Establishment and Preliminary Application of One-step Reverse Transcriptase Droplet Digital PCR Assay for Bovine Viral Diarrhea Virus

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Abstract **[Objective]** The paper was to establish a one-step reverse transcriptase droplet digital PCR (RT-ddPCR) assay for bovine viral diarrhea virus (BVDV). **[Method]** Based on one-step real-time quantitative PCR (RT-qPCR) assay, BVDV-specific primers and probes were designed in this study. The reverse transcriptase, annealing temperature, primer and probe concentrations and reaction conditions of RT-ddPCR assay were optimized. Meantime, the specificity, sensitivity and repeatability of RT-ddPCR assay were evaluated. **[Result]** The optimal reverse transcription system for the established RT-ddPCR assay was as follows: commercial one-step reverse transcriptase droplet digital PCR kit with matching reagents, a final primer concentration of 900 nmol/L, a final probe concentration of 250 nmol/L and an optimal annealing temperature of 57 ℃. The results were negative when the method was used to detect other common epidemic viruses; the minimum detection limit was 3.2 copies/μL with good repeatability, and the coefficient of variation was less than 5%. RT-ddPCR and RT-qPCR assays were used to test 24 bovine swab samples and the test results showed that the established RT-ddPCR assay was superior to RT-qPCR assay. **[Conclusion]** The RT-ddPCR assay established in this study has strong specificity, high sensitivity and good repeatability, and is suitable for nucleic acid detection of clinical samples. This study provided a technical support for early detection and quantitative diagnosis of BVDV infection.

Keywords Bovine viral diarrhea virus; One-step procedure; Droplet digital PCR; Quantitative detection

Bovine viral diarrhea-mucosal disease (BVD-MD), also known as bovine mucosal disease, is a globally prevalent bovine infectious disease caused by bovine viral diarrhea virus (BVDV), and often triggers respiratory, digestive and reproductive diseases in cattle, causing sustained economic losses to the cattle industry worldwide[1–2]. In 1980, Li Youmin *et al.*[3] found the first case of BVD in Jilin Province of China and successfully isolated the virus. Afterwards, the disease spread rapidly, and the occurrence and prevalence of BVD were found in cattle in all regions of China. The virus can invade the body through the respiratory tract and digestive tract, and can also be vertically infected through the placenta. The virus carried by the semen of bulls can also be transmitted and lead to the spread of the disease, inducing abortion, stillbirth, and persistent infection (PI) of cattle[4]. Studies have found that the herd of cattle has the highest infection rate of BVDV, being the main source of BVDV infection. Pig, deer, buffalo, yak, sheep and goat can also be infected, and most of them are clinically recessive infection, but can also become a source of infection. According to whether BVDV can produce cytopathic effect after cell inoculation, BVDV can be divided into two biological types: cytopathic biotype (CP) and non-cytopathic biotype (NCP), and the serotypes of the two biological types are identical[5]. Infection of cattle herds by NCP BVDV will lead to large area of PI, and the presence of PI cattle makes it difficult to manage BVD. The disease is easily co-infected with other viruses and bacteria, causing strong comprehensive harm to the cattle industry. The weaned PI cattle have lower average body weight than normal cattle, with the survival time less than 2 years. In addition, BVDV can also cause immunosuppression, thus increasing the risk of other pathogen infection in cattle[6]. The screening of BVDV in cattle herds is particularly important for the healthy development of cattle farms. Therefore, early prevention and control is of great significance to the healthy development of cattle industry, and efficient and sensitive detection technology is the key to disease diagnosis. At present, the methods for BVDV detection in clinical samples mainly include immunofluorescence assay, PCR technology, TaqMan-PCR, ELISA, *etc.*[7–9]. These commonly used methods are criti-

Received: 2023–05–18 Accepted: 2023–07–26
Supported by Science and Technology Development Plan Fund Project of Jilin Province (20210202101NC); YDZJ202203C G Z H 050.
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cized by misdiagnosis, time-consuming and laborious, and cross-contamination. Although RT-PCR and qPCR are featured by high sensitivity, good repeatability, better specificity and high degree of automation compared with previous methods, these methods can only achieve qualitative and semi-quantitative detection, and can not accurately quantify viral nucleic acid, with certain limitations in sensitivity and specificity. Digital PCR is an absolute quantitative analysis technology developed in recent years following fluorescence quantitative PCR technology. By transferring a single DNA molecule into an independent reaction chamber, the fluorescence signal is detected and analyzed after PCR amplification, so as to achieve the absolute quantification of a single molecule and get rid of the dependence on standard curve[10–11]. In this study, a digital PCR assay with high sensitivity, high accuracy, high specificity and accurate quantification was established for the quantitative detection of BVDV.

1 Materials and Methods

1.1 Materials and reagents BVDV strains, MDBK cells, BVDV standard positive plasmids and identification primers of BVDV were all preserved by the Key Laboratory of Molecular Biology, Institute of Special Animal and Plant Sciences, Chinese Academy of Agricultural Sciences. DNA/RNA extraction kits were purchased from Beijing TransGen Biotech Co., Ltd. One-Step RT-ddPCR Advanced Kit for Probes was purchased from Bio-Rad (USA). GoTaq Probe 1-Step RT-qPCR system was purchased from Promega (USA). Droplet digital PCR reaction system, droplet generation card slot and rubber pad and other related reagents were purchased from Thermo Fisher Scientific (USA).

1.2 Design and synthesis of primers and probes The conserved sequence of BVDV (Accession No.: AY278459.1) 5'-UTR non-coding gene was downloaded from NCBI database, and specific primers and fluorescent probes were designed for this sequence using Primer 5.0 software (Tab.1). The primers and probes were synthesized by Sangon Biotech (Shanghai) Co., Ltd.

1.3 Optimization of reverse transcription conditions AMV reverse transcriptase, SuperScript III enzyme and One-Step RT-ddPCR Advanced Kit for Probes were selected. Using RNA with the same concentration of BVDV as the template, digital PCR assay was carried out by adding AMV reverse transcriptase and SuperScript III enzymes to 2×ddPCR supermix for Probes system. (1) AMV reverse transcriptase reaction system: 2 ×ddPCR supermix for Probes 10.0 μL, AMV 0.2 μL, 10 μmol/L primer 1.8 μL, 10 μmol/L probe 0.5 μL, ultrapure water 5.5 μL, and template 2.0 μL. The reaction was performed in the following conditions: 42 ℃ 60 min; 95 ℃ 10 min, 95 ℃ 30 s, 55 ℃ 1 min, 40 cycles; 98 ℃ 10 min; the reaction was terminated by dropping to 4 ℃, and the heating and cooling rate was 2.5 ℃/s. (2) SuperScript™ III enzyme system: 2×ddPCR supermix for Probes 10 μL, SuperScript III 1 μL, 10 μmol/L primer 1.8 μL, 10 μmol/L probe 0.5 μL, ultrapure water 4.7 μL, and template 2.0 μL. The reaction was performed in the following conditions: 50 ℃ 60 min; 95 ℃ 10 min, 95 ℃ 30 s, 55 ℃ 1 min, 40 cycles; 98 ℃ 10 min; the reaction was terminated by dropping to 4 ℃, and the heating and cooling rate was 2 ℃/s. (3) One-Step RT-ddPCR Advanced Kit for Probes system: One-step ddPCR supermix 5.0 μL, Reverse transcriptase 2.0 μL, 300 mmol/L DTT 1.0 μL, 10 μmol/L primer 1.8 μL, 10 μmol/L probe 0.5 μL, ultrapure water 7.7 μL, and template 2.0 μL. The reaction was performed in the following conditions: 42 ℃ 60 min; 95 ℃ 10 min, 95 ℃ 30 s, 55 ℃ 1 min, 40 cycles; 98 ℃ 10 min; the reaction was terminated by dropping to 4 ℃, and the heating and cooling rate was 2 ℃/s. By comparing the number of droplet generation after three types of reverse transcription, the appropriate reverse transcription system was selected.

1.4 Optimization of primer and probe concentration and temperature Three sets of different concentration gradients were set to optimize the forward and reverse primers and probes, and RT-ddPCR assay was performed using RNA with the same concentration of BVDV as the template (Tab.2). According to the results generated by the droplet detector, the optimal primer and probe reaction concentrations were screened. The annealing temperature was set as 50, 52, 55, 57 and 60 ℃, and the reaction conditions and procedures were the same as described in Section **1.3**.

1.5 Sample loading and assembly operation of reaction system All components of the kit, probes and primers were taken out and placed on ice to melt naturally. After vortex oscillation for 5 s and instantaneous dissociation for 3 s, various components were placed on ice for later use. Ultraviolet cross-linking agent syringes

Tab.1 Probe and primer sequences of droplet digital PCR

Primers and probes	Sequences (5'-3')
BVDV-F	CGAAGGCCGAAAAGAGGCTAGC
BVDV-R	TGGGCATGCCCTCGTCCAC
BVDV-P	FAM -TCGAACCAYTGACGACTRCCCTGTACTCAGGG-BHQ

Tab.2 Concentrations of primers and probes nmol/L

Primers and probes	Group 1	Group 2	Group 3
BVDV-F	600	900	1 200
BVDV-R	600	900	1 200
BVDV-P	150	250	350

and oil sealing syringes were installed under dark conditions, and used immediately within 1 h. The chip, brush head and chip cover were placed on digital PCR chip loading instrument correctly. All the reaction liquid of 15 μL was added to the sampling hole, and the chip loading instrument was started to smear the chip; 10–15 drops of sealing oil were added to seal the chip. Finally, the chip was inserted into the chip loader, and the chip QR code was placed facing forward in the Proflex 2 × Flat PCR System instrument for PCR amplification. The reaction conditions were the same as described in Section **1.3**. After the reaction, the chip was taken out and placed in the QS 3D instrument to automatically read the chip result. Finally, QS 3D AnalysisSuit Software was used to analyze the secondary data.

1.6 Specificity test With the genomes of common respiratory tract and gastrointestinal virus pathogens of cattle, including bovine respiratory syncytial virus (BRSV), bovine parainfluenza virus type 3 (BPIV3), infectious bovine rhinotracheitis virus (IBRV), bovine coronavirus (BCV), bovine adenovirus type 3 (BAV3) and bovine viral diarrhea virus (BVDV), as the templates, the specificity of primers was detected using optimized RT-ddPCR assay and RT-PCR assay, to verify the specificity of the established RT-ddPCR assay.

1.7 Sensitivity test Standard positive control plasmids were extracted, and the concentration and dilution calibration of the standard plasmids were determined using NanoPhotometer nucleic acid testing instrument. The recombinant plasmids were diluted in multiple copies (1×10^{5}–1×10^{-2} copies/μL) and used as templates. The RT-ddPCR and RT-qPCR assays established in this study were used for detection, and each concentration was repeated 3 times. The primers and probes of RT-qPCR assay were the same as that of RT-ddPCR assay to evaluate the sensitivity of the established RT-ddPCR assay. RT-qPCR was performed in the following system: GoTaq Probe qPCR dUTP Master Mix 10 μL, GoScript RT Mix for 1-step RT-qPCR 0.5 μL, 10 μmol/L each forward and reverse primer 1.2 μL, 10 μmol/L probe 0.6 μL, ultrapure water 5.7 μL, and template 2 μL. The amplification conditions were 45 ℃ 15 min; 95 ℃ 2 min, 95 ℃ 15 s, 60 ℃ 1 min, 40 cycles. The fluorescence signals were collected at annealing of each cycle.

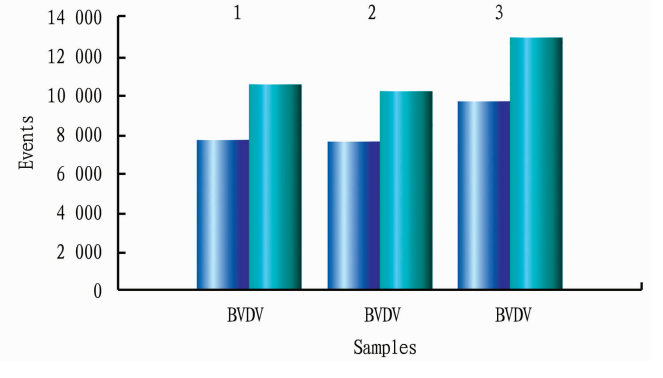
1.8 Repeatability test The BVDV positive standard plasmids were randomly selected as the samples, and the same BVDV sample was repeatedly tested 10 times by the optimized RT-ddPCR assay. The coefficient of variation was calculated according to the detected copy number to further verify the stability of the establish method.

1.9 Clinical sample testing The 24 samples of bovine clinical disease materials (feces and nasal swabs) collected and stored in the laboratory were freeze-thawed. With the genome of the disease samples obtained by the nucleic acid extraction kit as the template, the samples were detected by the established RT-ddPCR and RT-qPCR assays, and the detection results of the two methods were compared and analyzed.

2 Results and Analysis

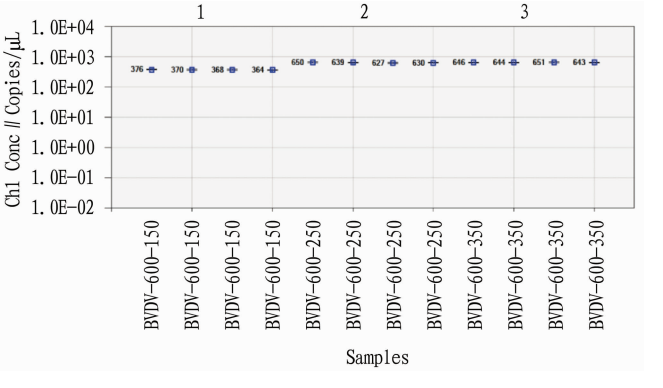
2.1 Optimization of reverse transcription conditions Three different reverse transcription methods were tested and the results were compared (Fig.1). The number of droplets generated by AMV reverse transcriptase was (7 294/9 960); the number of droplets generated by SuperScript III enzyme was (7 214/9 636); and the number of droplets generated by One-Step RT-ddPCR Advanced Kit for Probes was the largest of (9 155/12 247). It was proved that the enzyme reaction of the first two reverse transcription systems could not match well with the droplet oil produced by Bio-Rad, so the number of droplets generated decreased. Therefore, One-Step RT-ddPCR Advanced Kit for Probes was eventually selected as the kit in the following tests.

2.2 Optimization of primer and probe concentrations Three sets of different primer and probe concentrations were designed for RT-ddPCR, and the results showed that there were differences in inten-



Note: 1. AMV reverse transcriptase; 2. SuperScript III enzyme; 3. One-Step RT-ddPCR Advanced Kit for Probes reagent.

Fig.1 Optimization of reverse transcription conditions

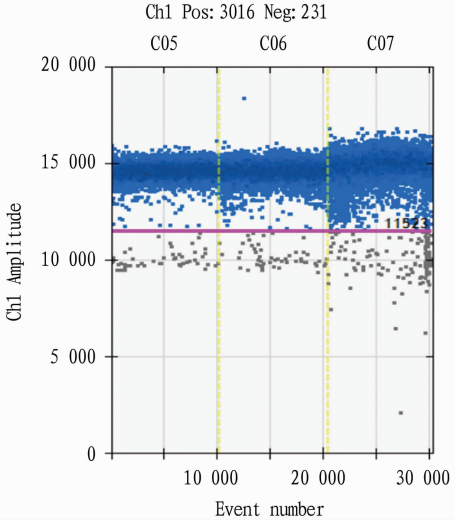


Note: 1. Primer concentration 600 nmol/L, probe concentration 150 nmol/L; 2. Primer concentration 900 nmol/L, probe concentration 250 nmol/L; 3. Primer concentration 1 200 nmol/L, probe concentration 350 nmol/L.

Fig.2 Optimization of primer and probe concentrations

sity of amplified fluorescence signal among the three groups. The copy number of the first group was significantly lower than that of the second group, and the concentration of primer and probe in the third group increased compared to the second group, but the copy number did not increase (Fig.2). Therefore, the second group with a primer concentration of 900 nmol/L and a probe concentration of 250 nmol/L was selected. Comprehensive evaluation showed that group 2 had the most suitable concentrations of primer and probe for practical application.

2.3 Optimization of annealing temperature The annealing temperatures of 50, 52, 55, 57, 60 ℃ were tested, and the optimization results are shown in Fig.3. When the annealing temperature was 60 ℃, the signal intensity decreased and the copy number reduced. Therefore, the annealing temperature was set at 57 ℃. Through the optimization of the above reaction conditions, the optimal reaction system of the established method was finally determined: Supermix 5.0 μL, Reverse transcriptase 2.0 μL, 300 mM DTT 1 μL, primer 1.8 μL (900 nmol/L), probe 0.5 μL (250 nmol/L), ultrapure water 7.7 μL, and template 2.0 μL. The reaction was performed in the following conditions: 42 ℃ 60 min; 95 ℃ 10 min, 95 ℃ 30 s, 57 ℃ 1 min, 40 cycles; 98 ℃ 10 min; 4 ℃ 60 min. The heating and cooling rate of the reaction was 2 ℃/s.



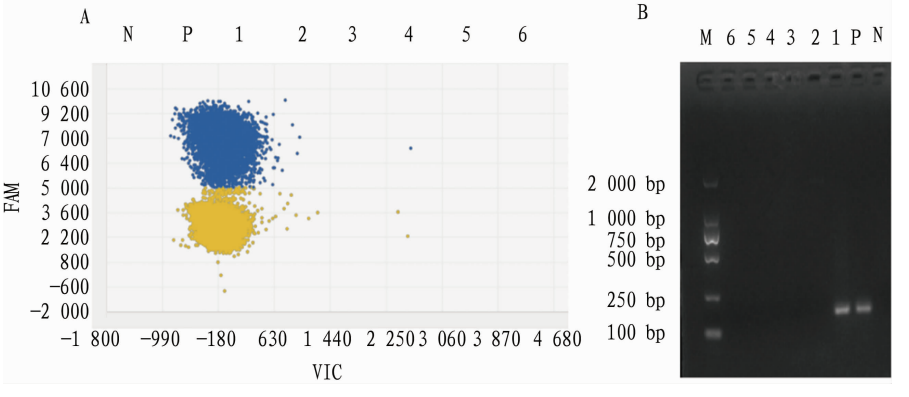
Note: The annealing temperatures of C05–C07 are 55, 57 and 60 ℃, respectively.

Fig.3 Optimization of annealing temperature

2.4 Specificity test The specificity test showed that there were positive droplets when the positive control and BVDV were amplified, while the negative control, BRSV, BPIV3, IBRV and BCV genomes all showed negative reactions (Fig.4). Meanwhile, RT-PCR assay with this primer showed that the positive standard control and BVDV had bands, while the negative control and other pathogens had no amplification bands, indicating that the designed BVDV primers and the established RT-ddPCR assay had strong specificity.

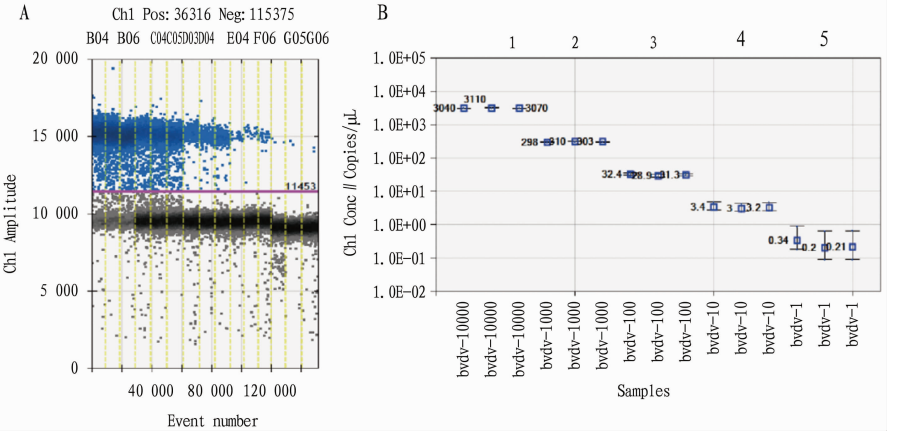
2.5 Sensitivity test The optimized RT-ddPCR assay can stably detect the standard BVDV as low as 3.2 copies/μL, and the coefficient of variation (CV) was 5.1%, less than 25%. It can also be detected when the concentration was as low as 0.25 copies/μL, but the CV value was 25.5%, close to 25%. Therefore, the minimum stable detection limit of this method was finally determined to be 3.2 copies/μL (Fig.5). Compared to RT-qPCR, the minimum detection limit was 1×10^{-1} copies/μL and the results were consistent in 3 repetitions (Fig.6), indicating that the established RT-ddPCR assay was more sensitive.

2.6 Repeatability test The positive standard plasmids were randomly selected as the samples, and the same positive sample was repeatedly tested by RT-ddPCR assay for 10 times. The repetitive detection results (Fig.7) showed that the coefficient of variation was 2.4%, less than 5% (Tab.3). The results demonstrated that the digital PCR assay had good repeatability and was stable and reliable.



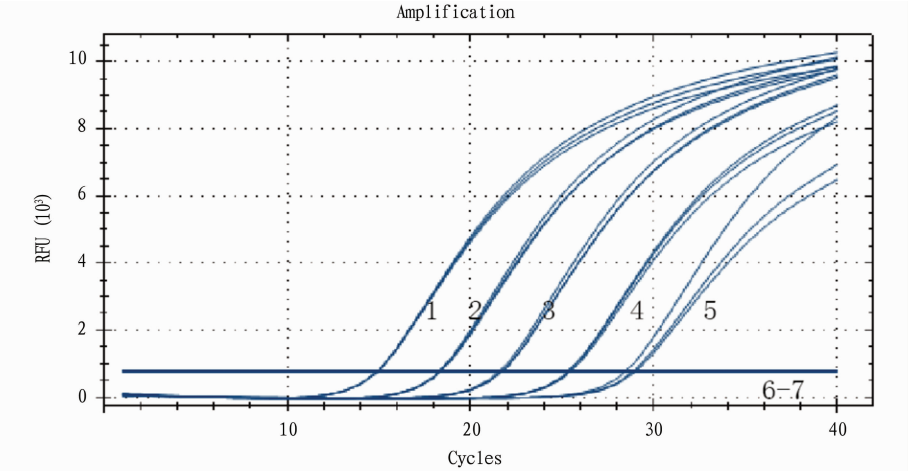
Note: M. DL2000 DNA Marker; N. Negative control; P. Positive control; 1. BVDV; 2. BRSV; 3. BPIV3; 4. IBRV; 5. BCV; 6. BAV3.

Fig.4 Specific detection of RT-ddPCR (A) and RT-PCR (B) assays



Note: 1–5. The template concentrations are 1×10^{3}–1×10^{-1} copies/μL.

Fig.5 Serial dilution amplification plot (A) and copy number plot (B)



Note: 1–7. The template concentrations are 1×10^5–1×10^{-1} copies/μL.

Fig.6 Sensitivity of RT-qPCR assay

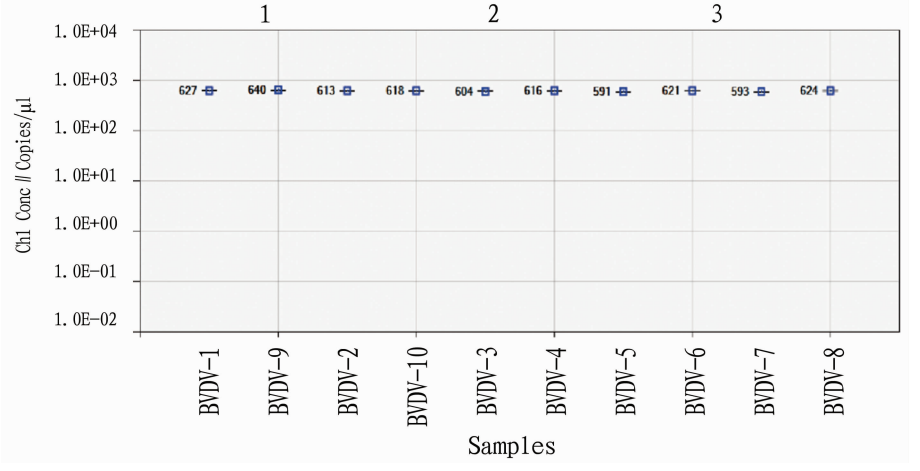


Fig.7 Copy number of repeatability test

Tab.3 Repeatability analysis of RT-ddPCR assay for BVDV

Sample	1	2	3	4	5	6	7	8	9	10
Measured value//copies/μL	627	613	604	616	591	621	593	624	640	618
Average value//copies/μL					614.70					
Standard deviation					14.42					
Coefficient of variation//%					2.34					

2.7 Testing of clinical samples The pathogens of 24 bovine nasal and fecal swabs collected were detected by RT-ddPCR and RT-qPCR assays. The detection rate of RT-ddPCR assay was 62.5% (15/24), while that of RT-qPCR assay was 45.8% (11/24). The results showed that the RT-ddPCR assay established for BVDV detection had higher sensitivity than RT-qPCR assay, and was more suitable for early clinical investigation and detection of samples with low virus content.

3 Discussion

With the transformation of China's cattle industry from natural grazing to intensive and large-scale development, there has been a high frequency of cross-region flow in beef cattle and dairy cattle breeding, whereas cattle transportation and herd links often bring opportunities for disease. BVD-MD is a disease common in cattle herds, caused by multiple factors. The complex interaction of environmental factors, feeding methods, pathogen transmission and other factors with the host makes the diagnosis, prevention and control of the disease more difficult[12]. The outbreak of PI diseases such as BVD in free-range or large-scale farms should be vigilant, and early warning and emergency treatment should be done in the prevention, control and purification of the epidemic. Therefore, a lot of detection and diagnosis methods have emerged in recent years. At present, the past diagnosis and detection methods can not found such hidden and persistent pathogens quickly and efficiently, making it difficult to completely purify the disease in cattle herds in the early stage. As one of the most common infectious diseases in cattle, BVDV harms cattle, which not only is reflected in the widespread prevalence of the disease, but also can form persistent infection and immunosuppression, thereby increasing the severity of the disease in cattle herds and increasing mixed infections. The serological test of PI cattle showed negative antibody and no obvious clinical symptoms, but the secretion, excreta, semen and milk of PI cattle could carry the virus. Studies have found that lifelong detoxification of PI animals is an important source of BVDV transmission, and PI cattle can further develop into fatal mucosal disease, so PI cattle must be removed from cattle herds to reduce the outbreak of the disease and purify the herd[4,13]. The traditional screening method of PI cattle is to collect otica and detect BVDV by immunohistochemistry or RT-PCR. However, it is difficult to collect samples and the test is complicated with poor operability. Meanwhile, traditional PCR assay has low detection sensitivity of serum samples, and can not meet the current detection requirements[14]. In recent years, with the vigorous development of China's cattle industry, the disease has spread in most areas of China, accompanied by an increasing trend year by year, which causes immeasurable economic losses to the cattle industry ev-

ery year. The screening of PI cattle requires qualitative and direct quantitative methods, especially it is very important to detect trace nucleic acid in samples.

Based on the counting principle of "single molecule template amplification reaction", digital PCR sufficiently separates the target sequence in 20 000 microreactors via chip dispersion system, and directly identify the copy number of nucleic acid target molecules in an absolute quantitative way through identification of the proportion of positive and negative amplification results and special algorithm statistics. It is suitable for the quantitative detection of trace nucleic acids in samples, the quantitative detection of rare base sequences in complex samples, and the accurate identification of small differences in nucleic acid copy number, and has better detection sensitivity, amplification specificity and quantitative accuracy than ordinary real-time quantitative PCR[15]. Zhang Yanfang *et al.*[16] established ddPCR that could quantitatively detect aviangyrovirus2 (AGV2) by using the characteristics of droplet digital PCR. Xu Renrui *et al.*[17] used droplet digital PCR to establish a RT-ddPCR assay for detecting rotavirus (RV), which improved the sensitivity and accuracy of detection. Catia Mio *et al.*[18] established a RT-ddPCR assay to detect the RNA of SARS-CoV-2 in nasopharyngeal swabs, which had higher accuracy and precision, especially at low concentrations, so as to facilitate early epidemic prevention and control[18]. Clarner *et al.*[19] used RT-ddPCR to quantify carrier expression in vitro and in vivo, which has simplified operation and powerful monitoring ability.

In this study, a one-step digital PCR assay for BVDV detection was established. Specific primers and probes were designed using the 5'-UTR non-coding gene conserved region, and an efficient, specific and sensitive RT-ddPCR assay for the detection of BVDV was established. This method can be used for the accurate detection of BVDV and the accurate quantification of viral nucleic acid in clinical samples, laying a foundation for the development of nucleic acid standard materials for diagnosis. The RT-ddPCR assay achieves the resolution and precise quantitative effect that traditional RT-PCR and fluorescent quantitative real-time PCR technology can not achieve, establishes an advanced technical detection method for BVDV trace nucleic acid detection, and provides a technical support for the early detection and purification of BVDV.

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