Polish Journal of Veterinary Sciences Vol. 22, No. 2 (2019), 405–413

DOI 10.24425/pjvs.2019.129300

Original article

TaqMan real-time PCR for detecting bovine viral diarrhea virus

H. Liang[1], J. Geng[2], S. Bai[1], A. Aimuguri[1], Z. Gong[3], R. Feng[2], X. Shen[4], S. Wei [1,2]

[1] Life Science and Engineering College, Northwest Minzu University, Lanzhou 730030, China
[2] Medicine College, Northwest Minzu University, Lanzhou, 730030, China
[3] Animal Cell Engineering Center of Gansu Province, Lanzhou 730030, China
[4] School of Karst Science, Guizhou Normal University, Guiyang, 550001, China

Abstract

The present study was aimed to establish a novel TaqMan real-time PCR (RTm-PCR) for detecting and typing bovine viral diarrhea virus (BVDV), and also to develop a diagnostic protocol which simplifies sample collection and processing. Universal primers and TaqMan-MGB probes were designed from the known sequences of conserved 5′ - and 3′-untranslated regions (5'UTR, 3'UTR) of the NADL strain of BVDV. Prior to optimizing the assay, cDNAs were transcribed in vitro to make standard curves. The sensitivity, specificity and stability (reproducibility) were evaluated. The RTm-PCR was tested on the 312 feces specimens collected from persistently infected (PI) calves. The results showed the optimum conditions for RTm-PCR were 17.0 µmol/L primer, 7.5 µmol/L probe and 51.4°C annealing temperature. The established TaqMan RTm-PCR assay could specially detect BVDV without detecting any other viruses. Its detection limit was 1.55×10^{0} copies/µL for viral RNA. It was 10000-fold higher than conventional PCR with excellent specificity and reproducibility. 312 samples were tested using this method and universal PCR from six dairy farms, respectively. Positive detections were found in 49 and 44 feces samples, respectively. The occurrence rate was 89.80%. In conclusion, the established TaqMan RTm-PCR could rapidly detect BVDV and effectively identify PI cattle. The detection limit of RTm-PCR was 1.55 copies/µL. It will be beneficial for enhancing diagnosis and therapy efficacy and reduce losses in cattle farms.

Key words: bovine viral diarrhea virus, quantitative real time PCR, TaqMan probe

Introduction

Bovine viral diarrhea virus (BVDV), one virus of Pestivirus genus in Flaviviridae, possesses a single-stranded RNA genome of 12.3 kb. It has a large open reading frame (ORF) that is flanked by 5′ and 3′ untranslated regions (5′ UTR, 3′ UTR) (Gaede et al. 2005, Francisco et al. 2017). BVDVs are classified into two genotypes, BVDV-1 and BVDV-2 (Francisco et al. 2017) according to the 5'UTR sequence difference. Up to date, at least 21 genetic groups of BVDV-1 (named BVDV-1a to BVDV-1r) and four groups in BVDV-2 (BVDV-2a to BVDV-2d) have been found (Alpay and Weşilag 2015, Zoccola et al. 2017, Peddireddi et al. 2018). Some strains of BVDV are highly virulent and produce severe hemorrhage or a mucosal illness

Correspondence to: Z. Gong and S. Wei, e-mail: weisc668@163.com, tel.: 0086-931-2937773

Table 1. Primer and probe sequences for qRT-PCR.

Primers	Primer sequences (5'→3')	Product length/bp
BVDV-F1	TCCTCYTYMGCGAMGGCC	681
BVDV-R1	CTRCCAGTYAYTCTCCCDATYC	
BVDV-probe	AGAGGAACATCTACAACCAC	
BVDV-F2	CCACTGTATCGCTACTAA	197
BVDV-R2	CCCCTCTTTTATGTGGTA	

(Bauermann et al. 2013). Understanding the variability of viral strains existing in a population provides valuable information of BVDV, particularly for control programs development and vaccination (Francisco et al. 2017). BVDV may cause various diarrhea symptoms in cattle, especially calves, which leads to the mortality increase in infected animals, resulting in significant economic losses (Bauermann et al. 2012, Bauermann et al. 2013). Persistently infected (PI) animals remain viremic throughout their life, and serve as viral reservoirs in cattle herds (Smirnova et al. 2012, Dubey et al. 2015).

Currently, several methods have been utilized to detect BVDV antigens, including immunohistochemistry (IHC), antigen ELISA, RT-PCR and RT-qPCR and the recently developed RT-ELISA and Cross-priming amplification techniques (Bhudevi and Weinstock 2003, Dubey et al. 2014). However, each method has its own strengths and weaknesses in terms of sensitivity and turnaround time (Kuta et al. 2015). Real-time PCR offers the same advantages as conventional PCR assays, but it is even more rapid and sensitive due to real-time quantification of the data and visualization (Baxi et al. 2006). Commonly used RT-PCR protocols require more than 1 h and a specialized PCR instrument (Sakurai et al. 2011, Zhang et al. 2015). Therefore, many efforts have been made to develop strategies that reduce reaction time to less than 20 min.

Recently, a single-step TaqMan® RT-qPCR was developed for the detection of BVDV-1. The assay was based on the conserved 5'UTR sequences of Italian BVDV-1 isolates. RT-qPCR, whose lower limit of detection was 100 copies of viral RNA (1 TCID50), correctly identified all PI animals (Zoccola et al. 2017). However, the oligonucleotides crossreacted with high titer BVDV-2 samples (Decaro et al. 2013).

Real-time PCR is now being used to identify, genetype and quantify many viral pathogens. RT-PCR methods for genotyping BVDV have been described previously (La Rocca and Sandvik 2009, Zhang et al. 2015). However, this differs in the efficiency of RNA extraction, reverse transcription or PCR reactions, leading to the unstable sensitivity and specificity of this technique (Yan et al. 2016). In order to overcome these limitations, in this study we put forth effort to develop a quantitative real time PCR (qRT-PCR) based on the TaqMan probe, additionally to establish accurate and rapid method for detecting the bovine viral diarrhea virus (BVDV) in different biological specimens.

Materials and Methods

RNA extract from BVDV NADL strain

NADL strain of BVDV that was provided by Animal Cell Engineering Center of Gansu Province (Lanzhou, China) was subcultured at 37°C and 5% CO_2 in MDBK cells using Dulbecco's modified Eagle medium (DMEM, Gibco, Shanghai, China) and 8% newborn bovine serum (Lanzhou Minhai Bioengineering Co., Ltd.) for 5-7 days. The supernatant was separated at 2000×g for 10 min from medium fluids harvested on days 5-7.

Total RNA was extracted from the supernatant using the TIANamp Virus RNA Kit (Tiangen Biochemical Technology Co., Ltd, Beijing, China) according to the manufacturer's instruction. The extracted RNAs were stored at -80°C for the subsequent tests.

Primers and probe design

The full-length genome of reference strains of BVDV was obtained from the GenBank database. Based on the earlier reports (Barlic-Maganja and Grom, 2001), we designed one pair of universal primers (BVDV-F1/BVDV-R1), one pair of specific primers (BVDV-F2/BVDV-R2) and a specific TaqMan probe (BVDV-probe) from the 5′-untranslated region (5'UTR) according to gene sequence of BVDV NADL strain (NC001461, AM709624.1, GI156622318, AJ133738.1) using DNAStar and Primer 5.0 software. The 5' end and 3' end of the BVDV-probe were labeled with 5-Carboxyfluorescein (FAM) and quencher fluorescent dye Eclipse, respectively. The specificity of the designed primers and probes was verified with Primer-BLAST on NCBI online. The primers and probes were synthesized by the TAKARA Bio INC (Beijing, China) (Table 1).

Construction of standard plasmids and copies

The extracted RNAs were reversely transcribed into cDNAs. The cDNAs were amplified by PCR using universal primers. 50 µL PCR reaction system included 0.5 µL Takara LA Taq (5 U/µL), 10×LA PCR Buffer II (Mg^{2+} plus, 8 µL dNTP Mixture (2.5 mM) 5µL, BVDV-F1 1 µL, L BVDV-R1 1 µ, template cDNAs 5 µL and diethyl pyrocarbonate (DEPC) water. The reaction conditions were 94°C 5 min, 94°C 30 s, 60°C 45 sec, 72°C 1 min, 35 cycles and 72°C 10 min. Then, 4.5 µL reaction supernatants were electrophoresed on 1% agarose gel to identify the amplified products.

The electrophoresis-verified PCR products were harvested utilizing EasyPure® Quick Gel Extraction Kit (Transgen Biotech Company, Beijing, China) and ligated into pMD18-T vector (Takara, Shanghai, China) for 2 h at 16°C. BL-21 competent cells were transformed with the ligated complex at 37°C overnight.

The selected monoclonal colony was inoculated into Amp/LB liquid media (5.0 mL), and gently shaken for 10-14h at 220 r/min and 37°C. Then the recombinant plasmids were extracted using the EasyPure® Plasmid MiniPrep Kit (Transgen Biotech Company, Beijing, China) and sequenced. The concentrations of plasmids were determined using Ultramicro nucleic acid protein analyzer (Thermo Fisher, USA) after the sequencing results were identical with the sequences documented in GenBank. DNA copies were calculated based on the formula: DNA copies = $[6.02\times10^{23}\times$plasmid concentrations (ng/µL) $\times10^{-9}]$ / (660×bp of standard plasmids).

Following amplification of the recombinant plasmids, the positive plasmids were sequenced (Shanghai Sangon Biotec, Shanghai, China). The homology of these sequences was compared with those in Genbank online of NCBI.

Optimization of PCR reaction conditions and creation of standard curve

RTm-PCR was performed in a 20 µL reaction system [containing Premix Ex Taq™ 10µL, forward primer (17.5 µM) 0.4 µL, reverse Primer (17.5 µM) 0.4 µL, TaqMan Probe (7.5 µM) 0.8 µL, ROX Reference Dye II (50×) 0.2 µL, DEPC water 6.2 µL, plasmid template (25 ng/µL) 2 µL].

The optimum schemes of qRT-PCR were selected from the different reaction conditions including the varying primer concentrations (15.0, 15.5, 16.0, 16.5, 17.0, 17.5, 18.0, 18.5, 19.0, 19.5 and 20.0 µmol/L), probe concentrations (5.00, 6.25, 7.50, 8.75 and 10.00 µmol/L) and annealing temperatures (51.0°C, 51.2°C, 51.4°C, 52.2°C, 52.9°C, 53.4°C, 53.8°C, 54.0°C).

Subsequently, the different concentrations of recombinant plasmids were amplified with the optimized qRT-PCR at the different concentrations (from 1.55×10^{9} to 1.55×10^{3} copies/µL) to acquire the amplification melting curve, standard curve and regression equation.

Specificity test

In order to evaluate the specificity of the designed TaqMan RTm-PCR, this method was utilized to synchronously detect reference strains and other six viruses as BVDV, such as Japanese encephalitis virus (JEV), Classical Swine Fever Virus (CSFV), Rabies virus (RABV), Bovine Rotavirus (BRV), Bovine Parvovirus (BPV), bovine foot and mouth disease virus (FMDV). Total RNAs were extracted with the methods described above. All these viruses were provided or gifted by the State Ley Laboratory of Biological Engineering and Technology of the Northwest Minzu University and Lanzhou Veterinary Research Institute of the Chinese Academy of Agricultural Sciences (Lanzhou, China). They were cultured in the common cells suitable to them, respectively. Total RNAs or DNAs were extracted using commercial RNA or DNA kits, respectively.

Sensitivity verification

The sensitivity of the qRT-PCR assay was evaluated using 10-fold dilutions (1.55×10^{11}~1.55×10^{0} copies/µL DNA) of the constructed recombinant plasmids, respectively under the optimum reaction conditions. The correlated CT values were used to set the standard curves for respective absolute quantifications (Decaro et al. 2005). The detection results were compared with those of common PCR so as to determine the minimum detection limit. The distilled water was used as negative controls and blank. The tests were implemented in triplicate.

Repeatability tests

In order to verify the stability of RTm-PCR assay, the intra-assay and inter-assay repeatability were evaluated testing at least three times the same plasmids in one experiment. Intra-assay and inter-assay coefficients of variation (CVs) were calculated by dividing the standard deviation of each tested plasmid by its mean and multiplying that result by 100, respectively.

Clinical application

Special emphasis was placed on the suitability of the tests for rapid and reliable detection of viral infections in the field. Clinical feces samples from 312 diarrhea calves from six different cattle farms in Gansu Province of China were detected with both TaqMan RTm-PCR and common PCR to verify the repeatability

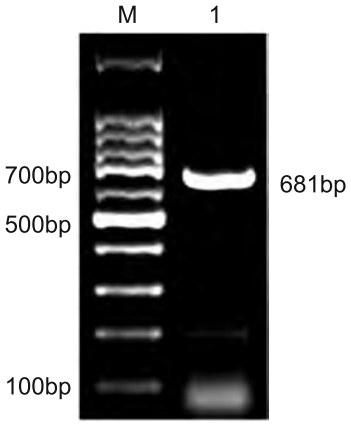

Fig. 1. The PCR amplification result of BVDV 5′ UTR.
M: 100bp Marker; 1: BVDV 5′ UTR

of this qRT-PCR method. The diarrhaois calves were less than 6 months old. They showed successively diarrhea symptoms for over 3 days. The feces samples were collected during days 3-5 of the diarrhea.

Suspensions were prepared by diluting the feces samples according to operation instruction described above. Then, total RNA was extracted from suspension of feces using the TIANamp Virus RNA Kit (Tiangen Biochemical Technology Co., Ltd, Beijing, China).

Results

PCR amplification of BVDV RNA extracts

After the 5′ UTR extracts of BVDV were amplified, the bands in agarose gel electrophoresis indicated the PCR product was 681bp, which was consistent with the expected product sizes (Fig. 1). The coincidence rate of the sequence of positive plasmids was 99% with the sequences documented in Genbank.

Construction and optimization of qRT-PCR based on TaqMan probe

Optimization of primer concentrations

In order to find an optimum primer concentration for this TaqMan RTm-PCR, varying concentrations of specific primer (BVDV-F2/BVDV-R2) were used in the experiment evaluation from 5.0 to 20.0 μmol/L at the gradient difference of 2.50 (5.0, 7.5, 10.0, 12.5, 15.0, 17.5 and 20.0) μmol/L. The better bands were found within the primer concentrations from 15.0 μmol/L to 20.0 μmol/L. Afterwards, the further refinement optimization was performed with 11 primer concentrations at the gradient difference of 0.50 μmol/L (15.0, 15.5, 16.0, 16.5, 17.0, 17.5, 18.0, 18.5, 19.0, 19.5 and 20.0 μmol/L) (Fig. 2A).

As shown in Fig. 2A, we found the optimum primer concentration for the RTm-PCR was 17.5 μmol/L.

Optimization of the probe concentration

The probe concentrations were optimized by two step process. The first step of probe optimization was performed in the gradient of 2.50 μmol/L (2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5 and 20.0μmol/L). As per the preliminary results of the first step, the further refinement selection was done in the gradient of 1.25 μmol/L (5.00, 6.25, 7.50, 8.75 and 10.00 μmol/L) (Fig. 2B).

The best amplification efficacy was acquired when probe concentration was 7.5 μmol/L.

Optimization of annealing temperature

The optimum reaction temperature was selected from nine annealing temperatures (50.0°C-60.0°C) in the tests. Then, the annealing temperatures were further optimized within 51.0°C and 54.0°C **(**51.0, 51.2, 51.4, 52.2, 52.9, 53.4, 53.8 and 54.0°C). The results showed the amplification efficacy was the greatest when annealing temperature was at 51.4°C.

Establishment of optimum conditions

On the bases of the screen results of three parameters (primer concentration, TaqMan probe concentration and annealing temperature), we obtained that optimum conditions were 17.0 μmol/L primer, 7.5 μmol/L probe and 51.4°C annealing temperature. Plasmid concentrations had an excellent linear relationship with cycle threshold (Ct) values.

Dynamic curves and standard curves are shown in Fig. 3. The Regression equation of standard curve is: $Y= -3.464 \log x +45.807$, $R^2=0.9998$. Amplification efficiency was 96.394%.

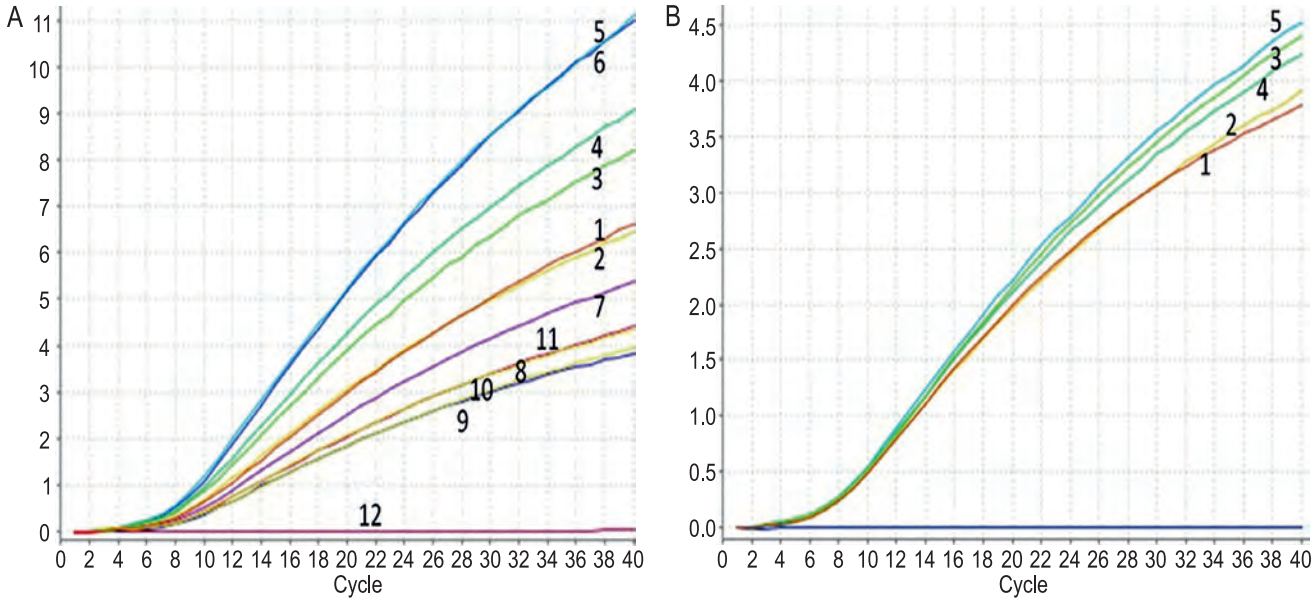

Fig. 2. Optimization of the primers and probe concentrations.
A. Optimization of the primers concentrations
Lines 1-11: The primers concentrations were 15.0, 15.5, 16.0, 16.5, 17.0, 17.5, 18.0, 18.5, 19.0, 19.5 and 20.0 μmol/L, respectively;
Line 12: Negative control.
B. Optimization of the probe concentration
Lines 1-5: Probe concentrations were 5.00, 6.25, 7.50, 8.75 and 10.00 μmol/L, respectively.

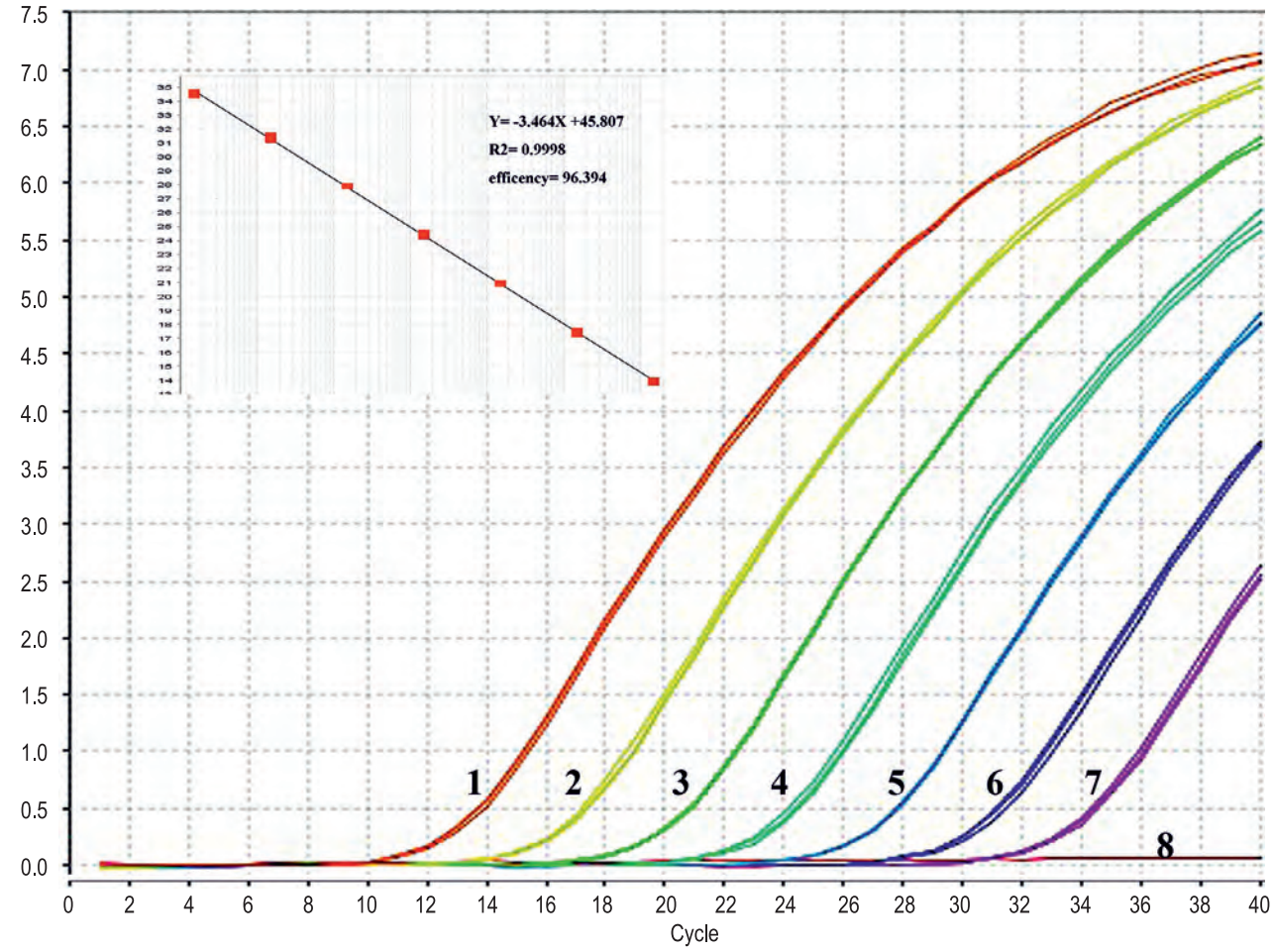

Fig. 3. Dynamic curves and standard curves (small graph) of RTm-PCR.
Lines 1-7: 1.55×10^{9} to 1.55×10^{3} copies/μL, respectively; Line 8: Negative control.

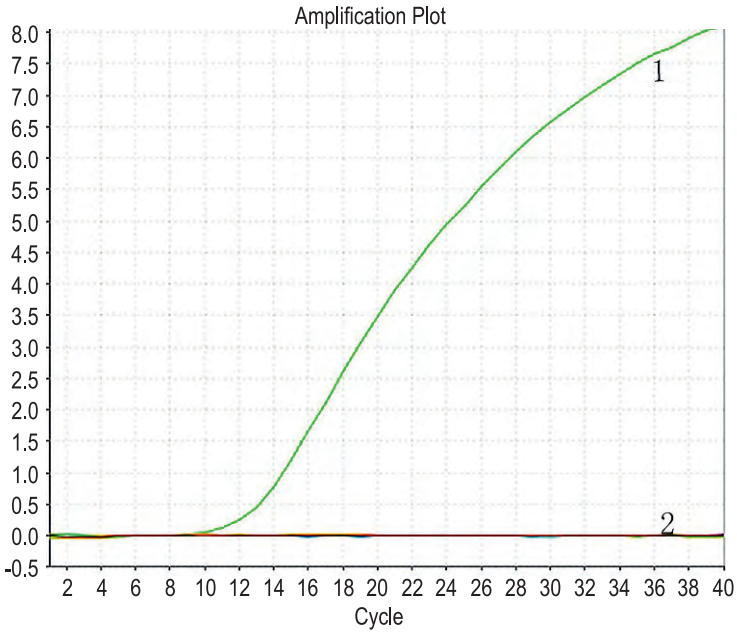

Fig. 4. The specificity test for TaqMan RTm-PCR of BVDV.
Line 1: BVDV; Line 2: Control group (JEV, RABV, CSFV, BRV, BPV and FMDV and Negative control).

Specificity assessment

As shown in Fig. 4, the qRT-PCR could clearly detect BVDV. However, all other viruses displayed no signal bands, including JEV, RABV, CSFV, BRV, BPV and FMDV. The findings indicated that the established Taqman RTm-PCR had a high specificity.

Sensitivity

After the standard plasmids were detected with this qRT-PCR assay, all plasmid concentrations from 1.55×10^{11} to 1.55×10^{0} copies/μL displayed positive amplification signals. But, common PCR assay only detected 1.55×10^{4} copies/μL. The results demonstrated that the least detection limit of the Taqman RTm-PCR was 1.55×10^{0} copies/μL, or 1.55 copies/μL (Fig. 5). That was 10000-fold higher than common PCR.

Repeatability

The results of repeatability tests indicated the intra--assay and inter-assay variation coefficient (VC) was 0.04%-0.25% and 1.02%-1.64%, respectively. This demonstrated that qRT-PCR shows an excellent repeatability, or stability.

Diagnostic validation of qRT-PCR

The qRT-PCR validation was done on 312 feces samples harvested from six dairy farms for the presence of BVDV in infected animals. The results showed that the positive results were found in 49 feces samples by the established qRT-PCR compared to 44 samples by universal PCR. The sensitivity and specificity of qRT-PCR were higher than that of universal PCR.

Discussion

The bovine viral diarrhea virus (BVDV) plays an important role in bovine respiratory disease by exerting the immunosuppressive effect (Decaro et al. 2014). The mechanism of BVDV-induced immunosuppression has not been fully determined (Fredericksen et al. 2015). Nowadays, the lack of specific antibodies for the detection of the molecules produced by BVDV-1 infection strongly suppresses evaluating these effects (Fredericksen et al. 2015).

The common RT-PCR has been reported for the detection of several animal and human viruses (Chaharaein et al. 2009; Santos et al. 2008). Currently, real-time fluorescence PCR (qPCR) has been utilized to identify, genetype and quantify many viral pathogens since it is a highly sensitive and rapid method for detecting viral nucleic acid sequences in clinical specimens (La Rocca and Sandvik 2009, Zhang et al. 2015). Generally, normalization is important to adjust the variation introduced by various steps of qRT-PCR assay, such as quantity and quality of RNA samples, cDNAs, fluorescence fluctuations, and well-to-well volume variations (Kuta et al. 2015, Mohamed et al. 2017) . However, these differ in the efficiency of RNA extraction, reverse transcription or PCR reactions which are crucial to the sensitivity of this method. The technical assay varies due to normalization control of the signal with an internal standard, typically a housekeeping

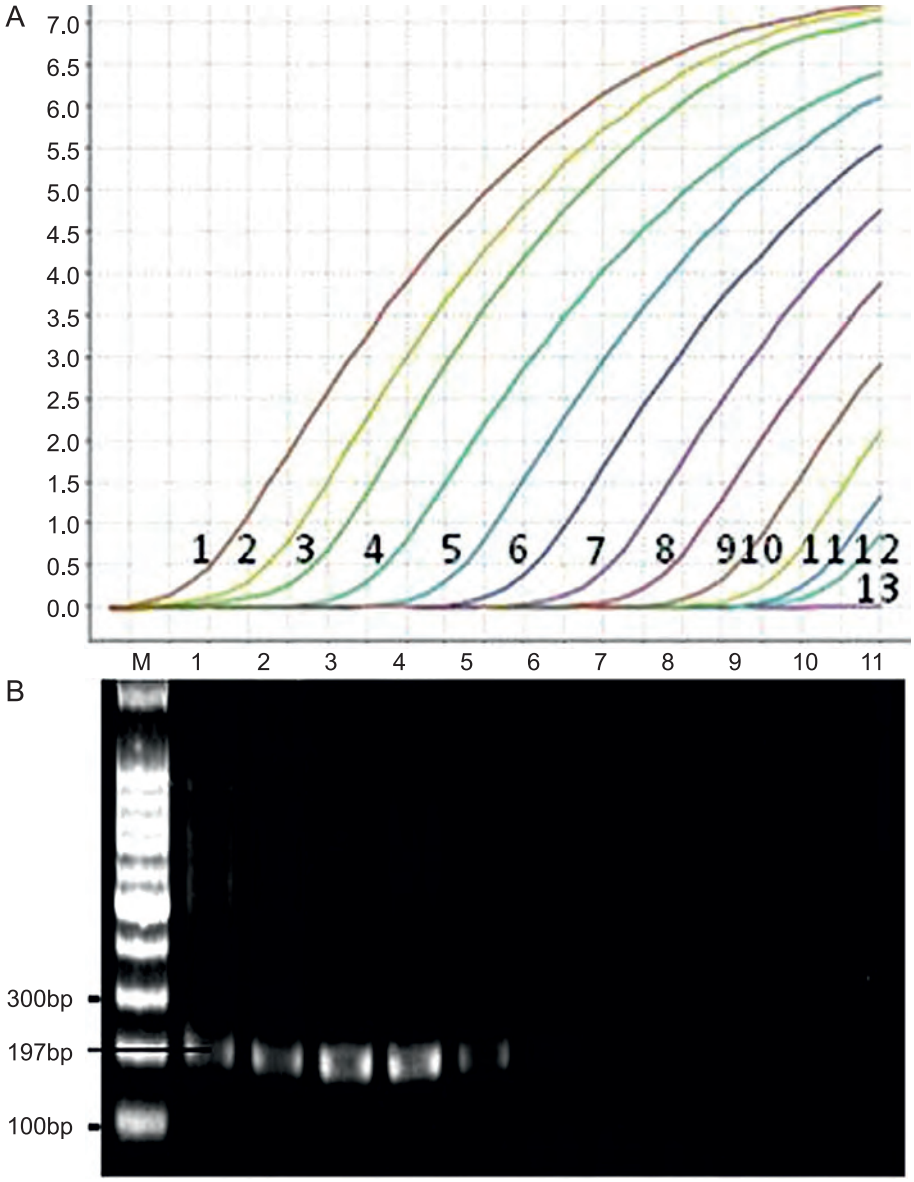

Fig. 5. Sensitivity assessment of the established TaqMan RTm-PCR and conventional PCR.
A. TaqMan RTm-PCR
M: 1000bp DNA Marker; Lines 1-12: 1.55×10^{11} to 1.55×10^{0} copies/μL, respectively;
Line 13: Negative control.
B. Conventional PCR assay
M: 1000bp DNA Marker; Lines 1-10: 1.55×10^{9} to 1.55×10^{0} copies/μL, respectively;
Line 11: Negative control.

gene (such as GAPDH and ACTB, etc). To circumvent these problems, we developed a quantitative real time PCR (qRT-PCR) in this study based on the TaqMan probe so as to accurately and rapidly detect bovine viral diarrhea virus (BVDV) in the feces.

In the present TaqMan qRT-PCR method, we selected and used one pair of specific primers (BVDV-F2/BVDV-R2) and a specific TaqMan probe (BVDV-probe) targeting the highly conserved 5′-untranslated region (5'UTR) (Barlic-Maganja and Grom 2001, Dybkaer et al. 2003). The specificity of primers and probes was validated with Primer-BLAST.

The specificity of the qRT-PCR was confirmed by the negative control and other six viruses, including JEV, RABV, CSFV, BRV, BPV and FMDV, which are the same family or genus viruses as BVDV.

The results verified that TaqMan qRT-PCR could only detect BVDV. This demonstrated the qRT-PCR had an excellent specificity. It is evident that this TaqMan qRT-PCR assay can detect BVDV with higher sensitivity and reproducibility than other published real-time RT-PCR methods (Dybkaer et al. 2003). The sensitivity test indicated the least detection limit of the qRT-PCR method was 1.55 copies/μL. The sensi-

tivity of TaqMan qRT-PCR assay was increased by 10000-fold as compared to universal PCR with a good inter-assay and intra-assay reproducibility. Our results are consistent with a previous study (Zhang et al. 2014). Additionally, the diagnostic sensitivity and specificity were determined using 312 clinical samples from six cattle farms. The qRT-PCR showed a higher sensitivity and specificity in comparison with the conventional PCR and 89.80% concordance between the two tests was found. Our findings were consistent with earlier studies (Dybkaer et al. 2003; Zhang et al. 2015).

Further, on the basis of the high sensitivity of the assay, we speculate that the qRT-PCR can be applied to test many kinds of biological specimens and thereby be a cost-effective method to diagnose BVDV infections and PI animals in dairy farms by providing a reliable clinical examination scheme to determine the BVDV infection rate.

Conclusion

We have established an assay method that could detect 1.55 copies/μL of BVDV from the feces. It was 1000-fold higher than common PCR with excellent specificity and reproducibility. The qRT-PCR could rapidly detect BVDV and effectively identify all persistently infected (PI) cows. It is probably applied to test many kinds of biological specimens and thereby be a cost-effective method to diagnose BVDV infections and PI animals in dairy farms. This novel qRT-PCR is beneficial for enhancing diagnostic efficiency and effectively controlling the spread of BVDV infections and reduce losses in cattle farms.

Acknowledgements

The work was supported by the Project of National Key research and Development Program of China in 13th Five-year Plan (2016 YFC0502601), the Science and Technology Support Program project of Gansu province of China (Grant No: 1604NKCA044), the innovative research team of Yak Reproductive Regulation Mechanism and Plateau Adaptability (31920190024), the Special Fund Project for Fundamental Research Business Expenses of Central Universities, the Science and Technology Planning Project of Lanzhou, China (Grant No: 2016-3-120), Chengguan district Science and Technology Planning Project of Lanzhou, China (Grant: 2016-1-1), the Innovative Team Development Project of Ministry of Education of China (IRT-17R88); the Postgraduate Student Research Innovation Project of Northwest Minzu University (Yxm2016140).

References

Alpay G, Weşilag K (**2015**) Serological relationships among subgroups in bovine viral diarrhea virus genotype 1 (BVDV- 1). Vet Microbiol 175: 1-6.

Barlic-Maganja D, Grom J (**2001**) Highly sensitive one-tube RT-PCR and microplate hybridisation assay for the detection and for the discrimination of classical swine fever virus from other pestiviruses. J Virol Methods 95: 101-110.

Bauermann FV, Flores EF, Ridpath JF (**2012**) Antigenic relationships between Bovine viral diarrhea virus 1 and 2 and HoBi virus: possible impacts on diagnosis and control. J Vet Diagn Invest 24: 253-261.

Bauermann FV, Ridpath JF, Weiblen R, Flores E (**2013**) Hobi-like viruses: an emerging group of pestiviruses. J Vet Diagn Invest 25: 6-15.

Baxi M, McRae D, Baxi S, Greiser-Wilke I, Vilcek S, Vilcek S, Amoako K (**2006**) A one-step multiplex real-time RT-PCR for detection and typing of bovine viral diarrhea viruses. Vet Microbiol 116: 37-44.

Bhudevi B, Weinstock D (**2003**) Detection of bovine viral diarrhea virus in formalin fixed paraffin embedded tissue sections by real time RT-PCR (Taqman). J Virol Methods 109: 25-30.

Chaharaein B, Omar AR, Aini I, Yusoff K, Hassan SS (**2009**) Detection of H5, H7 and H9 subtypes of avian influenza viruses by multiplex reverse transcription polymerase chain reaction. Microbiol Res 164: 174-179.

Decaro N, Lanave G, Lucente MS, Mari V, Varello K, Losurdo M (**2014**) Mucosal disease-like syndrome in a calf persistently infected by Hobi-like pestivirus. J Clin Microbiol 52: 2946-2954.

Decaro N, Losurdo M, Lucente MS, Sciarretta R, Mari V, Larocca V, Elia G, Cavaliere N, Martella V, Fasanella A, Buonavoglia C (**2013**) Persistent infection caused by Hobi-like pestivirus. J Clin Microbiol 51: 1241-1243.

Decaro N[1], Martella V, Ricci D, Elia G, Desario C, Campolo M, Cavaliere N, Di Trani L, Tempesta M, Buonavoglia C (**2005**) Genotypespecific fluorogenic RT PCR assays for the detection and quantitation of canine coronavirus type I and type II RNA in faecal samples of dogs. J Virol Methods 130: 72-78.

Dubey P, Mishra N, Rajukumar K, Behera SP, Kalaiyarasu S, Nema RK (**2014**) Development of a RT-PCR ELISA for simultaneous detection of BVDV-1, BVDV-2 and BDVin ruminants and its evaluation on clinical samples. J Virol Methods 213: 50-56.

Dubey P, Mishra N, Rajukumar K, Behera SP, Kalaiyarasu S, Nema RK, Prakash A (**2015**) Development of a RT-PCR ELISA for simultaneous detection of BVDV-1, BVDV-2 and BDV in ruminants and its evaluation on clinical samples. J Virol Methods 213: 50-56.

Dybkaer K, Munch M, Handberg KJ, Jorgensen PH (**2003**) RT-PCR-ELISA as a tool for diagnosis of low-pathogenecity avian influenza. Avian Dis 47 (Suppl 3): 1075-1078.

Francisco JD, Manuel C, Eduardo Y (**2017**) Bovine viral diarrhea virus (BVDV) genetic diversity in Spain: A review. Spanish J Agri Res 15(2): 2171-9292.

Fredericksen F, Delgado F, Cabrera C, Yanez A, Gonzalo C, Villalba M, Olavarria VH (**2015**) The effects of reference genes in qRT-PCR assays for determining the immune

response of bovine cells (MDBK) infected with the Bovine Viral Diarrhea Virus 1 (BVDV-1). Gene 569(1): 95-103.

Gaede W, Reiting R, Schirrmeier H, Depner KR, Beer M (**2005**) Detection and species-specific differentiation of pestiviruses using real-time RT-PCR. Berl Munch Tierarztl Wochenschr 118: 113-120.

Kuta A, Woźniakowski G, Polak MP (**2015**) Cross-priming amplification for detection of bovine viral diarrhoea virus species 1 and 2. J Appl Microbiol 119(3): 632-639.

La Rocca SA, Sandvik T (**2009**) A short target real-time RT-PCR assay for detection of pestiviruses infecting cattle. J Virol Methods 161(1): 122-127.

Mohamed FF, Mansour SM, El-Araby IE, Mor SK, Goyal SM (**2017**) Molecular detection of enteric viruses from diarrheic calves in Egypt. Arch Virol 162(1): 129-137.

Peddireddi L, Foster KA, Poulsen EG, An B, Hoang QH, O'Connell C, Anderson JW, Thomson DU, Hanzlicek GA, Bai J, Hesse RA, Oberst RD, Anderson GA, Leyva-Baca I (**2018**) Molecular detection and characterization of transient bovine viral diarrhea virus (BVDV) infections in cattle commingled with ten BVDV persistently infected cattle. J Vet Diagn Invest 30(3): 413-422.

Sakurai A, Nomura N, Nanba R, Sinkai T, Iwaki T, Obayashi T, Hashimoto K, Hasegawa M, Sakoda Y, Naito A, Morizane Y, Hosaka M, Tsuboi K, Kida H, Kai A, Shibasaki F (**2011**) Rapid typing of influenza viruses using super high-speed quantitative real-time PCR. J Virol Methods 178: 75-81.

Santos N, Honma S, Timenetsky Mdo C, Linhares AC, Ushijima H, Armah GE, Gentsch JR, Hoshino Y (**2008**) Development of a microtiter plate hybridization based PCR-enzyme-linked immunosorbent assay for identification of clinically relevant human group A rotavirus G and P genotypes. J Clin Microbiol 46: 462-469.

Smirnova NP, Webb BT, Bielefeldt-Ohmann H, Campen HV, Antoniazzi AQ, Morarie SE, Hansen TR (**2012**) Development of fetal and placental innate immune responses during establishment of persistent infection with bovine viral diarrhea virus. Virus Res167(2): 329-336.

Yan L, Pace LW, Baughman B, Wilson FD, Zhang S, Zhang MZ (**2016**) Failed detection of Bovine viral diarrhea virus 2 subgenotype a (BVDV-2a) by direct fluorescent antibody test on tissue samples due to reduced reactivity of field isolates to raw anti-BVDV antibody. J Vet Diagn Invest 28(2): 150-157.

Zhang SQ, Tan B, Li P, Wang FX, Guo L, Yang Y, Sun N, Zhu HW, Wen YJ, Cheng SP (**2014**) Comparison of conventional RT-PCR, reverse-transcription loop-mediated isothermal amplification, and SYBR green I-based real-time RT-PCR in the rapid detection of bovine viral diarrhea virus nucleotide in con-taminated commercial bovine sera batches. J Virol Methods 207: 204-209.

Zhang Y, Liu H, Wang X (**2015**) A novel real-time RT-PCR with TaqM an-MGB probes and its application in detecting BVDV infections in dairy farms. J Integrative Agri 14(8): 1637-1643.

Zoccola R, Mazzei M, Carrozza ML, Ricci E, Forzan M, Pizzurro F, Giammarioli M, Bandecchi P, Tolari F (**2017**) A newly developed BVDV-1 RT-qPCR Taqman assay based on Italian isolates: evaluation as a diagnostic tool. Folia Microbiol 62(4): 279-286.