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Development of multiplex PCR for simultaneous detection and differentiation of six DNA and RNA viruses from clinical samples of sheep and goats

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A B S T R A C T

Multiplex reverse transcription-polymerase chain reaction (RT-PCR) and PCR protocols were developed and subsequently evaluated for its effectiveness in detecting simultaneously single and mixed infections in sheep and goats. Specific primers for three DNA viruses and three RNA viruses, including foot and mouth disease virus (FMDV), Bluetongue virus (BTV), peste des petits ruminants virus (PPRV), sheeppox virus (SPPV), goatpox virus (GTPV) and orf virus (ORFV) were used for testing procedure. A single nucleic acid extraction protocol was adopted for the simultaneous extraction of both RNA and DNA viruses. The multiplex PCR consisted with two-step procedure which included reverse transcription of RNA virus and multiplex PCR of viral cDNA and DNA. The multiplex PCR assay was shown to be sensitive because it could detect at least 100 pg of viral genomic DNA or RNA from a mixture of six viruses in a reaction. The assay was also highly specific in detecting one or more of the same viruses in various combinations in specimens. Thirty seven clinical samples collected from sheep and goats were detected among forty three samples tested by both uniplex and multiplex PCR, showing highly identification. As results of the sensitivity and specificity, the multiplex PCR is a useful approach for clinical diagnosis of mixed infections of DNA and RNA viruses in sheep and goats with a reaction.

1. Introduction

Sheep and goats may infected with more than two virus simultaneously in a population when they are reared communally (Venkatesan et al., 2014). This system is common in many parts of the world. Among the mixed infections of small ruminants, foot-and-mouth disease (FMD), bluetongue (BT) and peste des petits ruminants (PPR) are considered to be significant followed by sheeppox (SP), goatpox (GP) and orf (Qin et al., 2015; Bhanuprakash et al., 2011; Yan et al., 2010). BT, FMD, PPR, GP, and SP are notifiable diseases in OIE manuals (OIE, 2016).

SPPV and GTPV cause mild to severe local or systemic skin lesions in sheep and goats. ORFV causes local persistent proliferative skin lesions and infect affected hosts repeatedly due to its host immune evasive strategy (Venkatesan et al., 2014). FMDV causes fever, vesicular lesions and erosion in the mouth, particularly on the tongue, and on the muzzle, feet and teats (Jiang et al., 2011). BTV causes soreness of the mouth and feet, oral erosions and ulcers, lameness and coronitis, weakness and depression, facial edema, and shared similarities with FMD (Maclachlan, 2011). PPRV causes high fever, ocular and nasal discharges, pneumonia, necrosis and ulceration of the mucous membrane and inflammation of the gastro-intestinal tract leading to severe diarrhoea (Dhar et al., 2002; Balamuruganet al., 2006). Infections by SPPV, GTPV and ORFV are economically significant and next to PPRV, FMDV and BTV. Although GTPV and SPPV are often considered as host-specific, some strains do infect both sheep and goats such as Kenya sheep and goat pox isolated by Davies in 1976 (Hosamani et al., 2004). Since Capripoxvirus (CaPVs) are serologically identical, their unequivocal identification depends exclusively on molecular techniques (Le Goff et al., 2009). In addition, capripox can occur in orf infected sheep and goats in a mixed fashion simultaneously. The mixed infections of CaPV with orf or other disease/s (FMD, BT, infections pleuropneumonia and PPR) can increase the severity of either infection and is not uncommon (Hosamani et al., 2004), as reported recently from China (Chu et al., 2011). Moreover, FMDV exists in the form of seven different serotypes. Similarly, twenty-four BTV serotypes are recognized currently, with a likely 25th serotype recently identified in goats in Switzerland (MacLachlan,

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2010; Hofmann et al., 2008). Diagnosis of the infection is usually based on clinical symptom and serological methods in the past. (Hosamani et al., 2009; Bhanuprakash et al., 2011). It is apparent that there are many similarities among the six diseases in clinic, so it is difficult to diagnose and identify the diseases by clinical diagnosis technique and serological method. Now nucleic acid techniques like PCR (Inoshima et al., 2002) and real-time PCR methods were applied to diagnose these diseases frequently (Bora et al., 2011; Venkatesan et al., 2012). However, these molecular techniques are based on the detection of different pathogens using different PCR assays targeting species-specific genes but not simultaneously in a single tube. There are reports for the detection and differentiation of SPPV and GTPV strains by PCR-restriction fragment length polymorphism (RFLP) (Hosamani et al., 2004; Fulzele et al., 2006), PCR/real time PCRs (Orlova et al., 2006; Lamien et al., 2011), CaPV and ORFV by duplex PCR (Zheng et al., 2007), and also for simultaneous detection of BTV, FMDV, PPRV and vesicular stomatitis virus(VSV)(Qin et al., 2015).

However, the information is scanty on the use of multiplex PCR (mPCR) for simultaneous detection and differentiation of FMDV, BTV, PPRV, SPPV, GTPV, and ORFV in a single tube format. The aim of this study was to develop the multiplex RT-PCR and multiplex PCR for simultaneous detection of six sheep and goats DNA and RNA viruses in clinical specimens, including FMDV, BTV, PPRV, SPPV, GTPV, and ORFV. From our results, these assays developed in this study are considered to be useful methods for identifying viruses in specimens from sheep and goats with multiple infections.

2. Materials and methods

2.1. Viruses and cells

The CVCC AV41 vaccine strain of GTPV (Shandong Lvdu Biotechnique Co., Cat. no. 151824003), HCE vaccine strain of ORFV (Shandong TaiFeng Biological Products Co., Cat. no. 151784013), Nigeria/75/1 vaccine strain of PPRV (Xinjiang TianKang Biotechnique Co., Cat. no. 2007291), BTV (China veterinary culture collection management center, Cat. no.CVCC AV47), SPPV (China veterinary culture collection management center, Cat. no.CVCC AV1011), and FMDV type O (China veterinary culture collection management center, Cat. no.CVCC AV100) were purchased. Bovine viral diarrhea viruses (BVDV) Shaanxi strain was provided kindly by Professor Jing-Yu Wang, College of Veterinary Medicine, Northwest A&F University. These viruses were used as standard viruses for the multiplex PCR and maintained at −80 °C until testing. Bovine testis cells (BTC) was described previously (Tian et al., 2013). BHK-21 cells (Shanghai Gaining Biological Co., Cat. No. CMT-013), and Vero cell lines (ATCC no. CCL-81) were our laboratory stock. BTC, BHK-21 cells, and Vero cell lines, *Escherichia coli* (China veterinary culture collection management center, Cat. No. CVCC3798) as well as BVDV were also used in the specificity assays. GTPV, SPPV, and ORFV were propagated in the Bovine testis cells, BTV and PPRV were propagated in the Vero cells and FMDV was propagated in the BHK-21 cells. To evaluate the efficiency of the extraction method and to assess the multiplex PCR, virus samples from cells infected with each of FMDV, BTV, PPRV, SPPV, GTPV or ORFV were examined by multiplex PCR.

2.2. Clinical specimens

During the period of December 2014 to December 2015, 43 clinical specimens consisting of skin scabs, mouth lesions, blood, swabs, lungs, spleens, lymph nodes and serums were collected from sheep and goats. These samples were either collected from field outbreaks by the disease investigating team or submitted to laboratory for clinical investigation from local farms in some western provinces of China.

2.3. Extraction of RNA and DNA

Viral genomic DNA and RNA were extracted from cell cultures infected with each virus and clinical specimens using the Axygen RNA/DNA Mini Kit (Axygen, San Francisco, USA) according to the manufacture's protocol. A single nucleic acid extraction protocol was adopted for the simultaneous extraction of both RNA and DNA viruses. Clinical samples homogenized as 10% suspension using phosphate buffer saline were used for extraction of viral nucleic acid and stored at −20 °C until use. The infected cells and clinical samples were freeze-thawed three times before subjecting for genomic extraction. The viral nucleic acid samples were extracted from 500 μl volumes of tissue suspensions. The extracted viral nucleic acid samples was stored at −80 °C until use.

2.4. Designing the mPCR primers

The sequences of primer pairs used to amplify the 3D gene of FMDV were the same as previous reports (Qin et al., 2015). The sequences of primer pairs used to amplify the NS3 gene of BTV were the same as previous reports (Feng et al., 2014). The sequences of primer pairs used to amplify the N gene of PPRV were the same as previous reports (Mao et al., 2010). Primers for amplifying SPPV, GTPV or ORFV were designed using PRIMER PREMIER 5.0 software. Six pairs of primers were synthesized by Invitrogen Trading Co. (Shanghai, China). PCR primer pairs for each target gene and GenBank accession number with each targeted gene and the expected sizes of PCR products were summarized in Table 1.

2.5. Reverse transcription

The reverse transcription (RT) reaction was performed in a 20 μl volumes, which contains 5 μl equal ratio mixture of the viral nucleic acid samples, 5 μl 4 × FQ-RT Super Mix (FastQuant RT Enzyme, RNase inhibitor, Random primers, Oligo dT Primer, dNTP Mixture, reaction Buffer, Tiangen, TIANGEN Biotech, Beijing, China), and 10 μl DEPC water. The reaction contains two steps: the mixture for incubation at 42 °Cfor 15 min and terminated the reaction by heating at 95 °C for 3 min. The products were kept in 4 °C for uniplex and multiplex PCR.

2.6. The uniplex PCR

The uniplex PCR reaction for FMDV, BTV, PPRV, SPPV, GTPV, and ORFV was carried in a 25 μl mixture containing 12.5 μl 2 × Premix Taq™ (20 mM Tris-HCl pH 8.3, 100 mM KCl, Taq DNA polymerase 1.25 U/25 μl, 3 mM $MgCl_2$, 0.4 mM each dNTPs), 1 μl of each 10 pmol/μl primer (Table 1), 100 ng of virus DNA and cDNA template, and then added with distilled water to 25 μl volume in total. The distilled water was used as a negative control. The amplifications were performed in a Thermo Cycler K960 (Heal Force, Shanghai, China) amplifier using the following steps: after an initial denaturation at 95 °C for 5 min, 30 cycles at 95 °C for 30 s, 57 °C for 30 s and 72 °C for 45 s, followed by a final extension at 72 °C for 10 min. Amplicons were analyzed by electrophoresis through 2% agarose-TAE (40 mM Tris-aceate pH 8.0, 1 mM EDTA) gel, stained with ethidium bromide and photographed.

2.7. Optimization of the multiplex PCR conditions

A number of experiments were carried out to optimize the multiplex PCR protocol, including reagent concentrations and PCR cycling parameters as described previously (Venkatesan et al.,

Table 1
Virus-specific primers used to amplify each target gene.

Virus	Primer	Sequence(5′-3′)	Position	Product size	Anneal site	Accession no. (Strain)
DNA virus						
SPPV	SPPVF	ACTAAACTTGTTACATTGTGA	119555–119576	177 bp	ORF112	AY077832.1 (TU-V02127)
	SPPVR	AACTTCTCCATCAATACATGA	119731–119711			
GTPV	GTPVF	TTTCAAAGCTTGTTTTTAACGTAGG	110977–111001	413 bp	ORF142	AY077835.1(Pellor)
	GTPVR	AAGTGCCGTCAATGAAGAATGG	111386–111368			
ORFV	ORFVF	CGAACTTCCACCTCAACCACTCC	572–594	507 bp	H2L	KP339952.1 (SDLC)
	ORFVR	CCTTGACGATGTCGCCCTTCT	1078–1058			
RNA virus						
FMDV	FMDVF	GGACCATACAGGAGAAGTTGA	1253–1273	130 bp	3D	X85493.1 (A22-645)
	FMDVR	CGCAGGTAAAGTGATCTGTAGC	1382–1359			
BTV	BTVF	AGTGTCGCTGCCATGCTATCC	8–29	257 bp	NS3	KP696699.1 (BTV-23/IND2004/09)
	BTVR	GCGTACGATGCGAATGCAGC	264–245			
PPRV	PPRVF	ATTGTCCACTATTGAATCCTTGAT	983–1006	687 bp	N	KR140086.1 (Izatnagar/94)
	PPRVR	TTGTCGTTGTAGACCTGACTGTTG	1669–1646			

2014; Qin et al., 2015). In the present study, parameter variables such as number of PCR cycle and cycle temperatures were optimized. PCR products were analyzed by electrophoresis through 2% agarose-TAE (40 mM Tris-aceate pH 8.0, 1 mM EDTA) gel, stained with ethidium bromide and photographed. Each specific viral target fragment was cloned into the pEASY-T1 Cloning vector (TransGen, Beijing, China) and sequenced by BGI Co.

2.8. Multiplex PCR

The multiplex PCR was performed in a 25 μl volumes and carried out with all six primer pairs with optimized parameters. After cDNA synthesis of RNA viruses, multiplex PCR was performed in a 50 μl volume. The reaction contained 25 μl 2 × Premix Taq™ (20 mM Tris-HCl pH 8.3, 100 mM KCl, Taq DNA polymerase 1.25 U/25 μl, 3 mM MgCl2, 0.4 mM each dNTPs), 1 μl of 10 pmol/μl ORFV primer (final concentration of 0.2 pmol/μl), 3 μl of 10 pmol/μl SPPV and GTPV primer (final concentration of 0.6 pmol/μl), and 2 μl 10 pmol/μl FMDV, BTV and PPRV primer (final concentration of 0.6 pmol/μl), and 5 μl of combined equal ratio mixture of the viral DNA and cDNA template, and then added with DEPC-treated water to 50 μl volume in total. Water was used a negative control. The cycling protocol consisted of an initial denaturing at 94 °Cfor 5 min; followed by 35 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 45 s, followed by a final extension at 72 °C for 10 min. Negative controls were run with each test. The amplicons were analyzed by electrophoresis through 2% agarose-TAE (40 mM Tris-aceate pH 8.0, 1 mM EDTA) gel, stained with ethidium bromide.

2.9. Specificity of the uniplex and multiplex PCR

In the specificity studies of the uniplex and multiplex PCR, BVDV, *Escherichia coli*, BHK-21 cells, Bovine testis cells, and Vero cell lines, FMDV, BTV, PPRV, SPPV, GTPV, and ORFV were tested with primers for FMDV, BTV, PPRV, SPPV, GTPV, and ORFV. To verify the results of the uniplex and multiplex PCR protocols, the size-specific PCR products (130, 257, 687, 177, 413, and 507 bp) obtained from experiments were sequenced to evaluate the specificity of the assay.

2.10. Sensitivity and reproducibility of the multiplex PCR

The sensitivity of multiplex PCR was carried out as described previously (Venkatesan et al., 2014; Qin et al., 2015). All viral genomic DNA and RNA was extracted using the Axygen RNA/DNA Mini Kit and detected by Nano Genius (MAPADA, Shanghai, China). The viral genomic DNA and RNA was diluted to be a concentration of 100 ng per μl and mixed as the template for sensitivity assay of the multiplex PCR. A 10-fold serial dilution was carried out to evaluate the sensitivity of the multiplex PCR.

Inter-assay and intra-assay reproducibility tests were performed in triplicate by testing 3 different concentrations of selected positive samples containing all the target viral nucleic acid to evaluate the reproducibility of the multiplex PCR. The genomic DNA and RNA of the six target viruses were used for the evaluation of reproducibility of the multiplex PCR. Six viral genomic DNA and RNA were extracted and 10-fold serially diluted at the different concentrations (100, 10, and 1.0 ng per μl). One μl of each concentration of each target viral nucleic sample was mixed and amplified by multiplex PCR. Each concentration was repeated three times on three successive days.

3. Results

3.1. Optimization of multiplex conditions

To determine the optimum annealing temperatures for multiplex PCR, different temperatures (51, 53, 55, 57, and 59 °C) were tested in the same reaction that included DNA of SPPV, GTPV, and ORFV as well as cDNA of FMDV, BTV, and PPRV, as template. The primers for FMDV, BTV, PPRV, SPPV, GTPV, and ORFV produced amplicons of 130, 257, 687, 177, 413 and 507 bp, respectively. The optimum annealing temperature for multiplex PCR was 55 °C (Fig. 1), the other optimum condition for multiplex PCR were established in this study. The final concentration of primers specific to ORFV was 0.2 pmol/μl. FMDV, BTV and PPRV were 0.4 pmol/μl while SPPV and GTPV were 0.6 pmol/μl. The PCR products were analyzed by electrophoresis through 2% agarose-TAE (40 mM Tris-aceate pH 8.0, 1 mM EDTA) gel, stained with ethidium bromide (Fig. 2).

3.2. The specificity of the multiplex PCR

The primers in the multiplex assays showed specificity for the respective viruses. No amplification occurred with BVDV, *Escherichia coli*, BHK-21 cells, Bovine testis cells, and Vero cell lines or ddH_2O (Fig. 3).

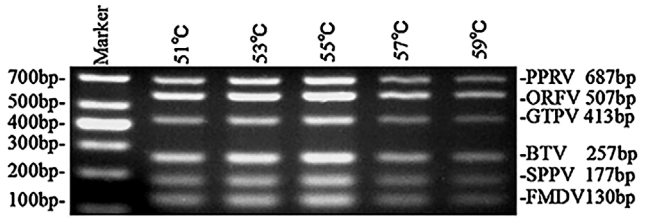

Fig. 1. Agarose gel electrophoresis of the multiplex PCR products (130 bp for FMDV, 257 bp for BTV, 687 bp for PPRV, 177 bp for SPPV, 413 bp for GTPV and 507 bp for ORFV) using different annealing temperatures. Marker: DL 1000 DNA ladder molecular weight marker.

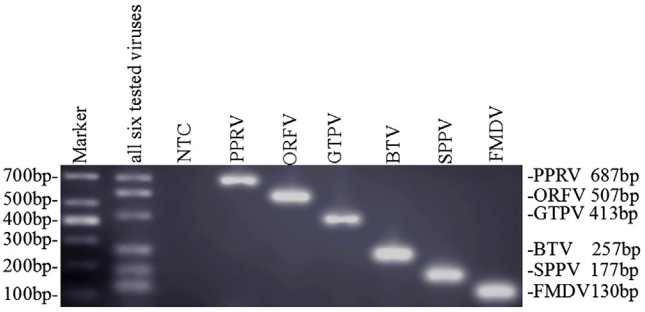

Fig. 2. Agarose gel electrophoresis of the uniplex and multiplex PCR products amplified with the optimum conditions. Marker, DL 1000 DNA ladder molecular weight marker; NTC, negative control: ddH_2O; all six tested viruses, FMDV, BTV, PPRV, SPPV, GTPV, and ORFV.

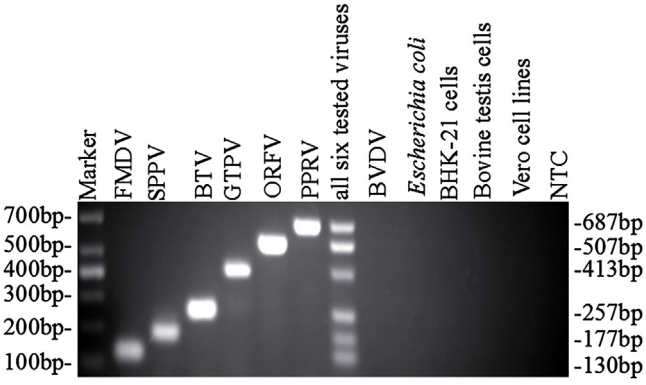

Fig. 3. Sensitivity of multiplex PCR. The viral DNA and RNA from six viruses were purified and mixed as the template, the template was 10-fold serially diluted. The concentrations of the viral DNA and RNA were indicated above each lane. Agarose gel electrophoresis of multiplex PCR products was carried out. NTC, negative control: ddH_2O. Marker, DL 1000 DNA ladder molecular weight marker.

3.3. The sensitivity and reproducibility of the multiplex PCR

To detect the sensitivity of the multiplex PCR, the viral genomic DNA and RNA was diluted to be a concentration of 100 ng/μl and mixed as the template for sensitivity assay of multiplex PCR. The multiplex PCR was able to detect at least 100 pg of six viral DNA and RNA (Fig. 4). In the reproducibility test of multiplex PCR, our results revealed that the target genes of six viruses were amplified, indicating that the multiplex PCR is highly reproducible (Fig. 5).

3.4. Multiplex PCR in positive samples

RNA and DNA were extracted from cell culture that was infected singly with FMDV, BTV, PPRV, SPPV, GTPV, and ORFV. Different combinations of viruses were amplified by six primers; all six viral DNAs and RNAs could be detected simultaneously. The PCR products were 130 bp (FMDV), 257 bp (BTV), 687 bp (PPRV), 177 bp (SPPV), 413 bp (GTPV), and 507 bp (ORFV), respectively (Fig. 6).

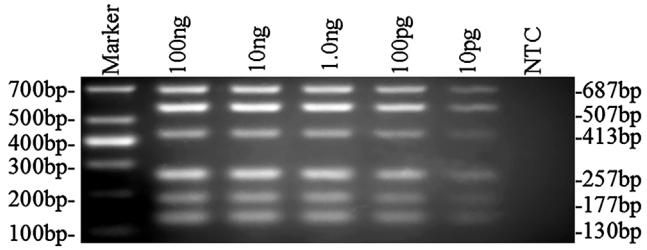

Fig. 4. Specificity of the uniplex and multiplex PCR. Six viruses (FMDV, BTV, PPRV, SPPV, GTPV, and ORFV) as well as unrelated viruses (BVDV), and *Escherichia coli*, BHK-21 cells, Bovine testis cells, and Vero cell lines were tested in this study to evaluate the specificity of the uniplex and multiplex PCR. The unrelated viruses, bacteria, BHK-21 cells,Bovine testis cells, and Vero cell lines were tested by multiplex PCR. The target viruses, unrelated viruses, bacteria, and cells are indicated above each lane. Marker, DL 1000 DNA ladder molecular weight marker; NTC, negative control (ddH_2O); all six tested viruses, FMDV, BTV, PPRV, SPPV, GTPV, and ORFV.

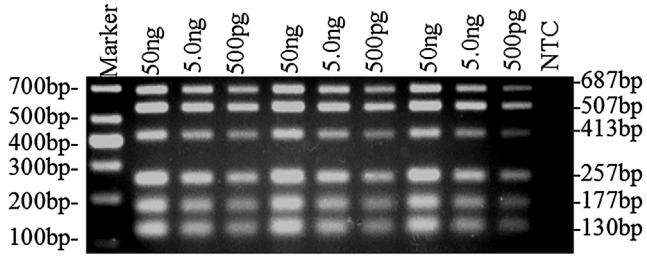

Fig. 5. The reproducibility of multiplex PCR. The genomic DNA and RNA of the six viruses were used for the evaluation of reproducibility of the multiple PCR. Agarose gel electrophoresis of multiplex PCR products from 10-fold serially diluted DNA and RNA samples from six tested viruses was carried. The concentrations of viral genomic DNA and RNA are indicated above each lane and the multiplex PCR was performed in triplicates. Marker, DL 1000 DNA ladder molecular weight marker; NTC, negative control (ddH_2O).

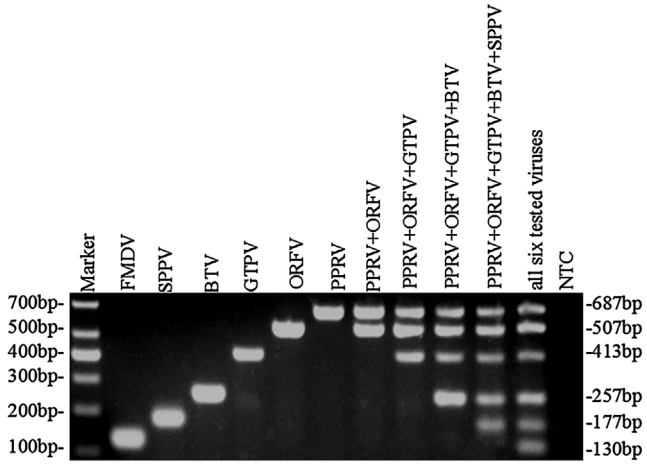

Fig. 6. Agarose gel electrophoresis of the uniplex and multiplex PCR products from single or multiple combinations of viruses. The tested viruses were indicated above each lane. Marker, DL 1000 DNA ladder molecular weight marker; NTC, negative control (ddH_2O).

3.5. Screening of clinical specimens by the uniplex and multiplex PCR

43 clinical sheep/goats specimens including specimens consisting of skin scabs, mouth lesions, blood, swabs, lungs, spleens, lymph

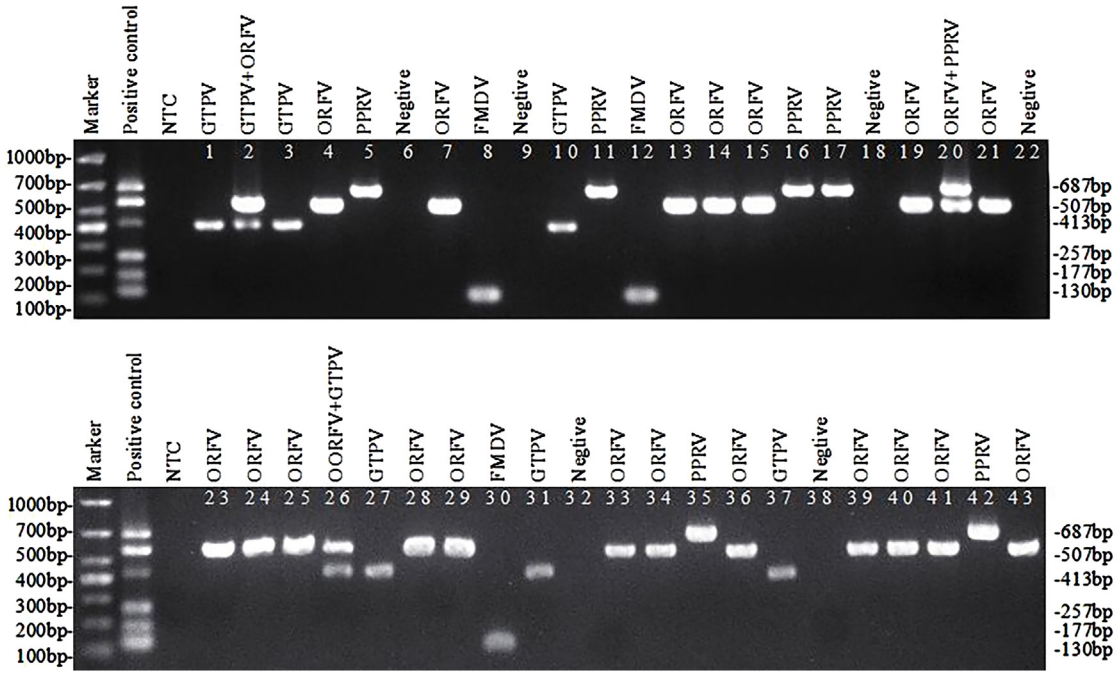

Fig. 7. Agarose gel electrophoresis of 43 clinical specimens detected by multiplex PCR. Each testing sample or result was indicated above each lane. Marker, DL 1000. DNA ladder molecular weight marker; NTC, negative control (ddH_2O); Positive control, FMDV + SPPV + BTV + GTPV + ORFV + PPRV, Negative, no virus detected by multiplex PCR.

Table 2
Detection of target viruses in 43 clinical specimens collected from sick sheep/goats by the uniplex and multiplex PCR.

Assay	Viruses					
	FMDV	SPPV	BTV	GTPV	ORFV	PPRV
Uniplex PCR/RT-PCR	3	0	0	8	22	7
Multiplex PCR	3	0	0	8	22	7

Table 3
Frequency of viruses alone or co-infection in 43 clinical specimens collected from sick sheep/goats.

Viruses	Number of infection	Positive rate (%)
FMDV	3	6.98
SPPV	0	0
BTV	0	0
GTPV	6	13.95
ORFV	19	44.19
PPRV	6	13.95
GTPV + ORFV	2	4.65
ORFV + PPRV	1	2.33

nodes and serums were collected from the some western provinces of China and detected by uniplex and multiplex PCR(Fig. 7). The results were identical with each other (Table 2). All PCR products were sequenced to validate the specificity of the uniplex and multiplex PCR. Among 43 clinical specimens, 37 specimens were positive by multiplex PCR or single PCR assays. Co-infection with two viruses was demonstrated in 3 samples (6.98%). Co-infection with three or more viruses was not detected in these clinical samples (Tables 2 and 3). The results obtained by the multiplex PCR were in consistent with morbidity of earlier reports, including the PCR for SPPV, GTPV and ORFV (Venkatesan et al., 2014; Xiao et al., 2012), and a multiplex PCR for BTV, FMDV, PPRV (Qin et al., 2015).

4. Discussion

The multiplex PCR and RT-PCR protocols were developed in this study for the simultaneous detection of single as well as mixed infections in sheep/goats. Respiratory and reproductive diseases, which have similar clinical signs, cause severe economic losses in sheep/goats. FMDV, BTV, PPRV, SPPV, GTPV, and ORFV were common viruses implicated in these diseases. Furthermore, multiple infections with pathogens are not uncommon in the sheep/goats industry worldwide. Therefore, a rapid and precise diagnostic detection of FMDV, BTV, PPRV, SPPV, GTPV, and ORFV is essential for early detection, surveillance, and prevention of spread of disease.

To date, a previous study has described in the detection of three DNA viruses and six RNA viruses, indicating that multiple PCR/RT-PCR has high sensitivity and specificity (Qin et al., 2015; Feng et al., 2014; Mao et al., 2010; Xiao et al., 2012; Zhu et al., 2007; Xiang et al., 2011). In the present study, the viral genomic DNA and RNA was extracted simultaneously and subjected to the multiplex PCR in a reaction. Therefore, a more cost effective and time saving mean for diagnosis, screening, and surveillance transmission of viruses was developed in this work. This multiplex PCR detects at least 100 pg of six tested viruses. Although, the sensitivity of the multiplex PCR is approximately 5–10 fold lower than established uniplex PCR (Qin et al., 2015; Feng et al., 2014; Mao et al., 2010; Xiao et al., 2012; Zhu et al., 2007; Xiang et al., 2011), the improvement of time saving would compensate for this minor reduction. No amplification was obtained from other pathogens and cells, such as BVDV, *Escherichia coli*, BHK-21 cells, Bovine testis cells, and Vero cell lines, indicating that the developed multiplex PCR has high specificity.

The examination of 43 clinical samples by multiplex PCR indicated that 6.98% of the samples were co-infected with two viruses. Co-infection with three or more viruses was not detected in these clinical samples. Because these clinical samples are representative of clinical samples in general in some western provinces of China, the results indicate that the relevant local departments should be alert to the epidemic of FMD, GP, ORF and PPR.

In summary, the developed assay allows for rapid, specific, and sensitive detection and surveillance of multiple viral infections in sheep/goats and fulfils all of the key requirements for clinical applicability.

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