RESEARCH ARTICLE

Establishment of dual LFD-RPA method for bovine coronavirus and bovine rotavirus

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Bovine coronavirus (BCoV) and bovine rotavirus (BRV) are important pathogens that cause diarrhea in calves. Both pathogens are transmitted through the fecal-oral route, which can lead to accumulation of the virus during the cold season. In severe cases, infected calves show large amounts of bloody feces, dehydration and weakness, or even death, which can seriously hamper the growth and development of calves. Mixed infections of the two pathogens often occur clinically, so early differential diagnosis of BCoV and BRV is the key to prevention and control of BCoV and BRV. This study focused on the establishment of a dual lateral flow dipstick-recombinase polymerase amplification (LFD-RPA) assay based on the conserved sequence of the N gene of the BCoV genome and the VP6 gene of the BRV genome, which was easy to operate, rapid in response, and could simultaneously detect BCoV and BRV, to provide technical support for rapid clinical diagnosis and effective prevention and control of single or mixed infections of BCoV and BRV. The results showed that the established dual LFD-RPA assay could be completed at 37°C for 11 mins. The lowest detection concentrations of BCoV and BRV plasmid nucleic acid were 5.82 copies/μL and 5.99 copies/μL, respectively. The lowest detection concentrations of BCoV and BRV genomic nucleic acid were 2.89 × 10^{-2} ng/μL and 1.35 × 10^{-2} ng/μL, respectively. The sensitivity of this method was higher than that of the basic PCR method. The dual LFD-RPA assay also demonstrated good specificity and stability. A total of 108 clinical samples were used to compare the LFD-RPA method and the basic PCR method, and the results confirmed that both methods were in good agreement and could be applied to clinical testing. The established dual LFD-RPA method for BCoV and BRV was characterized by rapid response, strong specificity, high sensitivity, and good reproducibility. The detection results could be visually observed through test strips, making it suitable for clinical rapid detection of BCoV and BRV.

Keywords: bovine coronavirus; bovine rotavirus; dual LFD-RPA.

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Introduction

Bovine coronavirus (BCoV) is an RNA virus belonging to the genus coronavirus in the family *Coronaviridae*. BCoV is an important pathogen that causes intestinal and respiratory diseases in cattle. It can also cause diarrhea in calves, winter diarrhea in adult cattle. The main symptom in newborn calves is the discharge of yellowish watery feces accompanied by fever [1, 2]. Bovine

rotavirus (BRV) is an RNA virus belonging to the genus rotavirus (RV) in the family *Eutheroviridae*. The clinical symptoms of BRV infection in cattle are mainly vomiting and diarrhea. Most infected calves excrete grayish-white or blackish-brown loose stools that are often mixed with undigested milk clots [1, 2]. Both viruses are transmitted through the fecal-oral route and are adapted to grow at low temperatures with severe disease showing large amounts of blood feces, dehydration, and even death. Mixed infections of these two viruses often occur, severely hampering the development of the cattle industry. Cui *et al*. tested 325 calf fecal samples from parts of southern Xinjiang, China, and the detection rates of BCoV, BRV, and mixed infection of both viruses were 9.54%, 15.38%, and 6.76%, respectively [3]. Gao *et al*. tested 76 fecal samples of cattle with clinical diarrhea collected from sick cattle in Shaanxi and Gansu Provinces, China by multiplexed fluorescence quantitative PCR assay. The detection rates of BCoV and BRV were 17.1% and 9.2% [1]. Sun *et al*. examined 220 yak diarrhea samples from the Northwest Grassland with the detection rate of BRV as 40.5% and no BCoV being detected [4]. The genome of BCoV is an approximately 31 kb, lipid-enveloped, single-stranded RNA, which is the largest currently known RNA virus. The viral genome encodes five major structural proteins in the order of nucleocapsid protein (N protein), envelope protein (M protein), small membrane protein (E protein), fibrillar protein (S protein), and hemagglutinin/esterase protein (HE protein) [5]. The N protein is highly conserved and is widely used for virus identification and detection [6]. Its role is to package the coronavirus genome into RNP complexes, ensuring that the virus can replicate in a timely manner and spread reliably, as well as to play a role in transcription and translation [7, 8]. BRV is a double-stranded, capsid-free RNA virus with a genome length of approximately 18.5 kb and belongs to group A rotaviruses [9]. BRV is composed of a core protein, an outer capsid, an inner capsid, and an unstructured protein [10]. Rotavirus VP6 constitutes the middle nucleocapsid protein of the virus and consists of 397 amino acids. Among the total structural proteins of coronaviruses, VP6 is the most abundant, accounting for about half of the total content, and maintains the stability of virus particles. The VP6 protein is the most conserved and at the same time better immunogenic and reactogenic protein among group A RVs, and it is the optimal choice for population and subpopulation antigens [11].

Recombinase polymerase amplification (RPA) is a new type of thermostatic amplification technology that utilizes recombinase, strand displacing enzyme, and single-stranded binding protein to synergistically achieve the specific binding of primer and template at room temperature to replace the denaturation and re-denaturation process of traditional polymerase chain reaction (PCR) [12, 13]. It is an emerging technology for rapid amplification of target fragments from minimally processed samples by portable instruments. The high amplification efficiency, simplicity, and low dependence on equipment make RPA highly advantageous for pathogen detection [14]. Lateral flow dipstick (LFD) typically consists of several components including a sample pad, a gold standard pad, a nitrocellulose (NC) membrane, an absorbent pad, and a PVC plate. LFD assay is implemented with the capillary effect as the driving force and the NC membrane as the reaction platform. When the target molecules are captured and deposited on the NC membrane through intermolecular interactions such as electrostatic interactions, hydrogen bonding, hydrophobic forces, *etc*., the labeled signaling molecules show the T line (detection line) and C line (control line), thus realizing the rapid detection of the target [15]. The combination of RPA and LFD produces the LFD-RPA assay, which has several advantages in sensitivity, specificity, simplicity, and efficiency [16, 17]. The biggest advantage of RPA technology is the fast detection speed compared to conventional detection methods such as PCR, real-time quantitative PCR (qPCR), loop-mediated isothermal amplification (LAMP), and enzyme linked immunosorbent assay (ELISA) [18]. It has been applied to the detection of pathogens such as avian influenza virus, cholera

isolate, trauma isolate, and leptospirosis [19-21], and has great potential for application in disease diagnosis and outbreak monitoring in resource-constrained areas. Yuan *et al*. established an LFD-RPA detection method based on the conserved sequence of the M gene of porcine epidemic diarrhea virus, and the target band could appear in 9 mins [22]. Zhang *et al*. proposed a rapid detection method for the detection of novel coronavirus nucleic acid by LFD-RPA, which could be completed in 15 mins [23]. In addition, a visual LFD-RPA technique for rapid detection of *Streptococcus suis* was proposed, which could be completed in 20 to 30 mins [24]. The LFD-RPA method has been fully developed for the rapid detection of bacteria and viruses.

In Ningxia region of China, the scale of cattle breeding is expanding, while the mixed infection of bovine rotavirus and bovine coronavirus is more serious. However, the detection of a single pathogen is much time-consuming and labor-intensive. It is necessary to find a fast, accurate, and simple method for the two pathogens detection. Currently, the commonly used detection methods for bovine rotavirus and bovine coronavirus include PCR, qPCR, LAMP, and ELISA. These methods are not able to realize the rapid detection of pathogens due to the complexity of the operation process, time-consuming, labor-intensive, and the dependence on instrumentation, which makes it difficult to be widely applied in the field and field detection [25]. This study applied RPA combined with LFD to establish a dual LFD-RPA detection method for BCoV and BRV, which would provide technical support for the clinical diagnosis, epidemiological investigation, and comprehensive prevention and control of BCoV and BRV.

Materials and methods

Sample source

Bovine rotavirus isolates and bovine coronavirus isolates were obtained from the Laboratory of Preventive Veterinary Medicine, College of Animal Science and Technology, Ningxia University, Yinchuan, Ningxia, China. Bovine viral diarrhea virus (BVDV)-1 standard strain (NADL) and Infectious bovine rhinotracheitis virus (IBRV) standard strain (HVRI-002) were purchased from China Veterinary Drugs Supervision Institute, Chinese Veterinary Microbial Strain Preservation and Management Center, Beijing, China. Bovine syncytial virus (BRSV), bovine origin *Escherichia coli* (*E. coli*), *Clostridium perfringens* type A (*C. perfringens*), *Salmonella typhimurium* (*S. typhimurium*), *Eimeria necatrix* (*E. necatrix*), *Cryptosporidium bovis* (*C. bovis*) were all provided by the Institute of Animal Science, Ningxia Academy of Agriculture and Forestry, Yinchuan, Ningxia, China. A total of 108 calf diarrhea samples were collected from diarrhea herds in Yinchuan City, Zhongwei City, and Qingtongxia City, Ningxia, China from 2022 to 2023. 0.5 g of each fecal sample was put into a 1.5 mL centrifuge tube and mixed well with 1 mL of physiological saline before centrifugation at 5,000 rpm for 10 mins. 200 µL of supernatant was transferred into a sterilized centrifuge tube and stored at -80℃.

Construction of recombinant plasmids

BCoV and BRV nucleic acids were extracted using TIANamp Virus RNA Kit (Tiangen Biochemical, Beijing, China) following kit's instructions. RNA was then reverse transcribed to cDNA using HiScript III RT SuperMix (Novizan Biotechnology, Nanjing, China) following manufacturer's instructions. The N gene and VP6 gene were amplified using BIO-RAD T100 Thermal Cycler (Bio-Rad, Hercules, California, USA) with the primers of BCoV-F/R and BRV-F/R (Table 1). The PCR reaction system consisted of 25 µL of 2× Taq PCR Master Mix II (Tiangen Biochemical, Beijing China), 2 µL of BCoV-F/R or BRV-F/R, 2 µL of BCoV/BRV cDNA template, 19 µL of ddH_2O. The reaction program was set as 95℃ for 3 mins followed by 35 cycles of 94℃ for 30 s, 52℃ (BCoV) or 54℃ (BRV) for 30 s, 72℃ for 1 min, and a final extension of 72℃ for 5 mins. PCR products were analyzed by gel electrophoresis and then recovered for cloning into pMD-18T vector (Takara, Beijing, China). The recombinant positive plasmid was transformed, cultured, and

Table 1. Basic-RPA primers.

Primer name	Primer sequence (5' → 3')	Clip size (bp)
PCR-BCV-F	CCC TAC TAT TCT TGG TTC TCT	939
PCR-BCV-R	CAA CAC CTT CAT TAT GGT CTC	
PCR-BRV-F	TCC CTT ATT CAG CGT CAT TC	829
PCR-BRV-R	TTA CTC TGT GTA GCG AAT ACG T	
RPA-BCoV-F1	GCT AAG GGG TAC TGG TAC AGA CAC AAC AGA	144
RPA-BCoV-R1	TCC GTC AAT GTC GGT GCC ATA CTG GTC TTT	
RPA-BCoV-F2	CTG AAG CTA AGG GGT ACT GGT ACA GAC ACA A	157
RPA-BCoV-R2	CAG AAG ACT CCG TCA ATG TCG GTG CCA TAC T	
RPA-BCoV-F3	TGG CAA TAG AAC CCC TAC CTC TGG TGT AAC AC	212
RPA-BCoV-R3	CTC TCT TCC CAA AAC ACT GCT GAA CAG TGC AT	
RPA-BRV-F1	ACA TAT TGT ACA GCT CCG AAG AGT GTT GAC T	199
RPA-BRV-R1	TGC TTG GTA AGT ATT TAT TAT CTG CCC GTT T	
RPA-BRV-F2	TTG AAC ATA TTG TAC AGC TCC GAA GAG TGT T	205
RPA-BRV-R2	CTT GCT TGG TAA GTA TTT ATT ATC TGC CCG T	
RPA-BRV-F3	CAT GAT AAT TTG ATG GGT ACG ATG TGG CTT A	164
RPA-BRV-R3	GGT AGA AGA GTT ATT GTA GCT GTA GTC AAC ACT C	

sequenced, and then stored at -20°C after a 10-fold multiplicative dilution.

Basic-RPA primer design and screening

The primers were designed using Premier 5 (http://www.premierbiosoft.com/primerdesign/index.html) software (Table 1). Screening of BCoV and BRV primers was performed using the TwistAmp™ Basic Kit (TwistDx, Ltd., Cambridge, UK) following kit's instructions. The Basic-RPA reaction system was prepared by adding 14.75 µL of rehydration buffer, 1.2 µL of each 10 µ forward and reverse primers, 5.6 µL of ddH_2O into the RPA lyophilized powder reaction tube. After mixing thoroughly, 1 µL of positive plasmid and 1.25 µL of Mg^{2+} were added to the reaction. Amplification was carried out at 39°C for 30 mins with 5 µL of amplified sample being removed every 5 mins. The reaction was terminated by placing reaction on ice. After the reaction was completed, the amplification products were diluted 5-fold with ddH_2O and analyzed by 2% agarose gel electrophoresis. The primer sets with clear production bands and no non-specific products were selected for subsequent experiments.

Dual Basic-RPA optimal primer concentration ratio

To determine the optimal primer ratios for BCoV and BRV dual Basic-RPA, the recombinant plasmids were used as templates for BCoV and BRV in a 50 µL reaction system containing 5.82×10^7 and 5.99×10^7 copies/µL, respectively. The reaction system consisted of 29.5 µL of rehydration buffer, 1 µL of each BCoV and BRV templates, 12 µL of ddH_2O, 2.5 µL of Mg^{2+}. Different primer ratios were set for the RPA reaction (Table 2). The reaction was carried out at 37°C for 25 mins and was terminated by placing the reaction tube on ice. The products were diluted 5-fold and analyzed by 2% agarose gel electrophoresis to determine the optimal primer concentration ratios of BCoV and BRV.

Table 2. Optimization of dual Basic-RPA primer ratios.

RPA-BCoV-F1/R1 (µL)	RPA-BRV-F1/R1 (µL)
0.0	2.4
0.3	2.1
0.6	1.8
0.9	1.5
1.2	1.2
1.5	0.9
1.8	0.6
2.1	0.3
2.4	0.0

Table 3. LFD-RPA primers and probes.

Primer/Probe name	Primer/Probe sequence (5' → 3')
BCoV-LFD-RPA-F	GCTAAGGGGTACTGGTACAGACACAACAGA
BCoV-LFD-RPA-R	Biotin-TCCGTCAATGTCGGTGCCATACTGGTCTTT
BCoV-LFD-RPA-Probe	Digoxin-CAGCCGATGGCAACCAGCGTCAATTGCTGCC/idSp/CGATGGTATTTTTAC-C3-space
BRV-LFD-RPA-F	ACATATTGTACAGCTCCGAAGAGTGTTGACT
BRV-LFD-RPA-R	Biotin-TGCTTGGTAAGTATTTATTATCTGCCCGTTT
BRV-LFD-RPA-Probe	FAM-GCAACTACATGGTACTTTAATCCAGTGATTC/idSp/TAGACCAAATAATGT-C3-spacer

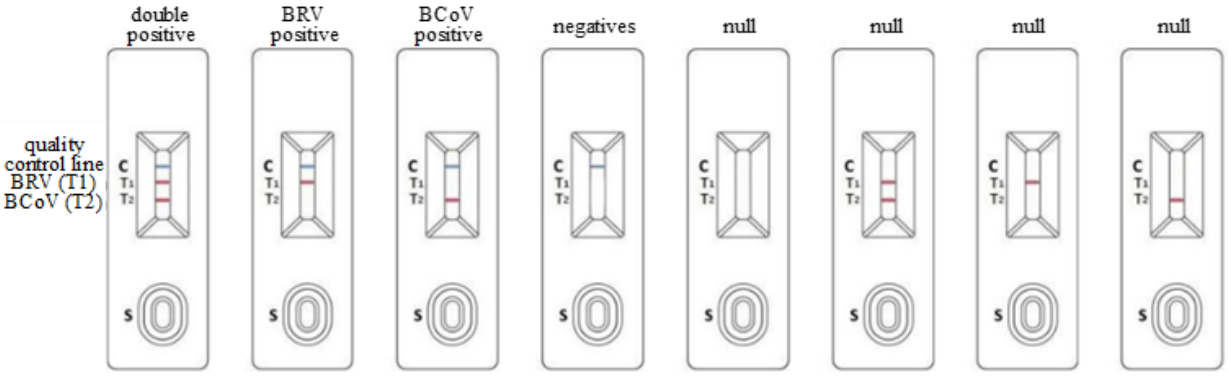

Figure 1. Interpretation of test strip results.

Establishment of dual LFD-RPA method and optimization of reaction conditions

(1) Design of dual LFD-RPA primers and probes

The design of primers and probes for BCoV and BRV dual LFD-RPA followed the instructions of Dual Nucleic Acid Detection Test Strips (Latex Chromatography) (Amplification Future, Weifang, Shandong, China) (Table 3). All primers and probes were synthesized by Sangon Biotech (Shanghai, China). A 50 µL LFD-RPA reaction system included 1.5 µL of each BCoV-LFD-RPA-F and BCoV-LFD-RPA-R primers, 0.3 µL of BCoV-LFD-RPA-Probe, 0.9 µL of each BRV-LFD-RPA-F and BRV-LFD-RPA-R primers, 0.6 µL of BRV-LFD-RPA-Probe, 29.4 µL of A buffer (Amplification Future, Weifang, Shandong, China), 4 µL of BCoV and BRV template, 4.4 µL of ddH_2O, and 2.5 µL of B buffer (Amplification Future, Weifang, Shandong, China). The interpretation of LFD results was shown in Figure 1.

(2) Dual LFD-RPA reaction time and reaction temperature optimization

The reaction time was optimized by incubating the reaction mixture at 39°C for 5, 7, 9, 11, 13, and 15 mins, respectively. The optimization of the reaction temperature was carried out at 20, 25, 30, 35, 37, 39, 41, and 43°C under the optimum reaction time. The reaction was terminated on ice and the reaction product was diluted 20-fold with sterile ddH_2O. A drop of 80 µL of diluted reaction product was aspirated into the spiked well of the LFD, and the results of the reading zone were recorded within 15 mins.

(3) Sensitivity testing

Dual LFD-RPA sensitivity assays were performed using BCoV and BRV recombinant plasmids as templates and BCoV and BRV genomes as templates, respectively. The purified recombinant plasmids pMD18T-N and pMD18T-VP6 were diluted 10-fold to 100 copies/µL. Genomic cDNAs of BCoV and BRV were 10-fold diluted to 10^{-4} ng/µL. Recombinant plasmid sensitivity test used 10^7 - 10^0 copies/µL as templates, while genome sensitivity test used 10^3 - 10^{-4} ng/µL as templates. Three replicates were

set for each sample and both tests using ddH_2O as negative control. Amplification was carried out at the optimal reaction time and optimal reaction temperature. The reaction was terminated by placing the reaction tube on ice. The amplification products were diluted and analyzed in conjunction with the LFD to determine the lowest detection limit of the dual LFD-RPA assay for BCoV and BRV. Meanwhile, the conventional PCR sensitivity test was performed. The PCR primers were designed based on the sequences of the N gene of BCoV and the VP6 gene of BRV. The specific primer sequences for N gene were F: 5'- CTG AAG CTA AGG GGT ACT GG -3', R: 5'- CAG AAG ACT CCG TCA ATG TC -3' with the product size of 156 bp. The specific primer sequences for VP6 gene were F: 5'- ATA ATT TGA TGG GGT ACG ATG -3', R: 5'- GAA GAG TTA TTG TAG CTG TA -3' with the product size of 158 bp. The PCR reaction volume was 25 µL including 1 µL of each 10 µM forward and reverse primers, 12.5 µL of 2× Taq PCR Master Mix II, 1 µL of template, 9.5 µL of ddH_2O. PCR reaction was performed at 95°C for 3 mins followed by 30 cycles of 94°C for 30 s, 55°C for 30 s (BCoV) or 45°C for 30 s (BRV), 72°C for 30 s, and then 72°C for 10 mins and 4°C for storage. The cDNA of BCoV and BRV genomes diluted at 10^3 - 10^{-4} ng/µL was selected as templates, and ddH2O was used as negative control. The results of PCR reactions were detected using 1.5% agarose gel electrophoresis.

(4) Dual LFD-RPA specificity test

Genomic DNAs of BCoV + BRV, BCoV, BRV, BVDV, IBRV, BRSV, *E. coli*, *C. perfringens*, *S. typhimurium*, *E. necatrix*, and *C. bovis* were used as templates, respectively. Three replicates for each sample were set and ddH_2O was used as a negative control. Amplification was carried out at the optimal reaction time and reaction temperature, and the reaction was terminated by placing the reaction tube on ice. The amplification products were diluted and analyzed in combination with the LFD to evaluate the specificity of the dual LFD-RPA assay for BCoV and BRV.

(5) Dual LFD-RPA repeatability test

Recombinant BCoV and BRV plasmids with the concentrations of 10^7 - 10^5 copies/µL were selected as templates, and three replicates were performed once a week for a total of three times. The amplification products were analyzed in combination with LFD.

(6) Clinical sample testing

A total of 108 diarrhea samples were tested using the established dual LFD-RPA assay for BCoV and BRV with ordinary PCR method detection. The standard strain and ddH_20 were employed as the positive and negative controls. The results were analyzed to compare the consistency of results between the two methods.

Results and discussion

Concentration of recombinant plasmids and genomes

The copy numbers of recombinant positive plasmids pMD18T-N and pMD18T-VP6 were 5.82 × 10^{10} copies/µL and 6.00 × 10^{10} copies/µL, respectively. The concentrations of BCoV and BRV genomic cDNA were 2.89 × 10^3 ng/µL and 1.35 × 10^3 ng/µL, respectively.

Basic-RPA primer screening

The results of the gel electrophoresis showed that BCoV F1/R1 amplified specific bands, which were consistent with the target bands, while BCoV F2/R2 showed non-specific bands, and BCoV F3/R3 showed multiple non-specific bands. BRV F1/R1 amplified a specific band, which was consistent with the target band. However, BRV F2/R2 showed a non-specific band, while BRV F3/R3 had a target fragment of 164 bp, but also showed a non-specific band. Therefore, primers BCoV F1/R1 and BRV F1/R1 were selected as LFD-RPA primers for BCoV and BRV, respectively.

Optimization of dual Basic-RPA optimal primer ratios

The results of the primer ratios of BCoV and BRV dual Basic-RPA showed that the best amplification effect of dual Basic-RPA was achieved when 1.5 µL and 0.9 µL of RPA-BCoV-

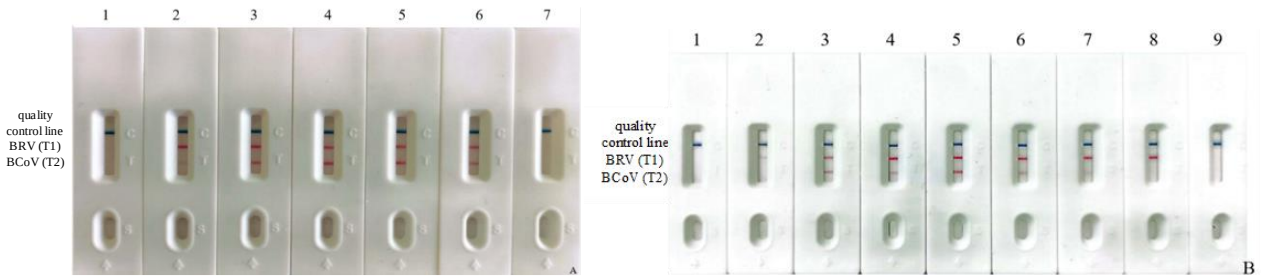

Figure 2. Dual LFD-RPA reaction time and temperature optimization. **A. 1:** 5 minutes, **2:** 7 minutes, **3:** 9 minutes, **4:** 11 minutes, **5:** 13 minutes, **6:** 15 minutes, **7:** negative control. **B. 1:** 20°C, **2:** 25°C, **3:** 30°C, **4:** 35°C, **5:** 37°C, **6:** 39°C, **7:** 41°C, **8:** 43°C, **9:** negative control.

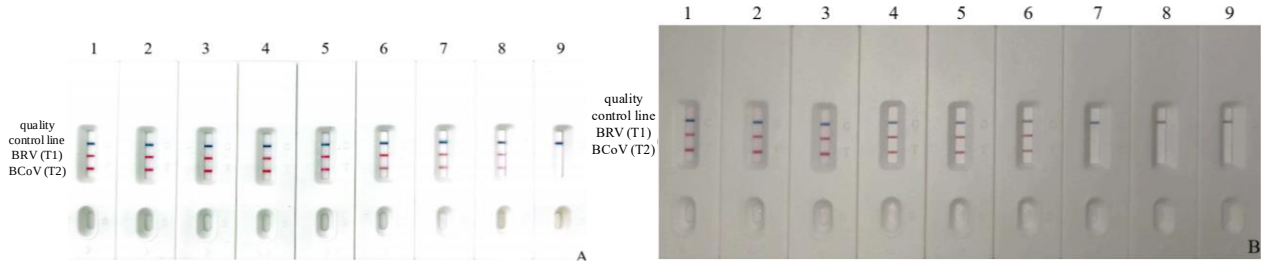

Figure 3. BCoV and BRV recombinant plasmids and genomes dual LFD-RPA sensitivity. **A.** BCoV/BRV plasmids. **1-8:** 5.82×10^{7}/5.99×10^{7}-5.82×10^{0}/5.99×10^{0} copies/μL, **9:** negative control. **B.** BCoV/BRV genomes. **1-8:** 2.89×10^{3}/1.35×10^{3}-2.89×10^{-4}/1.35×10^{-4} copies/μL, **9:** negative control.

F1/R1 and RPA-BRV-F1/R1 primers were used in the 50 μL reaction system. Therefore, RPA-BCoV-F1/R1 and RPA-BRV-F1/R1 primers at a ratio of 5:3 was selected as the primer concentration ratios for the subsequent LFD-RPA.

Dual LFD-RPA reaction time and temperature optimization

The results showed that there were two red bands at 7 mins of the reaction. The color of the bands was the brightest at 11 mins and 13 mins, then became lighter at 15 mins (Figure 2A). No red bands appeared at 20°C. However, a faint red band appeared at 25°C, 30°C, 35°C, and 37°C. The two red bands gradually deepened as the temperature got higher at 39°C and 41°C, then gradually became lighter as the temperature got higher. The two red bands were the deepest in color at 37°C (Figure 2B). To ensure that the reaction was carried out adequately, 11 mins was selected as the optimal reaction time and 37°C as the optimal reaction temperature. Since this reaction could be completed in 11 mins, the longer amplification time was prone to false positives. Zhang *et al* established a *B. burgdorferi* LFD-RPA assay with a reaction time of 12 mins, which was similar to the reaction time of this test [26]. The optimal reaction temperature was consistent with previous report that 37°C was the optimal temperature for the rapid diagnostic method of porcine crested virus LFD-RPA [27].

Sensitivity test of dual LFD-RPA

Dual LFD-RPA sensitivity test used BCoV and BRV recombinant plasmids, BCoV and BRV genomes as templates, respectively. Under the optimal reaction time and temperature, the BCoV and BRV recombinant plasmid demonstrated 2 red quality control (QC) lines that were weak colored when the concentration of BCoV/BRV recombinant plasmid was 5.82/5.99 copies/μL (Figure 3A). Therefore, the lowest detection limits for both BCoV and BRV were identified as 5.82 and 5.99 copies/μL, respectively. The 2 red QC lines were more faintly colored at BCoV/BRV genomic concentrations of 2.89×10^{-2} ng/μL and 1.35×10^{-2} ng/μL (Figure 3B). Therefore, the lowest detection limits for both BCoV and BRV

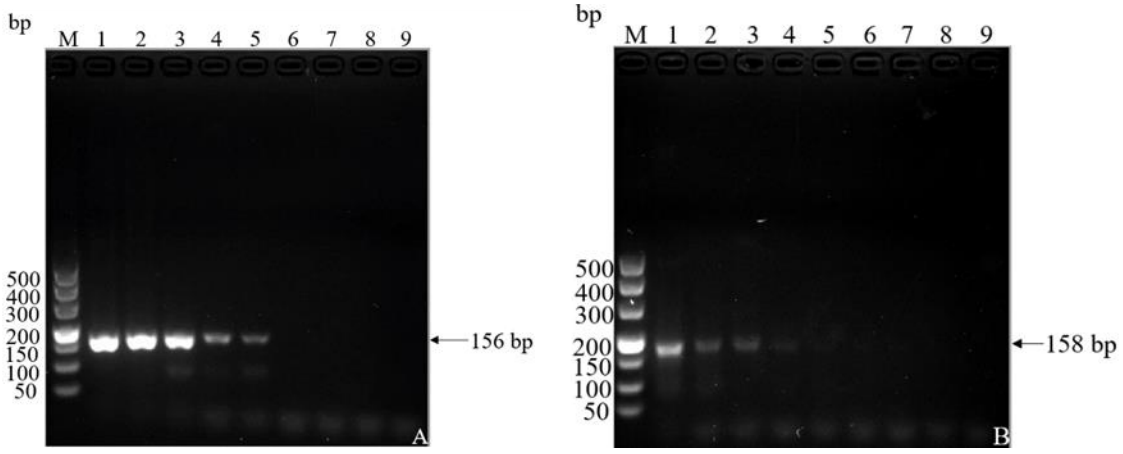

Figure 4. BCoV, BRV PCR sensitivity assay. **A.** BCoV sensitivity. **M:** DL-500 marker, **1-8:** 2.89×10^{3}-2.89×10^{-4} ng/μL, **9:** negative control. **B.** BRV sensitivity. **M:** DL-500 marker, **1-8:** 1.35×10^{3}-1.35×10^{-4} ng/μL, **9:** negative control.

Figure 5. Dual LFD-RPA specificity test. **1:** BCoV + BRV, **2:** BCoV, **3:** BRV, **4:** BVDV, **5:** IBRV, **6:** BRSV, **7:** *E. coli*, **8:** *C. perfringens*, **9:** *S. typhimurium*, **10:** *E. necatrix*, **11:** *C. bovis*, **12:** negative control.

were 2.89 × 10^{-2} ng/μL and 1.35 × 10^{-2} ng/μL, respectively. The sensitivity tests for both viruses were also performed using conventional PCR. The results showed that the lowest detection limit of genomic DNA of the BCoV in PCR assay was 2.89 × 10^{-1} ng/μL (Figure 4A). The BCoV LFD-RPA assay was 10-fold more sensitive than that of the BCoV PCR assay. The lowest detection limit for BRV VP6 gene in PCR assay was 1.35 × 10^{0} ng/μL (Figure 4B), and the BRV LFD-RPA assay was 100-fold more sensitive than the BRV PCR assay. The results indicated that the established LFD-RPA assay was more sensitive than the PCR assay. The lowest detection limits for both recombinant BCoV and BRV plasmids were 5.82 and 5.99 copies/μL, respectively, and the lowest detection limits for both BCoV and BRV genomes were 2.89 × 10^{-2} and 1.35 × 10^{-2} ng/μL, respectively. Zhang *et al*. established a multiplex fluorescence off quantitative PCR assay for BCoV and BRV plasmids with the lowest detection limits of 7.7 × 10^{2} and 1.74 × 10^{2} copies/μL, respectively [28]. Gao *et al*. also established a multiplex fluorescence quantitative PCR assay for four bovine diarrhea-causing viruses with the lowest detection limits of 9.1 × 10^{1} and 1.2 × 10^{1} copies/μL for BCoV and BRV, respectively. By comparing to previous studies, the LFD-RPA assay established in this research was more sensitive than the PCR method and qPCR assay.

Specificity test of dual LFD-RPA

Genomic DNAs of BCoV + BRV, BCoV, BRV, BVDV, IBRV, BRSV, *E. coli*, *C. perfringens*, *S. typhimurium*, *E. necatrix*, and *C. bovis* were used as templates to test the specificity of dual LFD-RPA, respectively. The results showed that red bands appeared in the detection line only when

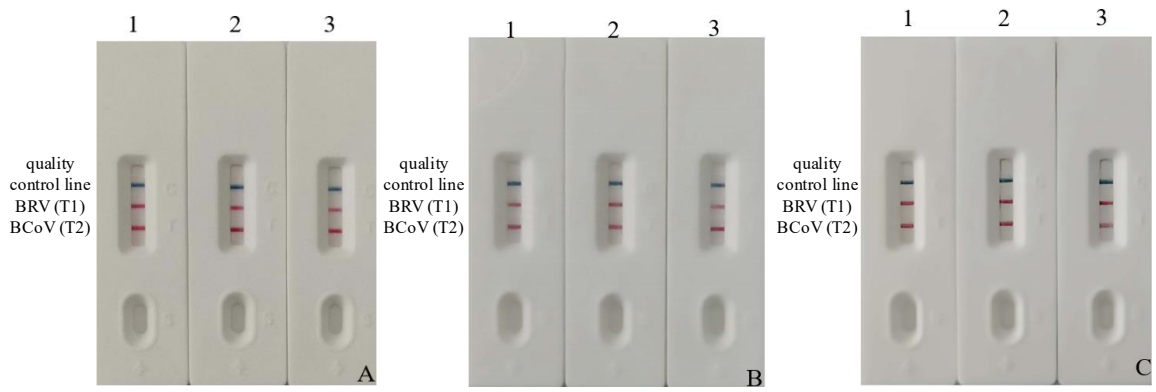

Figure 6. BCoV and BRV dual LFD-RPA repeatability test. **1-3:** 5.82×10^7/5.99×10^7-5.82×10^5/5.99×10^5 copies/μL.

BCoV + BRV, BCoV, and BRV were used as templates (Figure 5), indicating that the BCoV and BRV dual LFD-RPA test had good specificity.

Repeatability of dual LFD-RPA

The results showed that two red bands appeared in all three concentrations of BCoV and BRV, and the brightness of the red bands was basically the same in each concentration (Figure 6), which indicated that the LFD-RPA assay established in this research had good stability.

Clinical sample tests

The dual LFD-RPA assay and common PCR assay were applied to test 108 clinical samples. The results showed that the numbers of positive samples of BCoV and BRV detected by dual LFD-RPA were 38 (35.19% positive) and 48 (44.44% positive), whereas 37 and 46 were detected by common PCR assay. Twelve of them were identified as mixed infections of the two pathogens. The positive results of dual LFD-RPA were consistent with BCoV and BRV common PCR tests. However, the dual LFD-RPA assay detected one and two more positive BCoV and BRV samples on top of the positive samples detected by the ordinary PCR assay, which suggested the higher sensitivity of the dual LFD-RPA assay than that of the ordinary PCR assay, and also indicated that the dual LFD-RPA assay established in this study could be used for clinical sample detection.

In this study, a dual LFD-RPA assay for BCoV and BRV was established, which had the advantages of simple operation, completion of the assay in 11 mins at 37 ℃, and direct observation of the results with the bare eyes. The method is suitable for the rapid detection of BCoV and BRV and has a good application prospect. The results of clinical sample tests showed that BCoV and BRV had a high rate of infection in calf diarrhea in Ningxia, China and there is a need to pay continuous attention to the prevalence of BCoV and BRV and to do a good job of prevention.

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