RESEARCH ARTICLE

Development of a recombinase polymerase amplification combined with lateral flow dipstick assay for detection of bovine viral diarrhea virus

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Rapid detection of bovine viral diarrhea virus (BVDV) is a key method to control bovine viral diarrhea (BVD). Recombinase polymerase amplification (RPA) is a new nucleic acid isothermal amplification technology. This study established a lateral flow dipstick RPA (LFD-RPA) detection method for BVDV detection based on the conserved DNA sequence of 5' non-coding region (5'-NCR) of the BVDV genome. The optimum reaction time and the optimum reaction temperature were 15 mins and 35℃, respectively, and the minimum detection amount of BVDV cDNA was 5.8×10^{-2} ng/μL. The results showed that the LFD-RPA method had superior sensitivity, specificity, and reproducibility. Furthermore, the method was more rapid and convenient, and was suitable for the detection of BVDV in the field.

Keywords: bovine viral diarrhea virus; recombinase polymerase amplification; lateral flow dipstick.

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Introduction

Bovine viral diarrhea (BVD), namely bovine viral diarrhea/mucosal disease (BVD/MD), caused by bovine viral diarrhea virus (BVDV) infection of cattle is an infectious disease characterized by diarrhea, fever, cough, and abortion or birth of deformed fetuses in pregnant cow [1]. BVDV not only has high homology with classical swine fever virus (CSFV) and border disease virus (BDV), but also can break through host-specific cross-infection. Therefore, BVDV can infect not only cattle, but also sheep, goats, pigs, deer, and other ruminants and has a wide host range [2]. BVDV infection presents various clinical syndrome, ranging from transient detectable mild clinical symptoms to fatal mucosal disease [3]. In addition, persistent infection (PI) animals are the main source of virus transmission, causing significant economic losses to the global livestock industry [4]. Therefore, the establishment of rapid and accurate diagnostic methods to remove infected animals from the herd is critical for the control and eradication of BVD.

BVDV is an enveloped positive-stranded RNA virus with obvious fibers on the virion surface. It belongs to the genus *Pestivirus* of the family *Flaviviridae* [5, 6]. The length of the BVDV genome is 12.3 - 12.5 kb, and consists of an open

reading frame (ORF) coding for 3,900 amino acids, a 5' non-coding region (5'-NCR), and a 3' non-coding region(3'-NCR) [7]. The 5'-NCR of BVDV consists of about 372 - 385 nucleotides and is highly conserved among BVDV strains. Therefore, primers are often synthesized based on the sequence of 5'-NCR to detect BVDV or to type BVDV [8]. Nucleic acid detection is the main method to diagnose animals in the infection stage. At present, BVDV detection methods mainly include technologies such as enzyme-linked immunosorbent assay (ELISA), conventional reverse transcription polymerase chain reaction (RT-PCR), and quantitative real-time PCR (qRT-PCR). However, these techniques require complex equipment or experimental procedures, making their application outside specialized laboratories greatly limited. Therefore, developing a rapid, specific, and convenient nucleic acid detection method for BVDV is crucial for the detection of clinical BVDV and the reduction of economic losses.

Recombinase polymerase amplification (RPA) is a new nucleic acid isothermal amplification technology developed by the British company TwistDx Inc. in 2006 [9]. The technique mimics the nucleic acid replication mechanism in T4 phage, relying on the recombinase UvsX to bind to oligonucleotide primers to form a complex and search for the target site in the double-stranded DNA template. The recombinase opens the double-stranded DNA, and the primers undergo a strand-switching reaction with the homologous sequences to form a D-loop, whereby a single-stranded DNA binding protein (SSB) then binds to the replaced DNA strand. The 3' end of the primer is exposed and recognized by the strand replacement DNA polymerase, and the DNA amplification reaction is initiated by the DNA polymerase for replication and extension. The results are analyzed by gel electrophoresis. This method can complete nucleic acid amplification under same temperature conditions with high sensitivity and specificity. The optimum temperature is 37 - 42°C. At a temperature lower than the optimum range, the RPA can also react, but the reaction rate is lower. However, at a higher temperature, the enzyme will gradually become inactive and affect the reaction. The entire reaction process can be completed within 10 - 20 minutes, which is faster than PCR and other isothermal amplification methods. Pathogens that can be detected by RPA technology include viruses, bacteria, parasites, and chlamydia, *etc*. [10]. The lateral flow dipstick (LFD) is a simple test device for semi-quantitative or qualitative detection of oligonucleotides. RPA-LFD is a combination of RPA technology and LFD to create a fast and sensitive on-site detection system based on the principle of RPA amplification, using biotin-labelled primers and 6-carboxyfluorescein (FAM) labelled probes to amplify the target nucleic acid, resulting in an amplicon that has both a FAM group and biotin label on it. The gold nanoparticles with FAM antibody and biotin antibody were at the front and the detection line of the LFD, respectively. By dropping the product onto a test strip, the FAM and biotin groups on the amplicon react with the FAM and biotin antibodies on the LFD. When it is positive, the result is visualizable by seeing a red line in the detection zone. The aim of the present study was to establish an RPA nucleic acid detection method for BVDV. Based on the convenience, rapidity, and specificity of RPA technology, a visual RPA detection method, the lateral flow dipstick RPA (LFD-RPA), was established and could be applied in the prevention, control, and elimination of BVD.

Materials and methods

Virus strains, plasmid, and clinical samples

The BVDV/NADL strain was derived from China Veterinary Culture Collection Center (Beijing, China). Bovine rotavirus (BRV), bovine coronaviruses (BCoV), bovine kobuvirus (BKoV), bovine astrovirus (BAstV), bovine torovirus (BToV), and positive samples of *Salmonella*, *Escherichia coli* from bovine were all identified and preserved in our laboratory. The positive plasmid pUC57-BVDV1254 containing 5'-NCR and N^{pro} gene of BVDV was constructed by our laboratory. A total of 107 clinical blood samples

Table 1. RPA primers and probe information.

Assay	Primers/Probe	Primer sequence (5'-3')	Target fragment (bp)
Basic RPA	5'-NCR-1F	5'-GGGTAGCAACAGTGGTGAGTTCGTTGGATG-3'	172
	5'-NCR-1R	5'-GTCGGTTAAAACTGCTTTTACCTGGGCGAC-3'	
	5'-NCR-2F	5'-GTAGCAACAGTGGTGAGTTCGTTGGATGGC-3'	200
	5'-NCR-2R	5'-GCAGCACCCTATCAGGCTGTATTCGTAACA-3'	
	5'-NCR-3F	5'-AGCAACAGTGGTGAGTTCGTTGGATGGCTT-3'	220
	5'-NCR-3R	5'-AGTAGCAATACAGTGGGCCTCTGCAGCACC-3'	
	5'-NCR-4F	5'-CCTGAGTACAGGGTAGTCGTCAGTGGTTCGAC-3'	180
	5'-NCR-4R	5'-CAATACAGTGGGCCTCTGCAGCACCCTATCAGGC-3'	
LFD-RPA	LFD-F	5'-(Biotin)-GGGTAGCAACAGTGGTGAGTTCGTTGGATG-3'	
	LFD-R	5'- GTCGGTTAAAACTGCTTTTACCTGGGCGAC -3'	
	LFD-Probe	5'-(FAM) CCTTTATTCCAAGGCGTCGAACCACTGACGA (THF) TACCCTGTACTCAGG (C3 Spacer)-3'	

were randomly collected from dairy farms across Ningxia Province of China with 5 mL of blood per sample.

DNA/RNA extraction and cDNA synthesis

RNAs of BRV, BCoV, BKoV, BAstV, and BToV were extracted using E.Z.N.A.® SE Viral DNA/RNA Kit (Omega Bio-Tek, Norcross, GA, USA). RNA was eluted with 50 µL of nuclease-free water. The extracted RNA was used as the template for cDNA synthesis using HiScript® III 1st Strand cDNA Synthesis Kit (Novizan Biotechnology, Nanjing, Jiangsu, China) for reverse transcription. The genomic DNAs of *Salmonella* and *Escherichia coli* were extracted with TIANamp Bacteria DNA Kit (Tiangen Biochemical, Beijing, China). All products were stored at -20°C for later use.

Design and synthesis of primers and LFD probe

Referring to the genome sequence of BVDV/NADL strains (GenBank accession code NC_001461), the primers and LFD probe sequences were designed using TwistDx (TwistDx, Ltd., Cambridge, UK) according to the instruction manual. The specificity of primers and probe were analyzed by using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). All primers and probes were synthesized by Sangon Biotech (Shanghai, China). Information of primers and probes were shown in Table 1.

Screening of RPA Primers

The four groups of primers were dissolved according to the TwistAmp™ Basic kit (TwistDx, Ltd., Cambridge, UK). 29.5 µL of rehydration buffer, 2.4 µL of each 10 µmol/L forward and reverse primers, 11.2 µL of ddH_2O were mixed in the RPA lyophilized powder reaction tube thoroughly, and then 2 µL of the positive plasmid pUC57-BVDV1254 (1.9×10^{10} copies/µL) and 2.5 µL of Mg^{2+} were added to each tube. The amplification conditions were 39°C for 30 mins with 5 µl of the amplification samples being drawn every 5 mins. The samples were placed on ice to terminate the reaction. After the whole reaction was completed, the amplified product was detected and analyzed by 1.5% agarose gel electrophoresis, and the primer sets with clear destination bands and no non-specific products were screened out for subsequent experimental studies.

BVDV basic RPA assays

After determining the best primers, the reaction conditions were optimized. The best reaction conditions were determined by optimizing the basic RPA reaction time (5 min, 10 min, 15 min, 20 min, 25 min, and 30 min) and the reaction temperature (28°C, 30°C, 33°C, 35°C, 37°C, 39°C, and 42°C). Final reaction conditions were determined based on electrophoretic bands.

BVDV LFD-RPA assays

The reaction mixture contained 29.4 µL of A buffer, 2 µL each of 10 µmol/L forward and reverse primers, 0.6 µL of 10 µmol/L probe, and 12.5 µL of ddH_2O was mixed in the RPA lyophilized powder reaction tube thoroughly following the instructions of DNA thermostatic rapid amplification kit (Amp-future Biotechnology, Weifang, Shangdong, China). 1 µL of template (the positive plasmid pUC57-BVDV1254, 1.9×10^{10} copies/µL) and 2.5 µL of B buffer were added to each tube. After mixing and centrifugation briefly, based on the temperature and time determined by previous experiments, the amplification reaction was performed before 10 µL of amplification product was removed and added to a centrifuge tube containing 190 µL of ddH_2O. 50 µL of diluted solution was aspirated and added to the sample well of the lateral flow test strip. The results of control and detection lines was visually observed within 5 min. If the test strip showed 1 red control line in the quality control area and 1 red detection line in the detection area, the result was positive. If the test strip shows 1 red control line in the quality control area and no red detection line in the detection area, the result was negative. If the strip showed 1 red line in the detection zone and no red line in the quality control zone, the result was invalid.

Analysis of sensitivity and specificity

The positive plasmid pUC57-BVDV1254 was serially diluted ten-fold and took the dilution concentrations of 1.9×10^8 to 1.9×10^0 copies/µL as the templates to determine the minimum detection limit. At the same time, a traditional PCR comparison test was carried out with 2×Taq PCR MasterMix II (Tiangen Biochemical, Beijing, China). The PCR primers recommended by Office International Des Epizooties (OIE) for BVDV nucleic acid detection were used [11]. The reaction conditions were 95℃ for 30 s followed by 34 cycles of 95℃ for 5 s, 65℃ for 30 s, 60℃ for 30 s. The sensitivity of the LFD-RPA method was evaluated using the BVDV cDNA as a template. The RNA of the BVDV/NADL strain was extracted with E.Z.N.A.® SE Viral DNA/RNA Kit (OMEGA, Norcross, GA, USA) and reversed transcribed into cDNA using HiScript® III 1st Strand cDNA Synthesis Kit (Novizan Biotechnology, Nanjing, Jiangsu, China). The average cDNA concentration of 5.8×10^2 ng/µL was used as the initial concentration and was ten-fold diluted to 5.8×10^{-4} ng/µL. Reactions were performed under optimal reaction conditions to determine the minimum detection amount of BVDV cDNA. To evaluate the specificity of the basic RPA and LFD-RPA methods, the cDNAs of BRV, BCoV, BKoV, BAstV, BToV, and the genomic DNAs of bovine *Salmonella* and *Escherichia coli* were used as templates. Additionally, 1.9×10^6 copies/µL of BVDV-positive plasmid standard and BVDV-free samples were used as a positive and negative control, respectively.

Analysis of repeatability

The LFD-RPA method was used to conduct three repeatability experiments. Six different concentrations of the positive plasmid from 1.9×10^6 to 1.9×10^1 copies/µL were used as the templates, and the obtained amplification results were analyzed.

Applicability assays

107 blood samples were collected from cattle farms across Ningxia, China. 250 µL serum sample was separated for RNA extraction. The extracted RNA was dissolved in 50 µL of nuclease-free water and used as a template for cDNA synthesis by reverse transcription. The cDNA synthesized from each sample was used as a template in the BVDV basic RPA and LFD-RPA methods and compared with the results of the traditional PCR method.

Results

Screening of basic RPA primers

The candidate primers for the basic RPA assay were screened by performing with TwistAmp Basic reactions. The results showed that, although the third set of primers first amplified specific bands at 5 mins and all four sets of

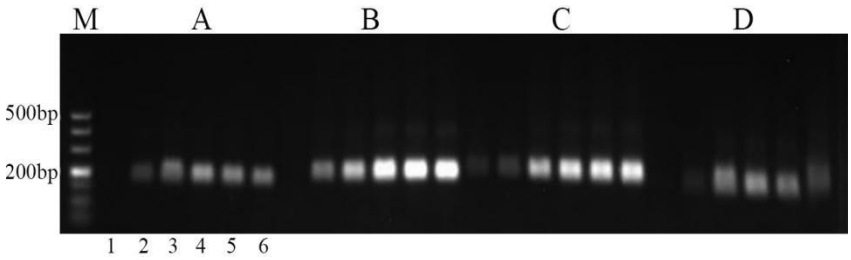

Figure 1. The primers selection for basic RPA. M: D500 marker. A: 5'-NCR-1F/5'-NCR-1R primers. B: 5'-NCR-2F/5'-NCR-2R primers. C: 5'-NCR-3F/5'-NCR-3R primers. D: 5'-NCR-4F/5'-NCR-4R primers. 1-6: reaction times of 5, 10, 15, 20, 25, and 30 mins at 39°C.

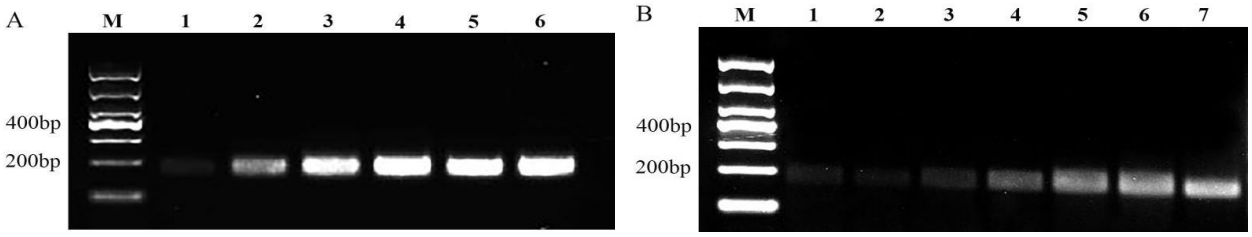

Figure 2. Determination of reaction temperature and time for basic RPA. **A.** Determination of reaction time. M: D1000 marker. Lanes 1-6: reaction times of 5, 10, 15, 20, 25, and 30 mins at 39°C. **B.** Determination of reaction temperature. M: D1000 marker. Lanes 1-7: reaction temperatures of 28, 30, 33, 35, 37, 39, and 42°C for 20 mins.

primers amplified specific bands at 10 mins, the second, third, and fourth sets of primers all showed non-specific bands after 15 mins of amplification (Figure 1). Therefore, the first set of 5'-NCR-1F/5'-NCR-1R primers was selected as the best primer for subsequent research. In addition, the different reaction temperature and reaction time were optimized. The results showed that, when the amplification time was 15 mins or longer, the product could be detected more efficiently (Figure 2A), while the brightest band was observed at 37°C (Figure 2B). Therefore, the optimum reaction conditions of the basic RPA detection method were set as 37°C for 20 mins.

Analysis of sensitivity and specificity of basic RPA

To determine the sensitivity of basic RPA, the assays with template concentrations from 1.9×10^8 to 1.9×10^0 copies/μL were performed. The result showed that the minimum detection limit of the positive plasmid was 1.9×10^2 copies/μL by basic RPA (Figure 3A), while the minimum detection limit of the positive plasmid by PCR was 1.9×10^3 copies/μL (Figure 3B). Under the same conditions, basic RPA was more sensitive than PCR. The basic RPA specificity test showed that only the positive plasmid amplified specific bands, and there was no cross-infection with other pathogens, indicating that the established basic RPA method had good specificity.

Optimal reaction conditions of LFD-RPA

The optimization results of LFD-RPA reaction time showed that a red band appeared on the detection line at 10 mins, and the band became brighter and tended to be stable at 15 mins. Therefore, 15 mins was selected as the optimal reaction time for this test (Figure 4A). The temperature optimization results showed that no bands appeared at 28°C and 30°C. The red bands appeared on the detection line at 33°C, and the bands gradually became brighter from 35°C to 42°C. In order to use the human body temperature for the reaction in an outdoor environment lack of equipment, 35°C was chosen as the optimum reaction temperature in this study (Figure 4B). Then, the complete LFD-RPA reaction system was finalized.

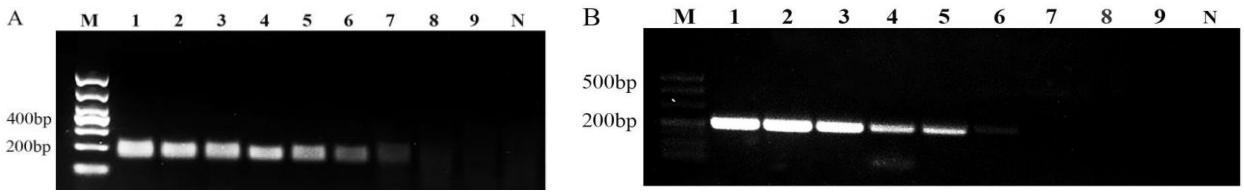

Figure 3. Comparison of the sensitivities of the basic RPA and PCR. **A.** Basic RPA. M: D1000 marker. **B.** PCR. M: D500 marker. Lanes 1 to 9: BVDV positive plasmid from 1.9×10^{8} to 1.9×10^{0} copies/μL, respectively. N: negative control.

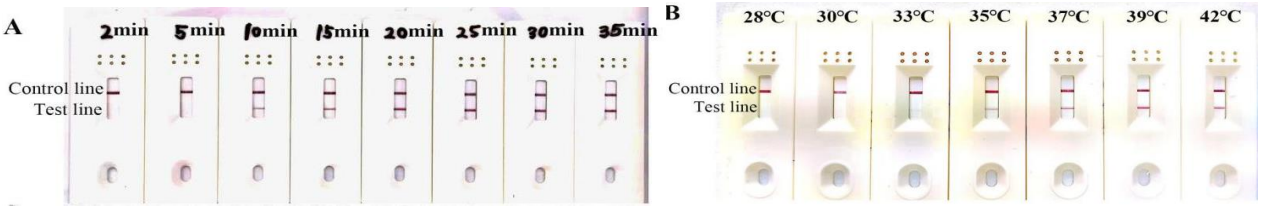

Figure 4. Determination of reaction temperature and time of LFD-RPA. **A.** The LFD-RPA amplification can be visible on the LFD for 15 mins or longer. **B.** The LFD-RPA worked effectively in a broad range of constant reaction temperatures.

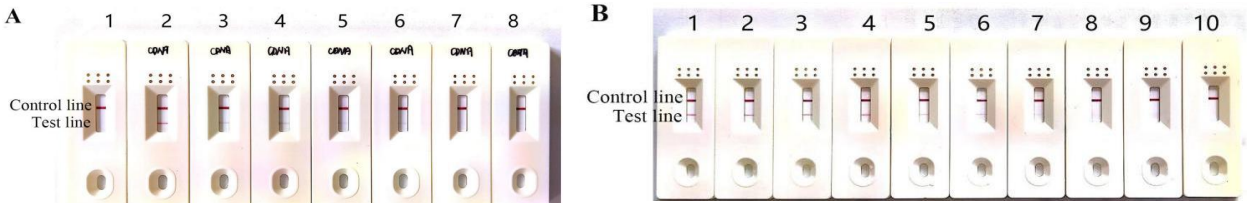

Figure 5. Analysis of sensitivity of the BVDV LFD-RPA assay. **A.** Sensitivity of LFD-RPA. Lane 1: negative control. Lanes 2-8: cDNA concentrations from 5.8×10^{2}-5.8×10^{-4} ng/μL, respectively. **B.** Positive plasmid sensitivity of LFD-RPA. Lanes 1-9: BVDV positive plasmid concentrations from 1.9×10^{8} to 1.9×10^{0} copies/μL, respectively. Lane 10: negative control.

Analysis of sensitivity and specificity of LFD-RPA

The sensitivity of the LFD-RPA method was evaluated with different concentrations of cDNA and the copy number of positive plasmids as templates. The results showed that the minimum detection amount of cDNA was 5.8×10^{-2} ng/μL (Figure 5A), and the minimum detection limit of positive plasmid was 1.9×102 copies/μL (Figure 5B), which was consistent with the minimum detection limit of the basic RPA method and was more sensitive than PCR under the same conditions. The LFD-RPA specificity test showed that only the detection line of the positive plasmid appeared red band, and there was no cross infection with other pathogens, indicating that the established LFD-RPA method has good specificity (Figure 6).

Analysis of repeatability of LFD-RPA

Six different concentrations of positive plasmid from 1.9×10^{6} to 1.9×10^{1} copies/μL were used as the templates and performed three repeatability experiments of the LFD-RPA. The results showed that the minimum limit of the three repeated detections were 1.9×10^{2} copies/μL. There was no red band at 1.9×10^{1} copies/μL, which could be used as a negative control, indicating that the established LFD-RPA detection method of BVDV had good stability (Figure 7).

Evaluation of the BVDV RPA methods with clinical samples

The previously collected bovine serum samples were used to evaluate the established RPA methods in this study. The positive detection rates of traditional PCR, basic RPA, and LFD-RPA

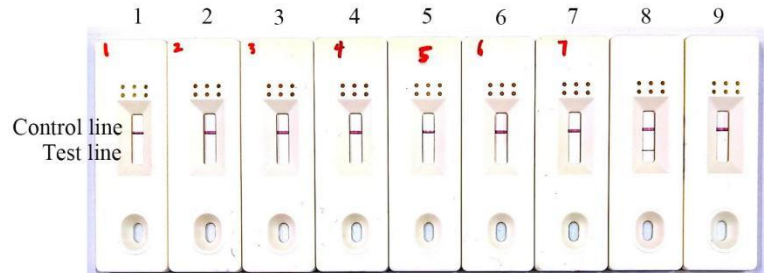

Figure 6. Analysis of specificity of the BVDV LFD-RPA assay. Samples 1-9 were BToV, BKoV, BAstV, BCoV, BRV, *Salmonella*, *E. coli*, positive plasmid, and negative control.

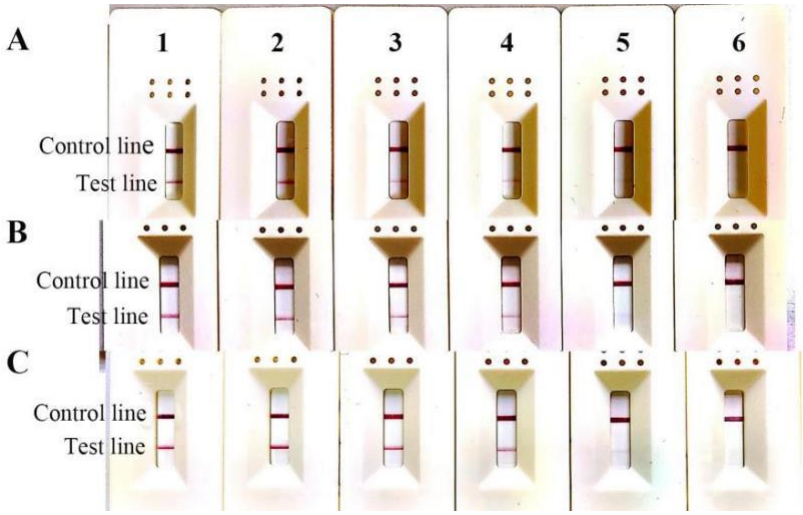

Figure 7. Analysis of repeatability of LFD-RPA. Samples 1-6 were the concentrations of 1.9×10^6 -1.9×10^1 copies/μL, respectively.

were 1.87% (2/107), 2.80% (3/107), and 2.80% (3/107), respectively. Therefore, basic RPA and LFD-RPA had high sensitivity compared with traditional PCR method and were suitable for detection of clinical serum samples.

Discussion

BVDV is one of the most important pathogens of cattle, causing severe economic losses to livestock industries around the world. The establishment of a rapid detection method for BVDV is essential for the control and eradication of BVD infection. In this study, a visualization method for rapid detection of BVDV (LFD-RPA) based on basic RPA was established. Compared with agarose gel electrophoresis, the operation was simple with rapid interpretation, and it could produce visible results without using high-precision equipment. Basic RPA laid the foundation for the establishment of a visual RPA detection method for BVDV. The ultimate goal was to establish a fast, convenient, and visual nucleic acid detection method for production practice and provide the technical means for the prevention and control of BVD. Relevant studies have shown that molecular biology is the main method for detecting BVDV. Zhang *et al*. applied a new TaqMan-MGB probe combined with real-time RT-PCR to identify BVDV-1 and BVDV-2 with the detection limits of 1.72×10^2 copies/μL for BVDV-1 and 2.14×10^2 copies/μL for BVDV-2 [12]. Mungthong *et al*. [13] developed a one-step LAMP method to detect BVDV1 and BVDV2 and the detection limit of the LAMP method was 10^3

copies of DNAs. Zhang *et al*. developed a triplex TaqMan real-time RT-PCR assay for the differentiation of wild-type CSFV, HCLV strains, and BVDV-1. The detection limit for BVDV-1 was 3.2 $TCID_{50}$ [14]. A one-step quantitative SYBR Green I RT-PCR method for the detection of BVDV-1 was reported with the detection limit as low as 10^2 copies/mL of BVDV RNA, which was 10-fold more sensitive than conventional RT-PCR [15]. Although those previous studies could detect BVDV with high sensitivity and high specificity, they all required expensive and complex instruments such as fluorescence quantitative PCR instrument, and complicated operations, which were not suitable for field detection. In addition, the detection times of those methods were longer than that of RPA. In contrast, the advantages of the RPA method were more obvious. In recent years, LFD-RPA detection technology has been widely used for the rapid detection of various viruses, bacteria, and parasites [16-18]. However, there are few reports on the rapid detection of BVDV. Yang *et al* reported the establishment of a rapid detection method for BVDV RPA-LDF [19] with the detection limit of 60 copies/μL, which was consistent with the performance of RT-qPCR assays. In addition, a rapid detection method for BVDV and bovine parainfluenza virus type 3 (BPIV-3) based on RT-RPA combined with LFD was investigated [20]. This method could detect BVDV with a minimum detection limit of 50 RNA molecules and no cross-reactivity with other viruses. Aebischer *et al*. investigated the rapid detection of *Schmallenberg* virus and BVDV genomes using isothermal amplification (LAMP and RPA) and RT-qPCR methods and evaluated their suitability for field applications [21]. Hou *et al*. used RPA amplification combined with lateral flow test strip method to rapidly detect BVDV in bulk milk with a detection limit of 20 copies per reaction, which achieved rapid, sensitive and specific detection of BVDV in bulk milk [22].

Different basic RPA primers were designed in this study. First, a basic RPA detection method for BVDV was established by screening effective primers, and then the reaction temperature and time were optimized. The results showed that the basic RPA reaction could achieve the amplification of target DNA by incubating at 37°C for 20 mins in a simple water bath, which was faster than other detection methods such as PCR. Specificity studies showed that the test had no cross-reaction with other viruses that infected cattle. In terms of analytical sensitivity, the detection limit of the basic RPA established in this study was 1.9×10^2 copies/μL, which was higher than that of ordinary PCR method and consistent with the results of other RPA studies. Wang *et al*. established an RPA assay for African swine fever virus and the detection limit was 10^2 copies [23]. Xu *et al*. established a real-time RPA detection method for rapid detection of genetically modified crops, which could detect as few as 10^2 copies of the target molecule [24]. Poulton *et al*. combined lateral flow chromatography strips with an RPA assay to develop a method for detecting *Schistosoma mansoni* infection with a minimum detection rate generally consistent with this test [25].

Conclusion

The RPA assay was successfully established based on the conserved sequence of the 5'-NCR of the BVDV genome. The established LFD-RPA assay could be used to directly observe the results with the naked eyes and was easy and fast to operate without the requirement of other instruments, which made it suitable for rapid detection in the field. The results of this study had broad application prospects.

Acknowledgements

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References

1. Tautz N, Tews BA, Meyers G. 2015. The molecular biology of pestiviruses. Adv Virus Res. 93:47-160.
2. Bachofen C, Vogt HR, Stalder H, Mathys T, Zanoni R, Hilbe M, *et al*. 2013. Persistent infections after natural transmission of bovine viral diarrhea virus from cattle to goats and among goats. Vet Res. 44(1):32.
3. Larson RL. 2015. Bovine viral diarrhea virus–associated disease in feedlot cattle. Veterinary Clinics of North America: Food Animal Practice. 31(3):367-380.
4. Rodning SP, Givens MD, Marley MS, Zhang Y, Riddell KP, Galik PK, *et al*. 2012. Reproductive and economic impact following controlled introduction of cattle persistently infected with bovine viral diarrhea virus into a naive group of heifers. Theriogenology. 78(7):1508-1516.
5. Lanyon SR, Hill FI, Reichel MP, Brownlie J. 2014. Bovine viral diarrhea: pathogenesis and diagnosis. Vet J. 199(2):201-209.
6. Hou P, Xu Y, Wang H, He H. 2020. Detection of bovine viral diarrhea virus genotype 1 in aerosol by a real time RT-PCR assay. BMC Vet Res. 16(1):114.
7. Falkenberg SM, Dassanayake RP, Neill JD, Ridpath JF. 2017. Improved detection of bovine viral diarrhea virus in bovine lymphoid cell lines using PrimeFlow RNA assay. Virology. 509:260-265.
8. Ridpath JF, Bolin SR, Dubovi EJ. 1994. Segregation of bovine viral diarrhea virus into genotypes. Virology. 205(1):66-74.
9. Piepenburg O, Williams CH, Stemple DL, Armes NA. 2006. DNA detection using recombination proteins. PLoS Biol. 4(7):e204.
10. Daher RK, Stewart G, Boissinot M, Bergeron MG. 2016. Recombinase polymerase amplification for diagnostic applications. Clin Chem. 62(7):947-958.
11. Hoffmann B, Depner K, Schirrmeier H, Beer M. 2006. A universal heterologous internal control system for duplex real-time RT-PCR assays used in a detection system for pestiviruses. J Virol Methods. 136(1-2):200-209.
12. Zhang YQ, Liu HS, Wu XD, Wang XZ, Li JM, Zhao YG, *et al*. 2015. A novel real-time RT-PCR with TaqM an-MGB probes and its application in detecting BVDV infections in dairy farms. Nong Ye Ke Xue Xue Bao. 14(08):1637-1643.
13. Mungthong K, Khaing ST, Otsubo T, Hatanaka C, Yoneyama S, Hisamatsu S, *et al*. 2021. Broad detection and quick differentiation of bovine viral diarrhea viruses 1 and 2 by a reverse transcription loop-mediated isothermal amplification test. J Vet Med Sci. 83(8):1321-1329.
14. Zhang XJ, Han QY, Sun Y, Zhang X, Qiu HJ. 2012. Development of a triplex TaqMan real-time RT-PCR assay for differential detection of wild-type and HCLV vaccine strains of classical swine fever virus and bovine viral diarrhea virus 1. Res Vet Sci. 92(3):512-518.
15. Zhang N, Liu Z, Han Q, Qiu J, Chen J, Zhang G, *et al*. 2011. Development of one-step SYBR Green real-time RT-PCR for quantifying bovine viral diarrhea virus type-1 and its comparison with conventional RT-PCR. Virol J. 8:374.
16. Yin F, Liu J, Liu A, Li Y, Luo J, Guan G, *et al.* 2017. Rapid diagnosis of *Theileria annulata* by recombinase polymerase amplification combined with a lateral flow strip (LF-RPA) in epidemic regions. Vet Parasitol. 237:125-129.
17. Kersting S, Rausch V, Bier FF, von Nickisch-Rosenegk M. 2014. Rapid detection of *Plasmodium falciparum* with isothermal recombinase polymerase. Malar J. 13(99):1475-2875.
18. Jaroenram W, Owens L. 2014. Recombinase polymerase amplification combined with a lateral flow dipstick for discriminating between infectious *Penaeus stylirostris densovirus* and virus-related sequences in shrimp genome. J Virol Methods. 208:144-151.
19. Yang S, Qian YW, Liu KX, Wang C, Tan B, Liu JF, *et al*. 2022. Rapid and sensitive recombinase polymerase amplification combined with lateral flow dipstick for visual detection of bovine viral diarrhea virus. Zhong Guo Shou Yi Ke Xue. 52(06):685-690.
20. Yang S, Wang QY, Tan B, Shi PF, Qiao LJ, Li ZJ, *et al*. 2022. A lateral flow dipstick combined with reverse transcription recombinase polymerase amplification for rapid and visual detection of the BVDV and BPIV3. J Virol Methods. 299:114343.
21. Aebischer A, Wernike K, Hoffmann B, Beer M. 2014. Rapid genome detection of Schmallenberg virus and bovine viral diarrhea virus by use of isothermal amplification methods and high-speed real-time reverse transcriptase PCR. J Clin Microbiol. 52(6):1883-1892.
22. Hou P, Zhao G, Wang H, He C, He H. 2018. Rapid detection of bovine viral diarrhea virus using recombinase polymerase amplification combined with lateral flow dipstick assays in bulk milk. Veterinarski arhiv. 88(5):627-642.
23. Wang J, Wang J, Geng Y, Yuan W. 2017. A recombinase polymerase amplification-based assay for rapid detection of African swine fever virus. Can J Vet Res. 81(4):308-312.
24. Xu C, Li L, Jin W, Wan Y. 2014. Recombinase polymerase amplification (RPA) of CaMV-35S promoter and nos terminator for rapid detection of genetically modified crops. Int J Mol Sci. 15(10):18197-18205.
25. Poulton K, Webster B. 2018. Development of a lateral flow recombinase polymerase assay for the diagnosis of *Schistosoma mansoni* infections. Anal Biochem. 546:65-71.