

## **Comparison of genes as target for molecular diagnosis of peste des petits ruminants in goats**

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### **Abstract**

Peste des petits ruminants (PPR) are an acute viral disease of sheep and goats. Rapid and accurate diagnosis is essential for successful control. Reverse transcriptase polymerase chain reaction (RT-PCR), a molecular diagnostic test based on amplification of the gene target is more sensitive than other tests. The study was to find an efficient primer set and structural gene, which would be more specific and sensitive for detecting PPR virus (PPRV) in field samples. Six primer sets for six structural genes of PPR were used. Primer against NP gene (np3/np4) was specific and sensitive. To ensure efficient amplification and detection of viruses in field samples, more than one set of primers should be used and F and N gene specific primers were the most suitable. (*Bangl. vet.* 2012. Vol. 29, No. 2, 56 - 62)

### **Introduction**

Peste des petits ruminants (PPR) is an acute viral disease of sheep and goats characterized by fever, catarrhal inflammation of ocular and nasal mucous membrane, erosive stomatitis, gastroenteritis and pneumonia. The causal agent is PPR virus (PPRV) an envelope, pleomorphic particle containing single-stranded RNA, approximately 16kb long with negative polarity genome (Barrett *et al.*, 2005). The genome of the virus codes for six structural (*N, P, M, F, H* and *L*) and two nonstructural (*C* and *V*) proteins in the order of 3'-*N-P(C/V) - M-F-H-L-5'* (Bailey *et al.*, 2005, Mahapatra *et al.*, 2006). The disease is prevalent in most African and Middle Eastern countries and the Indian subcontinent (Taylor, 1984). Morbidity and mortality of the disease can be as high as 100% and 90%, respectively (Abu-Elzein *et al.*, 1990, Dhar *et al.*, 2002). In Bangladesh, outbreaks of PPR were first reported in 1993 (Sil *et al.*, 1995; Islam *et al.*, 2001). Since then the disease has caused severe losses and is presently considered one of the major threats to about 22 million small ruminants in Bangladesh where mortality may reach 100%. At present, more than 1 billion sheep and goats in Africa and Asia are at risk of PPR (EMPRES, 2009). Rapid diagnosis is essential for successful control. Reverse transcriptase polymerase chain reaction (RT-PCR), a molecular diagnostic test based on amplification of the gene target offers a new strategy for diagnosis of PPRV, and is more sensitive than other tests. RT-PCR also offers the possibility of analysing the relationship between

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different PPRV strains for epidemiological studies (Shaila *et al.*, 1996; Ozkul *et al.*, 2002; Kwiatek *et al.*, 2007; Wang *et al.*, 2009; Balamurgan *et al.*, 2010). Although RT-PCR overcomes the limitations of conventional tests, the sensitivity varies depending on the primer used and the gene targeted. This may be due to high rate of nucleotide substitution error in RNA viruses (Steinhauer and Holland, 1986). The present study was undertaken to find an efficient primer set and gene, which would be specific and sensitive for detecting PPRV in field samples.

## Materials and Methods

### *Samples*

A total of 10 field samples were collected from dead PPR suspects. Mesenteric and bronchial lymph nodes and lung tissues were collected in sterile Falcon tubes. Pooled tissues were macerated using PBS as 20% (w/v) suspension, centrifuged at 800g for 10 minutes. The supernatant was collected in fresh sterile Falcon tubes, gentamycin was added 500 µg/mL and stored at -20°C. Freeze-dried live PPR vaccine from Livestock Research Institute (LRI), Department of Livestock Services and a previously identified isolate were used as the positive controls. When needed RNA was extracted from the same homogenate.

### *PCR*

RNA was isolated from the tissue suspension and vaccine virus using RNeasy Kit (Qiagen, Germany) as recommended by the manufacturer. Initially standardized RT-PCR specific for F and N genes were used individually to detect PPRV from each suspect. Samples found positive with F and N gene specific primers were used for further RT-PCR using P, M, H and L gene specific primers (Table 1). In all cases the reaction was set up as 5xQiagen One Step RT-PCR buffer 5 µL, dNTP Mix (containing 10 mM of each dNTP) 1 µL, Primer forward (100 pmol/µL) 0.5 µL, Primer reverse (100 pmol/µL) 0.5 µL, Qiagen One Step RT-PCR Enzyme Mix 1.0 µL, template RNA 5 µL, total reaction volume 25 µL. First the RNA was reverse transcribed by incubating at 50°C for 30 minutes, then the PCR started with an initial denaturation and activation of Taq polymerase at 95°C for 15 minutes. Annealing, elongation and final extension steps were standardized: Table 2 shows standardized temperature and time profile used. The expected band was visualized in 1.5% agarose gel stained with ethidium bromide. The result was viewed on the monitor; the image was printed and saved electronically.

### *Determination of sensitivity of primer*

RT-PCR was done further using diluted RNA and primer(s) that detected PPR virus in more field samples. Extracted RNA was quantified, and 10-fold dilution was made (Undiluted,  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$ ). One step RT-PCR was performed for each dilution. Judgment was made after electrophoresis on 1.5% agarose. The highest dilution that failed to amplify was considered as detection limit. Primers that

successfully amplified at highest dilution without background reaction were considered more sensitive.

Table 1. PCR primers used, sequences and specificity

Specificity	Primer name	Sequence	Position	Size	References
F gene	PPRV F1b	5'-AGTACAAAAGATTGCTGATC ACAGT-3'	760-784	448 bp	Ozkul, 2002
	PPRV F2d	5'-GGGTCTCGAAGGCTAGGCCC GAATA-3'	1207-1183		
N gene	NP3	5'-TCTCGGAAATCGCCTCACAG ACTG-3'	1232-1255	351 bp	Couacy- Hymann <i>et al.</i> , 2002
	NP4	5'-CCTCCTCCTGGTCCTCCAGA ATCT-3'	1583-1560		
L gene	pprl for2	5'-TGGAGCATTGTGGCATTGAC AC-3'	971-993	804bp	Muthuchelvan, 2005
	pprl rev2	5'-GTCATGCTCGTCTTTTGCCAT C-3'	1774-1753		
M gene	ppr_mfor1	5'-AGGAGCAAGGGCAACTGAG C-3'	1-20	780 bp	Muthuchelvan, 2006
	ppr_mrev2	5'-AGGCAAAACTAGACCCATC-3'	780-761		
P gene	pprp_for1	5'-TAGGACCCAGGTCAAGCAA C-3'	03-22	767 bp	Muthuchelvan, <i>et al.</i> , 2006
	pprp_rev2	5'-GACTGAGTCCCGTCTGTGC-3'	769-750		
H gene	Pprh_fr1	5'-TGTCATGTTCTTATAGAGTT-3'	1500-1519	328 bp	Balamurgan <i>et al.</i> , 2006
	Pprh_re2	5'-GACTGGATTACATGTTACCT-3'	1847-1828		

Table 2. PCR conditions for different primer sets used

Gene	Primer pair	PCR condition (s) at program step						Expected target size
		1	2	3	4	5	6	
F gene	PPRV F1b	95°C	94°C	50°C	72°C	35 cycles from step 2 to 4	72°C	448 bp
	PPRVF2d	15 min	1 min	1 min	2 min		7 min	
N gene	NP3	95°C	94°C	55°C	72°C	35 cycles from step 2 to 4	72°C	351 bp
	NP4	15 min	30 s	30 s	30 s		10 min	
L gene	pprl for2	95°C	95°C	50°C	72°C	30 cycles from step 2 to 4	72°C	804 bp
	pprl rev2	15 min	1 min	1 min	2 min		10 min	
M gene	ppr_mfor1	95°C	95°C	50°C	72°C	30 cycles from step 2 to 4	72°C	780 bp
	ppr_mrev2	15 min	1 min	1 min	2 min		10 min	
P gene	pprp_for1	95°C	95°C	50°C	72°C	30 cycles from step 2 to 4	72°C	767 bp
	pprp_rev2	15 min	1 min	1 min	2 min		7 min	
H gene	Pprh_fr1	95°C	94°C	55°C	72°C	29 cycles from step 2 to 4	72°C	347 bp
	Pprh_re2	15 min	30 s	30 s	30 s		7 min	

## Results and Discussion

Out of ten field samples, seven tested positive with one or more primers and three were negative with all primers (Figs. 1 - 6). The results showed that F gene specific

primer could detect six samples out of ten but gave non-specific band with some samples (Fig. 1). N gene specific primers detected PPRV in seven samples, with no non-specific bands (Fig. 2). The same six samples were positive in both cases. The 7th sample was positive with N specific primers but negative with F. The primers specific for L, M and P gene did not consistently detect PPRV (Figs. 3 - 5) and the primers specific for H gene failed to detect PPRV in any sample (Fig. 6). Dhar *et al.* (2002) noted that F1/F2 primer set did not work efficiently on a few samples. Three samples negative in conventional F gene based PCR were found positive by primers specific for N gene (Kerur *et al.*, 2008). None of the samples positive in F gene based PCR was negative by the two N gene based primers. N gene based primers were more sensitive than F gene based primers (Kerur *et al.*, 2008). The lower sensitivity with F gene specific primers might be attributed to nature of PPRV genome which shows higher rate of transcription of N-gene than F-gene. The target for PCR primers developed by Forsyth and Barrett (1995) was F gene, one of the external viral proteins. The N gene codes for an internal structural protein and mRNAs of N gene are the most abundant transcripts of the virus, making it attractive for development of a highly sensitive assay (Steinhauer and Holland, 1986).

In the present experiment, one isolate could not be amplified with F gene specific primer pair (F1b-F2d). This could be primer-template mismatch in the annealing site or low sensitivity of the test protocol. In the absence of sequence information conclusive inference could not be drawn on a possible mutation in the primer binding site. Failure of the other primers sets to detect PPRV in the field samples consistently could not be readily explained. RNA viruses are known to contain high rate of nucleotide substitution error (Steinhauer and Holland, 1986). To ensure efficient amplification and detection of PPR viruses, it is desirable to have more than one set of primers for diagnosis, and F and N genes are the most suitable (Kerur *et al.*, 2008). F and N specific primers could detect PPRV clearly until dilution  $10^{-2}$  ( $7.5 \text{ ng}/\mu\text{L}$ ) (Fig. 7 - 8). Although F gene specific primer could detect at higher dilution ( $10^{-5}$  or  $7.5 \text{ pg}/\mu\text{L}$ ) a strong non-specific band began to appear simultaneously at dilution  $10^{-3}$  and above (Fig. 7), which could not be readily explained. No such non-specific amplification was noticed with N gene specific primer (Fig. 8).

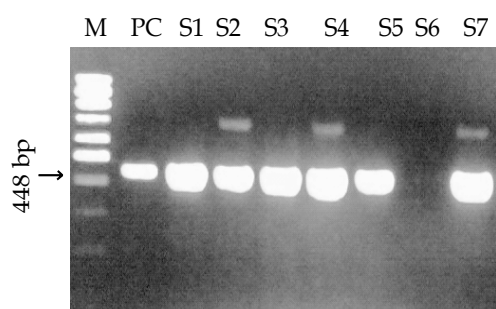


Fig. 1. Amplification of the fragment of F gene using specific Primer with different field samples

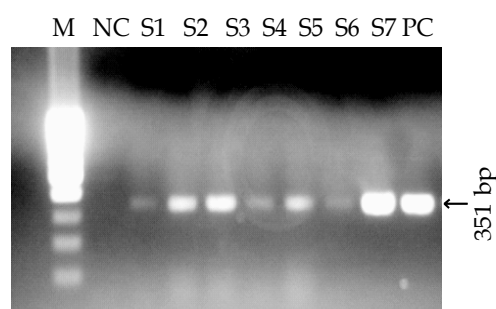


Fig. 2. Amplification of the fragment of N gene using specific Primer with different field samples

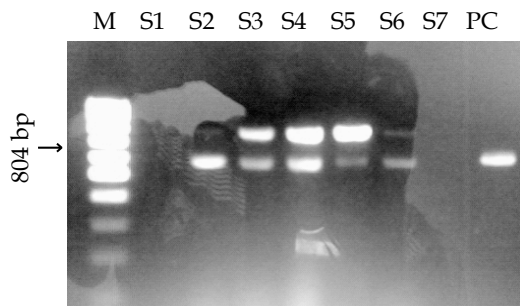


Fig. 3. Amplification of the fragment of L gene using specific primer with different field samples

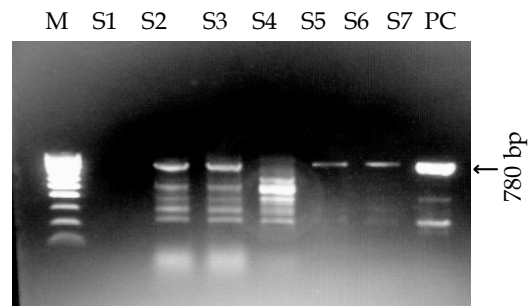


Fig. 4. Amplification of the fragment of M gene using specific primer with different field samples

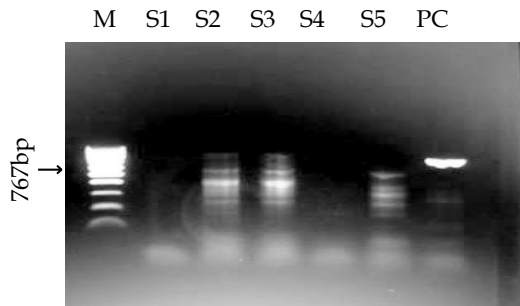


Fig. 5. Amplification of the fragment of P gene using specific primer with different field samples

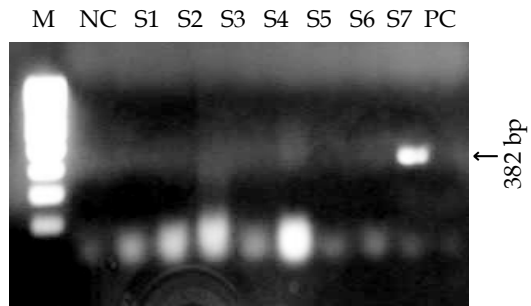


Fig. 6. Amplification of the fragment of H gene using specific primer with different field samples

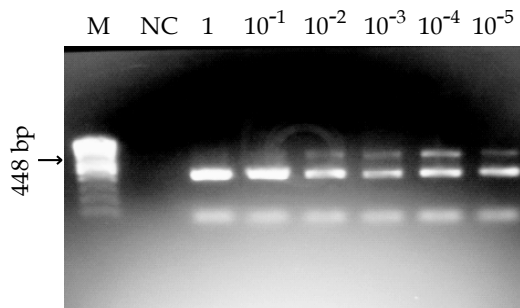


Fig. 7. RT-PCR with F gene specific primer at different dilution, Conc. of RNA : 1 = 750 ng/ $\mu$ L,  $10^{-1}$  = 75 ng/ $\mu$ L,  $10^{-2}$  = 7.5 ng/ $\mu$ L

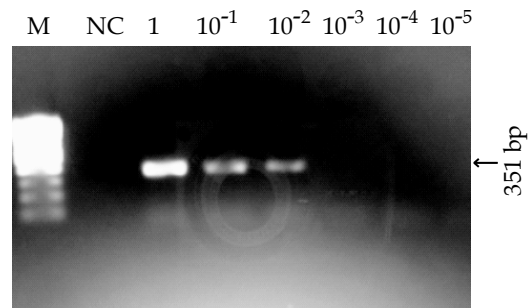


Fig. 8. RT-PCR with N gene specific primer at different dilution, Conc. of RNA : 1 = 750 ng/ $\mu$ L,  $10^{-1}$  = 75 ng/ $\mu$ L,  $10^{-2}$  = 7.5 ng/ $\mu$ L

## Conclusions

Primer against NP gene (np3/np4) was specific and sensitive for the detection of PPRV from field samples but for efficient amplification and detection of viruses in field samples, it is desirable to have more than one set of primers for diagnosis; and F and N genes are the most suitable.

## Acknowledgements

We thank IAEA, Vienna, Austria and PIU, BARC, Dhaka for funds and facilities

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