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Establishment and Preliminary Application of Multiplex Fluorescent Quantitative PCR for Simultaneous Detection of BVDV, BRV and BCV

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ABSTRACT

Background: In this study, we aimed to establish a multiplex fluorescence quantitative polymerase chain reaction (PCR) method for the identification and detection of bovine viral diarrhea virus (BVDV), bovine rotavirus (BRV) and bovine coronavirus (BCV).

Methods: Based on the highly conserved sequences of BVDV E2 gene, BRV VP6 gene and BCV N gene in GenBank, specific primers were designed to amplify the target gene fragments of each virus and the reaction conditions and system were optimized. Multiple fluorescence quantitative methods were established by fluorescence quantitative PCR.

Result: The minimum detection limits of plasmid standards for BVDV, BRV and BCV by multiplex fluorescence quantitative PCR were 1.19×10^2 copies/μL, 3.89×10^1 copies/μL and 3.74×10^1 copies/μL, respectively. The lowest sensitivity of the established method was 100 times higher than that of conventional PCR and had high sensitivity. Furthermore, BVDV, BRV and BCV were amplified specifically, with no cross-reactivity with *Escherichia coli* (*E. coli*), *Salmonella* and infectious bovine rhinotracheitis virus (IBRV). The intra-and inter-group coefficients of variation were less than 1%, showing good assay repeatability. Using the established method and ordinary multiplex PCR to simultaneously detect 150 clinical diarrheal disease material samples, the coincidence rate of samples with mixed infection of the three viruses was 83.3%. The results showed that the multiplex fluorescent quantitative PCR detection method established in this study provides a rapid, sensitive and specific technique for clinical diagnosis and epidemiological monitoring of BVDV, BRV and BCV.

Key words: Bovine viral diarrhea virus (BVDV), Bovine rota virus (BRV), Bovine coronavirus (BCV), Multiplex fluorescent quantitative PCR.

INTRODUCTION

Calf diarrhea (CD) is one of the most common symptoms of digestive tract disease in calves. If newborn calves are diagnosed and treated as soon as possible, this condition often leads to a large number of deaths. CD has been reported to be an important factor responsible for calf deaths before weaning in the United States, South Korea (Hur *et al.*, 2013) and Iran (Azizzadeh *et al.*, 2013). In recent years, with the expansion of the scale of dairy farming in China, the incidence of CD has been increasing year by year, with serious impacts on the early growth and development of late calves as well as the stability of late production performance. The mortality rate has been reported to reach 90%, which seriously affects the expansion of dairy farming (Gomez *et al.*, 2017; Ryu *et al.*, 2020).

At present, BVDV, BRV and BCV are detected mainly using pathogenic, immunologic and molecular biological detection techniques (Zhuang *et al.*, 2018). Real-time fluorescent quantitative polymerase chain reaction (FQ-PCR) is widely used as a detection method in the laboratory due to its advantages of operational simplicity and efficiency, high sensitivity and good repeatability (Letellier and Kerkhofs, 2003; Baxi *et al.*, 2006). There are two kinds of real-time FQ-PCR: the fluorescent dye method and the TaqMan probe method. The fluorescent dye method is the most commonly used (Zhang *et al.*, 2011) and allows the rapid detection of multiple genes or nucleic acid sequences within the same reaction tube. FQ-PCR overcomes the shortcomings of conventional PCR in terms of sensitivity, efficiency and pathogen content determination (Zhang *et al.*, 2014). The FQ-PCR methods that are currently available for the diagnosis of BVDV, BRV and BCV detect only single or double infections and cannot detect three viruses simultaneously, which limits the popularity and application of this technique in the clinic.

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MATERIALS AND METHODS

Viral and bacterial strains

BVDV, BRV, BCV, *E. coli*, *Salmonella* and IBRV were all preserved in the Parasite Laboratory of the College of Veterinary Medicine, Hebei Agricultural University (Baoding, Hebei, China). BVDV and IBRV were expanded in MDBK cells, BRV was expanded in MA-104 cells and BRV was expanded in HCT-8 cells. BVDV, BRV and BCV were used as standard viruses for the establishment of the multiplex PCR. The preserved *E.coli* and *Salmonella* strains were inoculated into Luria–Bertani (LB) liquid medium and cultured in a shaking incubator (220 r / min) at 37°C for 12-16 h.

Collection of clinical samples

In June 2020, 150 samples of fresh diarrheal feces were collected from 0-30-day-old calf. Each fresh stool sample was placed into a sterile centrifuge tube containing 5 mL of PBS (pH 7.2), shaken for 1 min and centrifuged at 5,000 r/min for 10 min. The supernatant was collected and stored at -20°C for later use.

Primer design and synthesis

Based on sequences of the BVDV (MK170077), BRV (MNo47454) and BCV (MK903505) reference strains published in GenBank, three pairs of primers were designed for specific amplification of the BVDV E2, BRV VP6 and BCV (N) genes using DNAStar (DNAStar, Madison, WI, USA) and Primer 5.0 (Premier Biosoft International, Palo Alto, CA, USA) software. The primers were synthesized by Changchun Kume Bioengineering Co. (China) and the sequences are shown in Table 1.

Extraction of viral RNA and bacterial DNA

Viral RNA was extracted using a genomic RNA Extraction Kit (TaKaRaBio, Dalian, China) (magnetic bead method) according to the manufacturer's instructions with an automatic nucleic acid extraction instrument. The extracted RNA was reverse-transcribed using a reverse transcription kit (TaKaRaBio, Dalian, China) and the cDNA product was stored at -20°C for later use.

Preparation of recombinant plasmid standards

The cDNA of BVDV, BRV and BCV were amplified by PCR using the designed specific primers. The primer concentration was diluted to 10 pmol/μL. 20-μL reaction system: 10 μL 2× ExTaq Master Mix, 7 μL dd H_2O, 0.5 μL upstream and 0.5 μL downstream primers and 2 μL DNA template. The reaction conditions were as follows: 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, with a final extension at 72°C for 7 min. Sterile deionized water was used as the negative control. The amplified PCR products were purified and ligated into the pUC57 vector for the transformation of DH5α competent *E. coli* cells. The cells were then plated on LB solid medium containing ampicillin and screened for positive clones, which were cultured and the recombinant plasmids were amplified by PCR. The PCR products were digested using restriction enzymes. The correct positive recombinant plasmid was identified by sequencing and its concentration was determined using a NanoDrop 2000 (Thermo, USA), The number of copies was calculated as follows:

Copy number = Plasmid concentration × 6.02×10^{23}/(660 × total length of plasmid) as a standard.

Table 1: PCR primer sequences.

Pathogen		Primer sequence (5′-3′)	Primer size (bp)
BVDV	F	GGTCATAGCTCTCGACACCA	280
	R	GAGCACGTATCTACCACCCA	
BRV	F	AGACAAAGAACGGGTTTCACA	151
	R	AGTCAAATCCAGCGACCTGA	
BCV	F	GCGTCCTCTGGAAATCGTTC	111
	R	AGCAGTTTGCTTGGGTTGAG	

Single FQ-PCR

Ten-fold serial dilutions of the three virus plasmid standards were prepared in triplicate at 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10^1 and 10^0 copies/μL for use as templates in single FQ-PCR. The amplification results were analyzed to generate the standard curves. The melting curves were analyzed to eliminate the interference of primer-dimers and nonspecific amplification. The 15-μL reaction system was as follows: 7.5 μL PerfectStart™ Green SuperMix, 0.5 μL upstream and 0.5 μL downstream primers, 2.0 μL template and 4.5 μL ddH_2O. The reaction conditions were as follows: 95°C for 5 min, followed by 45 cycles of 95°C for 20 s, 56°C for 20 s and 72°C for 20 s; sterile water was used as the negative control.

Optimization of reaction conditions for multiplex FQ-PCR

The multiple FQ-PCR method was optimized using a gradient of diluted standards (10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10^1 and 10^0 copies/μL) as templates, with various annealing temperature (52, 53, 54, 55, 56, 57 and 58°C) and the primer concentration was diluted to 10 pmol/μL,final primer concentrations (0.2, 0.3, 0.4, 0.5, 0.6, 0.7 and 0.8 μL). The reaction conditions resulting in a Ct value ≤45 cycles were defined as optimal for this FQ-PCR method.

Sensitivity test

Ten-fold serial dilutions of the three virus plasmid standards were prepared in triplicate at 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10^1 and 10^0 copies/μL for use as templates. The optimized reaction conditions were used for multiplex FQ-PCR detection to determine the sensitivity. Ordinary multiplex PCR detection was conducted in parallel for comparison of the results of the two methods.

Specificity test

The extracted genomic DNA of *E.coli, Salmonella* and IBRV was used as a template; a pool of BVDV, BRV and BCV genomic DNA was used as positive control and sterile water

was used as a negative control. The multiplex fluorescent quantitative PCR detection method was employed in this study using the optimized conditions. The specificity of the method was detected by fluorescent multiplex FQ-PCR.

Repeatability test

Ten-fold serial dilutions of the three virus plasmid standards were prepared at 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10^1 and 10^0 copies/μL and mixed in equal proportions. Plasmid mixtures at final concentrations of 10^6, 10^5 and 10^4 copies/μL were tested; sterile water was used as the negative control. The established FQ-PCR method was repeated three times to evaluate intra-group repeatability. Inter-group repeatability was evaluated using the same three standard dilutions (10^6, 10^5 and 10^4 copies/μL) prepared on separate occasions and the test results were analyzed statistically.

Clinical sample testing

According to the comparison between the established multiplex fluorescent quantitative PCR method and the conventional multiplex PCR method, BVDV, BRV and BCV were detected simultaneously in 150 clinical samples. The positive rate, sensitivity, specificity and coincidence rate of the two methods were compared.

The calculation formula is:

$$\text{Sensitivity} = \frac{\text{The number of positive copies consistent with the two tests}}{\text{The number of positive copies detected by routine PCR}}$$

$$\text{Specificity} = \frac{\text{The number of negative copies consistent with the two tests}}{\text{The number of negative copies detected by routine PCR}}$$

$$\text{The coincidence rate} = \frac{\text{The sum of the negative and positive copies of the two tests}}{\text{The total number of the tests}}$$

RESULTS AND DISCUSSION

Preparation of recombinant plasmid standard

Specific primers were designed for amplification of BVDV, BRV and BCV target fragments (280 bp, 151 bp and 111 bp, respectively) using the extracted cDNA as a template. As shown in Fig 1, the amplified target fragments were consistent with the expected results. The fragments were cloned into pUC57 vector and sequencing showed that the fragments were identical to those in the GenBank database. The concentrations of the recombinant plasmids containing the BVDV, BRV and BCV fragments were 0.05 ng/μL, 1.67 ng/μL and 1.60 ng/μL, respectively, corresponding to recombinant plasmid copy numbers of 1.19×10^{10} copies/μL, 3.89×10^{10} copies/μL, 3.74×10^{10} copies/μL.

Establishment of standard curves

The BVDV recombinant plasmid standard was detected in the range of 10^7 to 10^2 copies/μL and the BRV and BCV recombinant plasmid standards were detected in the range of 10^7 to 10^1 copies/μL. Automatic analysis software was used to construct FQ-PCR standard curves by plotting the logarithm of the copy number as the ordinate and the cycle threshold (Ct) value as the abscissa. As shown in Fig 2 (a-c), the amplification efficiency exceeded 90% for the BVDV standards in the range of 10^7-10^2 copies/μL and the BRV and BCV standards in the range of 10^7-10^1 copies/μL and the correlation coefficient R^2 was greater than 0.99, indicating a good linear relationship between the starting copy number of the templates and the Ct value of the various standards.

The linear equation of the copy number (x) and Ct value (y) was obtained as follows:

BVDV, $y = -3.911x + 40.21$, $R^2 = 0.995$.

BRV, $y = -3.657x + 37.64$, $R^2 = 0.992$.

BCV, $y = -3.647x + 37.73$, $R^2 = 0.997$.

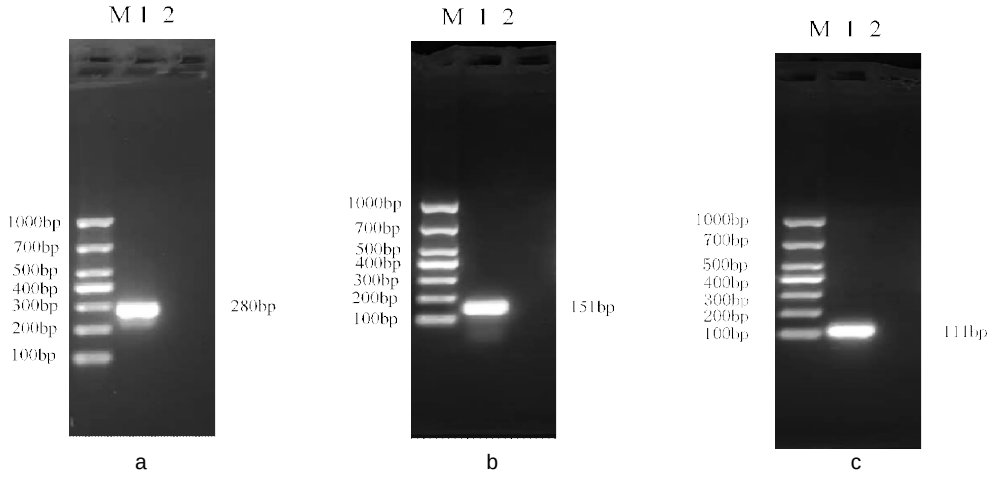


Fig 1: PCR amplification of recombinant plasmid

a: M. DL1000 Marker; 1. BVDV DNA; 2. Negative control.

b: M. DL1000 Marker; 1. BRV DNA; 2. Negative control.

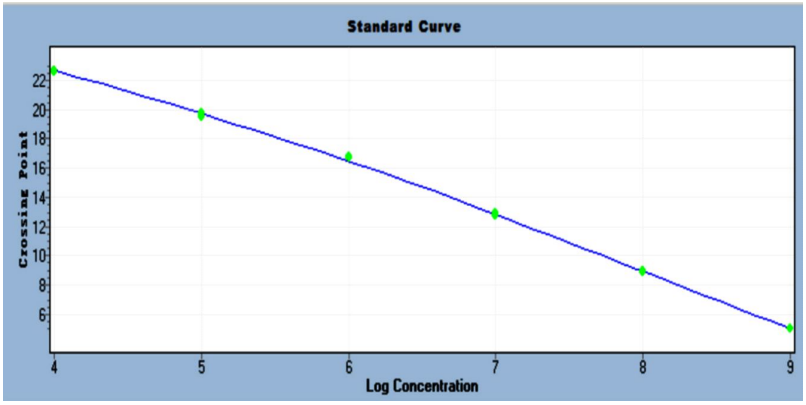
c: M. DL1000 Marker; 1. BCV DNA; 2. Negative control.

Determination of reaction conditions for multiplex FQ-PCR

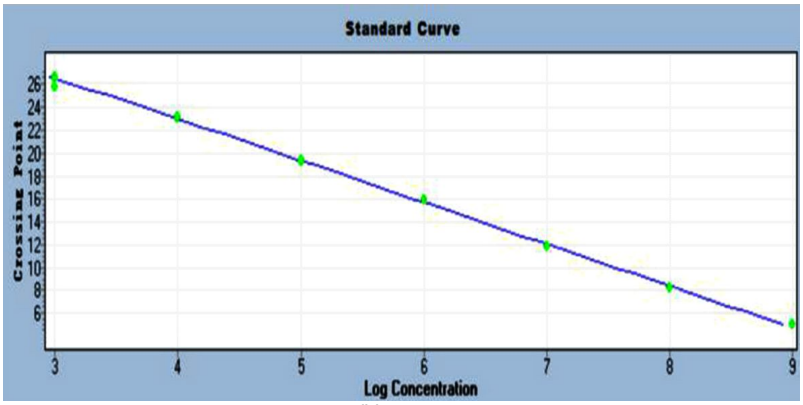
The conditions for FQ-PCR detection of BVDV, BRV and BCV were optimized. The optimal reaction system was identified as 25 µL containing 12.5 µL PerfectStart™ Green SuperMix, 0.5 µL upstream and 0.5 µL downstream primers of BVDV00.5 µL upstream and 0.5 µL downstream primers of BRV00.5 µL upstream and 0.5 µL downstream primers of BCV, 2.0 µL template of BVDV0BRV0BCV cDNA, respectively and 3.5 µL ddH_2O. The optimal reaction conditions were identified as 95°C for 5 min, followed by 45 cycles of 95°C for 20 s, 55°C for 20 s and 72°C for 20 s; sterile water was used as the negative control. In diagnostic real-time PCR assays, it was customary to regard values results between Ct 35 and 45 as equivocal, while those above Ct 45 were regarded as negative (Zhao *et al*., 2018).

Melting curve analysis

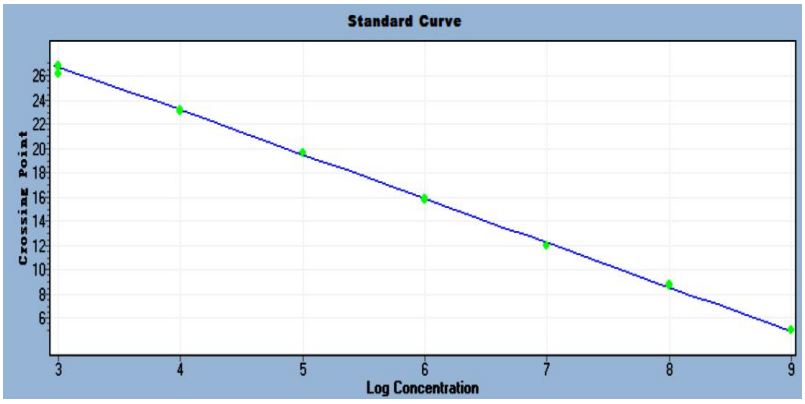
As shown in Fig 3 (a-c), a single peak was observed at each dilution for each of the melting curves for BVDV, BRV and BCV, which excluded primer-dimer interference. The melting temperatures for the BVDV E2, BRV VP6 and BCV N genes were approximately 85°C, 83°C and 84°C, respectively.



(a): BVDV

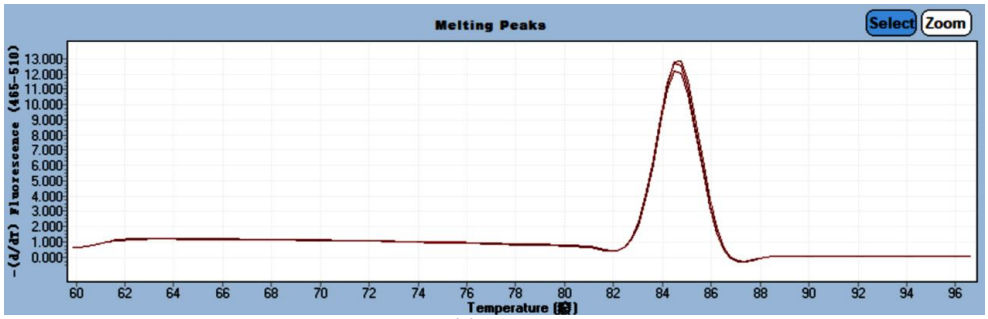


(b): BRV

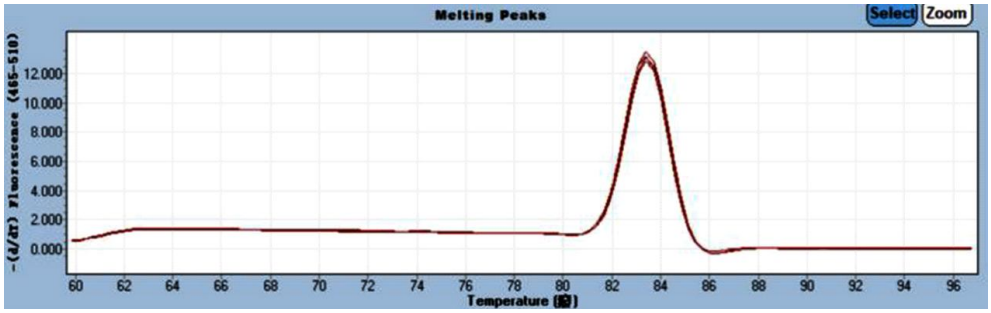


(c): BCV

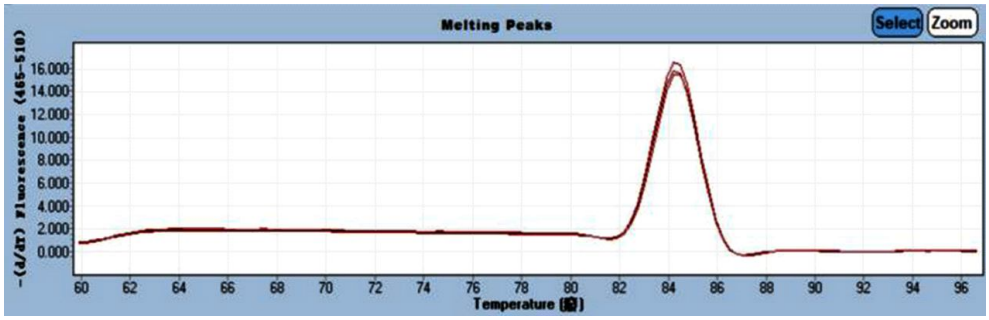
Fig 2: FQ-PCR standard curves.



(a): BVDV



(b): BRV



(c): BCV

Fig 3: Melting curves.

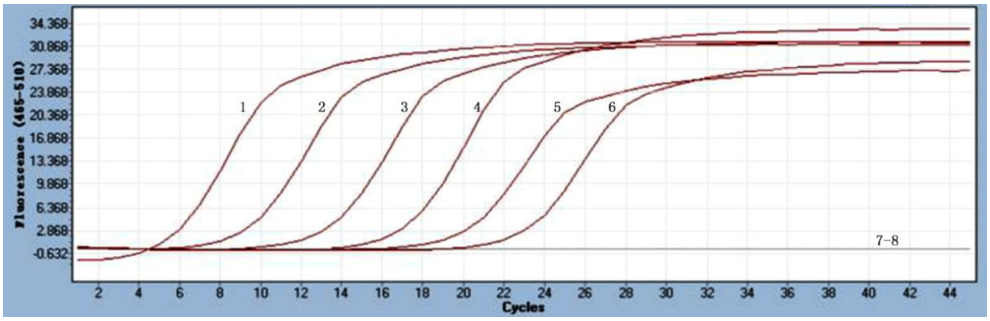
Cao (2018) reported that BRV amplified a single peak when the dissolution temperature was 77°C and BCV amplified a single peak when the dissolution temperature was 79°C, which was lower than that in this study. Jiang H H(2020) reported that BVDV amplified a single peak when the dissolution temperature was 84°C, It is similar to the results of this study.

Sensitivity analysis of multiplex FQ-PCR

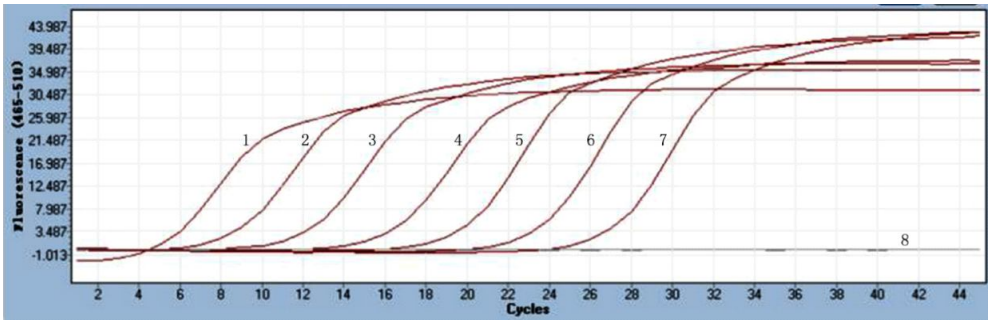
The sensitivity of the established multiplex FQ-PCR for the detection of BVDV, BRV and BCV was evaluated using 10-fold serial dilutions (10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10^1 and 10^0 copies/μL) of the recombinant plasmid standards analyzed under the established reaction conditions. For the multiplex FQ-PCR, the minimum detection limit for BVDV was 10^2 copies/μL and 10^1 copies/μL for both BRV and BCV (Fig 4a-c). For the conventional multiplex PCR, the detection limit for BVDV was 10^4 copies/μL and 10^3 copies/μL for BRV and BCV (Fig 5). Thus, the sensitivity of the established multiplex FQ-PCR method was much greater than that of the conventional multiplex PCR. It is similar to the real-time fluorescence quantitative detection method recommended by Cao (2018)and Ren (2019).

Specificity analysis of multiplex FQ-PCR

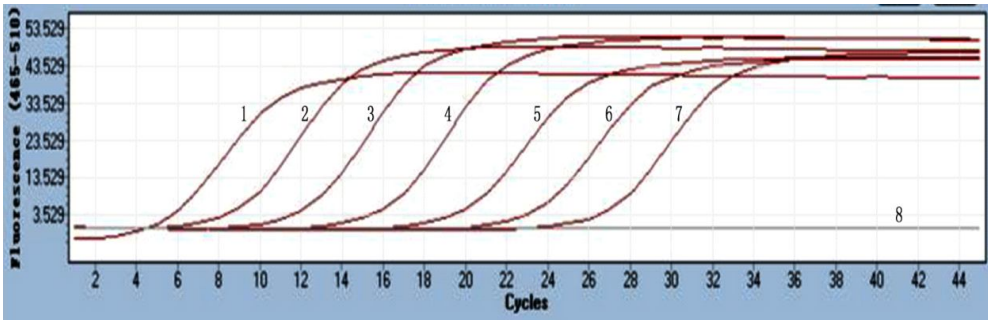
The specificity of the established FQ-PCR was evaluated using BVDV, BRV and BCV plasmid standards as positive controls and sterile water was used as the negative control. Genomic DNAs from *E. coli, Salmonella* and IBRV were used as templates and the FQ-PCR was performed under the established reaction conditions. As shown in Fig 6, the specific amplification curves of BVDV, BRV and BCV were detected respectively, while no fluorescence signal was detected in the negative control samples containing genomic DNA from *E. coli*, *Salmonella*, IBRV and sterile water as templates. These findings indicated that the established multiplex FQ-PCR method can be used to detect BVDV, BRV and BCV with high specificity.



(a): BVDV amplification curve



(b): BRV amplification curve



(c): BCV amplification curve

Fig 4: Sensitivity analysis of the established multiplex FQ-PCR.
1. 10^7 copies/μL; 2. 10^6 copies/μL; 3.10^5 copies/μL; 4.10^4 copies/μL; 5. 10^3 copies/μL; 6. 10^2 copies/μL; 7. 10^1 copies/μL; 8.10^0 copies/μL.

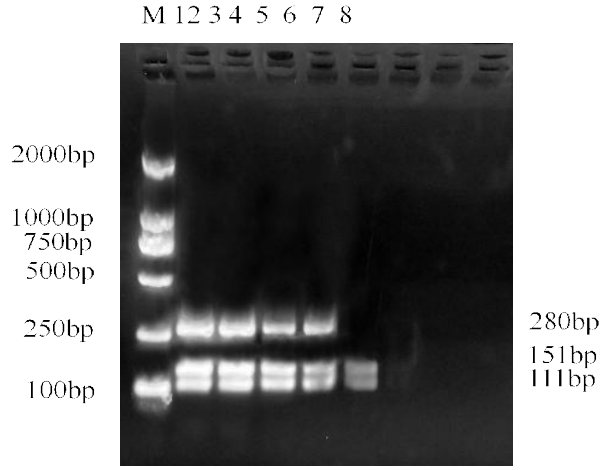


Fig 5: Conventional multiplex PCR sensitivity assay.
M. DL 2000 Marker; 1-8: 107 copies/μL-100 copies/μL.

Repeatability analysis of the multiplex FQ-PCR

Three different concentrations of the BVDV, BRV and BCV recombinant plasmid standards were selected as templates to test the repeatability of the established multiplex FQ-PCR. As shown in Table 2, the intra-and inter-group coefficients of variation were less than 1%, indicating that the multiple FQ-PCR established in this study can be used to detect BVDV, BRV and BCV with good repeatability.

Clinical sample testing

The established multiplex FQ-PCR and conventional multiplex PCR methods were compared for the analysis of 150 clinical fecal samples (Fig 7, Fig 8). As shown in Table 3, the mixed infection of the three viruses was positively detected in 12% (18/150) of samples by the multiplex FQ-PCR and 10.0% (15/150) of samples by the conventional multiplex PCR. The coincidence rate of the two methods was 83.3%. Comparison of the two detection methods revealed a sensitivity of 93.8%, a specificity of 81.8% and a total coincidence rate of 94.7%. Three additional mixed infection positive samples were detected by multiplex fluorescence quantitative PCR, indicating that the detection method established in this study is sensitive and can be applied to clinical detection. These results showed that the multiplex FQ-PCR method established in this study was more sensitive than the conventional multiplex PCR and is suitable for clinical detection.

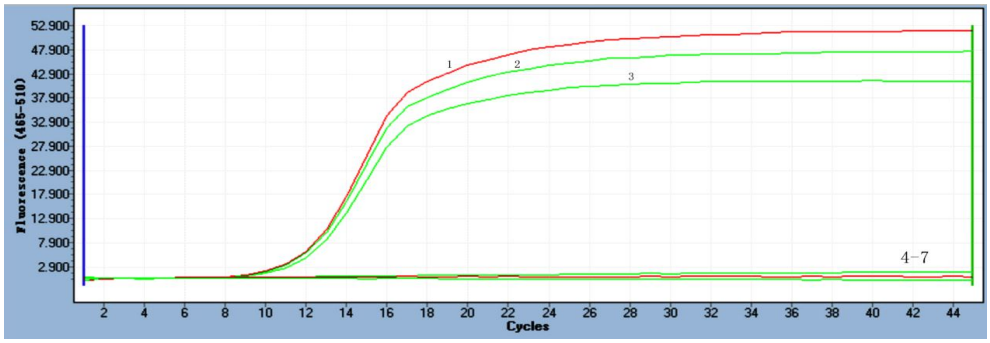


Fig 6: Specific detection using the multiplex fluorescent quantitative PCR method.
1. BVDV plasmid standard; 2.BRV plasmid standard; 3.BCV plasmid standard; 4-7. *E. coli* genomic DNA, *Salmonella* genomic DNA, IBRV genomic DNA and sterile water.

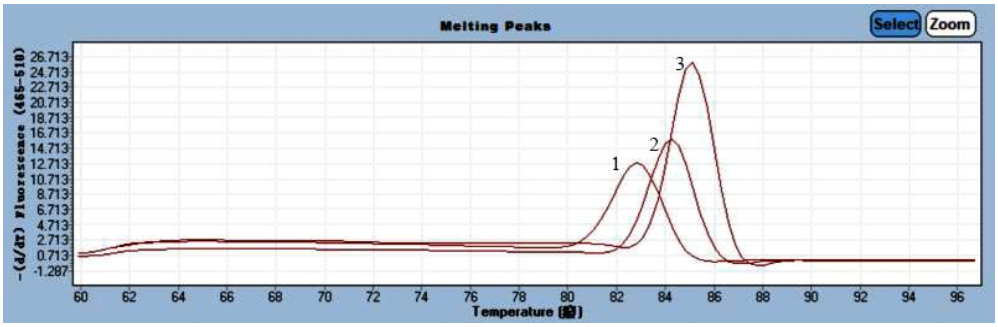


Fig 7: Melting curves of mixed infections in clinical diarrhea samples detected by multiplex FQ-PCR.
1. BRV; 2. BCV; 3. BVDV.

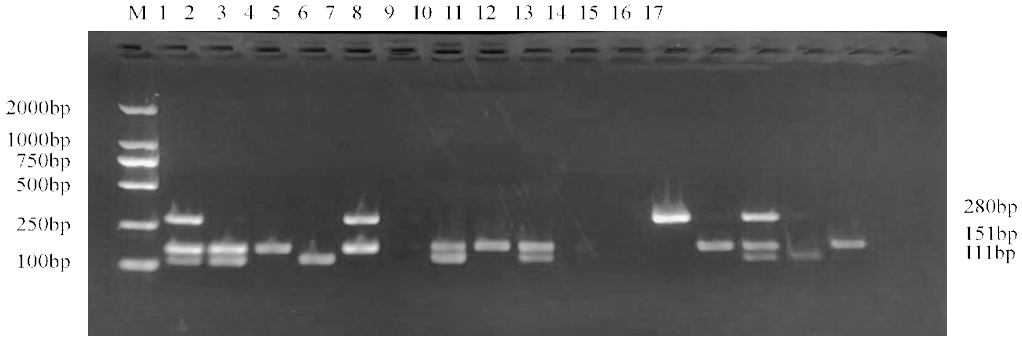


Fig 8: Analysis of clinical samples by conventional multiplex PCR.
M. DL 2000 marker; 1-17 Clinical sample test results.

Table 2: Repeatability results of multiplex fluorescent quantitative PCR.

Plasmid standard	Standard concentration (copies/μL)	n	Intra-group variation test		Inter-group variation test	
			Average AV	Coefficient of variation CV(%)	Average AV	Coefficient of variation CV(%)
BVDV	1.19×10^4	3	13.95±0.100	0.715	14.04±0.106	0.756
	1.19×10^5	3	9.48±0.062	0.650	9.44±0.062	0.653
	1.19×10^6	3	6.58±0.062	0.939	6.66±0.050	0.749
BRV	3.89×10^4	3	12.96±0.076	0.585	12.88±0.095	0.740
	3.89×10^5	3	8.71±0.063	0.728	8.51±0.069	0.816
	3.89×10^6	3	5.97±0.042	0.701	5.98±0.033	0.552
BCV	3.74×10^4	3	12.66±0.125	0.987	12.57±0.083	0.659
	3.74×10^5	3	8.60±0.049	0.573	8.74±0.057	0.654
	3.74×10^6	3	5.56±0.036	0.640	5.68±0.046	0.818

Table 3: Detection of clinical diarrhea samples.

Pathogen	Sample no.	Detection by multiplex FQ- PCR		Detection by conventional multiplex PCR	
		No. of positive samples	Positive rate	No. of positive samples	Positive rate
BVDV+BRV+BCV	150	18	12.0	15	10.0
BVDV+BRV	150	10	6.7	9	6.0
BRV+BCV	150	62	41.3	60	40.0
BVDV+BCV	150	9	6.0	7	4.7
BVDV	150	1	0.6	1	0.6
BRV	150	8	5.3	8	5.3
BCV	150	6	4.0	6	4.0
Total	150	114	75.9	106	70.6

CONCLUSION

In this study, this method was shown to amplify BVDV, BRV and BCV specifically, without cross-reaction with *E.coli*, *Salmonella* and IBRV, thus confirming the high level of specificity of the technique. The minimum detection limits of the BVDV, BRV and BCV plasmid standards by multiplex fluorescence quantitative PCR were 1.19×10^2 copies/μL, 3.89×10^1 copies/μL and 3.74×10^1 copies/μL, respectively and the sensitivity was 100 times higher than that of conventional PCR, with good assay stability. The intra- and inter-group coefficients of variation were both less than 1%, demonstrating the good repeatability of the assay. The coincidence rate of the two methods for the detection of samples with mixed infections was 83.3%. Thus, the method established in this study facilitates effective detection for early diagnosis and epidemiological investigation of CD caused by BVDV, BRV and BCV with high specificity and sensitivity.

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