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Comparative evaluation of different F and N gene based reverse transcription polymerase chain reaction for molecular detection of peste des petits ruminants virus from clinical samples

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ABSTRACT

In the present study the diagnostic value of different sets of F and N gene based primers currently used for diagnosis of Peste des petits ruminants (PPR) by reverse transcription polymerase chain reaction (RT-PCR) were assessed by comparing it with Sandwich ELISA (S-ELISA). A total of five primer pairs, consisting of two pairs (F1/F2 and Fb1/Fb2) amplifying two different regions of F gene and three pairs amplifying different regions of N gene (NP3/NP4, pprn_fr2/pprn_rev and N1/N2) were compared on 10 clinical samples (4 blood, 4 nasal swabs and 2 tissue samples) collected from animals suspected for PPR. The primer sets NP3/NP4 detected highest number of positive samples 6 out of 10 followed by N1/N2 (5/10). Both F-gene based primers (F1/F2 and Fb1/Fb2) detected 3 out of 10 samples as positive. Whereas the primer pair pprn_fr2/pprn_rev did not yield the desired amplicon in any of the samples tested. The maximum sensitivity and specificity of 100% was observed by NP3 and NP4 primer based RT-PCR whereas, 0% sensitivity was recorded by pprn_fr2/pprn_rev which fail to detect any positive sample. The overall agreement of 100% with kappa value 1.00 was highest between S-ELISA and NP3 and NP4 primer based RT-PCR suggesting an almost perfect agreement, followed by N1/N2, having kappa value of 0.800, suggesting a substantial agreement. Results thus obtained in the present study, suggest that F-gene primers based RT-PCR can be easily replaced by highly sensitive and specific N-gene primers based RT-PCR for detection of PPR virus nucleic acid.

Key words: evaluation, molecular detection, peste des petits ruminants, reverse transcription polymerase chain reaction

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Introduction

Ever since the first report of polymerase chain reaction (PCR) in 1985 (SAIKI, 1985) it has been increasingly applied for diagnosis of many diseases and same is the case with peste des petits ruminants (PPR). PPR is an acute, highly fatal disease of small ruminants caused by virus belonging to genus *Morbillivirus* of family *Paramyxoviridae* (GIBBS et al., 1979). The virus is closely related to Rinderpest, Canine distemper, and Measles virus (VAN MOL et al., 1995). The genome of PPR virus (PPRV) consists of un-segmented single stranded RNA of negative polarity with six transcriptional units that encodes six structural and two non-structural proteins (HAFFAR et al., 1999). The structural proteins include nucleoprotein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), haemagglutinin (H) and polymerase large (L) protein (BARON and BARRETT, 1995). Fusion protein (F) and Nucleoprotein (N) genes have been the target for PCR based specific diagnosis of PPR (FORSYTH and BARRET, 1995; COUACY-HYMANN et al., 2002).

Among the various PCR primers developed for detection of PPRV, F gene based primers F1/F2 developed by FORSYTH and BARRET (1995) have gained wide acceptance worldwide (SHAILA et al., 1996). But DHAR et al. (2002) observed that these F1/F2 primer set don't work efficiently on some samples so they used a set of another F gene based primers (F1b/F2b) located just outside the region of F1/F2 to amplify the region between 760 and 1207 nucleotides of F gene generating an amplicon of 448 bp. COUACY-HYMANN et al. (2002) on the other hand developed a nucleoprotein (N) gene based primers NP3/NP4 which produced an amplicon of 351 bp, amplifying a region of N gene between the nucleotide positions 1232 to 1583. KERUR et al. (2008) worked on genetic characterization of Indian PPRV and developed another N gene based primers N1/N2 targeting N gene position at 1208-1226 and 1670-1652, respectively producing and amplicon of 463 bp and reported that the newly designed N gene primers detected PPRV in more number of samples than F gene based primers (F1/F2).

For any disease control programme to be successful, prompt and specific detection of field outbreaks are important, thereby reducing the potentially serious economic damage which can result from the outbreak. Keeping in view the results of recent studies a need was felt to assess the suitability, sensitivity and specificity of primers which are currently in use for detection of PPRV infection in India. Moreover, literature appeared scanty with regards to the evaluation of efficacy of different primers for detection of PPRV.

Materials and methods

Clinical samples and standard virus. A total of 10 clinical samples including 4 nasal swabs, 4 blood samples and 2 tissue samples were collected during 2009-10 from sheep and goats with symptoms suggestive of PPRV infection from field outbreak in Jammu division of J&K state of India. For positive control, lyophilized freeze-dried live PPR

vaccine virus (Sungri/96) was obtained from the Division of Virology, IVRI, Mukteswar, India. A blood sample collected from an apparently healthy goat was used as negative control

Oligonucleotide primers. A total of five sets of primers were used in study which was obtained from Sigma Genosys and MWG -Biotech AG, Germany (Table1).

Table 1. Details of the primer used for RT- PCR detection of PPRV

Primer	Primer sequence	Reference
F1/F2	5'ATC ACA GTG TTA AAG CCT GTA GAG G 3'	FORSYTH and BARETT (1995)
	5' GAG ACT GAG TTT GTG ACC TAC AAG C 3'	
NP3 /NP4	5'TCT CGG AAA TCG CCT CAC AGA CTG 3'	COUACY-HYMANN et al. (2002)
	5'CCT CCT CCT GGT CCT CCA GAA TCT 3'	
N1/N2	5'GAT GGT CAG AAG ATC TGC A 3'	KERUR (2008)
	5'CTT GTC GTT GTA GAC CTG A 3'	
F1b /F2b	5'AGT ACA AAA GAT TGC TGA TCA CAG T-3'	DHAR et al. (2002)
	5'GGG TCT CGA AGG CTA GGC CCG AAT A-3'	
Pprn_fr2/ Pprn_rev	5'ACA GGC GCA GGT TTC ATT CTT 3'	GEORGE (2002)
	5' TGA TTT GGA CGG AGG GTG 3'	

Detection of PPRV by RT-PCR. For RT-PCR, Total RNA from blood, swab, tissue samples and from vaccine virus was extracted by GeNei™ TRIsoln (Bangalore Genei, India), according to the manufacturer's instructions.

Purity of RNA was judged on the basis of optical density ratio at 260:280 nm by using the formula suggested by SAMBROOK and RUSSEL (2001). RNA with acceptable purity was then amplified by using GeNei™ One Step AMV RT-PCR Kit (Bangalore Genei, India). Mastermix was prepared as per manufacture instruction. Reaction Mix I contains; RNasin, GeNeiTM 2X RT PCR Reaction Mix, GeNeiTM RT-PCR Enzyme Mix and Primers with final concentration of 0.6 µM.

Master Mix II containing template RNA was taken in separate 0.2 mL PCR tubes and heated at 65 °C for 5 minutes in water bath to denature the template RNA and then chilled on ice before adding it to the Master Mix I. Reverse transcription was done at 50°C for 30 min followed by 35 PCR cycles for each primers. The reaction condition for F1/F2, F1b/F2b, NP3/NP4, N1/N2 and Pprn_fr2/Pprn_rev primers were kept similar to that described previously by others (Table 1). 5 µL of PCR product from each tube was then mixed with 3 µL of 6X gel loading buffer and electrophoresed on 2.0% agarose gel containing ethidium bromide (1% 5 µL/100 mL) at constant Voltage of 75V for 30 min

in 0.5X TBE buffer. The amplified product was visualized as a single compact band of expected size under UV light.

Detection of PPRV by S-ELISA. PPR S-ELISA kit for PPRV antigen detection along with the user manual was obtained from Rinderpest Laboratory, Division of Virology, IVRI, Mukteswar. The test was preformed strictly as per the protocol outlined in the user manual supplied with the kit (SINGH et al., 2004). The ELISA plates showing proper colour development in control wells were read at 492 nm in ELISA plate reader (Multiskan plus, LabSystem).

Statistical analysis. The sensitivity, specificity and overall agreement between different types of primers were analyzed by comparing them with S-ELISA (Gold standard), as per the methods suggested by SAMAD et al. (1994). In addition, the positive and the negative predictive values along with Kappa statistics (κ) were analyzed as per the method suggested by THRUSFIELD (2006).

Results

Out of 10 sample tested, NP3/NP4 detected highest number of positive samples (06) by producing the desired amplicon of 351 bp followed by N1 /N2 which detected 5 samples as positive with both F-gene based primers (F1/F2 and Fb1/Fb2) detected 03 samples as positive. Whereas primer pair pprn_fr2/pprn_rev did not yield any sample as positive as it fail to produce the desired amplicon (365bp) in positive control as well as in any of the clinical samples tested (Table 2).

Table 2. Sample wise results of S-ELISA and different F and N gene based RT-PCR for detection of PPR virus infection

	S-ELISA		FI &F2		N1 & N2		Fb1&Fb2		NP3 & NP4		pprn_fr2 & pprn_rev	
Sample Type	ST	SP	ST	SP	ST	SP	ST	SP	ST	SP	ST	SP
Nasal Swab	4	3	4	1	4	3	4	1	4	3	4	0
Blood samples	4	2	4	1	4	2	4	1	4	2	4	0
Tissue samples	2	1	2	1	2	1	2	1	2	1	2	0
Total	10	6	10	3	10	6	10	3	10	6	10	0

ST- Sample Tested, SP- Sample Positive

Further statistical analysis of results revealed that the maximum sensitivity and specificity of 100% was observed by NP3 and NP4 primer based RT-PCR with overall agreement of 100% with kappa value 1 when compared with S-ELISA suggesting an almost perfect agreement (Table 3).

Table 3. Compassion of different F and N gene based RT-PCR with that of S-ELISA for detection of PPRV infection

S-ELISA Gold Standard	FI & F2	N1 & N2	Fb1 & Fb2	NP3 & NP4	pprn_fr2 & pprn_rev
Parameters	a = 3, b = 0, c = 3, d = 4	a = 5, b = 0, c = 1, d = 4	a = 3, b = 0, c = 3, d = 4	a = 6, b = 0, c = 0, d = 4	a = 0, b = 0, c = 6, d = 4
Sensitivity	50%	83.3%	50%	100%	0
Specificity	100%	100%	100%	100%	100%
Overall agreement	70%	90%	70%	100%	40%
Positive predictive value	1	1	1	1	-
Negative predictive value	0.571	0.800	0.571	1	0.400
Yule's Q	1	1	1	1	0
Kappa value	0.444	0.800	0.444	1	0
Overall fraction correct	0.700	0.900	0.700	1	0.400

a = Samples positive to both conventional and the gold standard tests, b = Samples positive to conventional but negative to the gold standard test, c = Samples negative to conventional but positive to the gold standard test, d = samples negative to both conventional and the gold. Kappa value > 0.81 Almost perfect agreement, 0.61 - 0.80 Substantial agreement, 0.41 - 0.60 Moderate agreement, 0.21 - 0.40 Fair agreement, 0.01 - 0.20 Slight agreement, 0.00 Poor agreement

Discussion

RT-PCR has greatly improved the rapid and specific diagnosis of PPR though out the world. It has been used as preferred method for PPR diagnosis in many laboratories worldwide. However, this assay may not always be suitable for diagnosis of every virus strain, variant or isolate, as changes at the 3´ end of the primer binding sites, as a result of variation between strains, may yield a false-negative result (BALAMURUGAN et al., 2006). In the present study, five sets of primers (two pairs of F-gene based primers and three pairs of N gene based primers) was evaluated and compared parallel to S-ELISA for detection of PPRV. The primer pair NP3/NP4 yielded a sensitivity of 100% by detecting 6 samples as positive followed by N1 and N2 detecting 5 samples as positive. Both F-gene primers sets F1/F2 and Fb1/Fb2 detected 3 samples as positive by RT-PCR. One N-gene based primer set pprn_fr2/pprn_rev failed to produce desirable amplicon in any of the sample tested as well in positive control.

The results of present study are in line with the study conducted by KERUR et al. (2008), who reported N gene primers more sensitive than that of F-gene based primers and observed that three samples negative by F-gene based RT-PCR were positive by N-gene based RT-PCR. The obvious reason for higher sensitivity of the N gene based primers than F gene based

primers can be attributed to the abundance of PPRV and is the first protein to be produced, N-gene transcripts than the F-gene (GHOSH et al., 1995), thus making N gene more suitable target for improving the sensitivity of RT-PCR for detection of PPRV from clinical samples.

In the present study primer set pprn_fr2/pprn_rev failed to detect PPRV in clinical samples positive with other sets of N-gene primers. When this primer was aligned with other N gene sequences, a mismatch at base 2 from the 3 end was found which was not in compliance with the minimal homology requirement defined by SOMMER and TAUTZ (1989) according to which at least three homologous nucleotides at the 3' end are required for successful priming. Also the success of PCR amplification is governed by complex set of factors. Due to the absence of sequence information of the annealing site of pprn_rev, no conclusive inference could be drawn about the behavior of this particular set of primers (pprn_fr2/pprn_rev).

Conclusion

In nut shell, it can be concluded that higher sensitivity and specificity shown by N-gene primers than F-gene based primers when compared with S-ELISA, suggest that F-gene primers can be easily replaced by highly sensitive and specific N-gene primers for detection of PPRV nucleic acid. Furthermore, the results of the study may prove useful to direct further studies like characterization of isolates which can enable in identifying possible genetic variation and the resulting changed behavior pattern of the virus in the field and correlating them to explore the new more sensitive and specific primers to detect PPRV by RT-PCR.

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SAŽETAK

Istražena je dijagnostička vrijednost različitih početnica za gene F i N koje se sada rabe za dijagnosticiranje kuge malih preživača lančanom reakcijom polimerazom uz prethodnu reverznu transkripciju u usporedbi sa sendvič imunoenzimnim testom (S-ELISA). Testirano je ukupno pet parova početnica: dva para (F1/F2 i Fb1/Fb2) specifična za različita područja gena F i tri para specifična za različita područja gena N (NP3/NP4, pprn_fr2/pprn_rev i N1/N2). Njihova vrijednost bila je uspoređena pretragom 10 kliničkih uzoraka (četiri uzorka krvi, četiri uzorka nosnog obriska i dva uzorka tkiva) uzetih od životinja pod sumnjom na kugu malih preživača. Uporabom početnica NP3/NP4 dokazan je najveći broj pozitivnih uzoraka (6/10), a potom početnicom N1/N2 (5/10). Objema početnicama za gen F (F1/F2 i Fb1/Fb2) dokazana su tri pozitivna uzorka (3/10). Par početnice pprn_fr2/pprn_rev nije dao željeni umnožak (amplikon) ni u jednom pretraženom uzorku. Osjetljivost i specifičnost od 100% dokazana je za početnice NP3 i NP4, dok pprn_fr2/pprn_rev nisu pokazale nikakvu reakciju te njihovom upotrebom nije dokazan nijedan pozitivan uzorak. Podudarnost od 100% s kappa vrijednošću 1,00 bila je najveća između S-ELISA-e te RT-lančane reakcije polimerazom upotrebom početnica NP3 i NP4 što govori o posvemašnjoj podudarnosti, dok je na drugom mjestu po podudarnosti bila početnica N1/N2 s kappa vrijednošću od 0,800. Rezultati pokazuju da se RT-lančana reakcije polimerazom upotrebom početnica za gen F može zamijeniti vrlo osjetljivom i specifičnom reakcijom uz upotrebu početnica za gen N za dokazivanje nukleinske kiseline virusa kuge malih preživača.

Ključne riječi: molekularni dokaz, kuga malih preživača, lančana reakcija polimerazom s prethodnom reverznom transkripcijom