THE M AND N GENES-BASED SIMPLEX AND MULTIPLEX PCRs ARE BETTER THAN THE F OR H GENE-BASED SIMPLEX PCR FOR PESTE-DES-PETITS-RUMINANTS VIRUS

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Summary. – Nucleocapsid (N), matrix (M) and hemagglutinin (H) genes-based simplex PCRs and an N and M genes-based multiplex PCR were developed for detection of Peste-des-petits-ruminants virus (PPRV). The M gene PCR was the most sensitive, followed by N, H and an already described fusion (F) gene PCRs, as they could detect the virus in samples with titers of 10^1, 10^2, $10^4$and 10^5 $TCID_{50}$/ml, respectively. The multiplex PCR was as sensitive as the M gene PCR, but it had the advantage of differentiating PPRV from Rinderpest virus (RPV).

Key words: Peste-des-petits-ruminants virus; PCR; RT-PCR; M gene; N gene; H gene; F gene; multiplex PCR

Introduction

Peste-des-petits-ruminants (PPR) is a highly contagious and economically important disease of sheep and goats reported from Ivory Coast, West Africa (Gargadennec and Lalanne, 1942). Morbidity and mortality rates can be as high as 100% and 90%, respectively (Abu-Elzein *et al*., 1990). The disease is considered one of the main constraints in improving small ruminant production (Stem, 1993). It is widespread in sub-Saharan Africa, Arabian Peninsula and south Asia (Taylor, 1984; Lefevre and Diallo, 1990; Shaila *et al*., 1996). Its etiological agent is Peste-des-petits-ruminants virus (PPRV, the genus *Morbillivirus*, the family *Paramyxoviridae*) containing a single-stranded genomic RNA (Fauquet *et al*., 2005). The virus is closely related to other morbilliviruses, namely Rinderpest virus (RPV), Canine distemper virus (CDV) and Measles virus (MeV). The symptoms of PPR are akin to RP in cattle, although the genomes of respective viruses are identical only to 66.98% (Bailey *et al*., 2005). The symptoms are sudden onset of high fever, mucopurulent nasal discharge, profuse catarrhal conjunctivitis, inflammation and necrotic lesions in the mouth and the gastrointestinal tract. Most animals manifest severe diarrhea and dyspnea, and bronchopneumonia in later stages, and die usually within 5–10 days. Pregnant animals may abort. As differential diagnosis of RP and PPR by clinical signs is not always accurate, laboratory tests are necessary to confirm and differentiate the two viruses. Competitive and immunocapture ELISAs based on PPRV-specific monoclonal antibodies have been developed for differential diagnosis of the two diseases (Libeau *et al*., 1994, 1995). Morbilliviruses consist of N, F, H, M, and polymerase (L) proteins (Grubman *et al*., 1988). Out of structural protein genes, only F gene was exploited for development of an RT-PCR for RPV and PPRV, which enabled a more sensitive diagnostics of PPRV (Forsyth and Barrett, 1995). The other important targets located either upstream (N and M genes) or downstream (H gene) of the F gene have not been tested yet for their suitability for RT-PCR. As the N protein is expressed to a very high level in morbillivirus-infected cells (Diallo, 1990), this made it a good antigen candidate for serological diagnostics of RPV and PPRV. In

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Abbreviations: PPR = peste-des-petits-ruminants; PPRV = PPR virus; N = nucleocapsid; M = matrix; H = hemagglutinin; F = fusion; RP = rinderpest; RPV = RP virus

Table 1. Primers and cycling conditions used in PCRs

PCR	Primers (forward and reverse)	Cycling conditions	Amplicon size (bp)
N gene	pprn_fr2 (5'-acaggcgcaggtttcattctt-3') pprn_rev (5'-tgatttggacgga gggtg-3')	95°C/3 mins, 30 cycles of 94°C/30 secs, 55°C/30 secs, and 72°C/30 secs, and 72°C/10 mins	365
M gene	mf-morb (5'-cttgatactccccagagattc-3') mr-ppr-3 (5'-ttctcccatgagccgactatgt-3')	95°C/3 mins, 30 cycles of 94°C/30 secs, 60°C/30 secs, and 72°C/30 secs, and 72°C/10 mins	191
F gene	F3 (5'-gggacagtccttcagcctattaagg-3') F4 (5'-cagccctagcttctgacccacgata-3')	95°C/3 mins, 30 cycles of 94°C/30 secs, 50°C/1 min, and 72°C/2 mins, and 72°C/10 mins	372
H gene	pprh_fr4 (5'-tcatcatcttacttctacccagtcc-3') ILR (5'-aactgagagggagtcca-3')	The same as for N gene PCR	320
β-actin gene	BA1 (5'-gagaagctgtgctacgtcgc-3') BA2 (5'-ccagacagcactgtgttggc-3')	The same as for N gene PCR	275
Multiplex (N-M genes)	pprn_fr2 and pprn_re1 (5'-gctgaggatatccttgtcgttgta-3') for N gene; mf-morb and mr-ppr-3 for M gene	95°C/3 mins, 30 cycles of 94°C/30 secs, 60°C/30 secs, and 72°C/1 min, and 72°C/10 mins	334 (N) 191 (M)

a study on regulation of RPV genome expression it has been reported that the mRNAs of the genes located towards the 3'-end are more abundant than those of the genes located towards the 5'-end of the genome (Ghosh *et al*., 1995).

Since the PPRV genome organization is 3'-N-P/C/V-M-F-H-L-5', which is similar to that of RPV and other morbilliviruses (Barrett *et al*., 1993), we made an attempt to use different gene targets such as N, M, and H genes of PPRV in developing a PCR that would be more sensitive than the hitherto used F gene PCR.

Materials and Methods

Viruses. Indian vaccine PPRV grown in Vero cells (Sungri 1996), RPV grown in Vero cells, lapinized RPV and a caprinized RPV were used (Parida and Bandyopadhyay, 1996). Indian vaccine PPRV together with the PPRV strains circulating in the Asian subcontinent belong to the phylogenetic lineage 4 of PPRV (Dhar *et al*., 2002).

Titration of infectious PPRV was carried out in a marmoset lymphoid cell line B95a (Sreenivasa *et al*., 2006).

Total RNA was extracted from 250 µl of tissue culture supernatant or 10% tissue homogenate on days 4–5 post infection using Trizol LS reagent (Life Technologies). Finally, RNA was dissolved in 20 µl of RNAse- and DNAse-free distilled water. Uninfected tissue cultures or tissues served as controls.

Reverse transcription. To generate cDNA, 5 µl of total RNA, 1 µl (100 ng) of random hexanucleotide primers, and 4 µl of nuclease-free water were mixed and incubated first at 70°C for 5 mins and then at room temperature for 10 mins. After incubation, 4 µl of 5x RT buffer, 1 µl of 10 mmol/l dNTPs, 1 µl (200 U) of MMLV reverse transcriptase, and 4 µl of nuclease-free water were added and the mixture was incubated first at room temperature for 5 mins and then at 37°C for 45 mins. The cDNAs (final volume of 20 µl) were kept at -20°C.

PCR primers. The forward and reverse primers for N, M and H genes were designed based on the published respective sequences (Table 1) (Diallo *et al.,* 1994; Hafar *et al*., 1999; Diallo, 1990). For F gene, the already published primer pair F3/F4 was used (Forsyth and Barrett, 1995). For β-actin gene (negative control) the primer pair BA1/BA2 was employed.

PCRs. For the N, M and H gene PCRs, the reaction mixture (final volume 25 µl) consisted of 2.5 µl of cDNA, 1x PCR buffer (20 mmol/l Tris-HCl pH 8.4 and 50 mmol/l KCl), 1.5 mmol/l $MgCl_2$, 200 µmoles dNTPs, 1.25 U of Taq polymerase (Promega), and 5 pmoles each of the primers. Optimal primer pairs and cycling conditions for each PCR are given in Table 1. The PCR products were separated by electrophoresis in 1.2% agarose gel and visualized by ethidium bromide staining.

Multiplex PCR with primer pairs for N and M genes was designed for differentiation of PPRV from RPV in single reaction. From four different primer pairs and cycling conditions tested optimal ones were chosen (Table 1). Optimal composition of the reaction mixture was as follows: 10 pmoles of the N gene primers and 5 pmoles of the M gene primers, 2.5 µl of cDNA, 1x PCR buffer (20 mmol/l Tris-HCl pH 8.4 and 50 mmol/l KCl), 1.5 mmol/l $MgCl_2$, 200 µmol/l dNTPs, and 1.25 U of Taq polymerase (Promega).

Results

N gene PCR

An N gene PCR specific for PPRV was developed using the primer pair pprn_fr2/pprn_rev (Table 1). A 365 bp PCR

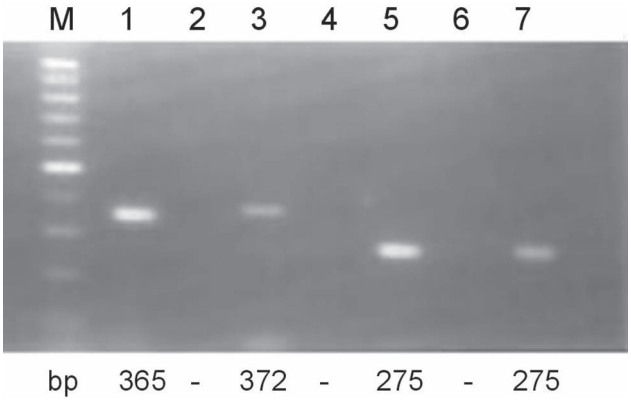

Fig. 1

N gene PCR

N gene PCR: PPRV (lane 1), RPV (lane 2), uninfected Vero cells (lane 4), and healthy goat tissue (lane 6). Positive controls: F gene PCR, RPV (lane 3); β-actin gene PCR, uninfected Vero cells (lane 5) and healthy goat tissue (lane 7). 100 bp DNA ladder (lane M).

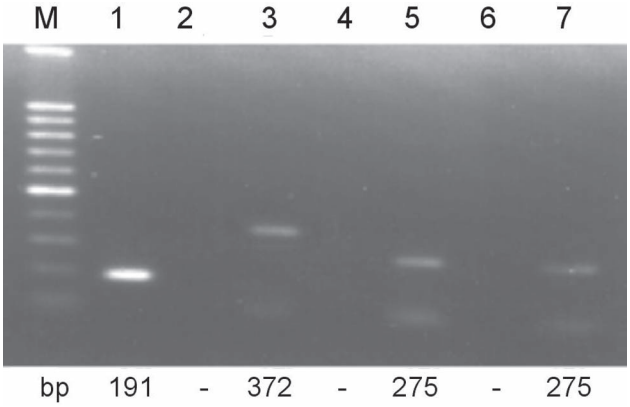

Fig. 2

M gene PCR

M gene PCR: PPRV (lane 1), RPV (lane 2), uninfected Vero cells (lane 4), and healthy goat tissue (lane 6). Positive controls: F gene PCR, RPV (lane3); β-actin gene PCR, uninfected Vero cells (lane 5) and healthy goat tissue (lane 7). 100 bp DNA ladder (lane M).

product was obtained for PPRV (Fig. 1) but none for closely related RPV, non-infected Vero cells, and healthy goat tissues. A good quality of the last two control cDNAs was checked by subjecting them to a PCR specific for β-actin gene with the primer pair BA1/BA2. Both gave a positive result, namely a 275 bp product. Also the cDNA from RPV was checked by a PCR specific for RPV F gene using the F3/F4 primer pair. It gave a positive results, namely a 372 bp product. These results thus confirmed that the N gene PCR was specific to PPRV.

Another primer pair, namely pprn_fr2/pprn_re1 yielded a 334 bp product for both PPRV and RPV (data not shown). This primer pair was regarded as unsuitable for an N gene PCR but appropriate for multiplex PCR (see below).

M gene PCR

An M gene PCR was developed for specific detection of PPRV. Of several primer pairs tested, mf-morb/mr-ppr-3 was found to be PPRV-specific, yielding a 191 bp product (Fig. 2). This PCR gave negative result for RPV, non-infected Vero cells and healthy goat tissues. As expected, the last two control cDNAs reacted positively in the PCR specific for the β-actin gene. Also the RPV cDNA gave positive result in the PCR specific for RPV.

H gene PCR

An H gene PCR specific for PPRV was developed using the primer pair pprh_fr4/ILR. It produced a 342 bp product with PPRV but not with RPV, non-infected Vero cells and healthy goat tissues (Fig. 3). Standard control PCR for β-actin gene with the last two cDNAs was positive. The control PCR for RPV was positive, giving a product of 372 bp.

Multiplex PCR

A multiplex PCR, based on N and M genes, was developed for detection and differentiation of PPRV from RPV. A total of four combinations of different primer pairs were tested. Optimal result was obtained with the PPRV/RPV cross-reacting N gene primer pair pprn_fr2/re1 and the PPR-specific M gene primer pair mf_morb/mr_ppr3. Multiplex PCR with these primer pairs amplified both the 334 bp N gene and the 191 bp M gene regions with PPRV cDNA (Fig. 4). In contrast, with RPV cDNA regardless of the virus strain, only a 334 bp N gene region was amplified. Summing up, such a multiplex PCR could be regarded as PPRV-positive in case of two products (191 bp and 334 bp), and as RPV-positive in case of one product (334 bp) only.

Sensitivity of the PCRs developed in comparison to F gene PCR

The sensitivity of the PCRs was evaluated using serial tenfold dilutions of PPRV corresponding to titers of 10^1–10^6 $TCID_{50}$/ml and expressed as the minimum titer that gave positive signal (Figs. 5 and 6).

The N, M, H and F gene and multiplex PCRs showed the sensitivities of 10^2, 10^1, 10^4, 10^5 and 10^1 $TCID_{50}$/ml, respectively. Thus the M gene and multiplex PCRs were equally and at the same time most sensitive, followed by the

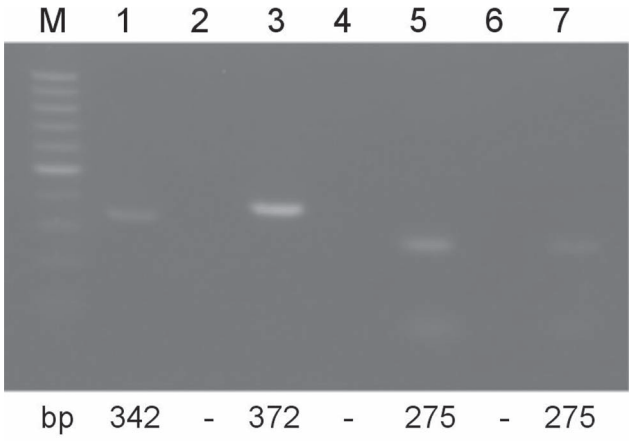

Fig. 3
H gene PCR

H gene PCR: PPRV (lane 1), RPV (lane 2), uninfected Vero cells (lane 4), and healthy goat tissue (lane 6). Positive controls: F gene PCR, RPV (lane 3); β-actin gene PCR, uninfected Vero cells (lane 5) and healthy goat tissue (lane 7). 100 bp DNA ladder (lane M).

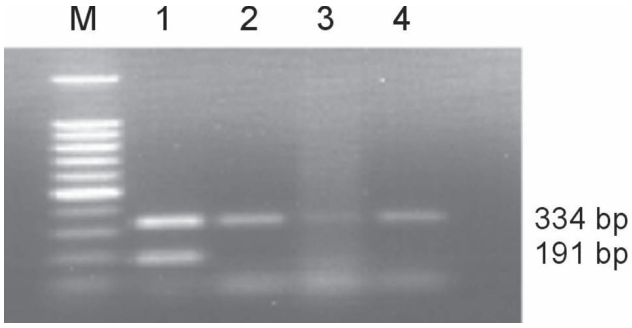

Fig. 4

Multiplex PCR

Whereas PPRV (lane 1) gave 2 bands of 334 bp and 191 bp, respectively, various strains of RPV yielded only single band of 334 bp: RPV grown in Vero cells (lane 2), lapinized RPV (lane 3), and goat strain of RPV (lane 4). 100 bp DNA ladder (lane M).

N, H and F gene PCRs (Table 2). The M gene and multiplex PCRs were 10,000 times more sensitive than the F gene PCR currently used in PPRV diagnostics. In comparing these two PCRs the latter has the advantage of differentiating PPRV from RPV in single reaction.

Discussion

In this study we developed N, M and H gene PCRs and an N-M gene multiplex PCR. The M gene and multiplex PCRs were found to be more sensitive than others tested as well as the currently used F gene PCR. Our strategy in developing the PCRs was based on an earlier report on RPV mRNAs, according to which the mRNAs of the genes located towards the 3'-end were more abundant than those of the genes located towards the 5'-end of the genome (Ghosh *et al*., 1995). As the PPRV genome organization is similar to that of RPV and other morbilliviruses (Barrett *et al*., 1993), we assumed that a PCR based on N or M gene located towards the 3'-end of PPRV genome would perform better than that based on F or H gene located more upstream of the 3'-end.

Table 2. Sensitivities of individual PCRs

PCR	$TCID_{50}$/ml						
	10^6	10^5	10^4	10^3	10^2	10^1	10^0
N gene	+	+	+	+	+	–	–
M gene	+	+	+	+	+	+	–
F gene	+	+	–	–	–	–	–
H gene	+	+	+	–	–	–	–
Multiplex (N-M genes)	+	+	+	+	+	+	–

(+)/(–) = positive/negative result.

The M and N gene PCRs turned out to be 10,000 and 1,000 times more sensitive than the F gene PCR, respectively. The higher sensitivity of the M gene PCR compared to the N gene PCR slightly opposed the above hypothesis that the N gene located more proximally to the 3'-end would be a better PCR target than the M gene. This discrepancy might be due to greater abundance of the M gene mRNA compared to the N gene mRNA in case of PPRV. A similar discrepancy was also observed with RPV; the P gene mRNA was more abundant than the M gene mRNA despite the fact that the M gene was located downstream of the P gene (Ghosh *et al*., 1995). Similarly, the H gene PCR was found to be 10 times more sensitive than the F gene PCR, although, the H gene was located downstream of the F gene.

The M gene PCR sensitivity of 10^1 $TCID_{50}$/ml for PPRV is comparable to that of an immunocapture ELISA, namely $10^{0.6}$ $TCID_{50}$/ml (Libeau *et al.,* 1994). The M gene PCR appears a suitable candidate for sensitive tool in PPRV diagnostics.

The N-M gene multiplex PCR developed in this study is the first of its kind, able to differentiate PPRV from RPV in single reaction. Several authors have reported multiplex PCRs for diagnostics of Goatpox virus (Markoulatos *et al*., 2000), foot and mouth disease virus (Callens and deClerq, 1997) and other human pathogens (Gregorie *et al.*, 1989). Out of four different combinations of primer pairs tested in the study presented here, only one worked. Our experience that the compatibility of the primer pairs in multiplex PCR is an important prerequisite of its success is in accord with a similar observation reported earlier (Edwards and Gibbs,

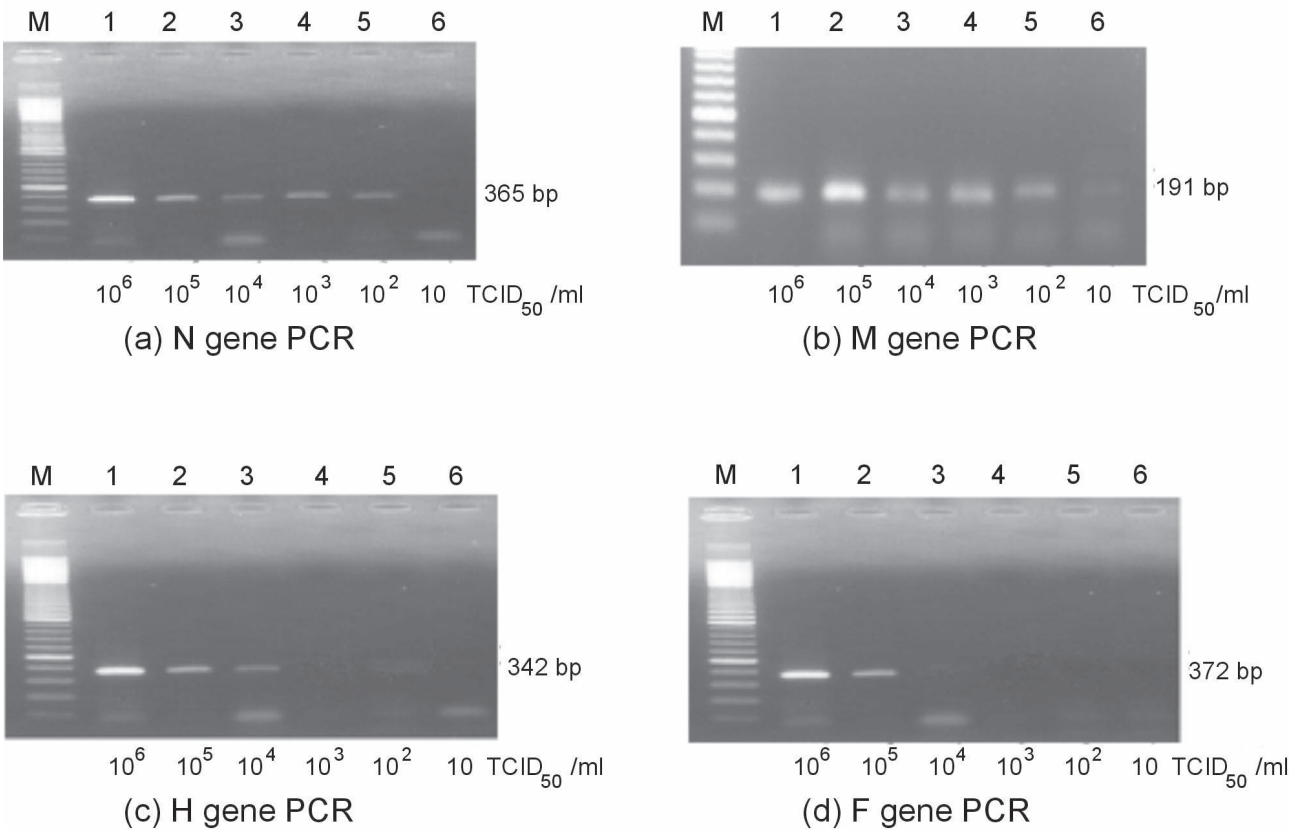

Fig. 5

Sensitivities of N, M, H and F gene PCRs in detecting PPRV

N gene PCR (a), M gene PCR (b), H gene PCR (c), and F gene PCR (d). 100 bp DNA ladder (lane M).

1995). Also the ratio of concentrations of the primer pairs for N and M genes was important; to achieve optimal performance of multiplex PCR, it had to be increased from 1:1 (5 pmoles each) to 2:1 (10 pmoles and 5 pmoles, respectively). Particularly, two bands of equal intensity corresponding to the two genes were thus obtained in gels after electrophoresis of the PCR products. A similar observation has also been reported for human retroviruses; namely, the primer concentration appears to be an important parameter of multiplex PCR provided there is a difference in the abundance of individual target sequences (Sunzeri *et al*., 1991).

Thus the M, N and H gene and multiplex M-N gene PCRs developed in this study turned out to be more suitable for diagnostics of PPRV than the F gene PCR, and among them, the M gene PCR was most sensitive, followed by the multiplex PCR. The M gene PCR has a potential to replace the currently used F gene PCR and thus to ensure a more efficient diagnostics of PPRV. Although some ELISA-positive samples were negative by some PCRs developed here, only very few ones were negative by the M gene and multiplex PCRs. Thus, the M gene PCR is comparatively better than the other PCRs developed in this study as well as the currently used F gene PCR. The higher sensitivity of

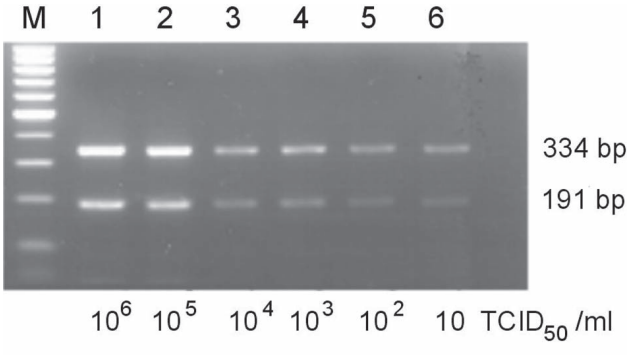

Fig. 6

Sensitivity of multiplex PCR in detecting PPRV

100 bp DNA ladder (lane M).

the M gene PCR as compared to others used in diagnostics of PPRV is being reported for the first time. In differentiating PPRV from RPV, the multiplex PCR is more sensitive, practical, time saving and cost effective than the F gene PCR with PPRV- and RPV-specific primers in two separate reactions. Thus the multiplex PCR has a potential to replace the currently used F gene PCR in differential diagnostics of PPRV and RPV.

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