Polish Journal of Veterinary Sciences Vol. 23, No. 4 (2020), 481–489

DOI 10.24425/pjvs.2020.134696

Original article

Triplex qRT-PCR with specific probe for synchronously detecting Bovine parvovirus, bovine coronavirus, bovine parainfluenza virus and its applications

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Abstract

Bovine parvovirus (BPV), bovine coronavirus (BCoV) and bovine parainfluenza virus (BPIV) are common etiologies causing gastrointestinal and respiratory diseases in dairy herds. However, there are few reports on the synchronous detection of BPV, BCoV and BPIV. The present article aimed to develop a quick and accurate RT-PCR assay to synchronously detect BPV, BCoV and BPIV based on their specific probes. One pair universal primers, one pair specific primers and one specific probe was designed and synthesized. After the concentrations of primer and probe and annealing temperature were strictly optimized, the specificity, sensitivity and repeatability of the established triplex probe qRT-PCR were evaluated, respectively. The results showed the recombinant plasmids of pMD18-T-BPV, pMD18-T-BCoV and pMD18-T-BPIV were 554bp, 699bp and 704bp, respectively. The optimal annealing temperature was set at 45.0°C for triplex qRT-PCR. The triplex probe qRT-PCR can only synchronously detect BPV, BCoV and BPIV. Detection sensitivities were 2.0×10^2, 2.0×10^2 and 2.0×10^1 copies/μL for BPV, BCoV and BPIV, being 1000-fold greater than that in the conventional PCR. Detection of clinical samples demonstrated that triplex probe qRT-PCR had a higher sensitivity and specificity. The intra-assay and inter-assay coefficient of variation were lower than 2.0%. Clinical specimens verified that the triplex qRT-PCR had a higher sensitivity and specificity than universal PCR. In conclusion, this triplex probe qRT-PCR could detect only BPV, BCoV and BPIV. Minimum detection limits were 2.0×10^2 copies/μL for BPV and BCoV, and 2.0×10^1 copies/μL for BPIV. The sensitivity of this triplex probe qRT-PCR was 1000-fold greater than that in the conventional PCR. The newly qRT-PCR could be used to monitor or differentially diagnose virus infection.

Key words: bovine parvovirus, bovine coronavirus, bovine parainfluenza virus, qRT-PCR, specificity, sensitivity

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Introduction

Diarrhea can cause losses into the production by decreased weight gain (Schoeman and Fielding 2019), reduce milk yield (Hall 2018, Hamilton and Cifu 2019). Bovine parvovirus (BPV), bovine coronavirus (BCoV) and bovine parainfluenza virus (BPIV) are commonly etiologies causing gastrointestinal and respiratory diseases in dairy herds. Diarrhea can negatively impact the quality of life and adherence to medication therapy, resulting in significant economic losses (Kailasan et al. 2015, Ribeiro et al. 2016, Gomez et al. 2017). Earlier reports indicated the existence of persistent infection of BCoV in cattle (Nemoto et al. 2017). The bovine parvovirus (BPV) is a member of the Bocaparvovirus genus with a non-enveloped capsid (Nemoto et al. 2017). The occurrence rate of diarrhea caused by bovine parvovirus (BPV) in cattle herds is from 83% to 100% over the world (de Souza et al. 2018, Chang et al. 2019).

Bovine respiratory disease complex (BRDC) results in higher levels of mortality and morbidity. Parainfluenza virus type 3 (PIV3) is a crucial BRDC pathogen in humans and animals. Bovine PIV3 (BPIV3) commonly causes the serious infection in cattle and sheep. It is estimated that BRDC accounted for 40-50% of mortality in all dairy farms. Parainfluenza virus type 3 (PIV3) is a crucial virus of BRDC (Headley et al. 2018). Early accurate detection and differential diagnosis is a key to effectively prevent and treat these diseases.

Currently, several methods have been utilized to detect BPV, BCoV and BPIV (Ljubisa et al. 2016, Gomez et al. 2017). However, each method has its own strengths and weaknesses in terms of sensitivity and turnaround time (Kuta et al. 2015). Currently, there are few documents on rapid and synchronous molecular detection of BPV, BCoV and BPIV (Gunn et al. 2015, Gomez et al. 2017). It is very urgent to early diagnose and control these diseases (Keha et al. 2019) to circumvent these weaknesses and to develop strategies that reduce reaction time (Thanthrige-Dona et al. 2018, de Los et al. 2019). The present study aimed to develop a quick and accurate RT-PCR assay to synchronously detect BPV, BCoV and BPIV in the dairy farms.

Materials and Methods

RNA or DNA extraction

The SWMU (Southwest Minzu University) strains of BCoV, Haden strain of BPV and Bovine parainfluenza virus 3 (BPIV3), which were harvested from the America type culture collection (ATCC) or gifted by other researchers, were utilized in this study. Viruses were propagated at 37°C and 5% CO_2 in Madin-Darby bovine kidney (MDBK) cells using Dulbecco's modified eagle medium (Tiangen Biochemical Technology Co. Ltd, Beijing, China) and 8% newborn bovine serum (TaKaRa, Dalian, China) for 5-7 days (JinJing Geng et al. 2019, Zhuandi GONG et al. 2020). Viral RNA (BCoV and BPIV) was extracted from the supernatant using the TIANamp virus RNA kit (Tiangen Biochemical Technology Co. Ltd, Beijing, China). Viral DNA (BPV) was also extracted using the QIAamp MinElute virus spin kit (Transgen Biotech Company, Beijing, China) following the manufacturer's instructions. DNA or cDNA was quantified using SYBR Green, and then stored at -80°C for the subsequent tests.

Primers and probe design

The full-length genomes of BPV (Gene Accession No: JN191349), BCoV (Accession: MK095166, KT318096.1) and BPIV3 (Accession: NC-002161) were obtained from the GenBank database. One pair universal primers, one pair specific primers and one specific probe were synthesized for every virus (Table 1). The specificity of primers and probes was validated with Primer-BLAST. Each primer-probe set was designed to detect a single target pathogen. The primers and probes were synthesized by the Takara Bio INC (Beijing, China).

Construction of recombinant standard plasmids of pMD18-T-BPV, pMD18-T-BCoV and pMD18-T-BPIV

Viral genomic DNA was also extracted from BPV. The extracted RNAs were reversely transcribed into cDNAs. Viral full length DNA or RNA was amplified by PCR using the universal primer, and then stored at -80°C for the subsequent tests. DNA or PCR products were ligated into pMD18-T vector for 2h at 16°C, respectively (Takara Biomedical Technology Co. Ltd. Beijing, China). Then, BL-21 competent cells were transferred with the recombinant plasmids of DNA/PCR products and pMD18-T at 37°C overnight. Detail methods were referred to earlier studies on these viruses by our team (Geng et al. 2019, Liang et al. 2019, Gong et al. 2020).

The conserved region of the VP2 gene of BPV, nucleocapsid phosphoprotein of BCoV and N gene of BPIV were cloned into pMD18-T-BPV, pMD18-T-BCoV and pMD18-T-BPIV, respectively, so as to establish the standard curve.

The recombinant plasmids were extracted using the EasyPure® Plasmid MiniPrep Kit (Transgen Biotech Company, Beijing, China) and sequenced (Shanghai Sangon Biotec, Shanghai, China). The concentrations of plasmids were determined using Ultramicro nucleic

Table 1. Primers and probes for triplex qRT-PCR.

Primers	Primer sequences(5′→3′)	NCBI Accession	Product length/bp
BPV-F	GCGAAAACACGACTTTGGCT	JN191349	554
BPV-R	GAGCCGTGTCACCAGTGTTA		
BPV-qF	CAAGCACATCCAATCAAC		198
BPV-qR	CCACAATGTTCTCGCTAA		
BPV-probe	CY5-CGTCCATCCGCCAGTTGAGTA-BHQ2		
BCoV-F	AGGCTATTCCGACTAGGTTTCCG	MK095166, KT318096.1, FJ938064	699
BCoV-R	GTCCRTTCTTCTGRCCRCGMTGA		
BCoV-qF	TGCTCCTAATTCCAGATC		194
BCoV-qR	CAGTCTGCTTAGTTACTTG		
BCoV-probe	CY3-CGCATCCAGTAGAGCCTCTAGTG -BHQ2		
BPIV3-F	ACACCCATCGCATAACTCCAGA	NC-002161	704
BPIV3-R	GAGAAAGACCCAGGAAGACAGA		
BPIV3-qF	ATCCCTTATATAGTTTCCTACA		118
BPIV3-qR	GTCTCAACAGAGCTTAGTA		
BPIV3-probe	ROX-ATCGTTGTCAAGTCGTTCCTACCT-BHQ1		

acid protein analyzer (Thermo Fisher, USA) after the sequences were identical with those sequences documented in GenBank. Following amplification of the recombinant standard plasmids, the positive plasmids were sequenced again (Shanghai Sangon Biotec, Shanghai, China). The homogeneities of these sequences were compared with those in Genbank online of NCBI. DNA copies per μL were calculated based on the plasmid concentrations.

Setting of triplex qRT-PCR reaction systems

On the bases of single qRT-PCR establishment for each BPV, BCoV and BPIV by our team as described previously (Geng et al. 2019, Gong et al. 2020), the optimization of the triplex qRT-PCR reaction was set and finished for BPV, BCoV and BPIV.

For triplex qRT-PCR, the reaction systems were 40 cycles of 95°C for 30 s, 95°C for 5 s and 45°C for 30 s. The annealing temperatures selected were from 41.7°C to 52.0°C so as to determine the optimal temperature.

Establishment of the dynamics curve and standard curve

Mixed plasmids of pMD18-T-BPV, pMD18-T-BCoV and pMD18-T-BPIV were diluted ten-fold into 2.0×10^8 copies/μL-2.0×10^2 copies/μL. They were amplified with triplex qRT-PCR based on the optimized reaction systems described above. The annealing temperature of qRT-PCR was set at 45.0 °C so as to establish the dynamics curve and standard curve, respectively.

Specificity tests of triplex probe qRT-PCR

The specificity of the triplex qRT-PCR was assessed by testing viral DNA of BPV or cDNA of BCoV and BPIV. The cDNAs of other viruses were used as the control, including Japanese encephalitis virus (JEV), Classical Swine Fever Virus (CSFV), Rabies virus (RABV), Bovine Rotavirus (BRV), Bovine Viral Diarrhea Virus (BVDV) and bovine foot and mouth disease virus (FMDV). All these viruses were provided or gifted by the State key laboratory of biological engineering and technology of the Northwest Minzu University and Lanzhou veterinary research institute of the Chinese Academy of Agricultural Sciences (Lanzhou, China).

Sensitivity verifications of triplex probe qRT-PCR

The sensitivity of triplex probe qRT-PCR was verified using 10-fold dilution of the mixed standard plasmids of pMD18-T-BPV, pMD18-T-BCoV, pMD18-T-BPIV (2.0×10^8-2.0×10^2 copies/μL). The standard curves were utilized to set for respective absolute quantifications (Decaro et al. 2005). The results were compared with those of universal PCR so as to determine the minimum detection limit. The tests were performed in triplicate.

Repeatability tests

In order to verify the repeatability of this triplex probes qRT-PCR assay, both intra-assay and inter-assay were evaluated by testing the same plasmids at least three times in one experiment.

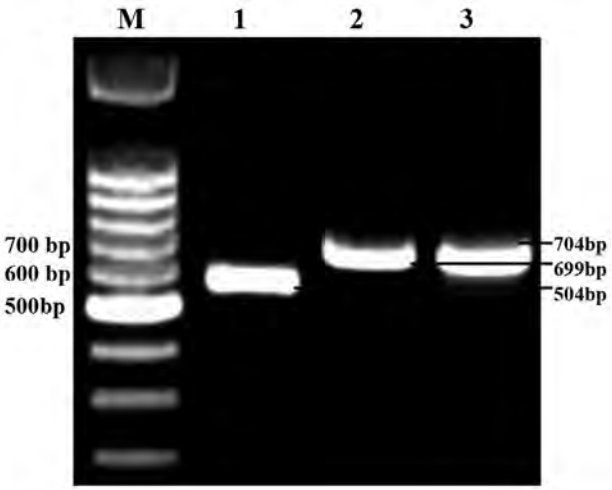

Fig. 1. Amplification of the recombinant standard plasmids.

Table 2. Optimization of the reaction conditions for qRT-PCR.

Virus	BPV PCR	BCoV PCR	BPIV PCR
Forward primer (μM)	14.5	14.5	19.5
Reverse primer (μM)	14.5	14.5	19.5
Probe contents (μM)	18	19.5	17.5
Annealing T (°C)	45.0	45.0	45.0
Reporting group	CY5	CY3	ROX
Quencher	BHQ2	BHQ2	BHQ2

Verification of clinical specimens

We have collected 312 feces samples from diarrheal calves in six different cattle farms during days 3-5 following diarrhea in Gansu Province of China. Two hundred and sixty-seven nasal swab samples were also collected from cattle farms in China. The suspension was prepared by diluting the feces and nasal swabs samples referring to the operation instruction described above. Then, viral DNA of BPV was extracted from the suspension of feces using QIAamp MinElute virus spin kit (Transgen Biotech Company, Beijing, China) following the manufacturer's instruction. Total RNA of BCoV and BPIV was also extracted from the suspension of feces and nasal swab samples using the TIANamp virus RNA kit (Tiangen Biochemical Technology Co. Ltd, Beijing, China) described above, respectively. They were detected with the triplex qRT-PCR and common PCR respectively, to test the practicability of this triplex qRT-PCR assay.

Results

The standard recombinant plasmids

The recombinant plasmids of pMD18-T-BPV, pMD18-T-BCoV and pMD18-T-BPIV were amplified with PCR after they were fully mixed and diluted into the final concentration of 2.0×10^{8}~2.0×10^{2} plasmids for each plasmid (Fig. 1). The sizes of pMD18-T-BPV, pMD18-T-BCoV and pMD18-T-BPIV were 554bp, 699bp and 704bp, respectively, which were the same as the single PCR. The results indicated we had successfully constructed the recombinant standard plasmids of pMD18-T-BPV, pMD18-T-BCoV, pMD18-T-BPIV.

The pMD18-T-BPV, pMD18-T-BCoV and pMD18-T-BPIV were amplified with PCR after three recombinant standard plasmids were fully mixed and diluted into the final concentration of 2.0×10^{8}~2.0×10^{2} plasmids for each plasmid.

M: DNA marker; 1: pMD18-T-BPV (554bp); 2: pMD18-T-BCoV (699bp); 3: pMD18-T-BPIV (704bp)

Optimization of the reaction systems for PCR of BPV, BCoV and BPIV

The results showed the least Ct and largest relative fluorescence units (RFU) were found at 45.0°C for triplex of BPV, BCoV and BPIV (Table 2). Therefore, the optimized annealing temperature was set at 45.0°C for qRT-PCR.

The primer and probe compound concentrations of the triplex RT-PCR were prepared based on the optimization of primer and probe concentrations for single PCR.

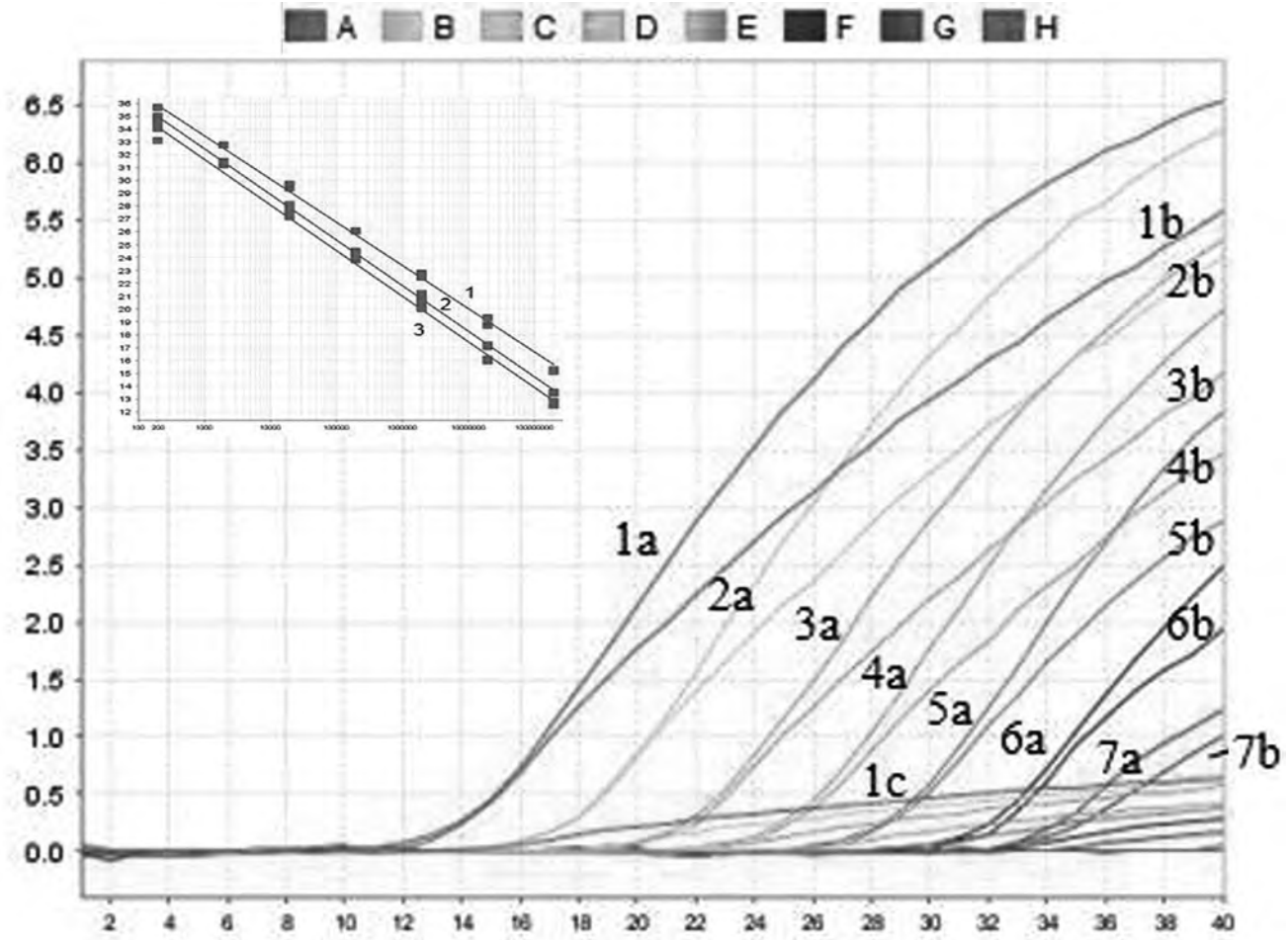

Fig. 2. Optimized dynamics curve and standard curve (small Fig.) of triplex probe qRT-PCT for BCoV, BPV and BPIV.

Dynamics curve and standard curve of triplex qRT-PCT

The mixed plasmids were diluted 10-fold into 2.0×10^8-2.0×10^2 concentration after three recombinant standard plasmids of pMD18-T-BPV, pMD18-T-BCoV, pMD18-T-BPIV were mixed equally. Both dynamic curves (Fig. 2 A) and standard curves (Fig. 2 B) were acquired with these plasmids on the bases of the optimized reaction systems for the recombinant standard plasmids. The results showed a good linearity for pMD18-T-BPV (R^2 = 0.9968), and pMD18-T-BCoV (R^2 = 0.9953) and pMD18-T-BPIV (R^2 =0.9992).

A: Curves 1-7 represent 2.0×10^8~2.0×10^2copies/μL of the recombinant standard plasmids, respectively.

Letters a, b and c represent BPIV, BPV and BCoV, respectively.

B: Numbers 1, 2 and 3 represent the standard curve of BCoV, BPIV and BPV, respectively.

$R^2_{(BPV)}$=0.9968, $R^2_{(BCoV)}$=0.9953, $R^2_{(BPIV)}$=0.9992, with the amplification efficiency of 90% -110%.

PCR amplifications of BPV, BCoV and BPIV

BPV, BCoV and BPIV were amplified with the established qRT-PCR, respectively. The products were 198, 194 and 118 bp for BPV, BCoV and BPIV, respectively (Fig. 3), each of which was the same size as the amplification of the single qRT-PCR.

The PCR products were 198, 194 and 118 bp for BPV, BCoV and BPIV, respectively.

M: DNA marker; 1: BPV; 2: BCoV; 3: BPIV

Specificity of the triplex probe qRT-PCR assay

This triplex qRT-PCR method can synchronously detect only BPV, BCoV and BPIV (Fig. 4). Non cDNA of other viruses including JEV, CSFV, RABV, BRV and FMDV displayed signal bands. The findings demonstrated that the established triplex qRT-PCR assay had a strong specificity for BPV, BCoV and BPIV.

Curves 1-3 represent BPIV, BPV and BCoV, respectively. Curve 4 represents other viruses including BVDV, BHV, BAV, PEDV, RABV and BRV, as well as a negative control.

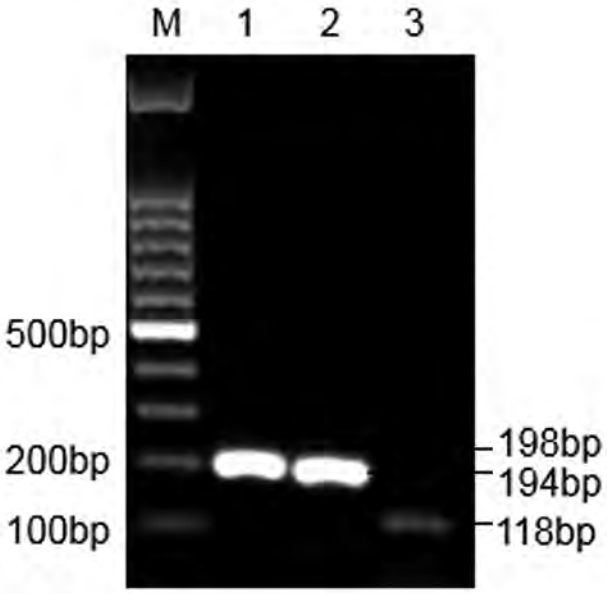

Fig. 3. PCR amplification of BPV, BCoV and BPIV.

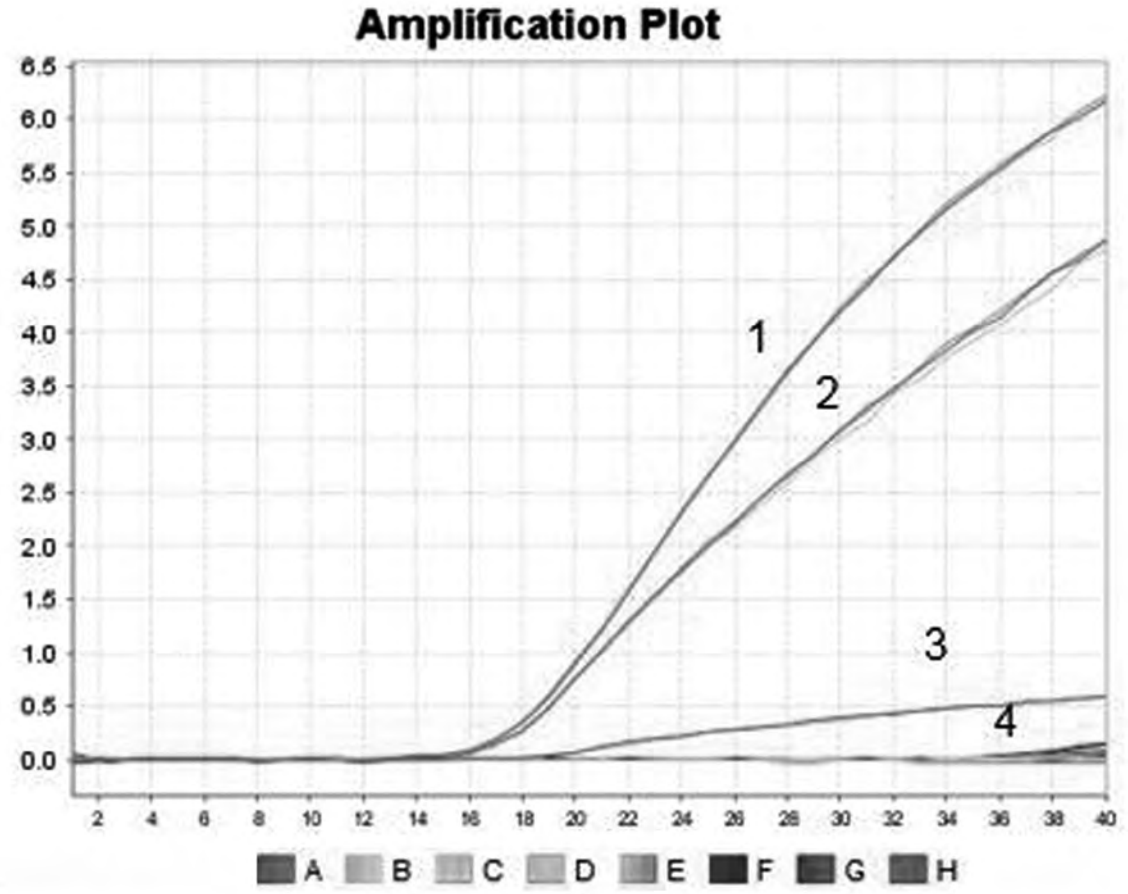

Fig. 4. Specificity of established triplex qRT-PCR.

Sensitivity of triplex probe qRT-PCR assay

The sensitivity assessment of three mixed plasmids showed that the least detection limits for BPV and BCoV were 2.0×10^2 copies/μL (or 200 copies/μL). Minimum detection limit for BPIV was 2.0×10^1 copies/μL (or 20 copies/μL) (Fig. 5). Detection limits of common PCR were 2.0×10^5 copies/μL, 2.0×10^5 copies/μL and 2.0×10^4 copies/μL for BPV, BCoV and BPIV, respectively. Analytical sensitivity of the developed triplex probe qRT-PCR was increased 1000-fold in comparison with the conventional PCR.

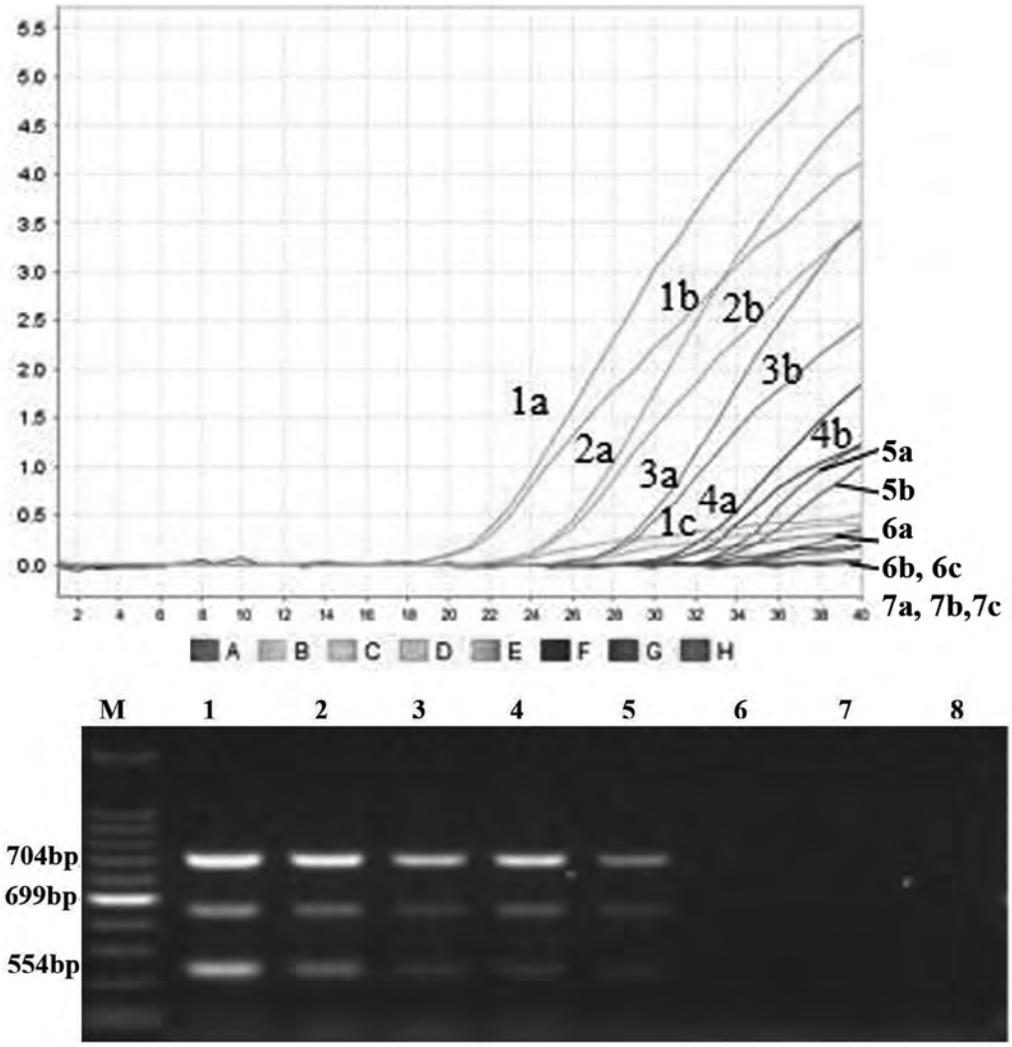

Fig. 5. Sensitivity of triplex probe qRT-PCR for BPV, BCoV and BPIV.

Table 3. Detection of 312 feces and 267 nasal swab specimens.

Virus	Triplex qRT-PCR	Single qRT-PCR	Universal PCR	Samples
BPV	36	36	32	feces
BCoV	43	43	59	feces
BPIV	99	99	84	nasal swab

Fig. A: Sensitivity of triplex probe qRT-PCR

Letters a, b and c represent BPIV, BPV and BCoV, respectively.

Numbers 1-7 represent 2.0×10^{8}-2.0×10^{2} copies/μL of the mixed standard plasmids, respectively.

The same color indicates the same concentration of the recombinant plasmids.

Fig. B: Sensitivity of universal PCR

M: DNA marke. Lanes 1-7: 2.0×10^{8}-2.0×10^{2} copies/μL of the mixed standard plasmids (704bp for pMD18-T-BPIV, 699bp for pMD18-T-BCoV, 554bp for pMD18-T-BPV), respectively.

Repeatability of triplex probe qRT-PCR assay

The results showed that intra-assay and inter-assay coefficient of variation (CVs) were lower than 2.0%, which indicated the developed triplex qRT-PCR for BPV, BCoV and BPIV had excellent repeatability.

Diagnostic validations of qRT-PCR for clinical specimens

As listed in Table 3, a total of 36 and 43 positive specimens of BPV and BCoV were detected from 312 feces samples of seven cattle farms using established triplex qRT-PCR assay. Ninety nine BPIV positive specimens were detected in 267 nasal swab samples.

All data were higher than those of universal PCR. The outcomes demonstrated that the sensitivity and specificity of the established triplex probe qRT-PCR were higher than those of universal PCR.

Discussion

BCoV plays a major role in bovine diarrhea symptoms and persistent infection in cattle (Mohamed et al. 2017, Schoeman and Fielding 2019). The occurrence rate of bovine parvovirus (BPV) infection is about 83% to 100% over the world (de Souza et al. 2018).

BRDC affects the health of dairy farms (Thanthrige-Don et al. 2018). BPIV-3 is one of the most important viral respiratory pathogens of cattle associated with BRDC (Sobhy et al. 2017, Comakli and Ozdemir 2019). Currently, few effective measures are offered to the treatment or prevention of BPV infections (Mohamed et al. 2018). Early accurate detection and differential diagnosis of calf diarrhea is key to effectively prevent and treat disease (Sharma et al. 2018, Miłek et al. 2019).

Multiplex RT-PCR assay can detect several target genes in a single reaction, which is applicable for simultaneous testing of the most important viral diseases in samples obtained from ruminants (Thanthrige-Dona et al. 2018). In this study, the authors put forth an effort to develop a novel triplex real-time PCR (qRT-PCR) for synchronously and quickly detecting BPV, BCoV and BPIV based on the specific probe of each virus.

The specificity of this triplex probe qRT-PCR was confirmed by the negative control and testing of other six viruses, including JEV, RABV, CSFV, BRV, BVDV and FMDV. The findings demonstrated that triplex probe qRT-PCR could detect only BPV, BCoV and BPIV. This verified that the qRT-PCR had an excellent specificity.

The sensitivity tests established the minimum detection limits of the triplex probe qRT-PCR of 2.0×10^2 copies/μL (or 200 copies/μL) for BPV and BCoV, and 2.0×10^1 copies/μL (or 20 copies/μL) for BPIV. The sensitivity was 1000-fold greater than that of conventional PCR (Mohamed et al. 2017). These outcomes are in agreement with previous studies (Alfieri et al. 2016, Gomez et al. 2017).

The newly developed multiplex qRT-PCR allows synchronously the specific and sensitive detection of three important diseases of digestive and respiratory systems in cows and could be used in the context of monitoring programs or for differential diagnostics of diarrhea syndrome in dairy farms (Keha et al. 2019).

Special emphasis was placed on the suitability of the tests for the rapid and reliable detection of viral infections in the field. Therefore, the diagnostic sensitivity and specificity were determined using 312 clinical samples. The outcomes demonstrated that the sensitivity and specificity of established triplex probes qRT-PCR were higher than those of universal PCR. These findings were in agreement with previous reports (Geng et al. 2019, Gong et al. 2020). The established triplex probes qRT-PCR could be applied to early detection and diagnoses of BPV, BCoV and BPIV infections in dairy farms.

Conclusions

In the present study, a specific triplex probe qRT-PCR has been established for synchronously detecting BPV, BCoV and BPIV. The minimum detection levels of this probe qRT-PCR were 2.0×10^2 copies/μL for BPV and BCoV, and 2.0×10^1 copies/μL for BPIV. The sensitivity was 1000-fold greater than that of the conventional PCR. This accurate detection assay is beneficial for enhancing the accuracy and reliability of determining BPV, BCoV and BPIV infections in cattle farms, which will reduce the cost and increase the economic benefits.

Ethical approval

All procedures referring to animal treatment were approved by the Experiment Animal Care and Use Committee of Gansu province, the People's Republic of China.

Acknowledgements

The work was supported by the Innovative Team Development Project of the Ministry of Education of China (IRT-17R88), the innovative research team of Yak Reproductive Regulation Mechanism and Plateau Adaptability (31920190024), the Science and technology support program project of Gansu province of China (1604NKCA044), and the innovation team for animal diseases in the plateau areas of Northwest Minzu University (2020-88-07-3).

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