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Development of a multiplex TaqMan qPCR assay for simultaneous detection and differentiation of four DNA and RNA viruses from clinical samples of sheep and goats

Xingang Xu[1], Feng Yang[1], Qi Zhang, Ying Xu, Jiali Huang, Mingzhe Fu, Weimin Zhang*

College of Veterinary Medicine, Northwest A&F University, Yangling, Shaanxi, 712100, China

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ABSTRACT

Mixed infections with different pathogens are common in sheep and goats under intensive production conditions. Quick and accurate detection and differentiation of different pathogens is necessary for epidemiological surveillance, disease management and import and export controls. Multiplex TaqMan qPCR protocols were developed and subsequently evaluated as effective tools in simultaneously detecting single and mixed infections in sheep and goats. Four pairs of primers and four probes labeled with Rox/BHQ2, Cy5/BHQ2, Hex/BHQ1 and Fam/BHQ1 for peste des petits ruminants virus (PPRV), foot and mouth disease virus (FMDV), goat pox virus (GTPV) and orf virus (ORFV), respectively, were used in the multiplex TaqMan qPCR assay. The assay was shown to be sensitive with detection limits of 9.17×10^1, 1.69×10^2, 9.41×10^1 and 7.46×10^1 copies/μL for PPRV, FMDV, GTPV and ORFV from a mixture of four viruses in a reaction, respectively. The assay was highly specific in its ability to detect one or more viruses in various combinations in the specimens. 38 clinical samples collected from sheep and goats were detected among 43 samples tested by multiplex TaqMan qPCR, showing highly effective identification. Overall, the multiplex TaqMan qPCR panel provides a fast, specific, and sensitive diagnostic tool for the accurate detection of multiple viral pathogens in sheep and goats.

1. Introduction

Sheep and goat husbandry is an important branch of livestock farming. However, with its intensification, simultaneous infection of one sheep or goat population with multiple viruses is becoming increasingly common in many parts of the world (G. Venkatesan et al., 2014) with severe economic consequence. Amongst common diseases in small ruminants, peste des petits ruminants (PPR), foot-and-mouth disease (FMD), and goatpox (GP) are notifiable diseases in OIE manuals (OIE, 2016). PPRV causes high fever, ocular and nasal discharges, pneumonia, necrosis and ulceration of the mucous membranes and inflammation of the gastro-intestinal tract leading to severe diarrhea (Balamurugan et al. (2006); Dhar et al. (2002)). FMDV results in fever, vesicular lesions and erosion of the mouth, particularly on the tongue, and on the muzzle, feet and teats (Jiang et al., 2011). GTPV causes mild to severe local or systemic skin lesions in goats. ORFV causes local persistent proliferative skin lesions and infection repeatedly affects hosts due to its host immune evasive capabilities. GTPV and ORFV infections are economically significant only next to PPRV and FMDV infections. Mixed infections of Capripoxvirus with orf or other diseases are common in China (FMD, PPR, et al.) (Chu et al., 2011), and mixed infections can increase the severity of either infection (Hosamani et al., 2004).

Nevertheless, it is significant challenging diagnose and identify some certain diseases based only on by the clinical symptoms. In recent years, nucleic acid detection techniques like PCR and real-time PCR methods have been used as a convenient and accurate detection method are frequently applied to diagnose these diseases. However, these molecular techniques are based on the detection of different pathogens using different PCR assays targeting individual species-specific genes but not simultaneously in a single tube. Previous reports have described the detection and differentiation of Sheep pox virus and GTPV strains using PCR-restriction fragment length polymorphism (RFLP) (Hosamani et al., 2004; Fulzele et al., 2006) or PCR/real time PCRs (Orlova et al., 2006; Lamien et al., 2011). Furthermore, detection and differentiation of Capripoxvirus, ORFV strains and the simultaneous detection of BTV, FMDV, PPRV and vesicular stomatitis virus (VSV) using duplex PCR have also reported (Zheng et al., 2007; Qin et al.,

* Corresponding author.
E-mail address: 313310625@qq.com (W. Zhang).
[1] These authors contributed equally to this work.

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2015). However, few reports have described the simultaneous detection and differentiation of FMDV, PPRV, GTPV, and ORFV in a single tube format by the multiplex TaqMan qPCR.

The aim of this study was to develop a multiplex TaqMan qPCR method for simultaneous detection of four DNA/RNA viruses in sheep and goats in clinical specimens, including FMDV, PPRV, GTPV, and ORFV. Our results showed that the assay developed in this study is useful for identifying viruses in specimens from sheep and goats with multiple infections.

2. Materials and methods

2.1. Viruses, cells, clinical specimens and primers

The CVCC AV41 vaccine strain of GTPV, HCE vaccine strain of ORFV, Nigeria/75/1 vaccine strain of PPRV, bluetongue virus (BTV, serotype 10), Bovine viral diarrhea viruses (BVDV, genotype 1) and FMDV type O were used as previously described (He et al., 2017). The Bartha-K61 vaccine strain of pseudorabies virus (PRV) (Shandong Lvdu Bio-technique Co., Cat. no. 151827018) were purchased. These viruses were used as standard viruses for the multiplex TaqMan qPCR 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, and ORFV were propagated in the Bovine testis cells, PPRV was 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 TaqMan qPCR, virus samples from cells infected with each of FMDV, PPRV, GTPV or ORFV were examined by multiplex TaqMan qPCR.

To evaluate and assess the efficiency of multiplex TaqMan qPCR, 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.

Primers and probes for amplifying PPRV, FMDV, GTPV and ORFV were designed using Beacon Designer 8 software. The primers and probes were synthesized by TsingKe Biological Technology Co. (Beijing, China). The primer pairs and probes used in the optimization of multiplex TaqMan qPCR are shown in Table 1, including GenBank accession number with each targeted gene and the expected sizes of PCR products.

2.2. 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 −80 °C until use. The infected cells and clinical samples were freeze-thawed three times before their subjection for genomic extraction. Each of the viral nucleic acid samples was extracted from a 500 μL volume of tissue suspensions. The extracted viral nucleic acid samples were stored at −80 °C until use.

2.3. Reverse transcription

The reverse transcription (RT) reaction was performed in a 20 μL volume, 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 °C for 15 min and the reaction terminated the reaction by heating at 95 °C for 3 min. The products were kept in 4 °C for uniplex and multiplex PCR.

2.4. Preparation of standard control plasmids

Standard plasmids containing specific viral target fragments were used as templates for optimization and sensitivity detection of each of the uniplex and multiplex TaqMan qPCR assays. The PCR amplification was performed with primers shown in Table 1 in a total reaction volume of 25 μL 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 μM primer (Table 1), 2 μL of DNA/cDNA template, and distilled water added to 25 μL volume in total. 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: initial denaturation at 95 °C for 2 min, 30 cycles at 95 °C for 30 s, 57 °C for 30 s and 72 °C for 30 s, final extension at 72 °C for 5 min. The PCR products were separated by agarose gel electrophoresis, purified with a PCR purification kit (Trans, Transgen Biotech, Beijing, China) and cloned into the pEASY-T1 system (Trans) according to the manufacturer's instructions. The constructed plasmid were confirmed by PCR and DNA sequencing (TsingKe Biological Technology Co., Beijing, China). The copy number of the extracted plasmids was calculated using the following formula.

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

Virus	Primer	Sequence(5'-3')	Product size	Anneal size	Accession No.(Strain)
RNA virus					
	PPRVF	ACAGGATTGCAGAAGATC			
PPRV	PPRVR	CGGCTTCTACAATATAGTTG	132	N	KR140086.1 (Izatnagar/94)
	PPRV-Probe	ROX-ATCACTTCGGCGGTTCATGGTATCTC-BHQ2			
	FMDVF	GGACCATACAGGAGAAGTTGA			
FMDV	FMDR	CGCAGGTAAAGTGATCTGTAGC	130	3D	X85493.1 (A22-645)
	FMDV-Probe	Cy5-CTCCGTGGCAGGACTCGCAGT-BHQ2			
DNA virus					
	GTPVF	CGTTCTCATCTGTATTATTATTAG			
GTPV	GTPVR	GCAGTTATGATGATTATATCGA	144	ORF103	AY077835.1 (Pellor)
	GTPVR-Probe	HEX-CCTCAACTAAGGACCATTCTTGCTCACA-BHQ1			
	ORFVF	CGTCAACTACTACAAGGTC			
ORFV	ORFVR	CCGAGGTTCTTGATAGTG	144	ORFV011	KP339952.1 (SDLC)
	ORFV-Probe	FAM-CGTCGGCAACCTTCTCGGCA-BHQ1			

For multiplex reactions, the concentration of each plasmid was used to make 10-fold serial dilutions to construct individual standard curves and to determine the limit of detection. Each standard plasmid was used to make 10-fold serial dilutions to construct multiplex standard curves for analytical validation.

2.5. Optimization of the uniplex and the multiplex TaqMan qPCR assay

Four uniplex qPCR assays, in which each probe and the corresponding primer pair were used separately for each pathogen, were initially carried out followed by multiplexing. All real-time PCR reactions were performed in a Thermo Cycler TL988 (TIANLONG, Shaanxi, China) capable of simultaneous detection of up to four different fluorescent dyes. Each uniplex and multiplex qPCR reaction was conducted in a 10 μL reaction volume that included cDNA/DNA, 2× Probe Master Mix (Vazyme, Nanjing, China), primers and probe(s). The conditions in the thermo cycler were: pre-denaturation at 95 °C for 10 min, 35–40 cycles of denaturation at 95 °C for 10 s, and annealing and extension at 57–61 °C for 30 s. Fluorescent measurements were carried out during the elongation step. The final concentrations of primers, probes and the amplification conditions were optimized to obtain the maximum reporter florescence and minimum threshold cycle (Ct) using the positive standards of different dilutions as template. No template negative controls (NTC) were included in each round.

2.6. Analytical specificity, sensitivity and reproducibility

To ensure the assay specificity, potential cross-detection within the primer pairs and the probes was first measured. Positive control plasmids of all 4 targets were mixed in equal amounts and used for specificity analysis as the target pool. Non-target pools were prepared the same way as target pool except that the target plasmids were not included in the plasmids pools. In all the reactions, no template control (NTC) was included always to check the cross contamination of test reagents. Amplified products at the end of run were also checked in agarose gel electrophoresis to confirm the expected size of amplicons and specificity of the assay. In addition, PPRV, FMDV, GTPV and ORFV clinical confirmed positive samples were used for diagnostic specificity analysis.

Limit of detection of the assay was determined by using10-fold serial dilutions of standard plasmids in the range 10^5-10˚copies/μL volume using TE buffer. Besides, the combination of four viruses mixed at equal ratios was also serially diluted and checked to assess the limit of detection. This was performed to know the sensitivity of the assay in case of mixed infections of viruses.

To determine and assess the repeatability and reproducibility of the multiplex TaqMan qPCR assay, the coefficient of variation (CV) was calculated by testing assay in three consecutive runs (inter-assay) in different days and three times in the same run (intra-assay) using 10^6-10^4copies/μL standard plasmids.

3. Results

3.1. Construction of standard plasmids DNA of PPRV, FMDV, GTPV and ORFV

The standard plasmids of PPRV (132bp), FMDV (130bp), GTPV (144bp) and ORFV (144bp) from reference viruses of respective species were confirmed for their specificity by restriction enzyme digestion and commercial sequencing. The expected size of these fragments was obtained (Fig. 1). The stock of these plasmids had a concentration of 9.17×10^{10}(PPRV), 1.69×10^{10}(FMDV), 9.41×10^{10}(GTPV) and 7.46×10^{10}(ORFV) copies/μL of solution with a purity of > 1.8 (ratio of 260/280 nm absorbance) when quantified using a NanoGenius Spectrophotometer (MAPADA, Shanghai, China).

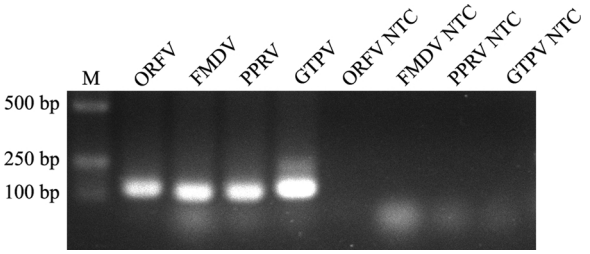

Fig. 1. Agarose gel electrophoresis of PCR products (144 bp for ORFV, 130bp for FMDV, 132 bp for PPRV, 144 bp for GTPV). M, DL 2 000 DNA ladder molecular weight marker. NTC, negative control.

3.2. Optimization of uni- and multiplex TaqMan qPCR conditions

After optimization, the following parameters for the four uniplex TaqMan qPCR assays and the multiplex TaqMan qPCR assays were chosen. Each uniplex TaqMan qPCR assay was conducted in a 10 μL reaction volume that included 2× Probe Master Mix, 0.4 μL of 10 μM primers, 0.2 μL of each 10 μM probe, and 0.5 μL of the viral cDNA/DNA template. The multiplex TaqMan qPCR assays were in a total volume of 10 μL included 2× Probe Master Mix, 0.3 μL of 10 u M PPRV and ORFV primers, 0.15 μL of 10 u M PPRV and FDMV probes, 0.2 μL of 5 μM GTPV and FDMV primers, 0.1 μL of 5 μM GTPV and FDMV probes, and 0.5 μL of each viral cDNA and DNA template. Reactions were carried out in a Thermo Cycler TL988 (TIANLONG, Shaanxi, China), and the cycling parameters were 10 min at 95 °C, 40 cycles of 10 s at 95 °C, and 30 s at 60 °C. Fluorescence data was collected at 60 °C.

3.3. Assay specificity

The specificity of the uni- and multiplex TaqMan qPCR panels was evaluated with clinical positive samples. The results show that only the intended target gene was amplified from its target templates, and no signal was detected in all non-target pools. The multiplex qPCR assay also correctly identified all of its four target genes from the mixed template and no cross-amplification was observed. Diagnostic specificity on the clinical positive samples was 100%, meanwhile, any positive signals were not be observed on the other non-target pathogens, suggesting that the panel was highly specific for detection of the target viruses (Fig. 2).

3.4. Linearity and analytical sensitivity of the assay

Standard curves were constructed using Ct values of the uni- and multiplex TaqMan qPCR panel. The dynamic range of the assay encompassed at least five orders of magnitude, with a strong linear relationship between the Ct values and the log10 of the input copy number. The uni- and multiplex TaqMan qPCR assays were linear with a dynamic range of detection between 10^7 and 10^3 copies/μL in 40 cycles. As shown in Fig. 3A–D, the uniplex TaqMan qPCR assays were linear for the standards of PPRV (correlation coefficient $R^2 = 0.999$, efficiency of amplification E = 95%), FMDV ($R^2 = 0.999$, E = 104%), GTPV ($R^2 = 0.999$, E = 93%), ORFV ($R^2 = 0.999$, E = 97%). The standard curves of the multiplex TaqMan qPCR for PPRV, FMDV, GTPV and ORFV, as shown in Fig. 3E, were plotted with slopes of -3.441, -3.243, -3.668 and -3.454, respectively, indicating that the amplification efficiencies were 95%, 103%, 87%and 95%, respectively. The correlation coefficients (R^2) were 0.997, 0.998, 0.997, and 0.999 for PPRV, FMDV, GTPV and ORFV, respectively. Both the uni- TaqMan qPCR and multiplex TaqMan qPCR showed high correlation between Ct value and template concentration, high degrees of accuracy and amplification efficiency. The detection limit of multiplex TaqMan qPCR was 9.17×10^1, 1.69×10^2, 9.41×10^1 and 7.46×10^1 DNA copies for PPRV, FMDV, GTPV and ORFV, respectively. The detection limit of

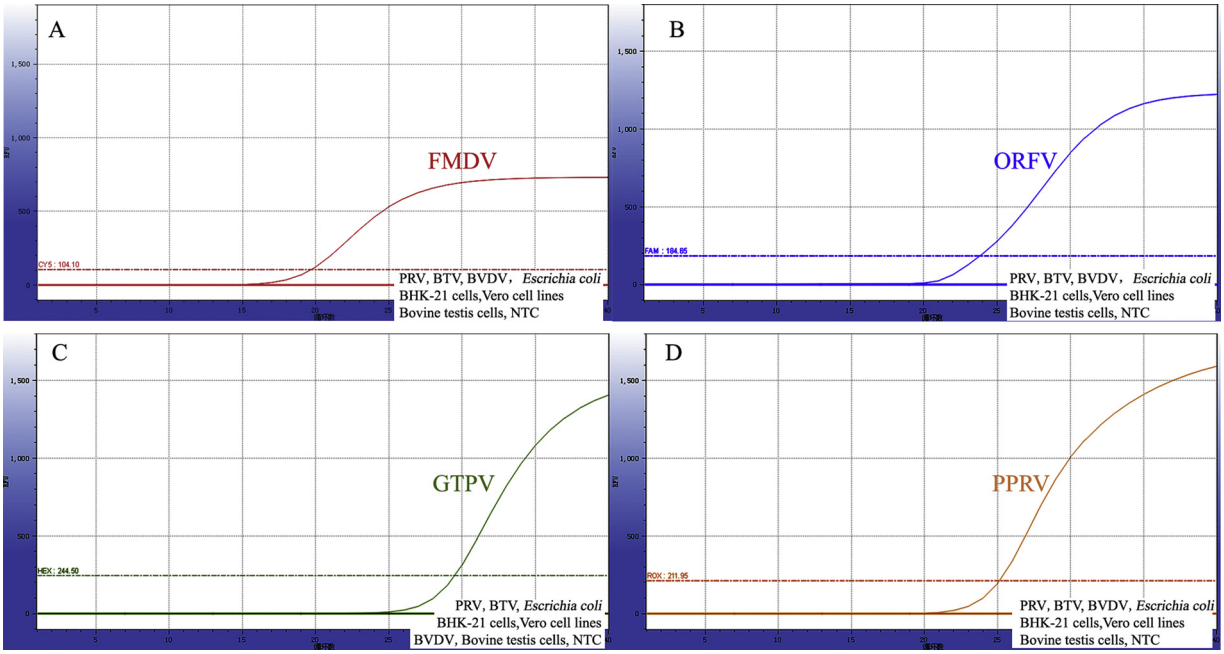

Fig. 2. The uniplex and multiplex TaqMan qPCR amplification plot showing specific amplification of clinical positive samples. (A), (B), (C) and (D) Specific amplification of FMDV, ORFV, GTPV and PPRV by the uniplex TaqMan qPCR, respectively, with no such signal in PRV, BTV, BVDV, BHK-21 cells, Vero cell lines, *Escherichia coli*, Bovine testis cells and NTC.

conventional PCR was 9.17×10^3, 1.69×10^4, 9.41×10^3 and 7.46×10^3 DNA copies for PPRV, FMDV, GTPV and ORFV, respectively (Fig. 4). The multiplex TaqMan qPCR was 100 times more sensitive than the conventional PCR.

3.5. Inter and intra-assay reproducibility of the multiplex TaqMan qPCR assay

To evaluate the reproducibility of the multiplex TaqMan qPCR, 10^6-10^4copies/μL plasmids DNAs of PPRV, FMDV, GTPV and ORFV were tested in triplicate and coefficient of variation (CV) was calculated, respectively. As shown in Table 2, the assay variability, calculated based on the CV of inter and intra-assay, was 1.06%–2.55% and 1.38%–4.05%, 0.78%–3.94% and 1.17%–3.45%, 3.62%–4.93% and 4.45%–4.93%, 1.46%–1.97% and 2.87%–3.70% for plasmids DNA of PPRV, FMDV, GTPV and ORFV, respectively. These data demonstrated good reproducibility of the assay for specific viruses was demonstrated.

3.6. Screening of clinical specimens by the multiplex TaqMan qPCR

43 clinical sheep/goats specimens including skin scabs, mouth lesions, blood, swabs, lungs, spleens, lymph nodes and serums were collected from western provinces of China and detected by the multiplex TaqMan qPCR assay. Among 43 clinical specimens, 38 specimens were tested positive by the multiplex TaqMan qPCR assay. Co-infection with two viruses was demonstrated in three samples (6.98%). Co-infection with three or more viruses was not detected in these clinical samples (Tables 3 and 4). The results show that the method is successful and can be used for clinical testing.

4. Discussion

In this study, a mulitiplex TaqMan qPCR protocol was developed for the simultaneous detection of single or mixed infections in sheep and goats. As the husbandry scale of these animals is intensive, the rapid development of diseases has become an important factor which can impact growth, hamper exports food quality and safety, especially PPRV, FMDV, GTPV, and ORFV. Moreover, mixed infectious multiple pathogens are common in the sheep and goat husbandry worldwide (He et al., 2017). Therefore, a rapid and precise diagnostic detection of PPRV, FMDV, GTPV, and ORFV is essential for early detection, surveillance, and for the prevention of spread of disease.

A previous study described in the detection of GTPV and ORFV, indicating that duplex real-time PCR has high sensitivity and specificity. In the present study, viral genomic DNA and RNA was extracted simultaneously and subjected to the multiplex real-time PCR in a single reaction. The process is a more cost effective and time saving means for diagnosis, screening, and surveillance transmission of viruses. Molecular tools specifically conventional multiplex PCR (He et al., 2017), have been reported for differentiating viruses of sheep and goats. However, PCR may not be sufficient to quantify small copy number targets and large scale clinical specimens. High sensitivity of the multiplex real-time PCR was demonstrated by its high detection limit compared to the gel based uniplex PCR method which had a sensitivity that was approximately 5˜10 fold higher than multiplex 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). Thus, the multiplex real-time PCR has a much greater advantage compared with multiplex conventional PCR. The intra and interrun variations of this assay were within the acceptable limits (< 5% CV) (Balamurugan et al., 2010), indicating the better repeatability and reproducibility of the developed multiplex real-time PCR. No amplification was obtained from other pathogens and cells, such as BVDV, PRV, BTV, *Escherichia coli*, BHK-21 cells, Bovine testis cells and Vero cell lines. These findings indicated that the developed multiplex real-time PCR has high specificity.

Examination of 43 clinical samples by multiplex real-time 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 the samples. The results obtained by the multiplex TaqMan qPCR were consistent with morbidity of earlier reports including the PCR for FMDV, PPRV, GTPV and ORFV (He et al., 2017; Qin et al., 2015; Venkatesan et al., 2014; Xiao et al., 2012). We successfully established a multiplex real-time PCR method for the detection of four common viruses in sheep and goats. In addition to clinical diagnosis this method could also be used for epidemiological investigations.

Recent years, FMDV, ORFV, GTPV and PPRV have erupted in

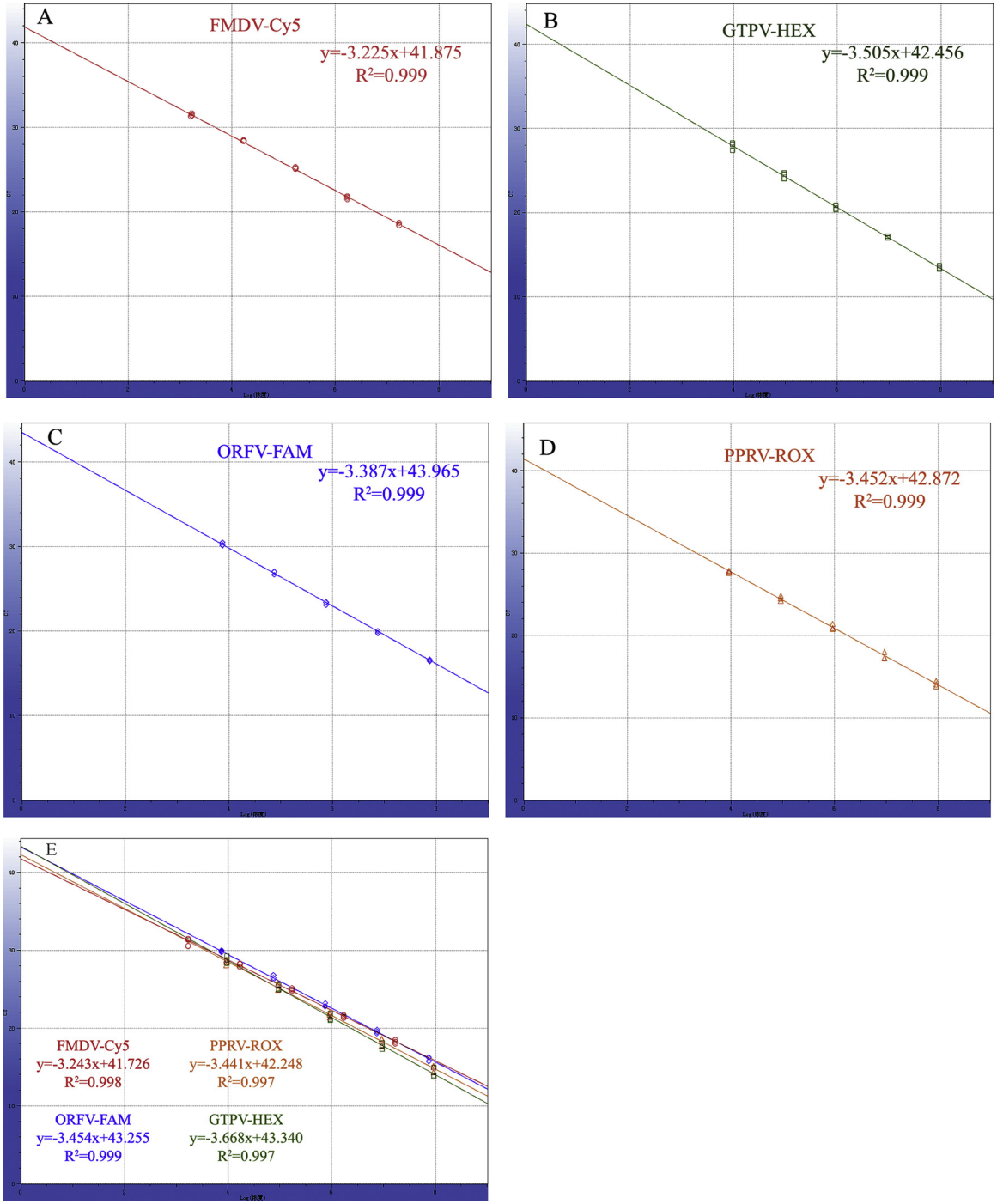

Fig. 3. Standard curves of the uniplex and multiplex TaqMan qPCR for PPRV, FMDV, GTPV and ORFV, respectively. (A) FMDV standard plasmid (1.69×10^7 to 1.69×10^3 copies/μL), (B) PPRV standard plasmid (9.17×10^7 to 9.17×10^3 copies/μL), (C) ORFV standard plasmid(7.46×10^7 to 7.46×10^3 copies/μL) and GTPV standard plasmid (9.41×10^7 to 9.41×10^3 copies/μL) used for establishing the standard curve by the uniplex TaqMan qPCR, respectively. (E) Standard serially diluted plasmid DNA (10^7 to10^3) of FMDV, PPRV, ORFV and GTPV used for assessing the linear correlation by the multiplex TaqMan qPCR.

several provinces in China especially in some western provinces. Among these four common goat and sheep diseases, ORFV has the highest incidence, followed by GTPV and PPRV, whereas FMDV has the lowest incidence according to published literature. (Bao et al., 2017; Yan et al., 2012; Yang et al., 2014; Jamal and Belsham (2018)). Our results showed that clinical specimens' detection rate was similar to the literature report, which come to a conclusion that the multiplex TaqMan qPCR assay we established can be used for clinical testing.

In summary, the developed multiplex real-time PCR assay allows for rapid, specific, and sensitive detection. It can be used for the surveillance of multiple viral infections in sheep and goats and can fulfill all of the key requirements for clinical applicability. This multiplex real-time PCR is a first trial that has not been reported yet. The multiplex TaqMan qPCR panel will be useful not only for diagnostics, and in ecological, epidemiological and pathogenesis studies. Our approach can also be used to investigated host/virus or virus/virus interactions, particularly during multiple infections.

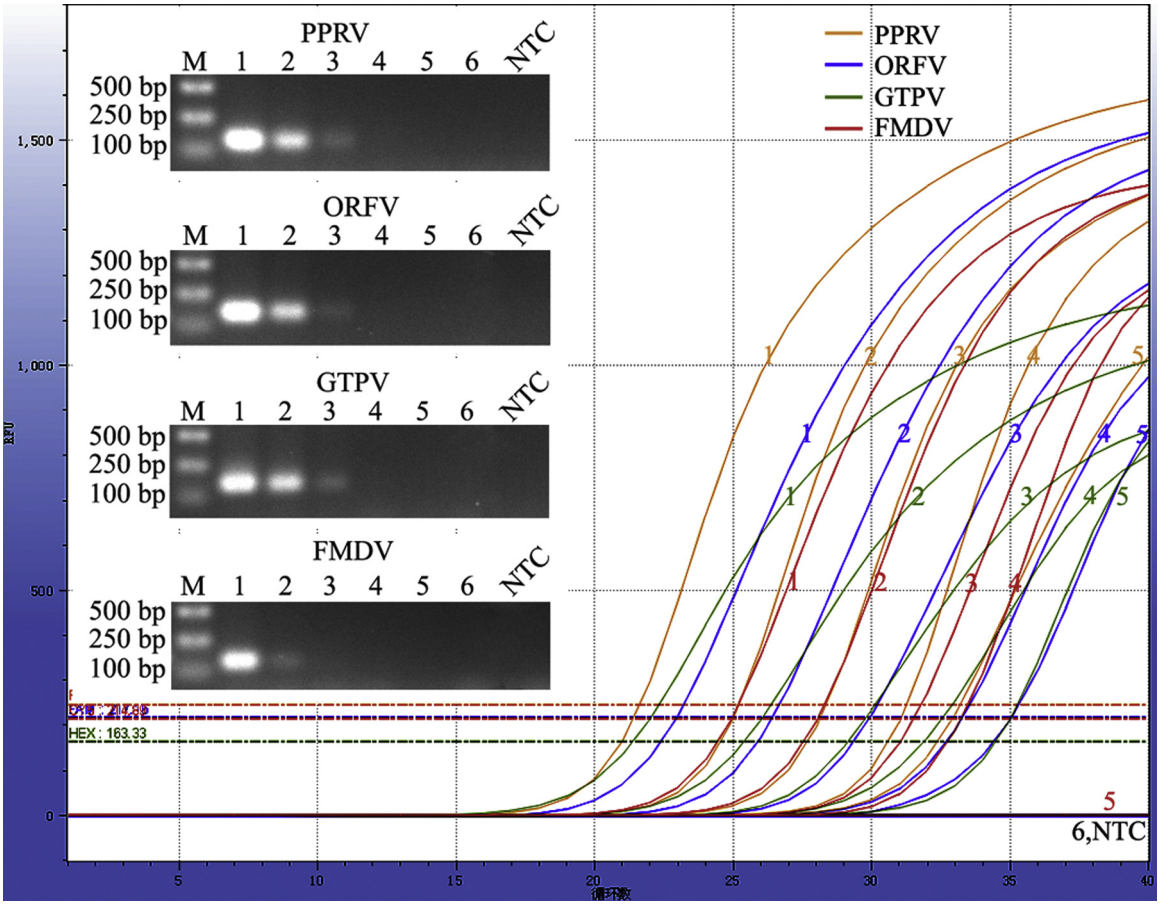

Fig. 4. Sensitivity of the multiplex TaqMan PCR and conventional PCR. Standard serially diluted plasmid DNA (10^5 to10˚) of FMDV, PPRV, ORFV and GTPV used for detection limit of the developed assay by the multiplex TaqMan PCR and conventional PCR, respectively. M, DL 2 000 DNA ladder molecular weight marker. 1–6, 10^5 to10 ˚Standard serially diluted plasmid DNA.NTC, negative control.

Table 2
Reproducibility of multiplex TaqMan PCR evaluated with plasmids of PPRV, FMDV, GTPV and ORFV, respectively.

Plasmid	Copy number (copies/μL)	Coefficient of variation (CV) (%)	
		Inter-assay	Intra-assay
PPRV	9.17×10^6	2.53%	4.05%
	9.17×10^5	2.55%	2.38%
	9.17×10^4	1.06%	1.38%
FMDV	1.69×10^6	0.78%	1.17%
	1.69×10^5	3.94%	3.45%
	1.69×10^4	3.15%	2.67%
GTPV	9.41×10^6	4.93%	4.93%
	9.41×10^5	3.62%	4.45%
	9.41×10^4	4.24%	4.48%
ORFV	7.46×10^6	1.67%	3.17%
	7.46×10^5	1.97%	3.70%
	7.46×10^4	1.46%	2.87%
NTC	None	None	

Table 3
Detection of target viruses in 43 clinical specimens collected from sick sheep/goats by the uniplex PCR and multiplex TaqMan PCR.

Assay	Viruses			
	FMDV	GTPV	ORFV	PPRV
Uniplex PCR	3	8	22	7
Multiplex TaqMan qPCR	3	8	23	7

Table 4
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
GTPV	6	13.95
ORFV	20	46.51
PPRV	6	13.95
GTPV + ORFV	2	4.65
ORFV + PPRV	1	2.33

Contributors and authorship

Xingang Xu and Feng Yang performed the majority of experiments and involved in manuscript preparation, Qi Zhang, Ying Xu and Jiali Huang participated in editing of the manuscript. Mingzhe Fu participated part of the experiments. Weimin Zhang conceived of the study, participate in its design and coordination, and revised the manuscript. All authors read and approved the final manuscript.

Acknowledgments

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