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Rapid visual detection of bovine viral diarrhea virus (BVDV) using recombinase polymerase amplification with SYBR green I

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Abstract

Background Bovine diarrhea virus (BVDV) is considered to be the most common pathogen causing severe diarrhea in cattle worldwide and can cause Bovine viral diarrhea (BVD). Clinical manifestations of fever, diarrhea, ulcers, and abortions, resulting in significant economic losses to the cattle industry. The development of an efficient, rapid and sensitive assay suitable for field conditions is of great significance for its early detection. Recombinase polymerase amplification (RPA) is a novel nucleic acid amplification method that has been widely used in the diagnosis of infectious diseases.

Results We developed a rapid assay (RPAS) combining RPA with SYBR Green I for the detection of BVDV. The BVDV RPAS assay was performed at 37 °C in 25 min. The minimum detection limit of the RPAS assay is 1×10^9 copies/μL in sunlight and 1×10^5 copies/μL in ultraviolet light, and there is no cross-reactivity with other viruses that cause gastrointestinal and respiratory infections in cattle. The coincidence rate of BVDV RPAS in clinical samples was higher than that of PCR.

Conclusions The BVDV RPAS assay established in this study has high sensitivity and specificity, and is expected to be a powerful tool for the prevention and control of BVD.

Keywords Bovine viral diarrhea virus, Recombinase polymerase amplification, SYBR green I, Visualization detection

Background

Bovine viral diarrhea virus (BVDV) is a positive-sense ribonucleic acid (RNA) virus belonging to the genus Pestivirus in the family Flaviviridae, which causes bovine viral diarrhoea (BVD) [1]. BVD is an acute, subacute, chronic or cryptogenic infection. The clinical manifestations include acute diarrhea, respiratory disease, immunosuppression, and reproductive disorders [2]. BVD is a worldwide epidemic, and is one of the major epidemiological diseases leading to the decline in cattle performance. In 1946, BVDV was first discovered and isolated by Olafson in the United States [3], and since then it has become widely prevalent all over the world. It was introduced into China in the 1980s. In addition to cattle, BVDV can infect sheep, goats [4, 5], pigs [6], deer [7] and members of the camelid family [8]. At present, BVDV is a serious threat to the development of animal husbandry in China and even globally, causing significant economic losses. Therefore, the detection and prevention of BVDV are of great significance.

Currently, the methods for detecting BVDV include virus isolation [9], PCR technique [10, 11], serum

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neutralization test [12], enzyme-linked immunosorbent assay [13, 14], and so on. The virus isolation relies on live viruses to determine a positive result, which is time-consuming and difficult to isolate [9]. The molecular biology assays detecting viral genomic DNA are either time-consuming or expensive or require sophisticated laboratory setup and skilled staff [11]. The serologic methods depend on high quality antisera and are likewise more expensive [14]. Therefore, there is an urgent need to establish a set of rapid, accurate and simple diagnostic methods to provide a new generation of detection technology and means for the on-site diagnosis of this disease.

Recombinase polymerase amplification (RPA) is a nucleic acid level detection technique that is different from PCR, and involves three main enzymes: single-stranded nucleic acid recombinase, single-stranded DNA-binding protein and strand-substituted DNA polymerase [15]. The RPA reaction requires low temperature, a short reaction time, simple operation, does not require special equipment [16], and is also very stable to reagents [17]. Currently, commonly used methods for RPA product detection include agarose gel electrophoresis (AGE) [15], real-time fluorescence [18], chemical color development [19], electrochemistry [20], and lateral flow test strips (LFD) [21]. Our RPAS assay avoids the requirement of an electrical assay. The SYBR Green I dye is embedded in the reaction system, and the amplified RPA product is interpreted by visual observation of the color change in sunlight or UV light, called RPAS. Although RPA assay has been widely used for the detection of various pathogens such as Mycobacterium tuberculosis [22], Heterotrichous nematodes [23], African swine fever virus [24], and Yersinia coli [25]. To date, no RPA assay has been developed for BVDV visual detection.

Therefore, we developed a rapid and sensitive BVDV RPAS assay for the rapid, specific, and sensitive visual detection of BVDV. The performance of the assay was further evaluated by testing nasal swabs, anal swabs and blood serum samples from clinical cattle.

Results

Construction and characterization of the PcDNA 3.1-BVDV Recombinant plasmid

The BVDV *5'UTR* was constructed into the pcDNA 3.1 vector using *Bam HI* and *Hind III* enzymes to obtain the pcDNA 3.1-BVDV recombinant vector, and the vector mapping is shown in Fig. 1A. The pcDNA 3.1-BVDV recombinant plasmid was digested by AGE (1%) to obtain a 365 bp target band (Fig. 1B), and sequencing was also as expected (Figure S1).

Establishment and optimization of reaction conditions for BVDV RPA assay

Candidate primers for the BVDV RPA assay were selected by Twist Amp ® Basic reaction. The AGE (2%) results showed that primer sets *5'UTR*-1 F/R, and *5'UTR*-2 F/R had specific amplification efficiency and produced a product with an expected size of 208 bp (Fig. 2A). The primer set *5'UTR*-3 F/R amplified a 206 bp fragment, but

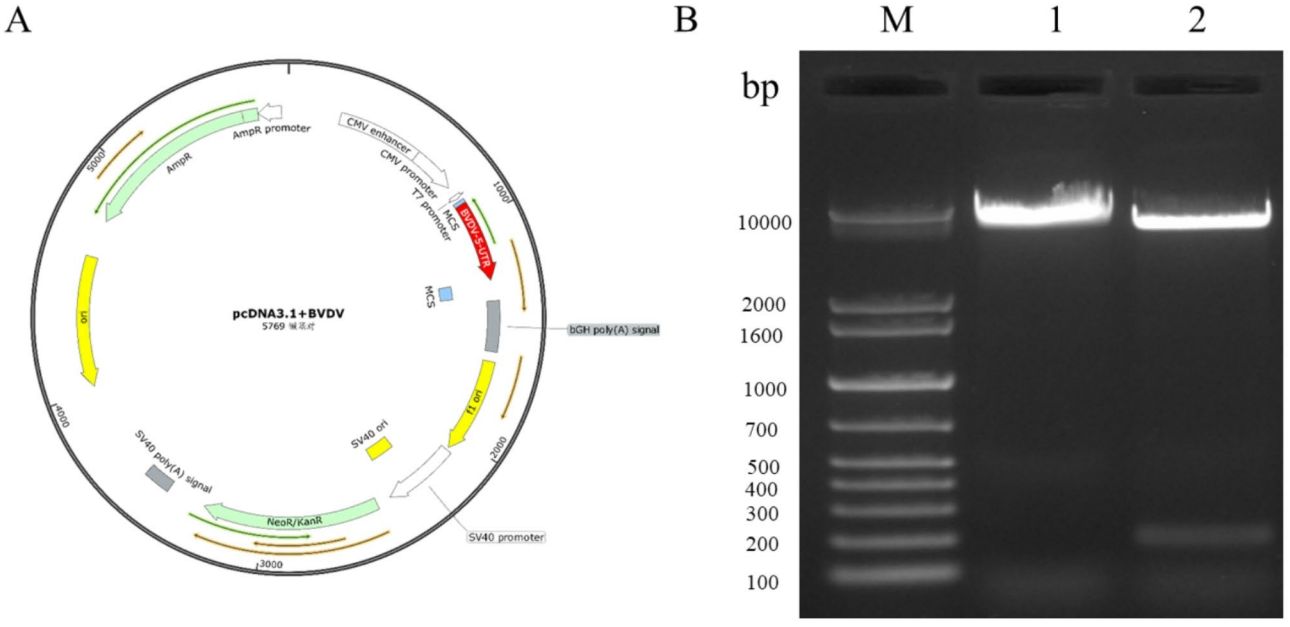

Fig. 1 pcDNA 3.1-BVDV recombinant plasmid construction and characterization. (**A**) pcDNA 3.1-BVDV recombinant plasmid mapping. (**B**) pcDNA 3.1-BVDV enzymatic identification. Lane M: molecular weight standard (1 kb plus DNA Marker); Lane 1: *Bam HI* single enzyme cleavage; Lane 2: *Hind III-Bam HI* double enzyme cleavage, and the expected size of the product was 365 bp

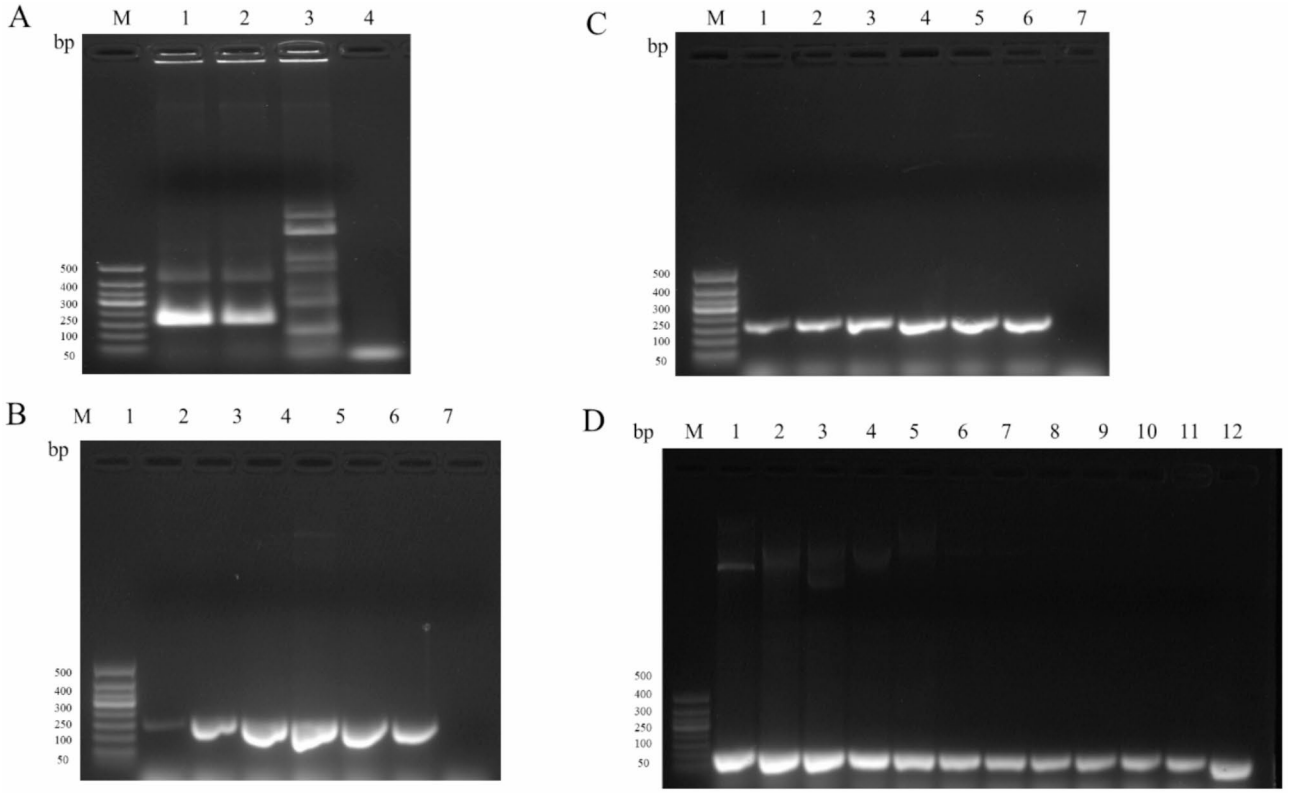

Fig. 2 Optimization of reaction conditions for BVDV-RPA reaction conditions. (**A**) BVDV-RPA primer screen, Lane M: molecular weight standard (50 bp DNA Marker); Lane 1: *5'UTR*-1 primers; Lane 2: *5'UTR*-2 primers; Lane 3: *5'UTR*-3 primers; Lane 4. Negative control. An amplification of 208 bp obtained using *5'UTR-1* primers primer. (**B**) BVDV RPA temperature condition reaction optimization, Lane M: molecular weight standard (50 bp DNA Marker); Lane 1: 30 ℃; Lane 2: 32 ℃; Lane 3: 35 ℃; Lane 4: 37 ℃; Lane 5: 39 ℃; Lane 6: 42 ℃; Lane 7: Negative control. (**C**) Optimization of BVDV RPA time conditions, Lane M: molecular weight standard (50 bp DNA Marker); Lane 1: 10 min; Lane 2: 15 min; Lane 3: 20 min; Lane 4: 25 min; Lane 5: 30 min; Lane 6: 40 min; Lane 7: Negative control. (**D**) Reproducibility of BVDV RPA assay. Lane 1 ~ 11: 10^{12} copies/μL ~ 10^{1} copies/μL

with more non-specific bands and primer dimerization. Under the same conditions, the *5'UTR*-1 F/R produced the brightest and best amplification of the target band. Therefore, it was subsequently used in the BVDV RPA assay.

The optimal conditions for BVDV RPA assay were determined by testing different temperatures (30 °C, 32 °C, 35 °C, 37 °C, 39 °C, 42 °C) and reaction times (10 min, 15 min, 20 min, 25 min, 30 min, 40 min). As shown in Fig. 2B, the target fragment gradually became brighter as the reaction temperature increased. The color change was the most intense at 37 °C. At 39 °C, the brightness of the target fragment gradually decreased. Therefore, 37 °C was determined to be the optimal reaction temperature. The same method was used to determine the optimal reaction time for the BVDV assay as 25 min (Fig. 2C). We performed 12 BVDV RPA assays on standard DNA and verified the good reproducibility of the assay (Fig. 2D).

Sensitivity and specificity evaluation of the BVDV RPAS assay

To optimize the concentration of SYBR Green I and to determine BVDV RPA assay sensitivity and reproducibility, we added different concentrations of SYBR Green I to the reaction system and performed three independent biological replicates. Under sunlight, the minimum detection limit of BVDV RPAS was 10×, with 50× being the most effective (Fig. 3A). Under UV, the minimum detection limit of BVDV RPAS was 0.16×, with 50× being the most effective (Fig. 3B). Therefore, 50× was selected for the BVDV RPAS determination.

The quantified pcDNA 3.1-BVDV recombinant plasmid was diluted in 11 gradients, i.e., 1×10^{6} copies/μL-1×10^{-4} copies/μL. The sensitivity of RPA assay was detected by the fluorescent dye technique or electrophoretic detection technique. Nuclease-free water was used as a negative control. Under sunlight, the minimum detection limit for BVDV RPAS visualization was 1×10^{3} copies/μL. Under UV, the lowest detection threshold for BVDV RPAS was 1×10^{1} copies/μL. The minimum detection limit for BVDV RPA-AGE was 1×10^{-1} copies/μL. The minimum detection limit for BVDV PCR-AGE was

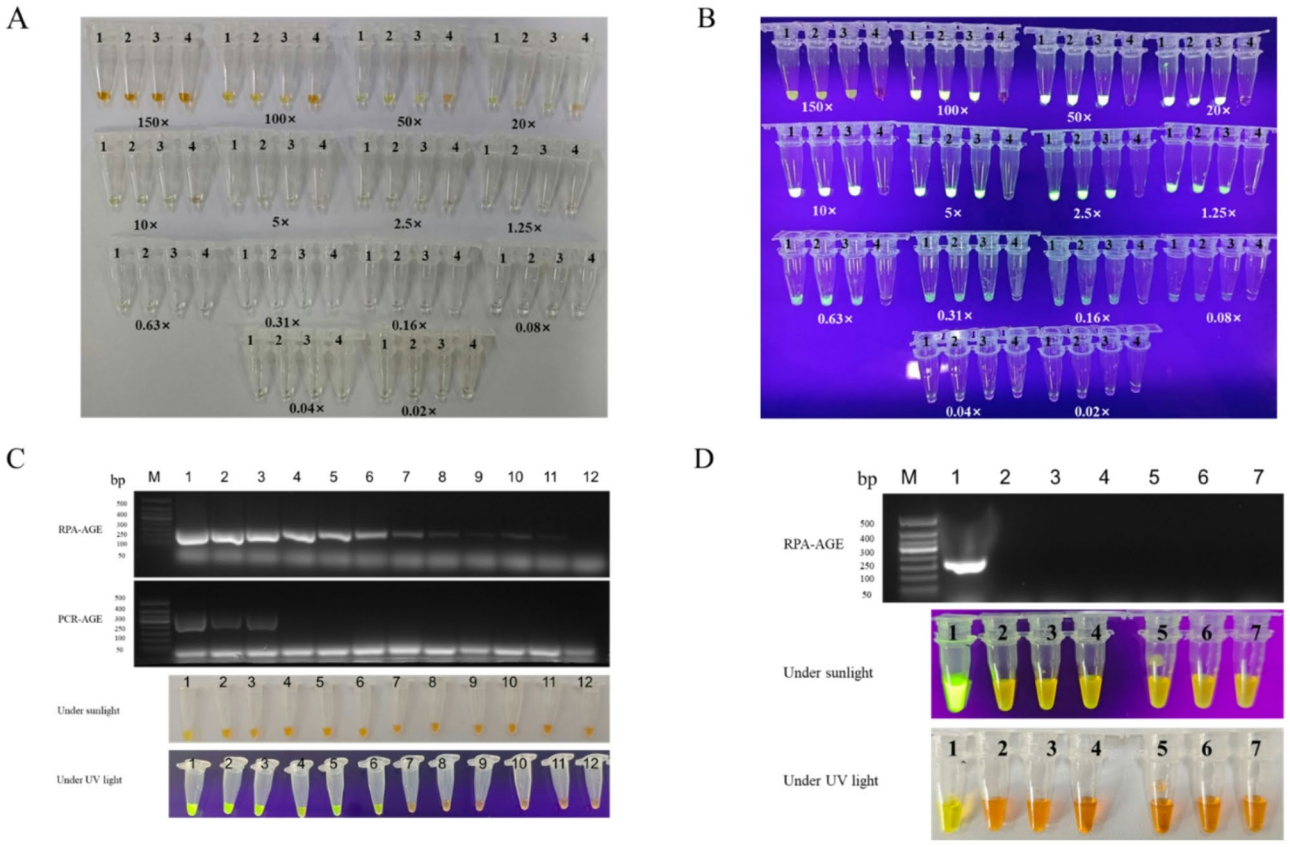

Fig. 3 Specificity and sensitivity evaluation of BVDV RPAS assay. (**A**) Under sunlight, BVDV RPA visualization graph. (**B**) Under UV light, BVDV RPA visualization graph. Tube 1 ~ 3: three replicates; Tube 4: negative control. (**C**) Visualization assay identification of the BVDV RPAS assay. Lane M: molecular weight standard (50 bp DNA Marker); Lane 1 ~ 11: 1×10^6 copies/μL ~ 1×10^{-4} copies/μL; Lane 12: Negative control. (**D**) Specificity of BVDV RPAS assay. Lane M: molecular weight standard (50 bp DNA Marker); Lane 1: BVDV; Lane 2: IBRV; Lane 3: BCoV; Lane 4: BRV; Lane 5: BNoV; Lane 6: BAstV; Lane 7: Negative control

1×10^4 copies/μL (Fig. 3C). The detection limit of BVDV RPAS was much higher than that of BVDV PCR.

The specificity of the BVDV RPA assay was evaluated using six bovine respiratory and diarrhoea-associated viruses. The results showed (Fig. 3D) that only the *5'UTR* gene purpose band appeared in the pcDNA 3.1-BVDV recombinant plasmid. The others (IBRV, BCoV, BRV, BNoV, BAstV, and nuclease-free water), demonstrating the high specificity of our assay.

Validation of the BVDV RPAS assays on clinical samples

To evaluate the practical clinical application of the BVDV RPAS assay, we detected cattle nasal swabs, anal swabs, and blood serum samples from cattle with significant respiratory and diarrheal disease. The results are shown in Fig. 4; Table 1, the positive rate of BVDV RPA-AGE and PRAS in cattle nasal swab and blood serum samples was 55% (22/40), while and 47.5% (22/40) for PCR-AGE. Two samples were negative for PCR and positive for RPAS (Fig. 4A). In cattle anal swabs samples, BVDV RPA-AGE and RPAS assays were consistent, with 55% positive rate (Fig. 4B) and 35% for PCR-AGE (Fig. 4C). Therefore, the proposed BVDV RPA/RPAS assay is feasible for BVDV detection in unknown samples.

Discussion

BVDV is an important pathogen in cattle that can cause BVD and is also an agent of bovine respiratory disease, which is distributed globally and can cause significant economic losses [26, 27]. Based on phylogenetic analysis of the *5'UTR* gene into two distinct genetic species, BVDV-1 (1a-1t) and BVDV-2 (2a-17c) [28]. BVDV-1 is predominantly prevalent in China [29]. The RPA assay is an emerging isothermal nucleic acid amplification technique that has been applied to detect pathogens in a variety of samples, such as blood [30], food [31], and feces [32]. In our study, we established BVDV RPAS assay based on BVDV-1 and validated it in cattle nasal swabs, anal swabs and blood serum samples.

The BVDV RPAS assay established in this study can be performed in only 25 min at 37 °C, without expensive or large instruments, with simple incubation conditions, even at body temperature [33]. In our study, the colour change was strongest at 37 °C and gradually decreased at 39 °C, which may be due to inactivation of the enzyme in

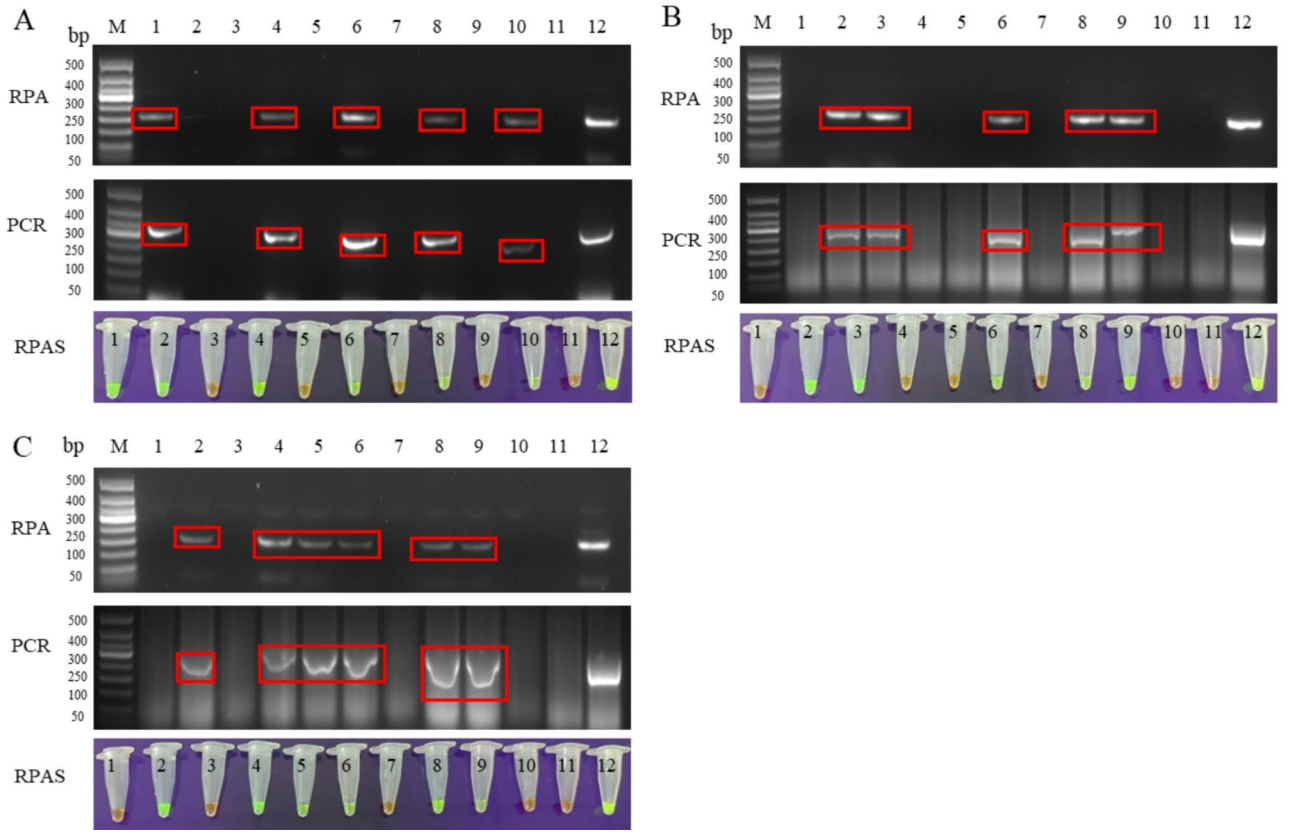

Fig. 4 Selected clinical samples for BVDV RPAS assay. (**A**) Cattle nasal swab sample testing; (**B**) Cattle anal swab sample testing; (**C**) Cattle blood serum sample testing. Lane M: molecular weight standard (50 bp DNA Marker); Lane 1 ~ 10: Cattle clinical samples; Lane 11: Negative control; Lane 12: Positive control. The expected size of the RPAS assay product is 208 bp, and the expected size of the PCR assay product for clinical samples is 354 bp. The testing of other samples is shown in Figure S2、S3、S4

Table 1 Comparison of RPA-AGE, PCR-AGE and RPAS using clinical samples

Sample Type	RPA-AGE(Positive/total)	PCR-AGE(Positive/total)	RPAS(Positive/total)
Nasal swab	22/40	19/40	22/40
Anal swab	20/40	19/40	20/40
Blood serum	22/40	14/40	22/40
Total	64/120	52/40	64/120

the system at high temperature, or the decrease of primer annealing efficiency by high temperature. The conventional PCR products are detected by AGE, whereas qPCR methods require a companion instrument. In terms of reaction time, the RPAS assay gives faster results (< 45 min) than the PCR/qPCR method (> 1 h) [34]. The cost of the RPAS platform is high (~$60.25) compared to conventional PCR (~$3.70) and real-time fluorescent quantitative PCR (~$2) [35, 36]. However, the RPA assay is resistant to a variety of known PCR inhibitors, including hemoglobin, heparin, undiluted serum, and ethanol [37]. In addition, we added SYBR Green I for endpoint monitoring, reducing the overall cost of the test and making it suitable for resource-limited areas. The SYBR Green I is a phorbol ester dye that has been used as a nucleic acid stain for the assay platform in several studies. It binds preferentially to double-stranded DNA, producing a DNA dye complex that emits a green light and is detectable by the naked eye [38–40]. Although there have been studies combining LFD with RPA assays, it is relatively costly compared to the SYBR Green I assay [41]. Meanwhile, we found that the minimum detection limit of RPAS was 100-fold higher than that of PCR-AGE under sunlight and 1,000-fold higher than that under UV.

The RPAS assay is expected to challenge the dominant position of PCR molecular detection with its advantages. However, it still has some limitations. False positives may exist in the RPAS assay, so the reaction tubes should be opened and closed carefully and gloves should be changed frequently. The SYBR Green I should be added to the tube cap in advance when visualizing the assay. Meanwhile, the technology is more costly and less

Table 2 Specific experimental strains of BVDV RPAS assay

Virus	GenBank ID	Gene
bovine rhinotracheitis virus(IBRV)	MK654723.1	*gB*
Bovine coronavirus (BCoV)	KT318096.1	*N*
Bovine rotavirus (BRV)	M92651.1	*VP6*
Bovine Norovirus (BNoV)	KX189094.1	*RdRp*
Bovine Astrovirus (BAstV)	MH123914.1	*ORF1ab*

popular, but the premixing of the RPA assay reaction system can be completed in advance, and the total volume can be reduced to 12.5 μL (1/4 of the original volume), thus reducing the cost of the assay. In addition, low-cost commercial nucleic acid extraction methods for field samples based on magnetic bead technology and the heated NaOH method can be used to further reduce the cost of the RPAS assay [42]. Furthermore, equipped with a miniature UV torch, it increases the sensitivity of sample detection and avoids the subjectivity of visual judgement.

In summary, the BVDV RPAS assays with high specificity and sensitivity is simple, rapid and reliable for BVD detection. It is suitable for rapid testing in under-equipped diagnostic laboratories as well as for BVDV diagnosis in quarantine stations and cattle farms, which is of great significance for controlling BVD.

Conclusions

The BVDV RPAS assay can be performed in 25 min at 37 °C with high specificity and no cross-reactivity with other pathogens. The detection limit of the RPAS assay was 1×10^3 copies/μL, which combines the sensitivity and specificity of traditional PCR with ease of operation and observable results. Therefore, the BVDV RPAS assay could be a potential solution for BVDV detection in resource-limited laboratories or in the field.

Methods

Viruses and clinical samples

The BVDV *5'UTR* gene information is based on the BVDV-1 (MA/101/05, GenBank ID: MW054940.1). Other bovine virus used for cross-reactivity testing included Bovine rhinotracheitis virus (IBRV), Bovine coronavirus (BCoV), Bovine rotavirus (BRV), Bovine Norovirus (BNoV), Bovine Astrovirus (BAstV). Information on the virus is given in Table 2.

The clinical samples were 10 nasal swabs, anal swabs, and blood serum samples of diarrheic cattle collected separately in the Ningxia region (July 2022).

Generation of the pcDNA3.1-5'UTR standard plasmid

Considering the applicability of the assay and the limitation by the source of the pathogen, we screened and artificially synthesised a 365 bp sequence of the 5'UTR gene (GenBank ID: MW054940.1) of BVDV as the target gene for amplification. The target gene was constructed into the pcDNA 3.1 vector using *Hind III* and *Bam HI* enzyme (NEB, item no. R0104/ R0136) restriction enzymes, and transformed into *E. coli* receptor cell Top10 to obtain the pcDNA 3.1-BVDV recombinant plasmid.

Table 3 Primer information for BVDV RPAS and PCR assay

Primer name	Primer sequence (5'-3')	Length (bp)
5'UTR-1 F	GAGTTCGTTGGATGGCTGAAGCCCTGAGTA	208
5'UTR-1R	AGTAGCATTACAGTGGGCCTCTGCAGCACC	
5'UTR-2 F	TGGTGAGTTCGTTGGATGGCTGAAGCCCTGA	208
5'UTR-2R	GCATTACAGTGGGCCTCTGCAGCACCCTAT	
5'UTR-3 F	GCATTACAGTGGGCCTCTGCAGCACCCTAT	206
5'UTR-3R	AGTAGCATTACAGTGGGCCTCTGCAGCACC	
PCR-F	TCTCGACCGGGGACATTATCT	354
PCR-F	CATTCTGCAACGCGAAGGTG	

Plasmid extraction was performed according to the instructions of the Axygen Plasmid Extraction Kit (item no. AP-MN-P) and measured using a Nanodrop ND-8000 spectrophotometer (Thermo Scientific, Dreieich, Germany). The DNA copy number was calculated by the following formula: DNA copy number = $(M \times 6.02 \times 10^{23} \times 10^{-9})/(n \times 660)$, where M is the amount of DNA in nanograms, and *n* is length of the plasmid in bp.

The RPAS primers were manually designed based on the conserved region of the *5'UTR* gene of BVDV according to the instruction provided by the RPA assay manufacturer Twist Dx (Cambridge, UK). The primers were screened by observation at 2% AGE. Additionally, common PCR primers were designed for the constructed plasmid sequences (Table 3). All primers were synthesized by Sangon Biotech (Shanghai, China).

Establishment and optimization of reaction conditions for BVDV RPA assay

Referring to the instructions of the Twist Amp ® Basic RPA kit (Twist Dx, No. 10270-106), RPA assay was performed using the extracted recombinant plasmid pcDNA 3.1-BVDV as a template. The RPA reaction system consisted of 2 μL of template, 11.2 μL of sterile deionized water, 29.5 μL of RPA reaction buffer, 2.4 μL each of the F and R primers, and 2.5 μL of magnesium acetate solution, which was mixed well and then fully solubilized in 4 mg of RPA Basic lyophilisate. The RPA reaction programme was 39 °C for 20 min. The products were electrophoresed on a 2% AGE. The results were visualized on a UV gel imaging system and photographed. A portion of the amplified product was mixed with l μL of SYBR Green I fluorescent dye (Solarbio, No. SY1020) and irradiated with UV light at 395 nm. To provide more visualization of the experimental results, we present a cropped gel image, which is free of samples and bands, and finally

showing only images of the gels containing the target bands and Maker.

The main parameters of RPA assay are amplification temperature and reaction time. To optimize the BVDV RPA assay, various reaction temperatures (30 °C, 32 °C, 35 °C, 37 °C, 39 °C, 42 °C), and times (10 min, 15 min, 20 min, 25 min, 30 min, 40 min) were tested according to the manufacturer's recommended protocol. The optimal reaction was determined by the most visible color on the naked eye detection line or a specific band in the agarose gel. Furthermore, the RPA assay was tested using the standard DNA in 12 replicates.

Sensitivity and specificity evaluation of the BVDV RPAS assay

The specificity of the assay was assessed in bovine respiratory or diarrhea virus pathogens with similar clinical signs. BVDV, IBRV, BCoV, BRV, BNoV, and BAstV recombinant plasmids were tested separately according to established RPA assays, and nuclease-free water was used as a nontemplate control in the assay.

To investigate the sensitivity of the RPAS assay, the quantified pcDNA 3.1-BVDV plasmid was subjected to 11 gradients of dilution, i.e., 1×10^6 copies/μL-1×10^{-4} copies/μL. The sensitivity of the RPAS assay was tested by fluorescent dye technique as well as electrophoretic detection technique. The negative control template was still nuclease-free water. Comparison was also made with normal PCR.

Validation of the BVDV RPAS assays on clinical samples

To effectively examine the detection effect of BVDV RPAS assay on clinical samples, we tested 120 samples (40 nasal swabs, 40 anal swabs, and 40 sera) of bovine respiratory syndrome and diarrhea syndrome with obvious clinical symptoms in Ningxia (collected in July 2022) by RPAS assay. The nuclease-free water was used as a negative control, synthetic recombinant plasmid pcDNA 3.1-BVDV was used as a positive control. The results were also compared with normal PCR to validate the RPAS assay.

Swab samples were resuspended in virus preservation solution, shaken for 10 min, centrifuged at 12,000 rpm for 10 min to remove the solid precipitate, and filtered in a sterile manner through a 0.22 μm filter membrane. Blood samples were centrifuged at 3,000 rpm for 10 min after an overnight incubation at 4 °C, and blood serum was extracted for subsequent tests. Viral genomic DNA/RNA was extracted using the TianGen Viral Genomic DNA/RNA Extraction Kit (Item DP315). The RNA was reverse transcribed into cDNA by the Prime Script™ II 1st Strand cDNA Synthesis Kit (No. 6210 A) from TAKARA. The reverse transcribed cDNA and extracted DNA were subjected to PPA assay or PCR assay. Amplification products were detected by 2% AGE or visualization.

Abbreviations

AGE	Agarose gel electrophoresis
BAstV	Bovine astrovirus
BCoV	Bovine coronavirus
BNoV	Bovine norovirus
BRV	Bovine rotavirus
BVD	Bovine viral diarrhea
BVDV	Bovine viral diarrhea virus
BVD-MD	Bovine viral diarrhea-mucosal disease
IBRV	Bovine rhinotracheitis virus
LFD	Lateral flow test
RNA	Ribonucleic acid
RPA	Recombinase polymerase amplification
RPAS	Recombinase polymerase amplification combined with SYBR Green I

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12917-025-04640-z.

Supplementary Figure S1: Sequencing results of BVDV ***5'UTR*** gene

Supplementary Figure 2: Results of BVDV RPAS for other samples from nasal swabs of beef cattle

Supplementary Figure 3: Results of BVDV RPAS for other samples from anal swab of beef cattle

Supplementary Figure 4: Results of BVDV RPAS for other samples from blood serum of beef cattle

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Not applicable.

Author contributions

Lingling Jiang study design, method establishment, data organization, manuscript writing and revision. GANG Zhang collaborated in data preparation, analysis and interpretation. Pu Wang, Xiaoxia Niu, Qiang Liu, Ruijin Liang, and Sinong Zhang participated in sample collection and preparation, article editing and proofreading. Yong Li researched design, analysis, funding acquisition and writing-review and editing. All authors contributed to the article and approved the submitted version.

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Data availability

The dataset analyzed during the current study is available from the corresponding author on reasonable request. The nucleotide sequences under the relevant accession numbers (MW054940.1; MK654723.1; KT318096.1; M92651.1; KX189094.1; MH123914.1) analyzed during the current study are available in the GenBank repository, https://www.ncbi.nlm.nih.gov/nucleotide/.

Declarations

Ethics and consent to participate declarations

Cattle nasal swabs, anal swabs and blood serum samples used in this study were collected at the cattle feedlot. The animal research protocol was approved by the Ningxia University Technology Ethics Committee (approval no. NXU-2024-003).

Consent for publication

Not applicable.

Competing interests
The authors declare no competing interests.

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