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Development of a two-step SYBR Green I based real time RT-PCR assay for detecting and quantifying peste des petits ruminants virus in clinical samples

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A two-step SYBR Green I based real time RT-PCR targeting the matrix (M) gene of *Peste des petits ruminants* virus (PPRV) was developed. The specificity of the assay was assessed against viral nucleic acid extracted from a range of animal viruses of clinical and structural similarities to PPRV including canine distemper virus, measles virus, bluetongue virus and Newcastle disease virus. But none of the viruses and no template control showed an amplification signal. Sensitivity of the same assay was assessed based on plasmid DNA copy number and with respect to infectivity titre. The lower detection limit achieved was 2.88 plasmid DNA copies/μl with corresponding Ct value of 35.93. Based on tissue culture infectivity titre the lower detection limits were $0.0001TCID_{50}$/ml and $1TCID_{50}$/ml for the SYBR green I based real time RT-PCR and conventional RT-PCR, respectively. The calculated coefficient of variations values for intra- and inter-assay variability were low, ranging from 0.21% to 1.83% and 0.44% to 1.97%, respectively. The performance of newly developed assay was evaluated on a total of 36 clinical samples suspected of PPR and compared with conventional RT-PCR. The SYBR Green I based real time RT-PCR assay detected PPRV in 32 (88.8%) of clinical samples compared to 19 (52.7%) by conventional RT-PCR. Thus, the two-step SYBR Green I based real time RT-PCR assay targeting the M gene of PPRV reported in this study was highly sensitive, specific and reproducible for detection and quantitation of PPRV nucleic acids.

1. Introduction

Peste des petits ruminants (PPR), also known as 'goat plague', is a viral disease of goats and sheep characterized by fever, sores in the mouth, diarrhoea, pneumonia, and sometimes death (OIE, 2010). PPR was first identified in West Africa in the 1940s, and can now be detected in a broad belt of sub-Saharan Africa, Arabia, the Middle East and Southern Asia (Dhar et al., 2002).

The causative agent *peste-des-petits ruminants virus* (PPRV) is classified as a member of the genus *Morbillivirus*, family *Paramyxoviridae* under the order *Mononegavirales.* It has a negative sense single-stranded RNA genome encoding eight proteins (Bailey et al., 2005).

Several conventional reverse transcription polymerase chain reactions (RT-PCRs) are available for detection of PPRV genomic material (Forsyth and Barrett, 1995; Couacy-Hymann et al., 2002; Balamurugan et al., 2006). However, these conventional RT-PCR assays are labour intensive, as they require gel analysis for the detection of PCR products with a consequent high risk of contamination (Bao et al., 2008).

Real time RT-PCR has gained wider acceptance over conventional RT-PCR because it is more rapid, sensitive and reproducible. Few real time RT-PCR assays have been described for detection and quantitation of PPRV in clinical samples using TaqMan chemistry (Bao et al., 2008; Balamurugan et al., 2010; Kwiatek et al., 2010; Batten et al., 2011). In comparison with TaqMan-PCR, SYBR Green I based real-time RT-PCR assay has the advantages of being more cost-effective, easy to design, more precise and produce a more linear decay plot (Schmittgen et al., 2000). The use of M gene based two-step SYBR Green I real time RT-PCR for molecular diagnosis of PPR has not been reported so far. The availability of such technique will provide an alternative to the N gene based real-time RT-PCR diagnostic assays that already exist (especially in terms of its sensitivity); thereby helping in rapid clinical diagnosis of PPR. In this regard, the present study developed a two-step SYBR Green I based real time RT-PCR assay for the detection and quantitation of PPRV in clinical samples.

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2. Materials and methods

2.1. Viruses, cells and clinical samples

Viruses used in this study are listed in Table 1. Vero cells were used for virus propagation and titration. A total of 36 field samples from PPR suspected outbreaks from different areas of the Indian state of Tamil Nadu were analyzed. Samples received for analyses from different vet clinics were ocular, nasal, oral and rectal swabs, spleen, kidney, lung, lymph nodes and dung.

2.2. Viral RNA extraction and cDNA synthesis

Viral RNA was extracted from tissue culture supernatant using QIAamp® Viral RNA Mini Kit (Qiagen®, Germany), following manufacturer's instructions. Trizol reagent (Invitrogen, CA, USA) was also used to isolate RNA from clinical samples. Synthesis of cDNA was carried out in 20 μl reaction using RevertAid™ H minus first Strand cDNA Synthesis kit (Fermentas, USA) following manufacturer's instructions. Briefly, 11 μl of the purified RNA were added to a mixture containing 4 μl RT buffer (5×), 1 μl of random hexamer, 2 μl 10 mM dNTP, 1 μl RNase inhibitor and 1 μl of Moloney murine leukaemia virus reverse transcriptase. The reaction was carried out at 25 °C for 5 min, 42 °C for 60 min and 70 °C for 5 min and stored at −40 °C until use.

2.3. Primer design and synthesis

The forward and reverse primers were designed according to the sequences of matrix protein (M) gene (GenBank accession no. GQ452014.1) of PPRV Sungri-96 strain by using FastPCR software. The designed primers (Forward M_3F: 5′GGAGTGATTGAGGATAACGACC-3′ (169–190) and Reverse M_3R: 5′-GCGTTAACAAGGACAGCGGAG-3′ (350–370) were validated by OligoAnalyzer 1.2 and synthesized commercially (Sigma Aldrich, Bangalore, India).

2.4. Real time RT-PCR

All reactions were performed using Realplex^4 real time PCR machine (Eppendorf, Germany) using SYBR premix Ex Taq, (TaKaRa Bio Inc., Japan). The PCR was set up in a 10 μl reaction volume containing 5 μl of 2× SYBR Premix Ex Taq master mix, 1.5 μl of cDNA, 1 μl (10 μM) of each primer and 2.5 μl nuclease free water. The optimized cycling conditions were as follows: initial denaturation at 94 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 20 s, primer annealing at 56 °C for 30 s, and extension at 72 °C for 20 s. The fluorescence was measured at the end of each cycle. A melt curve analysis was performed following amplification to verify the specificity of the amplified products. Melting curve analysis consisted of 70 °C for 15 s, and followed by temperature increase to 95 °C for 15 s at the rate of 1.25 °C/s with continuous reading of fluorescence.

2.5. Conventional RT-PCR

Conventional RT-PCR was performed on the cDNA preparations of the PPRV with a primer set of MF-Morb (5′-CTT GAT ACTC CCC AGA GATTC-3′) and MRPPR3 (5′-TTC TCC CAT GAG CCG ACT ATGT-3′) as described by Balamurugan et al. (2006) which yielded 191 bp of PCR product. Amplification of target sequences was performed in a 2720 thermal cycler (Applied Biosystems).

2.6. Generation of quantification standards

A fragment of 348 bp of M gene of PPRV containing the real time RT-PCR primers binding sites were amplified using the primer pair M_4F (forward primer): 5′-CCAGGTAAGGGTCATCGATCC-3′ (99–119) and M_4R (reverse primer): 5′-AAGCGGGACTAGGTTGACTGCA-3′ (426–447) (In house designed). The RT-PCR product was cloned into T&A cloning vector according to the manufacturer's instructions. Plasmid DNA was recovered from the transformed *Escherichia coli* BL-21 cells using AxyPrep Plasmid Miniprep Kit (Axygen Biosciences). The OD value of the plasmid DNA standard concentrations was measured at 260 nm/280 nm on Thermo Scientific NanoDrop™ 1000 Spectrophotometer (NanoDrop Technologies, LLC, Wilmington, DE, USA). Plasmid copy number was calculated using the formula described by Adams (2006). The concentration of the obtained plasmid DNA was 95 ng/μl which equates 2.88×10^{10} copies/μl.

2.7. Specificity and sensitivity of the real time RT-PCR

The specificity of the developed assay was assessed against viral nucleic acid extracted from a range of animal viruses of clinical and structural relevance to PPRV. The sensitivity of the assay was determined by running 10-fold serial dilutions of the plasmid standard in duplicates. Additionally, the sensitivity of the assay was evaluated by titrating PPRV Combatoire strain on Vero cells in a 96-well microtitre plate using standard cell culture procedure and the virus titre was calculated using Reed and Muench (1938) formula. PPRV having a titre of 10^5 $TCID_{50}$/ml was diluted 10-fold serially from 10^{-1} and 10^{-11}, and total RNA was extracted from each dilution, subsequently cDNA synthesis was performed.

2.8. Reproducibility

The DNA standard ranging from 2.88×10^7 copies/μl to 2.88×10^0 copies/μl was tested repeatedly. Three separate dilution series were assayed in a single run to evaluate intra-assay

Table 1
Viruses used in the study.

Virus	Strain	Source
Peste des petits ruminants virus (PPRV)	AR 87 vaccine strain	Dept. of Vet. Microbiology, Madras Veterinary College
	Sunguri vaccine strain	Dept. of Vet. Microbiology, Madras Veterinary College
	Coimbatore vaccine strain	Dept. of Animal Biotechnology, Madras Veterinary College
	Coimbatore field isolate	Dept. of Vet. Microbiology, Madras Veterinary College
	Recent field isolates	Dept. of Vet. Microbiology, Madras Veterinary College
Canine distemper virus (CDV)	Vaccine strain	Nobivac Puppy DP, Intervet
Measles virus (MV)	Vaccine strain	Serum institute of India LTD, Pune
Bluetongue virus (BTV)	Vaccine strain	Dept. of Vet. Microbiology, Madras Veterinary College
Newcastle disease virus (NDV)	D58	Dept. of Vet. Microbiology, Madras Veterinary College