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Development and application of reverse transcriptase droplet digital PCR technology for sensitive detection of BVDV-1 and BTV in bovine semen

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Abstract

Background Bovine viral diarrhoea virus genotype 1 (BVDV-1) and bluetongue virus (BTV) are potent viral pathogens that may be transmitted through semen, resulting in the spread of diseases via artificial insemination. Thus, establishing an early detection method for BVDV-1 and BTV infection is important for the trading of semen. In this study, we developed two RT–ddPCR methods to detect BVDV-1 and BTV, and each method was evaluated for repeatability, limit of detection and specificity. The sensitivity of these methods was compared with that of RT–qPCR (WOAH) by analysing clinical samples.

Results The RT–ddPCR results revealed that both methods exhibited good repeatability at low concentrations, with detection limits of 1.05 copies/μL and 0.662 copies/μL per reaction for BVDV-1 and BTV, respectively; additionally, both methods exhibited high specificity and did not exhibit cross-reaction with other important semen-transmitted pathogens. Eighty bovine semen samples and twenty mixed semen samples were tested. The results revealed that the positivity rates of BVDV-1 and BTV RT–ddPCR (25% and 23%, respectively) were greater than those of RT–qPCR (19% and 18%, respectively).

Conclusions RT–ddPCR was highly sensitive for detecting low concentrations of BVDV-1 and BTV in clinical samples and could be a good supplement for qPCR testing.

Keywords Bovine viral diarrhoea virus genotype 1, Bluetongue virus, Droplet digital PCR, Bovine semen

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Background

With the development of livestock farming, the demand for an increased quantity and quality of animal products is increasing. With the development of animal husbandry, cattle management has played a major role in the economies of farmers and nations worldwide [1]. One of the successful strategies for improving production efficiency and optimizing reproductive management in cattle farms is artificial insemination (AI) [2]. As a result, the trade of animal products between regions is increasing. However, the risk of the spread of transboundary diseases in animals has increased [3]. Therefore, the detection of semen from ruminants before admission to trade for use in animal products is necessary, especially because semen can potentially transmit viruses via trade [4, 5].

Bovine viral diarrhoea virus genotype 1 (BVDV-1) and bluetongue virus (BTV) are significant semen-transmitted viral pathogens. BVDV-1 can cause the clinical presentation of BVD in cattle, which is characterized by acute diarrhoea, mucosal disease, respiratory and reproductive disorders, immune suppression and persistent infections [6, 7]. BTV causes bluetongue, is an important infectious disease of ruminants and is characterized by high fever, oedema of the lips and tongue, excessive salivation, and nasal discharge [8, 9]. These viruses are excreted intermittently in the semen of BVDV/BTV-seropositive bulls, and BVDV/BTV may also be introduced through contaminated semen during AI. These viruses are responsible for considerable economic losses due to the trade restrictions of ruminants and are also distributed worldwide [10, 11].

Droplet digital PCR (ddPCR) is a novel detection technology based on water–oil emulsion droplets for nucleic acid detection and enables absolute quantification of target molecules without calibration curves, as in qPCR. The same primers and probes used for qPCR were used, but ddPCR has higher sensitivity [12, 13]. The ddPCR method has been applied for pathogen diagnosis because of its high sensitivity, especially at low target concentrations, compared with that of qPCR [14]. In this study, we established two RT–ddPCR assays for testing BVDV-1 and BTV in semen samples from cattle. We also evaluated the acceptable repeatability, sensitivity and specificity of the two methods and their effectiveness for clinical diagnosis.

Methods

Virus and clinical samples

BVDV-1, BTV, and bovine herpes virus type 1 (BoHV-1) culture media and *Mycobacterium bovis* (ATCC 27289), *Brucella* (CVCC 70502) and bovine semen samples were obtained from the Technology Center of Hohhot Customs District in China (Table S1).

RNA/DNA extraction and RNA reverse transcription

Two hundred microlitre samples were taken and added to 1.5 ml centrifuge tubes. RNA/DNA was extracted from the samples with a MiniBEST Viral RNA/DNA Extraction Kit (TaKaRa Bio, Inc., Shiga, Japan) according to the instructions. 1.0 μl of carrier RNA, 20 μl of proteinase K and 200 μl of Buffer VGB were added, and the mixture was thoroughly mixed at 56 °C in a water bath for 10 min. 200 μl of 97% ethanol was added to the lysate. The RNA from the virus samples was reverse transcribed at 42 °C for 5 min and 95 °C for 10 s via a RNA reverse transcription kit (Invitrogen, Carlsbad, CA, USA).

Primer and probe design

The BVDV-1 *5'-UTR* gene and BTV *NS3* gene sequences were obtained from GenBank (Table S2). After the consensus sequence was selected, primers and probes were designed with DNAMAN software (Lynnon Biosoft, San Ramon, CA, USA).

Construction of standard plasmids

The BVDV-1 *5'-UTR* and BTV *NS3* gene were amplified via PCR with primer pairs (Table S3). The reactions were carried out using 2×Taq Master Mix (Vazyme Biotech, Nanjing, China). The PCR products were collected and purified via an EasyPure PCR Purification Kit (TransGen Biotech, Beijing, China), and the target sequences were cloned and inserted into the Trans1-T1 vector (TransGen Biotech, Beijing, China). The recombinant plasmids were subsequently purified via a FastPure EndoFree Plasmid Mini Plus Kit (Vazyme Biotech, Nanjing, China). The concentration of the plasmid DNA was measured with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Delaware, USA). The initial concentrations of the BVDV-1 and BTV plasmids were 1.05×10^9 copies/μL and 6.62×10^9 copies/μL, respectively.

qPCR assay

qPCR analysis was performed via a QuantStudio 6 Flex Real-time PCR System (Thermo Fisher Scientific, Delaware, USA). The primers and probes used are listed in the additional file: Table S3. *PerfectStart*® II Probe qPCR SuperMix (TransGen Biotech, Beijing, China) was used to prepare a 20 μL reaction mixture comprising 10 μL of 2×*PerfectStart*® II Probe qPCR SuperMix, 1 μL of each of the primers (10 μM/L), 0.8 μL of the probe (10 μM/L), 5 μL of plasmid DNA, and 3.2 μL of nuclease-free water. The reaction conditions were as follows: 94 °C for 30 s, followed by 40 cycles of 94 °C for 5 s and 60 °C for 30 s.

RT–ddPCR assay

The primers and probes (Table S3) used for RT–ddPCR. The reaction was performed in a mixture containing 1 μL/0.5 μL of BVDV-1/BTV plasmid in a 20 μL reaction

volume containing 10 μL of 2×ddPCR SuperMix (Bio-Rad, Pleasanton, CA, USA), 1.6 μL (800 nmol/L) of each primer, 0.4 μL (200 nmol/L) of the probe and DNase/RNase-free H_2O to reach the final volume. To determine the optimal annealing temperature, the amplification reaction was performed with the following conditions: 95 °C for 10 min, 40 cycles of 95 °C for 30 s and a temperature gradient from 50 to 65 °C for 1 min, and 98 °C for 5 min (ramp 2.5 °C/s), ending at 16 °C and then amplification in a T100TM Thermal Cycler (Bio-Rad, Hercules, CA, USA). Next, each sample was transferred to a QX200 Droplet Generator (Bio-Rad, Hercules, CA, USA) to generate microdroplets. The droplets were transferred to a 96-well PCR plate, and the plate was subsequently sealed with foil via a PX1 PCR plate sealer (Bio-Rad, Hercules, CA, USA). The droplets from each well of the plate were read by a QX200 Droplet Reader (Bio-Rad, Hercules, CA, USA). QuantaSoft software (Bio-Rad, Hercules, CA, USA) was used to analyse the data.

Evaluation of the repeatability and sensitivity of qPCR and RT–ddPCR

The BVDV-1 and BTV plasmids were serially diluted 10-fold to concentrations ranging from 1.05×10^9 to 1.05×10^0 copies/μL and from 6.62×10^9 to 6.62×10^0 copies/μL, respectively, and were used to determine the repeatability (repeated three times) and sensitivity separately via a qPCR assay. For comparison, the RT-ddPCR assay was performed in parallel using suitable templates (1.05×10^3-1.05×10^{-1} copies/μL and 6.62×10^2-6.62×10^{-2} copies/μL).

Analytical specificity of RT–ddPCR

To determine the specificity of the established BVDV-1 and BTV RT-ddPCR assays, common bovine pathogens that can be transmitted through semen, including BVDV-1, BTV, BoHV-1, *M. bovis* (ATCC 27289) and *Brucella* (CVCC 70502), were tested.

Evaluation of the RT–ddPCR assay using clinical samples

The clinical samples were collected from 2017 to 2022. For BVDV-1, 80 bovine semen samples were tested via the established RT-ddPCR and RT-qPCR methods of the World Organization for Animal Health (WOAH). For BTV, 80 bovine semen samples were subjected to RT-ddPCR and RT-qPCR (WOAH). In order to reduce the concentration of positive samples, the negative and the positive samples were mixed according to the proportions (Table S4) into the mixed samples (No.1 ~ 4). To compare the sensitivity of these two methods for detecting low-concentration samples, 5 samples were respectively selected from the groups No.1 ~ 4 for detection.

Results

Thermal gradient optimization of the RT–ddPCR for BVDV-1 and BTV

As shown in Fig. 1A and B, the optimal annealing temperatures for BVDV-1 and BTV RT-ddPCR were 60 °C and 55.7 °C, respectively. The largest number of amplicons (positive droplets) was obtained, which was the greatest difference between the fluorescence values of the positive (blue) and negative (gray) droplets.

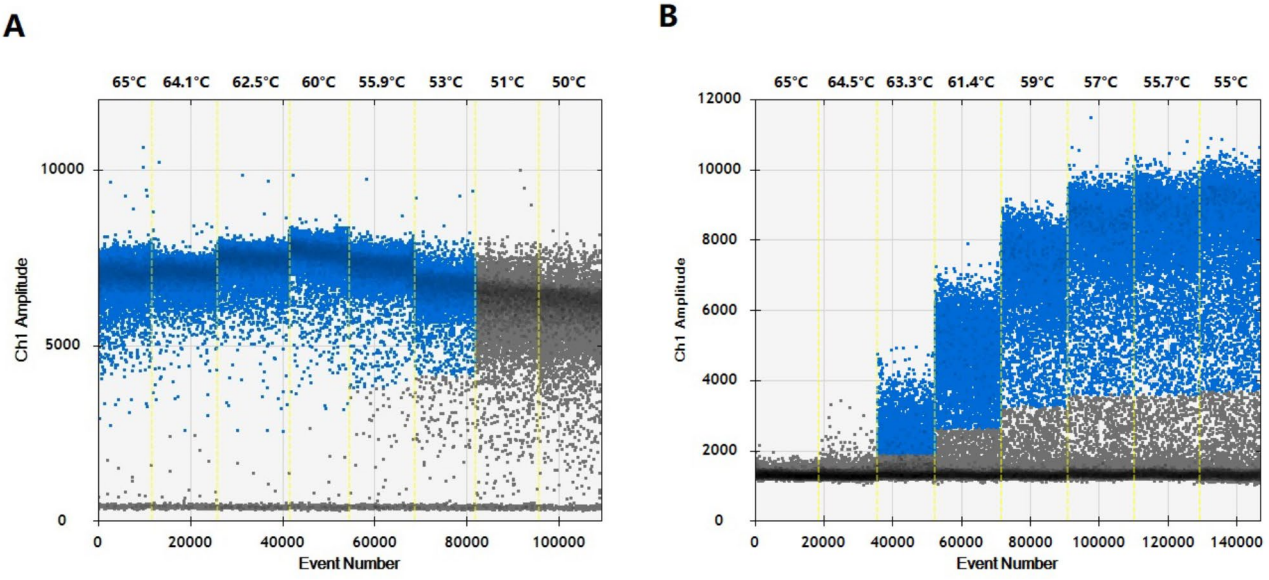

Fig. 1 Optimization of the annealing temperature. **A**: BVDV-1 RT–ddPCR. **B**: BTV RT–ddPCR

Analytical repeatability and sensitivity testing

In the repeatability analysis for BVDV-1, in comparison with qPCR, RT-ddPCR showed that good repeatability for testing low-concentration samples (Fig. 2A–B). The lower detection limit for qPCR was 1.05×10^2 copies/μL, whereas the limit for RT-ddPCR was 1.05 copies/μL (Fig. 2C–D).

For BTV, the RT-ddPCR showed good repeatability for detecting low-concentration samples (Fig. 3A–B). The lower detection limit for qPCR was 6.62×10^2 copies/μL, whereas the limit for RT-ddPCR was 6.62×10^{-1} copies/μL, which was lower than that for qPCR (Fig. 3C–D).

Specificity of the RT–ddPCR assay

For the specificity analysis, the DNA/cDNA templates of BVDV-1, BTV, BoHV-1, *M. bovis* and *Brucella* were detected via BVDV-1 RT-ddPCR and BTV RT-ddPCR. As shown in Fig. 4, the primers and probes used were specific. For BVDV-1 RT-ddPCR, only the BVDV-1 sample tested positive, and for BTV RT-ddPCR, only the BTV sample tested positive.

Evaluation of the RT–ddPCR assay with clinical samples

For comparison, RT-qPCR (WOAH) was used to test clinical samples to evaluate the sensitivity and accuracy of RT-ddPCR. For BVDV-1, 80 bovine semen samples were tested via RT-qPCR (WOAH) and RT-ddPCR. Both methods detected the same five positive samples, and the percentage of BVDV-1-positive samples was 6.25%. For BTV, 80 bovine semen samples were subjected to RT-qPCR (WOAH) and RT-ddPCR. Both methods detected the same three positive samples, and the percentage of BTV-positive samples was 3.75%. As shown in Table 1, the positivity rate of RT-ddPCR (25% and 23%) was greater than that of RT-qPCR (WOAH) (19% and 18%). The detection results of both methods were accurate for the semen samples. In addition, the kappa statistical method was used to analyse these two methods. For BVDV-1, the kappa value of approximately 0.790 indicates a high degree of consistency between RT-qPCR (WOAH) and RT-ddPCR. For BTV, the kappa value of approximately 0.879 indicates a high degree of agreement between the two detection methods. However, when the samples were mixed into groups, the rates of BVDV-1

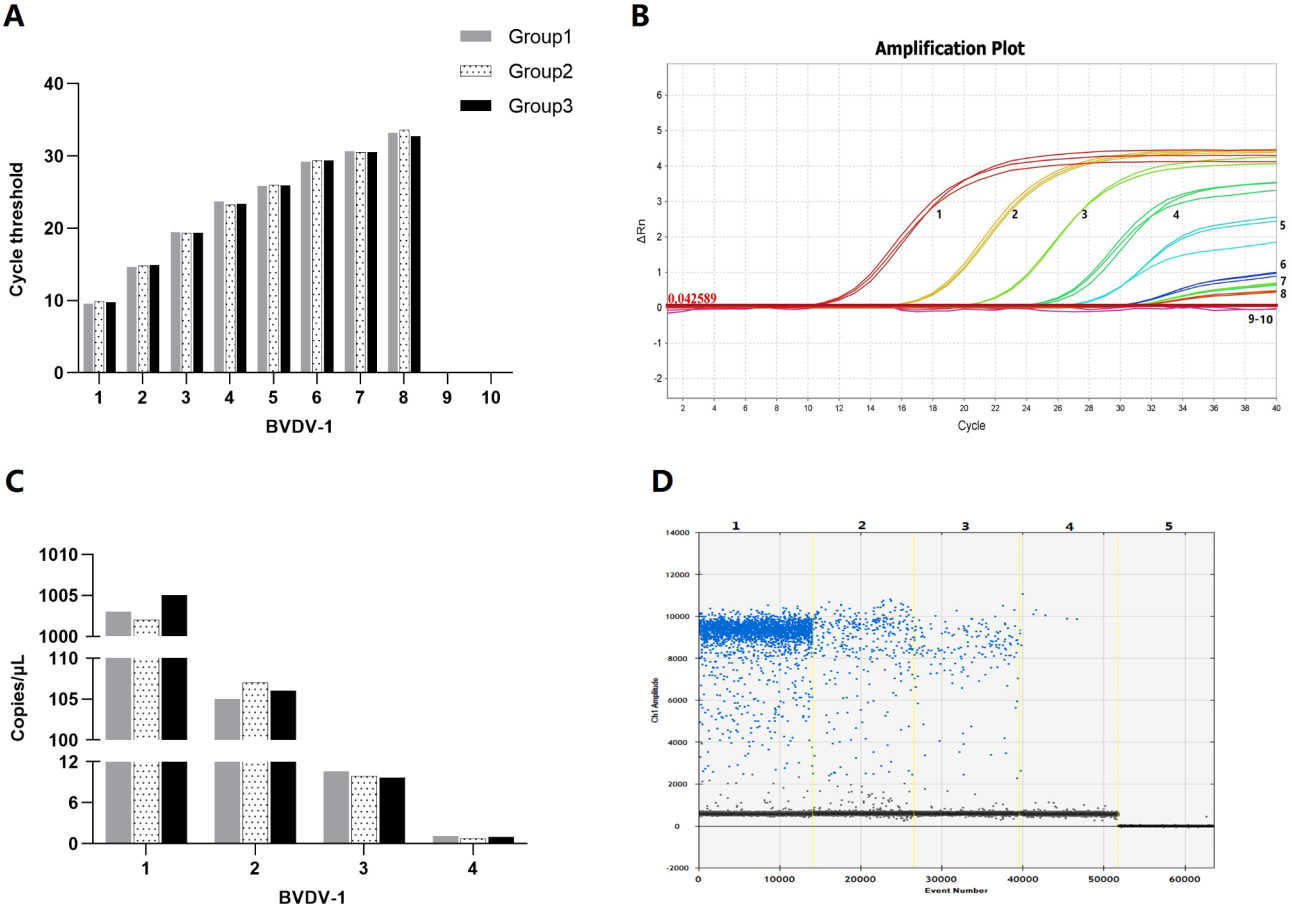

Fig. 2 Repeatability and sensitivity analysis of the qPCR and RT–ddPCR assays with different dilution samples. **A-B**: 1–10: 1.05×10^9-1.05×10^0 copies/μL dilution standards. **C-D**: 1–5: 1.05×10^3-1.05×10^{-1} copies/μL dilution standards

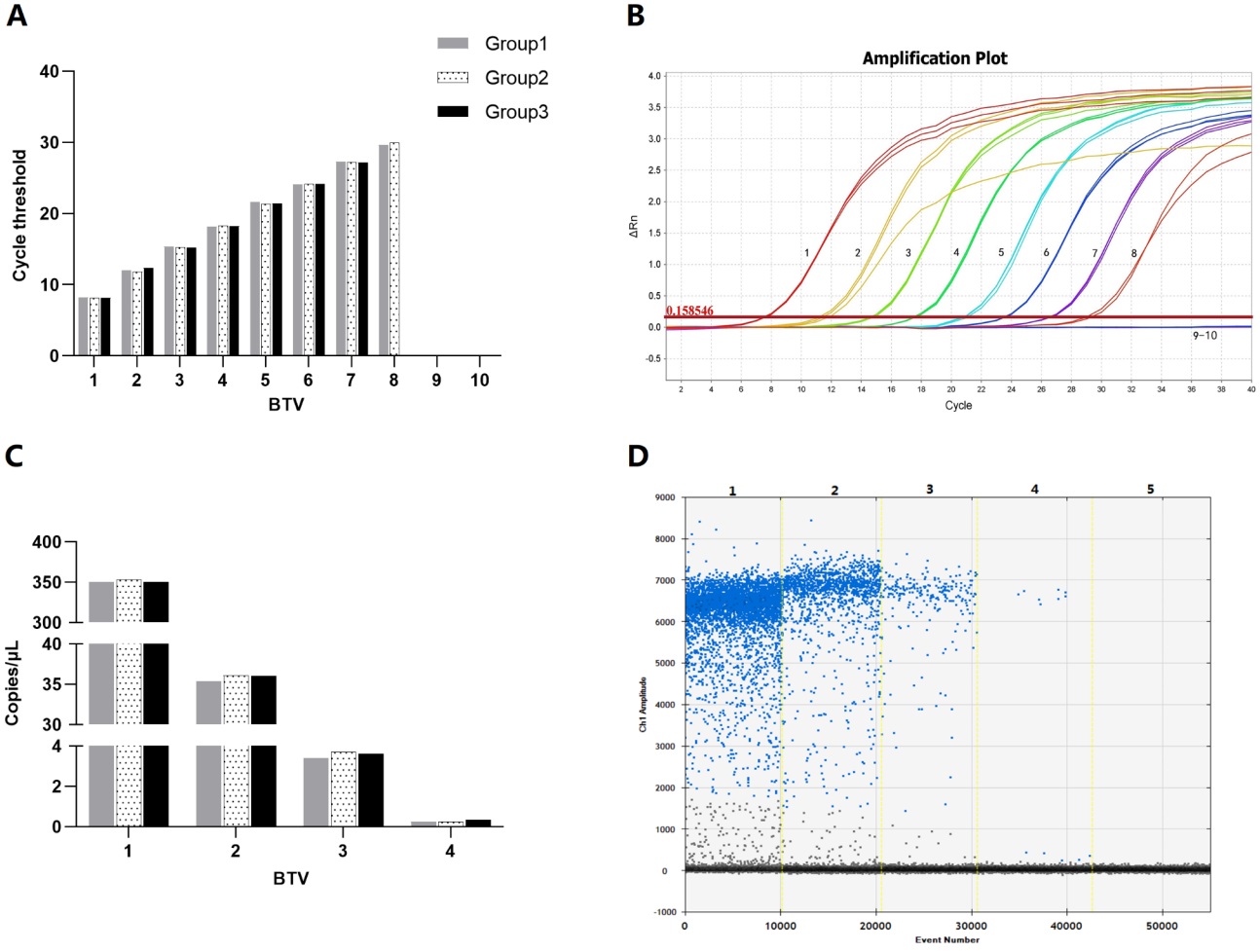

Fig. 3 Repeatability and sensitivity analysis of the qPCR and RT–ddPCR assays with different dilution samples. **A-B**: 1–10: 6.62×10^{9}-6.62×10^{0} copies/μL dilution standard. **C-D**: 1–5: 6.62×10^{2}-6.62×10^{-2} copies/μL dilution standard

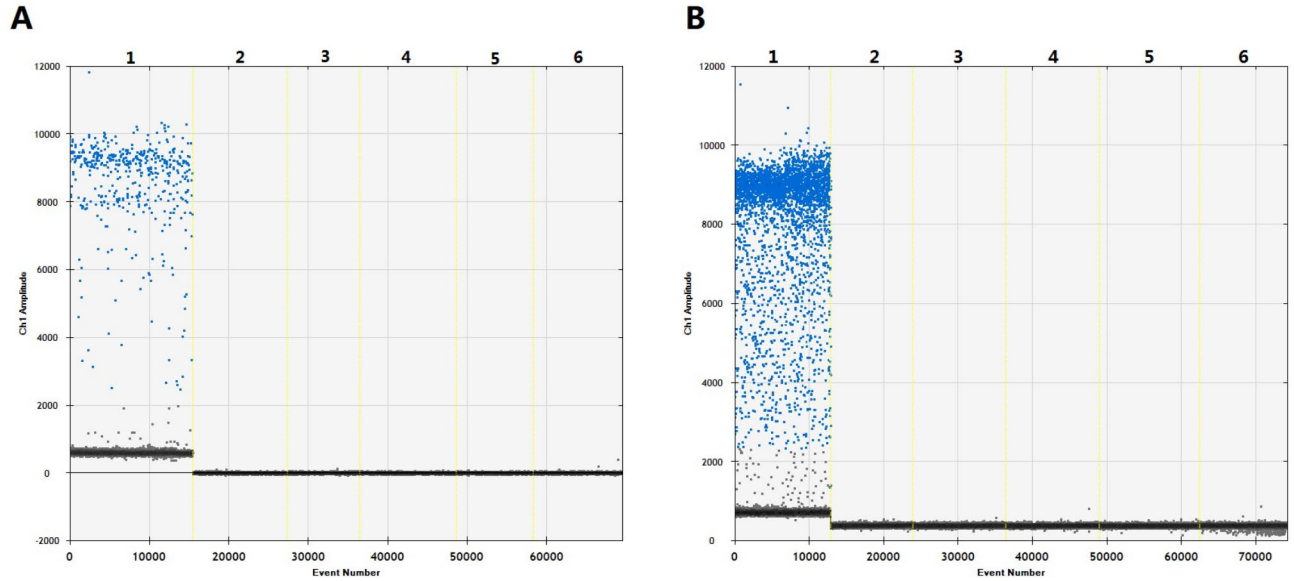

Fig. 4 Scatter plot of the specificity of the RT–ddPCR assay. **A**: 1–6: BVDV-1, BoHV-1, BTV, *Mycobacterium bovis*, *Brucella* and negative control. **B**: 1–6: BTV, BoHV-1, BVDV-1, *Mycobacterium bovis*, *Brucella* and negative control

Table 1 Detection results for clinical samples

Groups	Real-time RT–PCR (WOAH)			Droplet digital PCR	
	Positive	Negative	Suspicious	Positive	Negative
Semen samples	5	75	0	5	75
Mixed samples	14	0	6	20	0
Positive rate of BVDV-1	19%			25%	
Semen samples	3	77	0	3	77
Mixed samples	15	0	5	20	0
Positive rate of BTV	18%			23%	

and BTV positivity decreased, and suspicious (ambiguous) results were obtained via RT-qPCR (WOAH). These suspicious samples were retested via RT-ddPCR, and the results were positive. Therefore, all mixed samples were determined to be true positives by RT-ddPCR and false negatives by RT-qPCR (WOAH).

Discussion

In this study, we established RT-ddPCR assays to test BVDV-1 and BTV in bovine semen. We chose the *5'-UTR* and the *NS3* gene to design the primers and probes for the two RT-ddPCR methods, respectively. The *5'-UTR* is frequently targeted for the detection of BVDV because it is highly conserved in the genus *Pestivirus* [15]. The *NS3* gene is highly conserved in the genus *Orbivirus* and is thus useful for the development of reliable diagnostic tests for BTV [16]. We first developed a qPCR assay to determine primer and probe suitability because RT-ddPCR uses the same primers and probes. Visual inspection of the droplet readouts revealed a stable number of droplets and the most visible difference between the positive and negative droplets at 60 °C and 55.7 °C for the BVDV-1 and BTV RT-ddPCR platforms, respectively. Determining the greatest difference in fluorescence between negative and positive droplets is critical for establishing the RT-ddPCR method [17]. For further studies, annealing temperatures of 60 °C and 55.7 °C were chosen.

The repeatability and sensitivity of the two RT-ddPCR methods were evaluated via analysis of the BVDV-1 and BTV plasmids at serial 10-fold dilutions. The RT-ddPCR assays were more sensitive than qPCR for virus detection at low concentrations. In the RT-ddPCR assay, after a reaction mixture is partitioned into thousands of nanosized microdroplets, the target molecule is mixed with water–oil droplets. Every microdroplet is subjected to PCR in a microreactor, and the number of partitions in which the target gene is determined is counted. The data (the absolute number of target genes) on the basis of positive partitions are analysed via Poisson statistics [18]. Therefore, the initial quantity of template should be lower than 1×10^5 copies; otherwise, a high concentration of template will lead to nonlinear results [19]. Therefore, the RT-ddPCR method can achieve high precision for detecting target molecules when analysing samples with low copy number.

We described RT-ddPCR and demonstrated that the limit of detection was 1.05 copies/μL for BVDV-1 samples. Previous studies have reported methods for detecting BVDV via RT-qPCR. Zhang et al. (2015) used RT-qPCR with TaqMan-MGB probes for BVDV-1, with a limit of detection of 1.72×10^2 copies. Zoccola et al. (2017) established a BVDV-1 RT-qPCR, in which the detection limit was 100 copies of viral RNA [20, 21]. Liang et al. (2019) developed a specific and sensitive RT-qPCR, and its the detection limit was 1.55 copies/μL for viral RNA. Moreover, Hou et al. (2020) developed an RT-qPCR method for the detection of BVDV-1 in aerosol samples, and the lower limit of detection was 5.2 RNA molecules per reaction [22, 23]. In this study, the sensitivity of the RT-ddPCR method was greater than that of the RT-qPCR method. Several studies have reported techniques for detecting the BTV. Mulholland et al. (2017) used an rRT-PCR assay for BTV serotypes, which yielded a detection limit of 200 copies of RNA per reaction. Lakshmi et al. (2018) developed a qPCR method for detecting BTV in blood samples, and it was found to be very sensitive, with the lowest limit of 13 copies [24, 25]. Rocchigiani et al. (2020) established an RT-ddPCR for BTV in field samples. The primers and probe used for this method were published by WOAH; the *NS3* gene was amplified, and the optimized annealing temperature was 58.8 °C. The detection limit was 0.72 copies/μL, with a higher sensitivity than that of RT-qPCR [26]. To estimate the specificity of the RT-ddPCR, we selected several semen-transmitted cattle pathogens, such as BVDV-1, BTV, BoHV-1, *Brucella* and *M. bovis.* Both methods showed no cross-reactivity signals with other samples and good specificity.

Two RT-ddPCR methods were used to analyse 80 bovine semen samples, respectively. Similarly, these samples were subjected to RT-qPCR (WOAH) to evaluate the feasibility of RT-ddPCR. The results demonstrated that both assays provided similar results; the positive rates for BVDV-1 and BTV were 6.25% and 3.75%, respectively. In addition, through kappa analysis, we found that these two new methods each have high consistency with RT-qPCR (WOAH). However, when

the samples were mixed, the concentration needed for a positive result was lower, leading to questionable RT–qPCR results. Typically, samples are mixed for large-scale detection of pathogens, or different RNA extraction methods may be used, which might reduce the concentration of the pathogen [27, 28]. Therefore, RT–ddPCR is more suitable for detecting viral RNA in samples with lower concentrations. Currently, the most commonly reported method for detecting BVDV in semen samples is RT–qPCR. Mishra et al. (2018) used RT–qPCR to detect BVDV-2 in three semen samples from southern India. El-Mohamady et al. (2020) utilized raw semen samples for qPCR detection of BVDV and concluded that BVDV-infected bulls presented low semen quality. Using a qPCR assay, Read et al. (2020) detected BVDV in semen samples from five bulls three to eight months after infection and confirmed that this virus-contaminated semen presents a biosecurity risk. However, reports of ddPCR assays for detecting BVDV in bovine semen are lacking [29–31]. Gu et al. (2014) compared 5 RT–qPCR assays for the testing of BTV in semen serially collected from 8 bulls after experimental infection. Notably, the preferred qPCR assay has very high diagnostic sensitivity [32]. However, Vanbinst et al. (2010) validated RT–qPCR for the detection of semen for use in AI. The RT–qPCR analysis of 89 semen samples revealed a high number of 48 positive samples and 30 doubtful samples, and the authors confirmed that the uncertainty of the measurements was high around the detection limit [33]. In this study, we obtained the same results in mixed samples via RT–qPCR (WOAH). However, RT–ddPCR did not yield suspicious samples. Previous research has shown that ddPCR for clinical samples has high accuracy, but it lacks reported detection results for semen samples [26]. In addition, we found that the mixed sample was suspicious on the basis of RT–qPCR, in contrast to the positive results for these samples obtained by RT–ddPCR. Therefore, the use of RT–ddPCR as a supplement could further identify viral RNA at lower concentrations.

However, the present research has limitations. First, clinical semen samples lack other testing methods for verification. Second, different serotypes of BTV have not been detected via this method because of the lack of samples from them.

In conclusion, we demonstrated that the established BVDV-1 and BTV RT–ddPCR methods displayed good repeatability, sensitivity and specificity. Moreover, both methods accurately detected BVDV-1 and BTV in semen samples. Therefore, these methods have the potential to compensate for the viral RNA in semen samples and can be used as efficient detection methods for BVDV-1 and BTV.

Abbreviations

AI	Artificial insemination
BoHV-1	Bovine herpes virus type 1
BVDV	Bovine viral diarrhoea virus
BTV	Bluetongue virus
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
PCR	Polymerase chain reaction
qPCR	Quantitative real-time PCR
RT–ddPCR	Reverse transcriptase droplet digital PCR
WOAH	World Organisation for Animal Health

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12917-025-04506-4.

Supplementary Material 1: Table S1. Information on virus gene fragment sequences on the GenBank. Table S2. Information on BVDV-1 *5′-UTR* gene and BTV *NS3* gene fragment sequences on the GenBank. Table S3. Information on the primers and TaqMan probes. Table S4. The quantity and proportion of the bovine semen mixed samples in groups

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Author contributions
Y.ZC: methodology, conducted literature review, interpreted data, and drafted manuscript. Z.ZG: designed study and reviewed manuscript. C.LJ, C.Q and Y.H: performed study. L.JY, L.XP, Z.NML and L.YY: data collection and analysis. J.XH and Y, YH: reviewed manuscript. All authors have read and approved the manuscript.

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Data availability
The data supporting our findings of this article are included within the manuscript.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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