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Establishment of Multiplex PCR for Detection of Calf Diarrhea Associated Virus and Analysis of its Clinical Infection Status

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ABSTRACT

Outbreaks of calf infectious diarrhea caused by bovine viral diarrhea virus (BVDV), bovine rotavirus (BRV), and bovine coronavirus (BCV) have increased calves morbidity and mortality in Hebei Province. To detect these three pathogens simultaneously, we designed specific primers based on the conserved gene sequences of the three pathogens available in GenBank. After optimization of the reaction conditions and system, we successfully established a novel multiplex PCR method for detection of the aforementioned three pathogens. The results show that the amplified fragments of interest were 280 bp, 151 bp, and 111 bp for BVDV, BRV, and BCV, respectively. The method had no cross-reaction to *Escherichia coli, Salmonella,* and infectious bovine rhinotracheitis virus. Moreover, it detected the minimum limit of 1.19 × 10^3 copies/μL for BVDV, 3.89 × 10^2 copies/μL for BRV, and 3.74 × 10^2 copies/μL for BCV, indicating its high specificity and sensitivity. The results of the clinical detection of 150 samples, collected form calves in Hebei Province, by multiplex PCR were the same as those obtained by colloidal gold test paper detection. We discovered that the co-infection rate of BRV and BCV was 41.3% (62/150), of BVDV and BRV 8.0% (12/150), of BVDV and BCV 6.0% (9/150), and of BVDV, BRV, and BCV 10.0% (15/150). In our clinical samples, mixed infection of BRV and BCV was the main pathogen causing calf diarrhea. The developed multiplex PCR assay is a fast, sensitive, and specific, novel detection method for disease diagnosis, clinical monitoring, and treatment of BVDV, BRV, and BCV infections.

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Key words
Bovine viral diarrhea virus (BVDV), Bovine rotavirus (BRV), Bovine coronavirus (BCV), Multiplex PCR

INTRODUCTION

Diarrhea is the most common symptom of digestive tract diseases in calves. A large number of deaths occur in cases of delayed diagnosis and treatment of newborn calves (Cho *et al*., 2013; Lyoo *et al*., 2018). In recent years, with the further expansion of dairy farming industry, the yearly incidence rate of calf diarrhea has been steadily increasing, affecting seriously the growth and development of calves and the stability of their late production performance. Its high mortality rate of 90% seriously negatively affects the expansion of cattle production and the effective development of dairy farming (Ammar *et al*., 2014). The causative agents of calf diarrhea are complex, among which mixed virus infection is the predominant and most serious. Bovine viral diarrhea virus, bovine rotavirus, and bovine coronavirus are the most common pathogens causing calf diarrhea (Gebregiorgis and Tessema, 2016; Mohamed *et al*., 2017). However, it has not been possible to determine whether the disease is caused by single or mixed pathogens based only on its clinical symptoms. Therefore, a more economic, rapid, and high-sensitivity method needs to be established for detection and identification of the specific pathogen among a range of diarrhea causative pathogens. Such a methodological approach would technically facilitate timely detection, prompt diagnosis, and the establishment of effective prevention and control measures against various diarrhea pathogens.

Currently, the detection of calf diarrhea pathogen is based mainly on traditional pathogen isolation and detection by conventional single PCR and ELISA (Zhao *et al*., 2015; Zhang *et al*., 2012). Importantly, Tsunemitsu *et al*. (1999) established a RT-PCR method for detection of bovine rotavirus based on the nucleocapsid protein (N)

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gene of bovine rotavirus and verified its sensitivity and. However, the practical applications of single PCR are limited due to its capacity for amplification of only one target fragment in a single reaction, which is cumbersome, time-consuming and reagent-consuming. Ammar *et al.* (2014) employed antigen-ELISA and detected rotavirus and viral diarrhea virus in calves with diarrhea in western Algeria. The infection rates of bovine rotavirus and bovine viral diarrhea virus determined by these researchers were 14.63% and 20.73%, respectively. ELISA is widely used by at home and abroad for virus detection in diarrhea samples, but commercial ELISA kits are usually expensive. In addition, due to the specificity of ELISA, its detection range is small (Brar *et al*., 2017; Gomez *et al*., 2017). Furthermore, ELISA detection requires the availability of high-purity antigen, whose preparation is relatively difficult, which limits the use of ELISA as the main method for detection of calf diarrhea pathogens in grassroots laboratories (Cho *et al*., 2010). On the other hand, multiplex PCR can simultaneously detect multiple pathogens in a reaction system, and distinguish them by to the size of the amplified target fragment, achieving high specificity in clinical diagnosis (Fukuda *et al*., 2012). Therefore, multiplex PCR has been widely used for simultaneous detection of multiple pathogens in a single sample or multiple serotypes in a pathogen (Cho *et al*., 2010; Fan *et al*., 2011). This method is simple, time-saving, and suitable for large-scale epidemiological investigations of calf diarrhea, providing high accuracy, specificity, and sensitivity of detection.

Briefly, in this study, we established a novel, fast and sensitive, multiplex PCR method to simultaneously and timely detect the pathogen of calf viral diarrhea and distinguish among bovine viral diarrhea virus, bovine rotavirus, and bovine coronavirus in clinical samples. Furthermore, based on our results obtained through this new method, we determined the status of diarrhea-related virus infection in Hebei Province, China.

MATERIALS AND METHODS

Bacterial strain

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).

Collection of clinical samples

In June 2020, 150 samples of fresh diarrhea feces from 0–30-day-old calves were collected from 10 cattle farms in Shijiazhuang, Tangshan, Qinhuangdao, and other regions. Then, the fresh stool sample was placed into a sterile centrifuge tube containing 5 mL of PBS with pH 7.2, followed by shaking for 1 min and centrifugation at 5,000 r/min for 10 min. Finally, the supernatant was stored in a refrigerator at -20°C for later use.

Main reagents

DL1000 DNA Marker and DL2000 DNA were purchased from Marker, Takara Bio Co., Ltd, Dalian, China. Viral genome RNA extraction kit (magnetic bead method) was purchased from Jiangsu Shuoshi Biotechnology Co., Ltd. (Taizhou, China); DH5α competent cells were purchased from Takara Bio Co., Ltd., Dalian, China. Super Gel Red S2001 was purchased from US Everbright Inc. Co., Ltd, (San Francisco, CA, USA). 2×EsTaqMasterMix was purchased from Beijing Com Win Biotech Co., Ltd. (Beijing, China). Plasmid extract kit was purchased from BIOMIGA Co., Ltd. San Diego, California. Bovine rotavirus, bovine coronavirus, giardia, cryptosporidium, and E. coli five-linked colloidal gold test paperboards were purchased from Genesis Co., Ltd., Korea. Bovine viral diarrhea virus colloidal gold test paperboards were bought from Genesis Co., Ltd. Korea.

Primer design and synthesis

Base on the BVDV (MK170077), BRV (MNo47454) and BCV (MK903505) reference strain sequences published in GenBank, three pairs of specific primers were designed using DNA Star (DNAStar Inc., Madison, WI, USA) and Primer 5.0 (Premier Biosoft International, Palo Alto, CA, USA) software to amplify the BVDV E2, BRV VP6, and BCV (N) genes. The sequences of the primers, synthesized by Changchun Kume Bioengineering Co., Ltd., are presented in Table I.

Table I. PCR primer sequences.

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

Extraction of viral RNA and bacterial DNA

Following the instructions of the virus genomic RNA Extraction Kit (by the magnetic-bead method), we extracted the virus nucleic acid by an automatic nucleic acid extraction instrument. The extracted nucleic acid products were reverse-transcribed through a reverse

transcription kit, and the obtained cDNA was stored at -20°C until further use.

Bacterial DNA was extracted according to the instructions of the bacterial genomic DNA extraction kit and stored at -20°C.

Single PCR amplification

Using the extracted cDNA of the three viruses as templates, we performed PCR amplification with the corresponding primers; sterile deionized water was set as the negative control. A volume of 20 μL of the reaction system contained the following reagents: 10 μL of 2 × EsTaq MasterMix (ComWin Biotech, Beijing, China), 6 μL of ddH_2O, 1 μL of upstream and downstream primers, and 2 μL of cDNA template. The following reaction protocol was applied: 94 °C for 5 min; 94 °C for 30 s; 54 °C for 30 s; 72 °C for 30 s; and 72 °C for 7 min; a total number of 35 cycles. The amplified products of 5 PCR L were detected by electrophoresis in 1.5% agarose gel, and the results were analyzed. The PCR reaction products were then recovered, cloned into pUC57 vectors, and transformed into DH5α competent cells. Next the cells were coated with plates, and screening for positive clone plasmids was performed, followed by culturing and extraction of recombinant plasmids for PCR product conservation. The obtained PCR products were sent to Changchun Kumei Bioengineering Co., Ltd. for sequencing and analysis.

Establishment and optimization of multiplex PCR conditions

The system of multiplex PCR establishment encompassed the following major steps. First, three pairs of primers and templates were mixed for the amplification of BVDV, BRV, and BCV genes. Then, the reaction conditions for upstream and downstream primer concentration and annealing temperature were optimized. The optimal annealing temperature was 53°C–58°C, the primer concentration was 0.6–1.4 μL; sterile deionized water was used as negative control. A certain condition of multiplex PCR was optimized by determination of its best value under unchanged other reaction parameters. The 5μL PCR product was detected by electrophoresis in 1.5% agarose gel, and the optimal temperature and primer concentration were screened and determined.

Multiplex PCR specificity test

Based on the optimized PCR reaction system conditions, DNA from BVDV, BRV, BCV, *E. coli, Salmonella*, and IBRV was used as a template for amplification of the primer to its optimal concentration at the best annealing temperature. Next, the obtained 5μL PCR product was detected by electrophoresis in 1.5% agarose gel, and the specificity of the multiple PCR reaction, established in this experiment, was tested.

Multiplex PCR sensitivity test

The correct positive recombinant plasmids were determined by NanoDrop 2000. The concentrations of BVDV, BRV, and BCV recombinant plasmids were 0.05 ng/μL, 1.67 ng/μL and 1.60 ng/μL, respectively. The copy numbers of recombinant plasmids were 1.19×10^{10} copies/μL, 3.89×10^{10} copies/μL and 3.74×10^{10} copies /μL, correspondingly.

Ten-fold dilution of the recombinant plasmids of the three viruses was used in eight gradients, which were utilized as templates, gradient 1×10^7 copies/μL, 1×10^6 copies/μL, 1×10^5 copies/μL, 1×10^4 copies/μL, 1×10^3 copies/μL, 1×10^2 copies/μL, 1×10^1 copies/μL, 1×10^0 copies/μL gradient as template. PCR amplification was performed using the optimized conditions and system. A volume of 5 μL of the obtained product was next detected by electrophoresis in 1.5% agarose gel, and the sensitivity of the multiplex PCR reaction, established in this experiment, was determined.

Multiplex PCR repeatability test

Using the optimized multiplex PCR reaction system and conditions, three recombinant plasmid mixtures with a final concentration of 1×10^5 copies/μL were selected as templates to examine the stability of multiplex PCR detection results.

Clinical sample testing

The cDNA of 150 clinical diarrhea stool samples was analyzed. We compared the coincidence rate of the commercial colloidal gold test board and multiplex PCR methods, and analyzed the detection results were through the established triple-PCR method and the commercial colloidal gold test board.

RESULTS

Optimum conditions of multiplex PCR

Figure 1 shows the 280 bp, 151 bp, and 111 bp PCR product of the genomic cDNA of BVDV, BRV, and BCV. Figure 2 shows the optimization condition for annealing temperature and primer concentration of multiplex PCR. Annealing temperature of 55 °C for 30 second and 1.0 μL primer concentration were found optimum for multiplex PCR. Finally, the optimal reaction system of multiplex PCR was determined as 25 μL: 10 μL of 2 × EsTaqMasterMix, 1.0 μL of cDNA of each of the three viruses, 1.0 μL of upstream and downstream primers, and 3.0 μL of ddH_2O. The following reaction procedure was applied: 94 °C for 5

min; 94 °C for 30 s; 55 °C for 30 s; 72°C for 30 s; and 35 cycles of final elongation at 72 °C for 7 min.

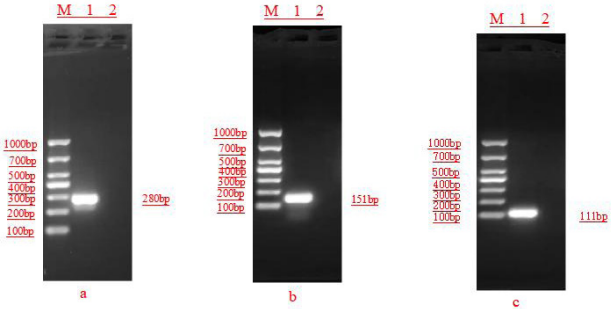

Fig. 1. PCR amplification of (a) BVDV DNA (b) BRV DNA and (c) BCV DNA. M, DL1000 Marker Lane 2 is Negative control.

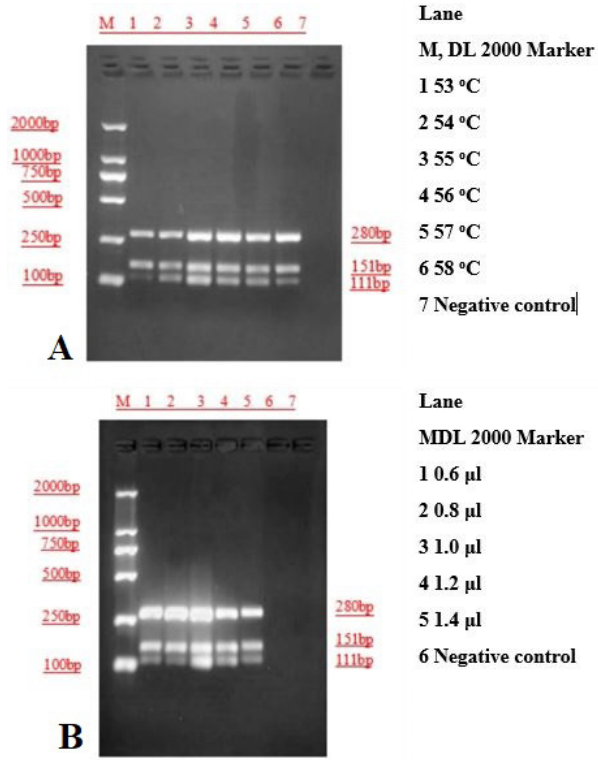

Fig. 2. Optimization of the annealing temperature (A), PCR primer concentraction; (B), multiplex PCR primer concentration.

Figure 3 shows multiplex PCR specificity and sensitivity tests. BVDV, BRV, and BCV DNA were successfully used as templates for amplification of the corresponding target fragments, but none of them could be amplified when genomic DNA of *E. coli, Salmonella*, IBRV, and water was used as a template, indicating that the multiplex PCR method examined in this experiment had good specificity (Fig. 3A). The minimum detection limit of BVDV was 10^3 copies/µL, and that of BRV and BCV was 10^2 copies/µL, showing that the established multiplex PCR method had good sensitivity (Fig. 3B).

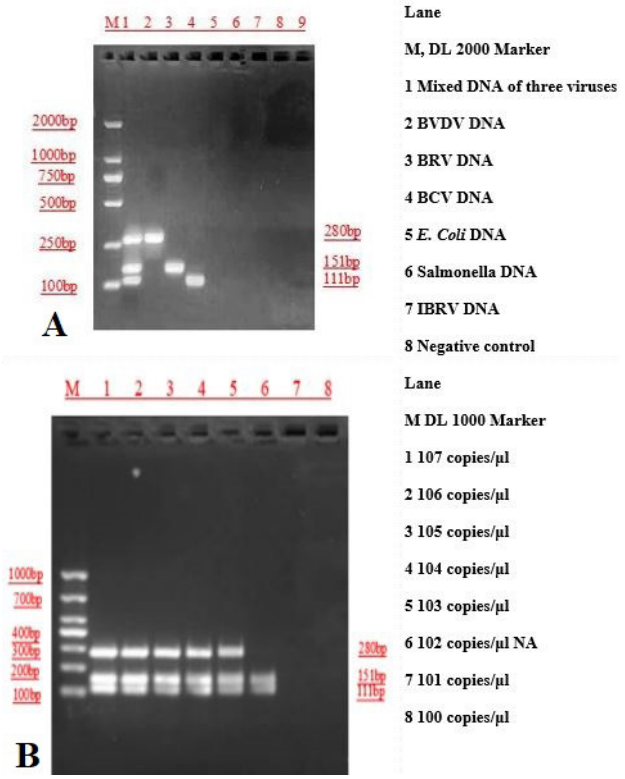

Fig. 3. Results of the multiplex PCR specificity (A), and sensitivity (B) test.

Infection status of the clinical samples

Table II shows infection of single mixed viral infection in the clinical samples. The positive rates of the single samples infected with BVDV, BRV, and BCV were 0.6% (1/150), 5.3% (8/150), and 4.0% (6/150), correspondingly. The positive rate of the mixed infections with BRV and BCV was 41.3% (62/150), of BVDV and BRV 8.0% (12/150), of BVDV and BCV 6.0% (9/150), and of mixed infection with the three pathogens of 10.0% (15/150). These results showed that the mixed infection of BRV and BCV was the main pathogen causing calf diarrhea in our clinical samples.

Multiplex PCR detection of 150 stool samples revealed that the highest positive rate (100%) of infection was in Shijiazhuang, followed in a descending order by those in Qinhuangdao (75%) and Tangshan (40%), whereas the lowest positive detection rate of 30% was established in Baoding (Table III).

Table II. Multiplex PCR results of the clinical samples.

Pathogen/ Region	Shijiazhuang	Baoding	Tangshan	Qinhuangdao	Number of positive samples (copies)	Positive rate (%)
BVDV+BRV+BCV	8	0	4	3	15	10.0
BVDV+BRV	6	0	3	3	12	8.0
BRV+BCV	56	0	5	1	62	41.3
BVDV +BCV	2	4	0	3	9	6.0
BVDV	0	0	0	1	1	0.6
BRV	8	0	0	0	8	5.3
BCV	0	2	0	4	6	4.0
Total	80	6	12	15	113	75.2

Table III. Results of the multiplex PCR amplification detection of clinical samples from different regions.

Area	Cattle farms	Number of fecal samples tested	Number of positive stool samples	Positive rate (%)
Shijiazhuang	4	80	80	100
Baoding	2	20	6	30
Tangshan	2	30	12	40
Qinhuangdao	2	20	15	75
Total	10	150	113	75.3

DISCUSSION

BVDV, BRV, and BCV are important pathogens causing single or mixed infection of bovine viral diarrhea (Gomez *et al*., 2017; Kuta *et al*., 2013; Khodaram-tafti and Farjanikish, 2017). Mixed infections not only aggravate the symptoms of calf diarrhea, but also hinder the accurate evaluation of prevention and control measures against single-pathogen infections, eventually leading to huge economic losses to dairy cattle breeding (Lu *et al*., 2020; Nguyen *et al*., 2020). Currently, there is no specific prevention and treatment for calf diarrhea caused by BVDV, BRV and BCV (Zhang *et al*., 2014). Therefore, an urgent need exists to establish a rapid method for simultaneous detection of the three viruses, which can facilitate the early diagnosis and control of cattle infections caused by BVDV, BRV, and BCV in China.

Briefly, multiplex PCR is performed by the addition of two or more specific primers into the same reaction tube for complementary base pairing with the target gene aimed at amplification of the corresponding DNA fragment (Ding *et al*., 2018; Liu *et al*., 2016). Based on the advantages of multiplex PCR, including its high specificity, sensitivity, and efficiency, we designed specific primers and optimized the reaction conditions to establish a novel method for identification and detection of BVDV, BRV, and BCV. Our findings provide an effective approach for laboratory detection and serve as a reliable basis for preliminary clinical diagnosis. In this study, we designed specific primers for the conserved sequences of the three examined viruses. The E2 gene belongs to the highly conserved sequence of BVDV which encodes BVDV proteins (Hou *et al*., 2020; Shu, 2013). The gene sequence of the VP6 protein of BRV is highly conserved, with good antigenicity and immunogenicity (Huang, 2016). On the other hand, E gene of BCV has high specificity and conservation (Singasa *et al*., 2017; Shin *et al*., 2019). Therefore, the target genes of the three viruses can ensure the high sensitivity, accuracy, and specificity of test results.

We successfully established the above-described triple-PCR method after optimization of the reaction conditions. Our results showed that this novel method had no cross reaction with *E. coli*, *Salmonella*, and IBRV, and was characterized by good specificity. The detection limits of the plasmid standards for BVDV, BRV, and BCV were 1.19×10^3 copies/μL, 3.89×10^2 copies/ μL, and 3.74×10^2 copies/μL, respectively.

A total number of 150 clinical samples were simultaneously detected by this method and the commercial colloidal gold test board. The total coincidence rate of the two methods was 100.0%. The results showed that the positive rate of mixed infection of BRV and BCV was

41.3% (62/150), of BVDV and BRV 8.0% (12/150), of BVDV and BCV 6.0% (9/150), and of the mixed infection of the three viruses 10.0% (15/ 50). The mixed infection of BRV and BCV in clinical samples caused more serious symptoms. The highest positive rate of infection in Shijiazhuang area was 100%, followed in a descending order by those in Tangshan and Qinhuangdao areas, where the positive detection rates were 40% and 75%, respectively, and the lowest positive rate in Baoding area was 30%. The infection status of the 150 clinical samples tested showed that BVDV, BRV, and BCV infections existed in calves from different regions, and the mixed-pathogen infection was the prevalent cause of calf diarrhea.

The novel multiplex PCR method established in this study is simple, specific, sensitive, and inexpensive. It is a valuable new diagnostic method for the preliminary clinical diagnosis and epidemiological monitoring of BVDV, BRV, and BCV infections, with significant application value for the monitoring, prevention, and control of viral pathogens causing calf diarrhea.

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Ethical approval

All animal studies have been reviewed by the appropriate ethics committees.

Statement of conflicts of interest

The authors have declared no conflict of interest.

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