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Detection of BVDV - Comparative Evaluation of RT-qPCR, PCR and RT-LAMP

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ABSTRACT

***Background*:** Bovine viral diarrhea virus (BVDV) causes various diarrhea symptoms in cattle, which leads to the mortality increase of infected animals. Therefore, this disease causes significant economic losses of cattle industry over the world. Recent advances in molecular diagnosis methods based on PCR technique allow rapid detection of BVDV in the clinical sample. However, each method has its weaknesses in the specificity and sensitivity. Therefore, it is highly necessary to develop a more specific and precise detection method. Loop-mediated isothermal amplification (LAMP) offers an alternative DNA amplification method, which amplifies DNA with high specificity, efficiency and rapidity. Compared with the PCR-based assays, LAMP is more resistant to PCR inhibitors and can be carried out in a shorter reaction time. The present study aimed to select an accurate, quick and cost-effective detection assay of bovine viral diarrhea virus (BVDV).
***Materials, Methods & Results*:** Specific primers were designed and synthesized using Primer Premier 5.0 based on NADL strain of BVDV and BVDV1/JL. The conventional PCR was established according to TaKaRa Ex Taq reagent manual and the reaction systems and reaction conditions were optimized for improving reaction specificity and amplification efficiency. Reverse transcription real-time fluorescence quantitative PCR (RT-qPCR) was constructed using according to the TB Green Premium Ex Taq reagent instruction. Five gradients of primer concentrations (6 μM, 8 μM, 10 μM, 12 μM, 14 μM and 16 μM) were set to select an optimum concentration. The appropriate annealing temperature was determined based on 8 gradients temperatures from 53°C to 61°C within 20 s - 80 s. The kinetics curve, standard curve and melting curve of RT-qPCR amplification were drawn based on the recombinant plasmids of 8.134×10^8 - 8.134×10^4 copies/μL. The specificity between 3 methods was evaluated on the bases of simultaneous detection of 6 viruses including BVDV, BPV, BPIV, BCoV, BRV and CSFV. Both sensitivity and repeatability of 3 assays were also compared according to determining different concentrations of plasmid standards from 8.134×10^{10} to 8.134×10^0 copies/μL and by testing plasmid standards in triplicate. The coefficient of variation of the intra-assay and inter-assay were calculated. Kappa consistency testing and pairing χ^2 test were performed to assess their clinical practicality. Three developed methods of PCR, RT-qPCR and RT-LAMP could only detect BVDV, indicating that the developed methods had excellent specificity. Eleven gradients of plasmid standards from 8.134×10^{10} to 8.134×10^0 copies/ μL were tested with these detection assays. Minimum detection limits were 8.134×10^4, 8.134×10^1 and 8.134×10^2 copies/ μL for PCR, RT-qPCR, and RT-LAMP, respectively. The RT-qPCR method has the highest sensitivity, which was 10-folds and 1,000-folds higher than RT-LAMP and PCR. In conclusion, the constructed PCR, RT-qPCR and RT-LAMP assays had excellent specificity, sensitivity and reproducibility. The sensitivity of RT-qPCR was 10-folds and 1,000-folds higher than RT-LAMP and PCR.
***Discussion*:** In the present study, the conventional PCR, RT-qPCR and RT-LAMP methods were established and optimized based on the specific primers of BVDV NADL strain. The results indicated the developed PCR had excellent specificity. Meanwhile, the comparisons between PCR, RT-qPCR and RT-LAMP assays indicated that these methods had excellent specificity only for BVDV. The minimum detection limits for PCR, RT-qPCR and RT-LAMP were 8.134×10^4, 8.134×10^1 and 8.134×10^2 copies/μL, respectively. RT-qPCR sensitivity was 10-folds and 1,000-folds higher than that of RT LAMP and PCR. Kappa and McNemar calibration verified and supported these results. Our findings provided the technical support for early screening and preventing of BVDV.

Keywords: Bovine viral diarrhea virus, RT-LAMP, RT-qPCR.

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INTRODUCTION

Bovine viral diarrhea virus (BVDV) are critical pathogenic factors causing viral diarrhea of calves [27]. The disease causes various diarrhea symptoms in cattle, especially calves leading to the mortality increase [3,6]. Therefore, it has caused significant economic losses of cattle industry over the world [17]. Recent molecular diagnosis allows rapid detection of BVDV [19,20]. Furthermore, bovine-derived biological materials (BDBMs) are often contaminated by BVDV [5]. It is highly necessary to exactly detect of BVDV in BDBMs [10,12]. Several methods are utilized to detect BVDV antigens including PCR [6]. However, each method has its weaknesses in specificity and sensitivity [11]. It is necessary to develop a more specific and precise protocol that may decrease operation time to less than 30 min [7,15]. Loop-mediated isothermal amplification (LAMP) offers an alternative DNA amplification method with high specificity, efficiency and rapidity [7,18]. Compared with PCR-based assays, LAMP is more resistant to PCR inhibitors with using a simple water bath and a shorter reaction time [25]. LAMP assay is more suitable than PCR assays (nested and real-time qPCR), thereby providing on-site detection of a pathogen without requiring sophisticated equipment [7,11,23].

In the present study, we established 3 detection methods including conventional reverse transcription PCR, reverse transcription real-time fluorescence quantitative PCR (RT-qPCR) and reverse transcription LAMP (RT-LAMP) for detecting BVDV nucleotide. The specificity, sensitivity and stability were compared expecting to cherry-pick an accurate, sensitive, quick and cost-effective assay.

MATERIALS AND METHODS

Viruses and clinical samples

NADL strain of Bovine viral diarrhea virus (BVDV), Bovine papillomavirus (BPV), Bovine parainfluenza virus 3 (BPIV3), Bovine coronavirus (BCoV), Bovine reovirus (BRV), and Swine fever virus (CSFV) were purchased from the America Type Culture Collection (ATCC) or preserved in our laboratory, which were used as additional controls to test specificity. In addition, clinical samples were collected from 67 clinical bovine diarrhea samples harvested in Zhangye city and Lanzhou city of Gansu province from June 2021 to June 2022, and stored at -80°C in the laboratory. The feces samples were thoroughly mixed and ground to form a suspension with 1 × PBS buffer (1:5), from which the supernatant was acquire by centrifuging at 4°C at 8,000 *g* for 10 min.

Construction of recombinant plasmid standards and copies

Viral total RNA was extracted from the virus culture supernatant and clinical samples using QIAamp MinElute Virus Spin Kit[1] referring to the manufacturer's instruction. The extracted RNAs of BVDV were reversely transcribed into cDNAs. The RNA and cDNA were resuspended in DEPC-treated water and stored at -80°C. The entire PCR sequence was analyzed using a 5'UTR non coding region primer (BVDV-5' UTR-F/R) at 94°C for 5 min; denaturation at 94°C for 30 s, annealing at 55.3°C for 45 s, and extension at 72°C for 30 s, a total of 35 cycles and extend at 72°C for 10 min.

DNA/PCR products were ligated into pMD18-T vector4 for 2 h at 16°C. Then, BL-21 competent cells were transferred with the recombinant plasmids of DNA/PCR products and pMD18-T at 37°C overnight. The recombinant plasmids were extracted using EasyPureR Plasmid MiniPrep Kit[2] and sequenced. Their sequence homology was compared with those of NCBI Genbank after they were sequenced by Shanghai Sangon Biotec[3]. The concentrations of constructed recombinant plasmids were determined utilizing the Thermo Fisher micro nucleic acid protein detection system[4].

Primers and design

Specific primers were designed and synthesized using Primer Premier 5.0 based on the nucleotide sequences in 5' non coding region (5' UTR) of NADL strain of BVDV (GenBank Accession: AM709624.1, NC001461.1, AJ133738.1) and BVDV1/JL (GenBank accession: KF501393) published in GenBank. One pair of primers (BVDV-5'UTR-F/R) was designed to construct standard plasmid (Table 1). One pair of primers (5'UTR-PCR-F/R) was designed to establish conventional PCR method. Another pair of primers (RT-qPCR-F/R) was also designed to built reverse transcription real-time fluorescence quantitative PCR (RT-qPCR). Three sets of primers (LAMP-1, LAMP-2, LAMP-3) were designed to establish RT-LAMP. NCBI Primer-BLAST and Primer Explorer V5 verification were performed for all primers. The designed primers were synthesized[5]. The specificity of primers was verified with Primer-BLAST at NCBI online.

Table 1. Primers used in the experiments for detection of BVDV.

Gene	Primers(5' → 3')	Product (bp)
BVDV-5'UTR	F: GTATACGAGAATTAGAAAAGGCACT R: GTGCCATGTACAGCAGAGATTT	385
PCR	F: AATGAGGGGGGTAGCAACAGTG R: CAGGTTAAGATGTGCTTTGGGCAT	143
RT-qPCR	F: AATGAGGGGGGTAGCAACAGTG R: CAGGTTAAGATGTGCTTTGGGCAT	143
LAMP-1	1F3: GCCATGCCCTTAGTAGGACT 1B3: AGCACCCTATCAGGCTGTA 1FIP:CGAACCACTGACGACTACCCTG-GGTAGCAACAGTGGTGAGTT 1BIP:AAGGTCTCGAGATGCCACGTG-CTGCTTTTACCTGGGCGAC 1LF: GGGCTTAAGCCATCCAACG 1LB: GACGAGGGCATGCCCAAA	160
LAMP-2	2F3: GCCATGCCCTTAGTAGGACT 2B3: AGCACCCTATCAGGCTGTA 2FIP:CGACTACCCTGTACTCAGGGCT-TAATGAGGGGGGTAGCAACA 2BIP:AAGGTCTCGAGATGCCACGTG-CTGCTTTTACCTGGGCGAC 2LF: CCATCCAACGAACTCACCAC 2LB: GCACATCTTAACCTGAGCGG	170
LAMP-3	3F3: ATGAGGGGGGTAGCAACA 3B3: AGCACCCTATCAGGCTGTA 3FIP:GCGTCGAACCACTGACGACT-GTGGTGAGTTCGTTGGATGG 3BIP:AAGGTCTCGAGATGCCACGTG-ACTGCTTTTACCTGGGCG 3LF: ACCCTGTACTCAGGGCTTAA 3LB: GCACATCTTAACCTGAGCGG	151

Establishment of conventional PCR and optimization of conditions

The conventional PCR for BVDV detection was established according to TaKaRa Ex Taq reagent manual[6] as described early [9,12]. The reaction systems and reaction conditions were optimized for improving reaction specificity and amplification efficiency. BVDV-5 'UTR, 5' UTR-PCR and LAMP-3 primers (F3/B3) were amplified with this method to verify their specificity by utilizing 25 μL reaction system at 94°C for 5 min, 98°C for 30 s, 55°C for 30 s, 72°C for 1 min, 35 cycles; at last 72°C for 10 min.

Establishment and optimization of RT-qPCR

RT-qPCR for BVDV was constructed using chimeric fluorescence method according to the TB Green Premium Ex Taq reagent instruction. The basic reaction systems and conditions included TB Green Premium Ex Taq (2X) ROX plus 10 μL, 0.4 μL (10μM) of each forward and reverse primers, 2 μL (< 100ng) of DNA, 7.2 μL dd-water with a total volume of 20 μL at 95°C for 30 s, 95°C for 5 s, 60°C for 34 s for 40 cycles.

Five gradients of primer concentrations (6 μM, 8 μM, 10 μM, 12 μM, 14 μM and 16 μM) were set to select at optimum concentration. On the base of the 1st optimization, another set of 6 gradients of primer concentrations at 1 μM gap was utilized to secondly screen an optimum concentration of primer.

Additionally, 5 volume gradients (8 μL, 9 μL, 10 μL, 11 μL, 12 μL) of TB Green Premium Ex Taq were optimized to screen an optimum reaction volume. The appropriate annealing temperature was determined

based on 8 gradients temperatures from 53°C to 61°C (at 1°C increment) within 20 s - 80 s.

The recombinant plasmids of 8.134×10^8-8.134×10^4 copies/μL were amplified using the optimized reaction systems and conditions so as to acquire the kinetics curve of PCR amplification. The standard curve and melting curve were drawn based on these data. Meanwhile, the regression equation was calculated.

The recombinant plasmid standards of 8.134×10^7, 8.134×10^5 and 8.134×10^3 copies/ μL were tested using the established RT-qPCR method in thrice repeats. Both inter-assay and intra-assay coefficients of variation were calculated.

Establishment of visualized RT-LAMP reaction systems

According to the Bst 3.0 DNA Polymerase reagent manual, RT-LAMP reaction was performed in 25 μL of a mixture at 65°C for 30 min to 60 min. The mixture contained 2.5 μL 10 × Isothermal Amplification Buffer II, 1 μL each inner primers FIP and BIP primers (1 μL each outer primers F3 and B3 Primers, 1 μL each Loop F/B primers, 1.5 μL MgSO4, 1 μLBst 3.0 DNA polymerase[7], 1 μL BVDV cDNA/RNA template, 5IU of AMV reverse transcriptase[6], Nuclease-free Water to 25 μL. The blank negative control was set simultaneously. The LAMP reaction products were identified by 1.2% agarose gel electrophoresis. The visual observation was performed under visible and ultraviolet light.

Moreover, the reaction system (dNTP, Mg^{2+} and primer concentration) and reaction conditions (reaction temperature and time) were optimized in order to strengthen the specificity and amplification efficiency of RT-LAMP.

Comparison of specificity, sensitivity and repeatability between 3 methods

-Specificity testing

In order to evaluate the specificity of the conventional PCR, RT-qPCR and visualized RT-LAMP, 6 viruses including BVDV, BPV, BPIV, BCoV, BRV and CSFV were simultaneously detected using these assays.

-Sensitivity testing

A total of 11 gradients of plasmid standards from 8.134×10^{10} to 8.134×10^0 copies / μL were respectively determined, so as to determine the minimum detection limits for 3 established method and compare the sensitivity under the optimized reaction system and conditions of these methods.

-Repeatability evaluation

The repeatability of the assay was determined by testing plasmid standards of 8.134×10^7, 8.134×10^5 and 8.134×10^3 copies/μL using RT-qPCR method in triplicate in the intra-assay and inter-assay. The mean, standard deviation (SD) and coefficient of variation (CV) were calculated separately using Microsoft Excel software for each plasmid standard.

-Detection of clinical samples

A total of 67 clinical diarrhea samples harvested from Lanzhou city and Zhangye city in Gansu province of China were detected by using these assays of PCR, RT-qPCR and RT LAMP. The detection results were subjected to Kappa consistency testing and pairing χ^2 test using SPSS Statistics 24 software (McNemar calibration test). The results of 3 methods were compared to assess their clinical practicality.

Statistical analysis

The experiment data were analyzed by SPSS version 22.0[8] and were presented as means ± SEM. Statistical differences between intra-assay and inter-assay were determined by using the Student's *t*-test and one-way analysis of variance (ANOVA). Tukey's test was used to estimate the significance of the results. Kappa test and McNemar calibration test were performed among 3 detection methods. $P < 0.05$ was considered to be significant.

RESULTS

The optimal reaction systems and conditions of conventional PCR

The optimized reaction systems and reaction conditions of routine PCR were acquired including 2.5 μL 10 × Ex Taq Buffer (Mg^{2+}plus), 0.5 μL Ex Taq (5 U/μL), 2.0 μL DNTP Mixture (2.5 mM), 1.0 μL forward primer, 1.0 μL reverse primer (10 μM), 1.5 μL plasmid standards, and DEPC water to 25 μL at 94°C for 5 min, 94°C for 30 s, Tm (F+R)/2 for 45 s, 72°C for 1 min in Positive recombinant plasmid standards and copies.

The conventional PCR was utilized to amplify recombinant pMD18T-BVDV plasmid with BVDV-5'UTR primer, PCR primer and LAMP-3(F3/B3) primers, respectively. The results showed that the product sizes were 385 bp, 143 bp and 207 bp for these primers, respectively (Figure 1). They were consistent with the expected sizes. Micro spectrophotometer analysis revealed the recombinant plasmid concentration was

274.40 ng/μL and 8.134×10^{10} copies/μL. The recombinant plasmid standards were diluted by 10-folds into 11 gradients from 8.134×10^{10} to 8.134×10^{0} copies/μL.

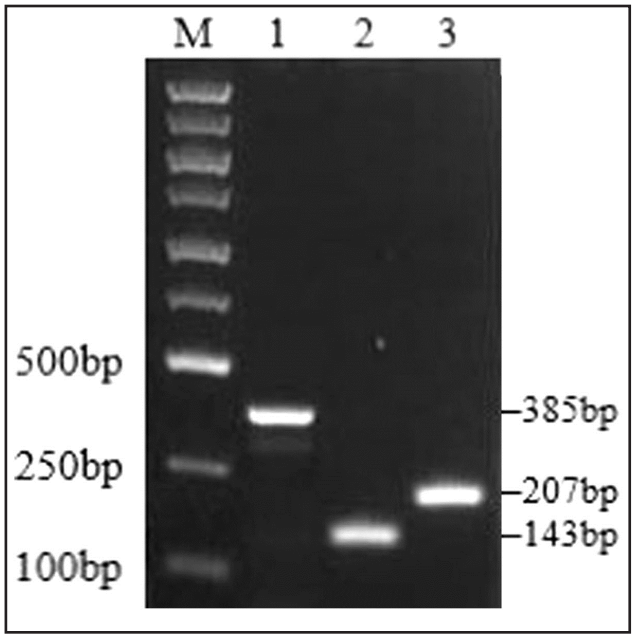

Figure 1. PCR amplification of target genes. [M:100bp DNA Marker; 1: BVDV-5'UTR; 2: 5'UTR-PCR; 3: LAMP-3(F3/B3)].

Standard curve and melting curve of RT-qPCR

The kinetic curve, standard curve, and melting curve of BVDV RT-qPCR were established under the optimized reaction system and conditions on the bases of 8.134×10^{8} - 8.134×10^{4} copies/ μL plasmid standards (Figure 2). The optimized reaction system and conditions of RT-qPCR were at 95°C for 30 s, 95°C for 5 s, 56°C for 30 s, 72°C for 30 s in 40 cycles, and at last 72°C for 10 min.

In addition, plasmid standards were detected using RT-qPCR method to determine the intra-assay and inter-assay coefficient of variation (CV) in triplicates. The results showed that the intra-assay CV was 0.28 to 0.94% and inter-assay CV was 0.49 to 1.06%. The outcomes confirmed the established RT-qPCR was high repeatability.

Establishment of RT-LAMP

-Screen and verification of primers for RT-LAMP

RT-LAMP reactions were performed on the 3 sets of LAMP-1, LAMP-2, and LAMP-3 primers, respectively. The positive reactions showed a light green under visible light and a green fluorescence under ultraviolet light, respectively (Figure 3). However, the negative reaction solution emitted an orange yellow under visible light and did not show green fluorescence under ultraviolet light. The results of agarose gel electrophoresis assay demonstrated the specific ladder-shaped bands were emerged in the positive reaction products, but no bands were observed in the negative reaction products. The optimum reaction primers were selected based on the outcomes of the gel electrophoresis imaging and visual detection analysis.

The detection results demonstrated 1-3 lanes (LAMP-1, LAMP-2 and LAMP-3 primers) showed excellent consistency. The lane 3 displayed the clearest visible green fluorescence (Figure 3), which could be applied to establish RT-LAMP assay.

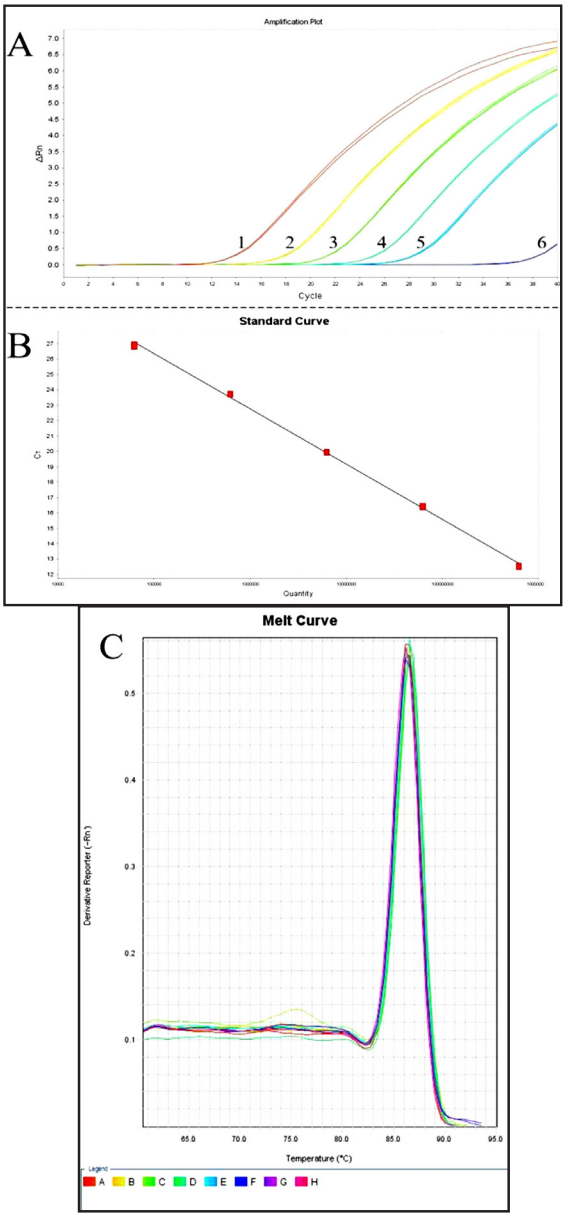

Figure 2. Kinetic curve (A), standard curve (B), and melting curve (C) of qpcr in kinetic curve A- 1, 2, 3, 4 and 5 represented 5 concentrations of plasmid standards from 8.134 × 107 to 8.134 × 103 copies/μL, respectively. In standard curve (B), the correlation coefficient R2 = 0.9990, and the amplification efficiency was 91.078%. The linear regression equation for the standard curve was Y= -3.485 lgX + 43.946. The melting curve (C) showed a single peak at the melting temperature of 86.5 ± 0.5°C, the findings confirmed the built qPCR method had excellent specificity.

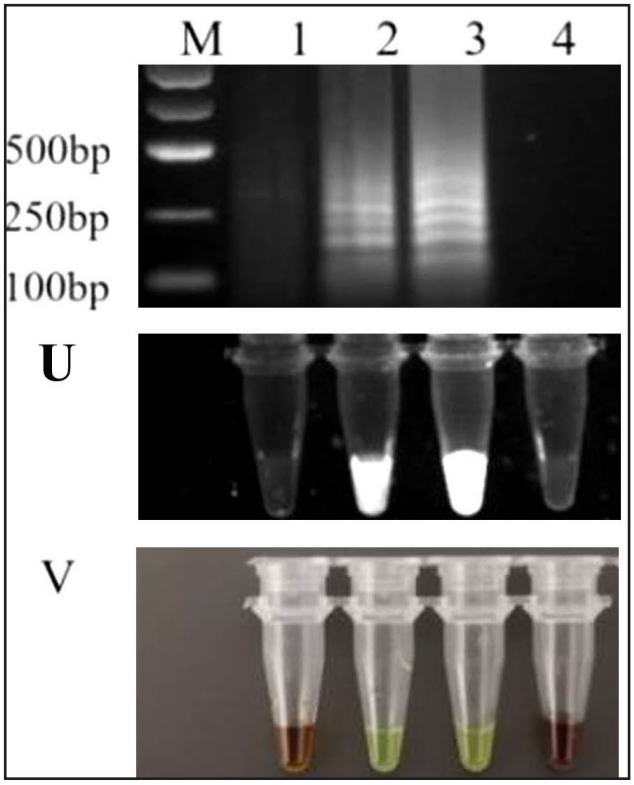

Figure 3. Validation results of 3 sets of RT-LAMP primers. [M: 100bp DNA standard; 1; LAMP-1 primer; 2: LAMP-2 primer; 3: LAMP-3 primer; 4: Blank negative control; U: Ultraviolet light visualization; V: Visible light visualization].

-Primers optimization in RT-LAMP assay

According to the design of RT-LAMP systems in Table 2, lane 6 showed a great visible fluorescence band (Figure 4A), which was consistent with the results in screen and verification of primers of LAMP primers (Figure 3). The optimum concentrations of three set primers were respectively 5 μM F3/B3, 15 μM LF/LB and 40 μM FIP/BIP with their ratio of 1:3:8.

-Optimization of amplification temperatures and reaction times for RT-LAMP

Six temperature gradients (60, 61, 62, 63, 64 and 65°C) were utilized to screen an optimum amplification temperature (Table 3). Besides, 7 gradients of reaction times (10, 20, 30, 40, 50, 60 and 70 min) were set to screen an optimum reaction time. Additionally, a blank control group was also set. The PCR amplification and visual detection analyses indicated that lane 5 showed a clearer specific band (Figure 4B and 4C). The results demonstrated the optimal reaction.

-Determination of optimal RT-LAMP system and conditions

After the optimization of primer concentrations and their ratio, reaction times temperature, dNTP mix, and $MgSO_4$ contents (Mg^{2+}), the optimal system and conditions for BVDV RT-LAMP (Table 3).

Comparisons between PCR, RT-qPCR and RT-LAMP

-Comparison of specificity

Three established conventional PCR, RT-qPCR and RT-LAMP assays were simultaneously utilized to detect several common viruses including BPV, BPIV, BCoV, BRV and CSFV in the same genus with BVDV. Moreover, a control group was set up. The detection results showed that only except for BVDV displayed a specific amplification band or fluorescence signals (Figure 5). No other viruses were amplified. These outcomes indicated that 3 established detection assays had excellent specificity for BVDV.

-Comparison of sensitivity

Eleven gradients of the plasmid standards from 8.134×10^{10}iL to 8.134×10^{0} copies/iL were detected with the conventional PCR, RT-qPCR and RT-LAMP assays, respectively.

Lanes 1-7 in the conventional PCR displayed obvious bands with the product size of 143bp (Figure 6A). But no bands were observed in lanes 8-11 and negative control. The minimum detection limit of the conventional PCR was 8.134×10^{4} copies/μL.

In RT-qPCR, lanes 1-10 emerged clear bands (Figure 6C). But no bands were observed in lanes 8-11 and negative control. The minimum detection limit of RT-qPCR was 8.134×10^{1} copies/μL.

In RT-LAMP assay, the specific ladder-shaped bands were observed in 1-9 lanes of the gel electrophoresis and visual images (Figure 6B, U). The green fluorescence in the visual bands became faded along with decrease of the concentration of the plasmid standards. Concurrently, the brightness of amplification bands and fluorescence were gradually reduced. The minimum detection limit of RT-LAMP was 8.134×10^{2} copies/μL.

The findings demonstrated that RT-qPCR method was more sensitive 1,000- and 10-folds higher than convention PCR and visual RT-LAMP, respectively. However, the sensitivity of RT-LAMP assay was 100-folds greater than conventional PCR.

-Repeatability evaluation

The repeatability of the assay was determined on the bases of the recombinant plasmid standards of 8.134×10^7, 8.134×10^5 and 8.134×10^3 copies/ μL. The intra-assay coefficients of variation (CV) were 0.88-0.94, and inter-assay CVs were 0.49-1.06. The outcomes indicated these detection methods were quite stable and had satisfactory.

-Detection of clinical feces samples

A total of 67 feces samples were detected by using the established PCR, RT-qPCR and RT-LAMP. The results indicated RT-qPCR showed the greatest positive rate (Table 4). Kappa test and McNemar calibration test demonstrated the detection rates for BVDV in feces samples had no significant difference between the 3 developed methods.

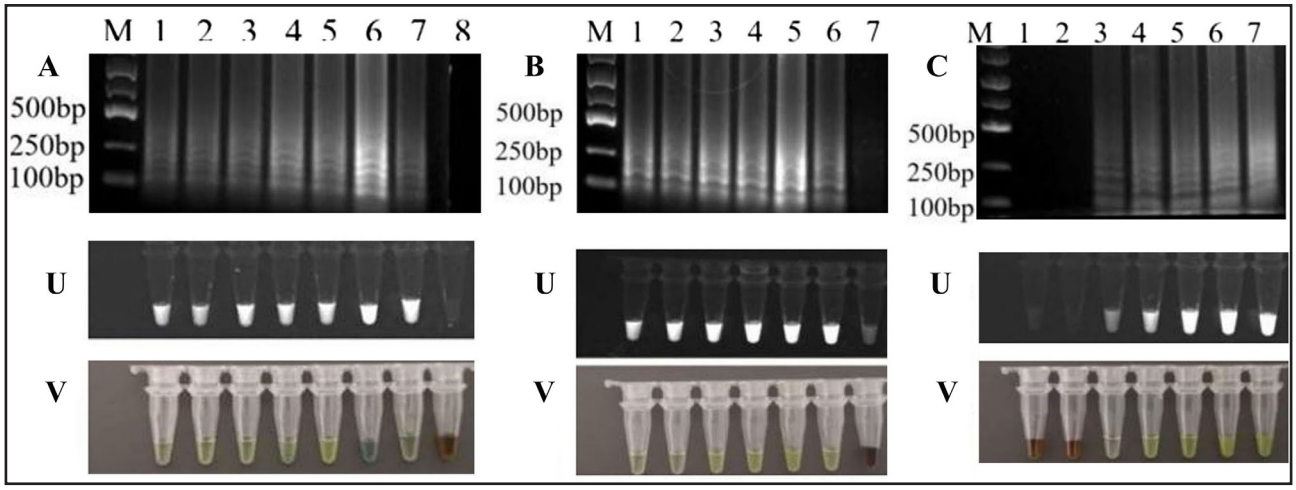

Figure 4. Optimization results of primer concentrations, reaction temperatures and times for RT-LAMP. [M: 100bp DNA standards; U: ultraviolet (UV) light visualization; V: visible light visualization; A: Primer concentrations rates, 1-7 represented 1:1:2, 1:2:4, 1:2:6, 1:2:8, 1:3:6, 1:3:8, 1:3:10, respectively. B: Reaction temperatures, 1-7 represented 60, 61, 62, 63, 64, 65°C and negative control, respectively; C: Reaction times, 1-7 represented 10, 20, 30, 40, 50, 60, 70 min, respectively].

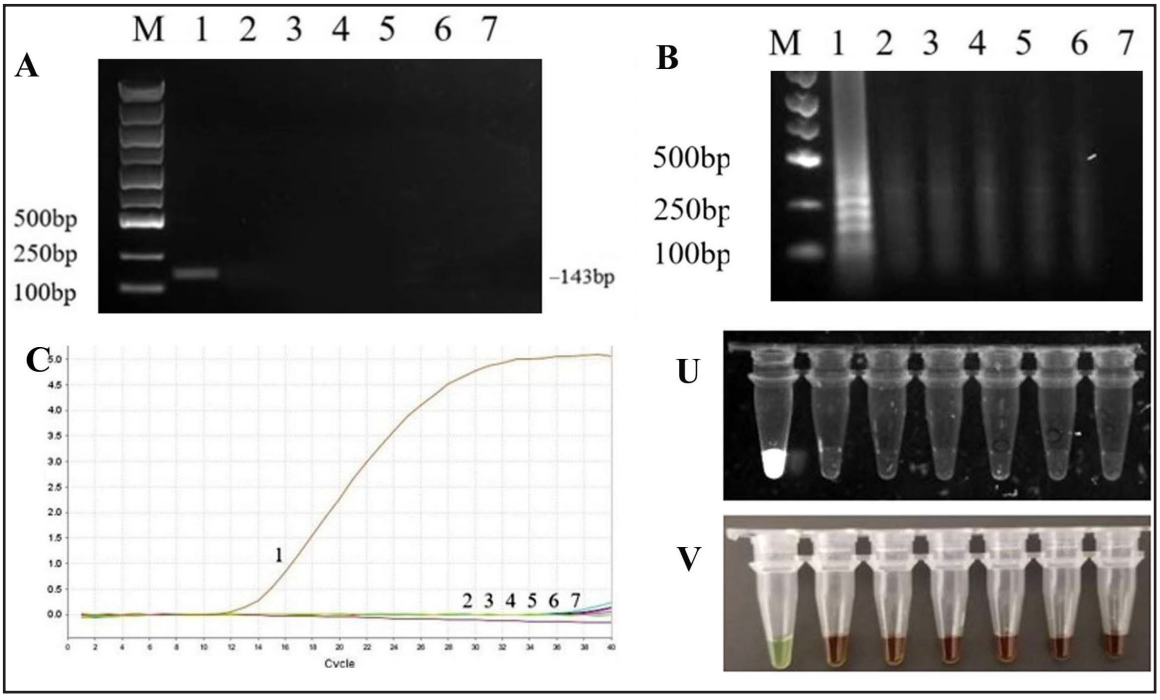

Figure 5. Specificity of PCR (A), qPCR (B) and RT-LAMP (C). [M: 100bp DNA molecular weight standard; A: convention PCR; B: qPCR; C: visual RT-LAMP; U: ultraviolet (UV) visualization; V: visible light visualization. Lanes 1-7 represented BVDV, BPV, BPIV, BCoV, BRV, CSFV and negative control, respectively].

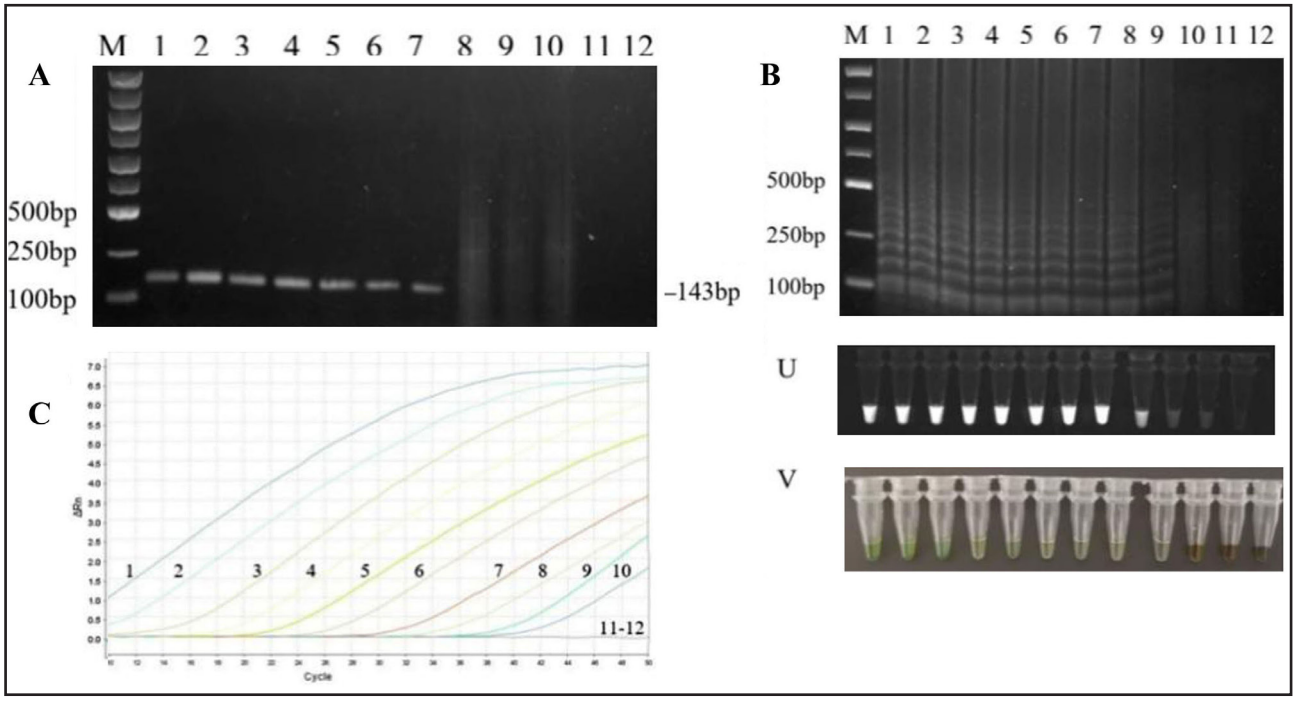

Figure 6. Sensitivity of PCR, qPCR and RT-LAMP. [M: 100bp DNA molecular weight standard; A: convention PCR; B: qPCR; C: visual RT-LAMP; U: UV visualization; V: Visible light visualization. Lanes 1-12 represented plasmid standards from 8.134×10^{10}/ µL to 8.134×10^{0} copies/ µL and negative control, respectively].

Table 2. Optimization results of RT-LAMP reaction systems and reaction conditions.

Gradients		1	2	3	4	5	6	7
dNTP Mix (10 mM)	Reagent volume (µL)	2.5	3.0	3.5	4.0	4.5	5.0	/
	Final concentration (mM)	1.0	1.2	1.4	1.6	1.8	2.0	/
$MgSO_4$ (100 mM)	Reagent volume (µL)	1.0	1.5	2.0	2.5	3.0	3.5	/
	Final concentration (mM)	4	6	8	10	12	14	/
Primer contents	F3 and B3 (µM)	5	5	5	5	5	5	5
	Loop F/B primers (µM)	5	10	10	10	15	15	15
	FIP and BIP (µM)	10	20	30	40	30	40	50
	Ratio of F3/B3: LF/LB:FIP/BIP primers	1:1:2	1:2:4	1:2:6	1:2:8	1:3:6	1:3:8	1:3:10
Reaction condition	Reaction temperature (°C)	60	61	62	63	64	65	/
	Reaction time (min)	10	20	30	40	50	60	70

Table 3. Reactions System and Reaction Conditions of visualized RT-LAMP assay.

Compositions	Doses	Final concentration
10×Isothermal Amplification Buffer II	2.5 µL	1 × (contains 2 mM) $MgSO_4$
$MgSO_4$ (100 mM)	2 µL	8 mM
dNTP Mix (10 mM)	4 µL	1.6 mM each
Inner primers FIP/BIP (25×)(40 µM)	1 µL	1.6 µM
Outer primers F3/B3 (25×)(5µM)	1 µL	0.2 µM
Loop F/B Primers (25×)(15µM)	1 µL	0.6 µM
Bst 3.0 DNA Polymerase (8,000 U/mL)	1 µL	320 U/mL
Template	1 µL	>10copies or more
Nuclease-free Water	25 µL	
Total Reaction Volume	25 µL	
Optimal reaction conditions: 64°C 50 min; 85°C 5 min.		

Table 4. Comparison of three methods for detection of bovine viral diarrhea virus (BVDV) from clinical feces samples.

Methods	Samples	Positive	(%)
PCR		20	29.85
RT-qPCR	67	23	34.33
RT-LAMP		22	32.84

Kappa tests indicated that Kappa value of PCR and qPCR was 0.898, $P < 0.001$; Kappa value of PCR and LAMP was 0.861, $P < 0.001$; The Kappa value of qPCR and LAMP was 0.900, $P < 0.001$. The outcomes revealed there was a good consistency among the paired diagnostic tests. McNemar calibration test showed 3 P values of PCR vs qPCR, PCR vs LAMP, qPCR vs LAMP were more than 0.05, which testified the detection rates between 3 developed methods had no significant difference.

DISCUSSION

The Bovine viral diarrhea virus (BVDV) is a key pathogenic factor in bovine diarrhea [1]. Differential diagnosis of viral diarrhea is often relied on virus detection by isolation and/or detection of serum antibody using ELISA. Currently, few effective measures are available for the treatment or prevention for BVDV and BPV infections in animals [13,27]. Real-time PCR is now being used to identify and quantify many viral pathogens and genotype BVDV as described previously [29]. However, this differs in the efficiency of RNA extraction, reverse transcription or PCR reactions, leading to unstable the sensitivity and specificity of this technique [24].

In the present study, the conventional PCR and RT-qPCR were established based on the specific primers according to the nucleotide sequences in 5' non coding region (5' UTR) of NADL strain of BVDV. After the optimization of reaction systems and reaction conditions, BVDV-5'UTR, 5'UTR-PCR and LAMP-3(F3/B3) displayed clear bands. The results indicated the developed PCR had excellent specificity. Meanwhile, the kinetic curve, standard curve, and melting curve of BVDV RT-qPCR were acquired on the bases of the optimized reaction system and conditions of RT-qPCR. In addition, the intra-assay and inter-assay coefficient of variation (CV) of RT-qPCR were assessed by detecting the different concentrations of pMD18-T-BVDV recombinant plasmid standards. Our findings demonstrated the RT-qPCR had a high repeatability with the stable and reliable detection results.

Reserve transcription real-time fluorescence quantitative PCR (RT-qPCR) includes the fluorescence probe method and SYBR GREEN dye method based on chemical fluorescence method. The fluorescence probe method has higher sensitivity and specificity compared to the dye method, but the probe cost is higher. BVDV qPCR methods established [16,26,28] had minimum detection limit of 5.0267 copies/μL, 10^1 copies/ μL, and 10^2 copies/μL, respectively. In our study, the minimum detection limit was 8.134×10^1 copies/ μL. Our findings were similar to the early report [29].

RT-LAMP technology has a lot of obvious advantages, such as its amplification temperature is constant, and the entire reaction can be completed in a large cycle with a high amplification efficiency and without the need for refolding and denaturation process. Due to its strong specificity and relatively less non-specific amplification, RT-LAMP can be carried out in small amounts samples [4,18,20]. The amplification results were diverse and visualized. Visual judgment can be achieved by observing white precipitates and adding dye indicators, which is suitable for general detection departments and cattle farms to promote and apply [27].

LAMP technology has been thoroughly improved over the world since the assay visualization of LAMP reaction was proposed in 2008 [22]. RT-LAMP has been used in detecting viral RNA molecules due to its simplicity and high sensitivity [2,14].

So far, a RT-LAMP could detect BVDV RNA within 60 min under isothermal conditions [6]. Another study reported that the LAMP method could efficiently detect and quickly distinguish BVDV-1 and BVDV-2 [21]. A visual one-step RT-LAMP detection method for BVDV was established with a minimum detection limit of 0.021 pg for BVDV RNA/ μL [30].

In the present study, a highly efficient and practical RT-LAMP method was established and optimized based upon 3 sets of designed primers (inner primers FIP and BIP, outer primers F3 and B3, as well as, loop F and loop B primers). The amplification reactions could be finished within 60 min. Our findings were consis-

tent with early reports [6,7,22]. Comparisons between PCR, RT-qPCR and RT-LAMP assays indicated that these methods had excellent specificity only for BVDV. The minimum detection limits for PCR, RT-qPCR and RT-LAMP were 8.134×10^4, 8.134×10^1 and 8.134×10^2 copies/μL, respectively. RT-qPCR sensitivity was 10-folds and 1,000-folds higher than that of RT LAMP and PCR. The intra-assay and inter-assay coefficient of variation (CV) analyses testified these assays were stable with outstanding reproducibility. Furthermore, the Kappa and McNemar calibration verified and supported these results. These findings were agreement with the previous reports [4,7].

CONCLUSION

In conclusion, the conventional PCR, RT-qPCR and RT-LAMP for detecting BVDV had been successfully established in the present study. They possessed excellent specificity, sensitivity and reproducibility. Comparison between three assays demonstrated that the sensitivity of RT-qPCR was 10-folds and 1,000-folds greater than RT-LAMP and PCR. Our findings provided the technical support for rapidly, simply, cost-effectively detecting BVDV and for differential diagnosing BVDV, which is beneficial for early screening and preventing of BVDV infection, also promoting prevention and treatment effectiveness.

MANUFACTURERS

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***Authorship contributions*.** Dr. Pei Mengyuan established the PCR, RT-qPCR and RT-LAMP. Dr. Li Dianyu did premier design. Professor Gong Zhuandi performed clinical samples tests and did the statistical analysis of data. Professor and Dr. Wei Suocheng designed the experiments and wrote the manuscript. All authors interpreted the data, critically revised the manuscript for important intellectual content, and approved the final version.

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***Ethical approval*.** All procedures referring to animal treatment were approved by the Experiment Animal Care and Use Committee of Gansu province, the People's Republic of China.

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