a confirmatory diagnostic tool for PPR virus. The virus was identified after a severe outbreak during 1993 from the south-western districts of Bangladesh and the disease is now endemic in this country [2]. PPR borne morbidity and mortality varies depending on the environment. The outbreaks of PPR caused 74.13 % morbidity and 54.83 % mortality in Black Bengal goats in Bangladesh [3], [4]. The influence of sex on PPR outbreaks was found to be higher in male (28.52 %) than female (13.04 %) goats. As regards to age, PPR was significantly higher in young (31.06 %) and adult (10.15 %) [5]. Rapid and specific diagnosis by RT-PCR has become possible with the techniques first developed by [6]. using F gene-specific. RT-PCR helps to study the possibility of analyzing the relationship between the different PPRV strains for molecular epidemiological studies [7].

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Partial sequencing of 372 bp RT-PCR product of F-gene was used by several researchers to identify nucleotides sequence variation and to delineate the PPRV isolates from different geographical locations [8]. About half crore of domesticated goats are dipped in a risk because many outbreaks remain unreported and undiagnosed so that disease is really hard to confirm. Goats are highly susceptible to PPRV and molecular (Quick confirmatory) diagnosis an epidemiological studies are crying need as beyond of doubt. It has assumed that further study is required to know details about the husbandry effects of PPR, morbidity & mortality rate of PPR infection during post outbreak period and molecular properties of currently circulating strain of PPR virus in Bangladesh. To mitigate aforementioned points, the present study was planned with the objectives- epidemiological investigation of PPR in goats, detection of PPRV by RT-PCR from field samples and sequence analysis of F gene for phylogenetic analyses.

2. Materials and Methods

A survey was conducted and 143 data (Such as-age, sex, distribution, time, clinical signs, morbidity and mortality rate) were collected from PPR suspected goats for epidemiological study of village Bakson & Medinipara, Daridaha cattle & goat market of Shibgonj, Bogra, Bangladesh. To conduct molecular epidemiology 72 nasal swab samples were collected from suspected goats with the history of fever, discharge (specially nasal) from veterinary hospitals of Dhaka (Dhamrai), Bogra (Shibgonj, Gabtoli, Sariakandi, Sonatola, Dhunot), Norshingdi (Shibpur), Chittagong (CVASU Vet Teaching Hospital), Jaypurhat (Kalai), Mymensingh (Sadar), Rajshahi (Sadar) during July-November, 2012. Sample was vortexed and filtered with 0.22 µm syringe filter and aliquote was used to extract DNA for virus detection using One-step RNeasy kit of QIAGEN (Germany) (Cat No. 74106) according to manufacturer's instruction followed by detection by RT-PCR, purification of PCR product followed by sequencing and phylogenetic analyses. Two sets of primer (PPRV F1b & PPRV F2d of 448 bp (Ozkul, 2002) and NP3 & NP4 of 351 bp) [9]. were used to detect virus genome. About 5 µl of extracted viral RNA was taken into the PCR tube the RT-

PCR for virus detection was performed as following: Reverse transcription at 50oC for 30 min, enzyme inactivation at 95°C for 15 min. PCR for F gene was performed as, initial denaturation for 1 min at 95°C, followed by 35 full cycles of amplification, with each cycle consisting of 1 min of denaturation at 94°C, 1 min of annealing at 50°C, and 2 min of extension at 72°C followed by final extension for 7 min at 72°C. For N gene, annealing was performed at 55°C for 30 sec and extension at 72°C for 30 sec and final extension was for 10 min. Other conditions were same as F gene. After the program was over PCR product was run in 1 % agarose-gel. PCR reaction mixture was transferred to the EZ-10 Column purified PCR product was sequenced by dideoxy terminating method [12]. using Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Perkin-Elmer Applied Biosystems) sequence reading by using an automated DNA sequencer 3110 Genetic Analyzer (Applied Biosystems) available at National Reference Laboratory for Avian Influenza (NRL-AI) in BLRI, edited with SeqEscape V2.6 software. Molecular and all the laboratory works were done at Animal Health Research Division of Bangladesh Livestock Research Institute (BLRI), Savar, Dhaka.

3. Results and Discussion

3.1 Overall survey results

During July 2012, a total of 143 goats information were collected. Under this study we found that about 19.79 % of goats were infected with PPR like disease and 81.21 % were not showing significant symptoms. Overall mortality was 11.51 % (18/143) (Table 1). The highest morbidity and mortality was in the village Bakson and male goats were more susceptible (15.07 %) than the females (9.14 %). The present study was performed to observe the differences and occurrences of PPR according to sex difference. Numbers of male and female goat were 91 and 52 respectively. In male, the morbidity was 15.07 % and mortality was 9.14 %. Considering the sex, the highest morbidity and mortality were found in male goats and infection rate was found higher in goats of age between 4-12 months than those of 13-24 months and over 24 months (Table 2).

3.2 Clinical sign wise distribution of affected goats

Clinically, PPR diagnosed tentatively by observing commonly manifested clinical signs such as high fever, diarrhea, nasal discharges, coughing, respiratory disturbances, loss of appetite and death history. When, any of the goats showing PPR representative one and/or more of above described clinical signs then nasal swab were taken by casting of goats with the help of animal assistant of veterinary hospitals. Among these signs the most predominant one was high fever (23.53%) and another more common was diarrhea (19.35%) whereas nasal discharge was (17.39%) and lowest was coughing (3.13%). This study can be interpreted as, the goat suffering from high fever slightly with or without diarrhea may incubate the disease.

3.3 Age and sex wise distribution of clinically affected goat

It was conducted to study the differences and occurrences of PPR between the age and sex of goat. Among the (n=72) samples, infection was found higher in goats between 4-12 months than those of 13-24 months and over 24 months of age. The percentage of infection was more in young animals (59.40%) as compared to adults (46.12%) in goats described by Kumar et al. [10]. It was found that the rate of infection was higher (29.87%) in goats of 4-12 months of age. The representing data can be interpreted as the females (18.05% (13/72)) are most susceptible to PPR infection. The prevalence of PPR in goats was higher (28.52%) in males than females (13.04%) described by Rahman et al. [11]. Also our survey result detected that in goats of both sexes highest morbidity and mortality were found in male (Male: 15.07 & 9.14 % and female: 4.71 & 2.35%).

3.4 Spatial and temporal distribution of clinically affected goat

It is known that occurrence of PPR infection strictly follow the distribution patterns and was found to depend upon the month of a year other than that the endemic disease indicates that the infection found throughout the year and rates of infection is in regular fashion. A total (n=72) of nasal swab (24 from Norshinghdi, 4 from Dhaka, 6 from Rajshahi, 8 from Chittagong, 2 from Mymensingh, 4 from Jaypurhat, 24 from Bogra) were collected and among all suspected nasal swab samples 12 in Bogra, 2 in Joypurhat, 4 in Norshingdi, 2 in Dhaka, 3 in Chittagong and one in Rajshahi were found positive which indicated that previous infection was observed as endemic pattern. The clinical prevalence of PPR was highest in the month of December (31.68%) and lowest in June (9.52%) described by Subir et al. [5]. The present study revealed that 33.33% cases were found to occur in July-August whereas 66.67% in Sept.-Oct.

3.5 Identification of PPRV in field samples by reverse transcriptase polymerase chain reaction (RT-PCR)

The samples were processed for virus detection by RT-PCR and two sets of primer targeting two different genes having the Amplicon size for F1b and F2d primer was 448 bp and NP3 & NP4 was 351 bp (PPRV-F1b:5´ AGTACAAAAGATTGCTGATCACAGT-3´(Forward), F2d:5´-GGGTCTCGAAGGCTAGGCCCGAATA-3´(Reverse) & NP3:5´-TCTCGGAAATCGCCTCACAGACTG-3(Forward), NP4: CCTCCTCCTGGTCCTCCAGAATCT-3´(Reverse)) of PPRV were used. RNA was extracted and the product was run on the ethidium bromide stained 1% agarose gel in TAE buffer at 100 volts for 40 minutes. Finally, DNA band was visualized by observing the gel under UV trans-illuminator. Out of 72 swabs, about 33.33 % (24/72) samples were found positive. Representative figures for RT-PCR result were presented in Figure No. 2, 3 and 4 and figure represents the detection of 351 bp & 448 bp DNA from field samples amplified by primer set. Our study confirmed that the district wise highest positivity rate was 50% found in those samples collected from Joypurhat and Dhaka followed by Bogra 40.55% (Table 3).

3.6 Comparison of two set of primers for detection of PPRV

PPRV detection was done by using two set of primer PPRVNP3 & PPRVNP4 and PPRVF1b & PPRVF2d. Among 72 clinical samples 24 samples were as positive by NP whereas 18 were detected as positive by F. By this study it is evident that N gene is more effective than the F gene in regards to quick confirmatory disease diagnosis of field sample in our investigation. This finding can be interpreted as the N primer is capable for detection of all positive nasal swabs except any type of assay faults.

3.7 Nucleotide sequencing of F gene and phylogenetic analyses

For molecular characterization F gene of three PPRV (two from Rajshahi and one from Bogra) obtained from goat were sequenced. Numbers of nucleotides sequenced from F gene of PPRV were 295 bp for PPRV/BD/Rajshahi-1/2012, PPRV/BD/Rajshahi-2/2012 and 289 bp for PPRV/BD/Bogra/2012. Nucleotides of sequenced three isolates range from adenine (A) 29.9-30.2 %, thymine (T) 23.1-23.2 %, guanine (G) 22.0-22.1 % and cytosine (C) 24.6-24.8 %. For phylogenetic analysis a total of 20 PPRV covering lineage I-IV are used and found that the average nucleotide contents of these viruses is A 29.9, T 23.2, G 23.9 and C 23.8 %. Sequenced viruses are found 99.7-100 % homologous to each other. Most closely related viruses obtained from Gene Bank are also isolated from Bangladesh. The viruses are; BD/Narayangonj/2009, Dhaka/2009,BAU Mymensingh/2010, Netrokona/2/2011andNerokona/2/2012. Interestingly, one of the virus namely Bogra/2012 sequenced in this study has 100% homology (in terms of partial sequence, 289bp of F gene) with the viruses namely BD/Narayangonj/2009 (Accession number JX094440), Dhaka/2009 (Accession number JX094438) and BAU Mymensingh/2010 (Accession number JX094436) sequenced earlier. Our isolates clustered with the viruses from Bangladesh, China, India, Turkey belongs to lineage IV (Figure:5) Tables

Table 1. *Overall survey result of Daridaha cattle & goat market, Bakson & Medinipara (n=143)*

Places	No. of goats			No. of Sp		No. of Dead		Mb (%)		Mt (%)		Overall Mb (%)	Overall Mt (%)
	Total	M	F	M	F	M	F	M	F	M	F		
Daridaha cattle & goat market	50	34	16	6	4	4	2	12	8	8	4	19.79 (29/143)	11.51 (18/143)
Bakson	65	45	20	10	4	8	2	15.38	6.15	12.30	3.07		
Medinipara	28	12	16	5	0	2	0	17.85	0	7.14	0		
Total	143	91	52	21	8	Average		15.07	4.71	9.14	2.35		

M=Male; F=Female; Md=Morbidity; Mt=Mortality; Sp=Suspected

Table 2. *Age and sex wise distribution of clinically affected goats*

Age group	sex group		Total (%)
	No. of male (%)	No. of female (%)	
Day old-3 months	8 (11.11)	5 (6.94)	13 (18.05)
4-12 months	13 (18.05)	18 (25.0)	31 (43.05)
13-24 months	17 (23.61)	10 (13.89)	27 (37.5)
≥24 months	7 (9.72)	2 (2.78)	9 (12.5)
Total	37 (51.39)	35(48.61)	72 (100)

Table 3.*Detection of PPRV from field samples by RT-PCR reaction (n=72)*

Name of District	Name of Upazila	No of Nasal swab sample	No. of RT-PCR positive	% positive
Bogra	Sadar	4	2	50.00
	Shibgonj	10	6	60.00
	Sonatola	6	2	33.33
	Gabtoli	2	2	100.0
	Sariakandi	1	0	00.00
	Dhunot	1	0	00.00
	Average			50.00
Joypurhat	Kahalu	4	2	50.00
Mymensingh	Sadar	2	0	00.00
Norsingdi	Shibpur	24	4	16.67
Dhaka	Dhamrai	4	2	50.00
Chittagong	CVASU campus	8	2	25.00
Rajshahi	Sadar	6	2	33.33
	Total	72	24	33.33

Figure 1.*Goats with nasal discharge (Left) and collection of nasal swab (Right)*

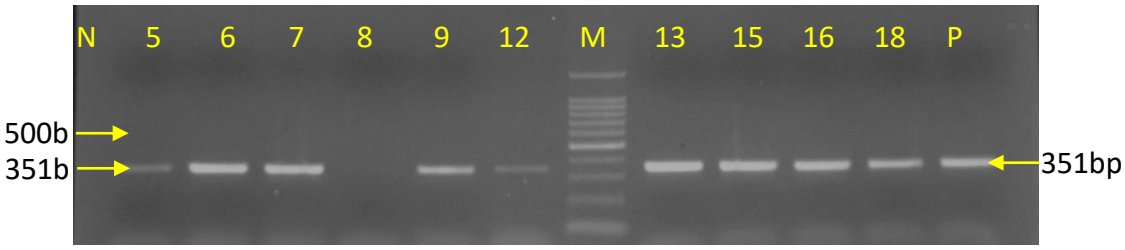

Figure 2. *Amplification of 352 bp fragment of N gene of a PPR virus from clinical samples by RT-PCR. (1% agarose gel stained with ethidium bromide, Lane M=Marker, Lane N= Negative control and P= Positive control and other lanes: represents field samples marked by RLDL No. 5, 6, 7, 8, 9, 12, 13, 15, 16 and 18)*

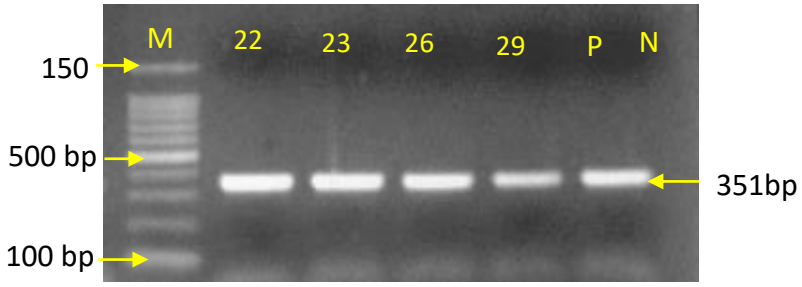

Figure 3. *Amplification of 352 bp fragment of N gene of a PPR virus from clinical samples by RT-PCR. (1% agarose gel stained with ethidium bromide, Lane M=Marker, P=Positive control, N=Negative control and other lanes: represents field samples marked by RLDL No. 22, 23, 26 and 29)*

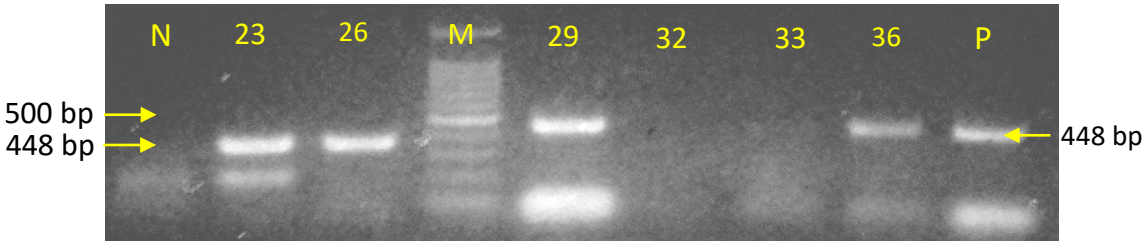

Figure 4. *Amplification of 448 bp fragment of F gene of a PPR virus from clinical samples by RT-PCR. (1% agarose gel stained with ethidium bromide, Lane M=Marker, Lane N= Negative control and other lanes: represents field samples marked by RLDL No. 23, 26, 29, 32, 33 and 36)*

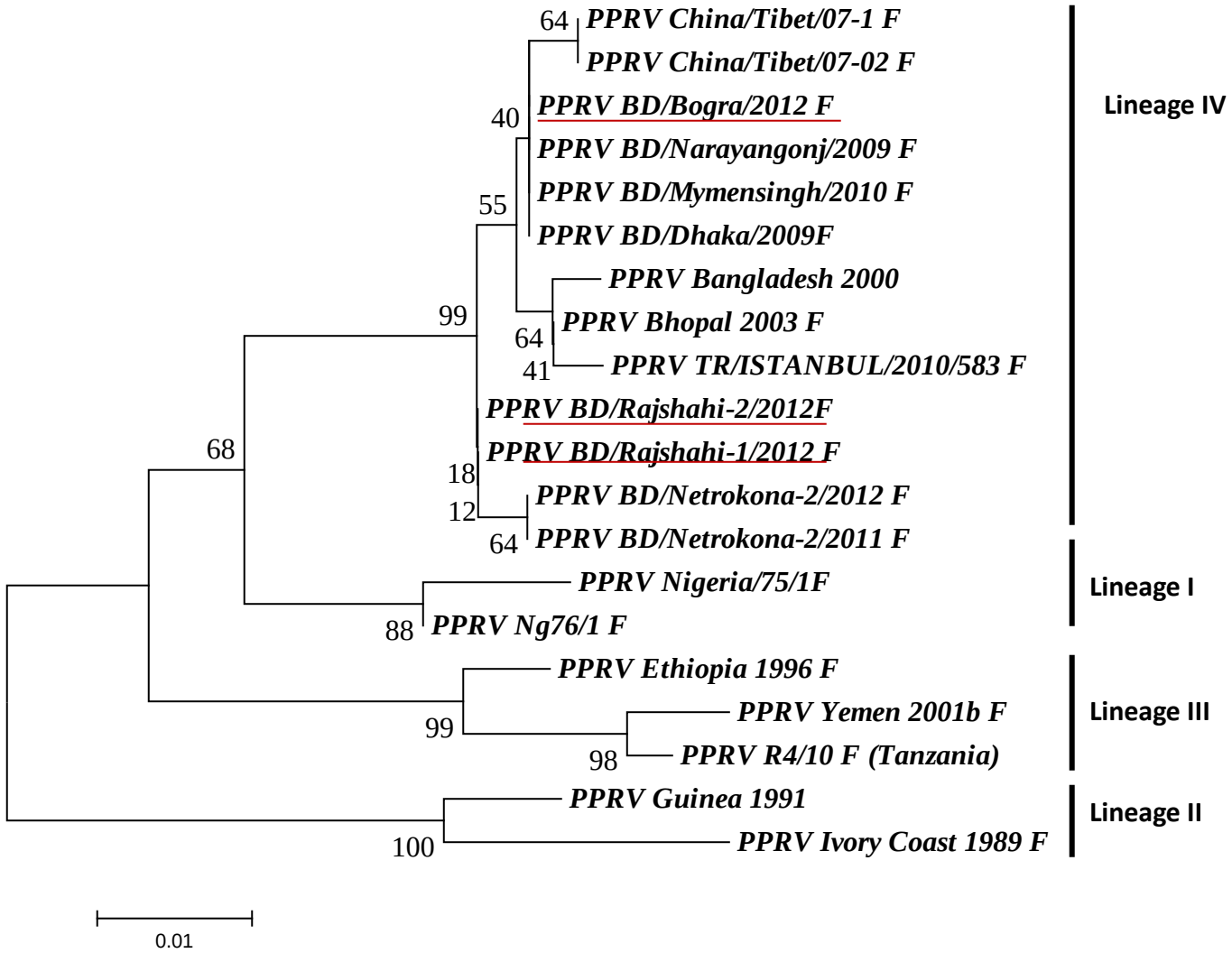

Figure 5. *Phylogenetic analysis of PPR viruses isolated from goat in Bangladesh. The tree was generated using neighbor-joining method and 1,000 replications of bootstrap re-sampling. The number at each branch point indicates percentage probability that the resultant topology is correct. About 289 bp from viral F gene of PPRV is employed to generate phylogram. Sequences generated through this study marked by red underlines in the tree.*

4. Conclusion

It can be concluded as after analyses data that PPR in Bangladesh maintains its existence endemically anywhere and round the year. Virus is shedding through nasal discharge from during and post infection of goats. Young goat and male are significantly getting higher frequency of infection than adults and females. Molecular studies revealed that it is the best confirmation as well as most of the isolates are under Lineage IV similarly has been found in south East-Asia, China, India and Pakistan from the years of this decade.

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