TaqMan probe qRT-PCR detects bovine parvovirus and applies clinically

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Abstract: The present study aimed to establish a TaqMan probe real-time PCR (qRT-PCR) for detecting Bovine parvovirus (BPV), and to develop a novel diagnostic method of BPV. TaqMan probe and primers were designed and synthesized from the sequences of conserved 5′-untranslated regions (5′-UTR) of strain Haden of BPV. Optimum reaction conditions were comparatively selected. The sensitivity, specificity, and reproducibility of TaqMan probe qRT-PCR were measured respectively. Feces specimens collected from diarrhea calves were detected using this assay. The results showed that optimized conditions for the probe qRT-PCR were 0.8 μL probes (18.0 μmol/L), 0.4 μL primers (10.0 μmol/L) and annealing temperature of 43.2 °C for 40 s. The probe qRT-PCR could specially detect BPV, but not for other viruses (JEV, CSF, RABV, BRV, BVDV, and bFMDV). The detection limit was 5.26 copies/μL. Both intraassay and interassay VCs were lower than 1.65%. Detection of 308 feces samples showed 36 positives for TaqMan probe qRT-PCR compared to 32 positives of universal PCR. Coincidence rate of the two methods was 88.89%. In conclusion, the established TaqMan probe qRT-PCR could specifically detect BPV. It had excellent sensitivity, specificity, and stability. The detection limit was 5.26 copies/μL. Its sensitivity was 10,000-fold over the conventional PCR. TaqMan probe qRT-PCR will help to enhance diagnosis and therapy efficacy of diarrhea calves.

Key words: Bovine parvovirus, TaqMan probe, real-time PCR, sensitivity, specificity

1. Introduction

Bovine parvovirus (BPV) was firstly identified in the 1960s in diarrhea calves [1]. BPV is a member of the genus *Bocaparvovirus* with a nonenveloped capsid [2]. The viral capsid plays a critical role in the infection of host cells by adhering the virus to specific receptors on the target cells [3]. The genome comprises single-stranded DNA (ssDNA) [4,5] and possesses three open reading frames (ORFs; ORF1, ORF2, and ORF3). ORF3 encodes two capsid viral proteins (VP; VP1 and VP2). VP1 (75 kDa) and VP2 (61 kDa) share a C-terminal end. VP2 region locates in the crystal structure, from residues 39 to 536 [6,7].

BPV may cause respiratory and gastrointestinal disease in cows [2]. It is also a possible contaminant of biologics production [8,9]. It was isolated from children with severe respiratory and gastrointestinal infections [5]. Currently, several methods have been utilized to detect BPV antigens [9,10]. However, each method has its own strengths and weaknesses in terms of sensitivity and turnaround time [11]. Real-time PCR offers the same advantages as conventional PCR assays, but it is much more rapid and sensitive due to real-time quantification of the data and

visualization [12]. Detection method for parvovirus has been described previously in human and animals [9,10]. Commonly used RT-PCR protocols require more than 1 h and a specialized PCR instrument [13–15]. It is highly necessary to develop strategies that reduce reaction time to less than 30 min [14,16]. In this study, we are expected to develop a novel real-time PCR (qRT-PCR) based on the TaqMan probe, additionally to provide accurate and rapid method for detecting BPV.

2. Materials and methods

2.1. DNA extracts of BPV

Strain Haden of BPV was purchased from the American type culture collection (ATCC). Viruses were propagated at 37 °C and 5% CO_2 in MDCK 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 from medium fluids by centrifuging at 2000 × *g* for 10 min.

Clinical feces samples were collected from 308 diarrhea calves in six different cattle farms in Gansu Province of

364

China. Collection of feces samples were done during days 3–5 following diarrhea. Suspension was prepared by diluting the feces samples as per the operation instruction described above.

Viral genomic DNA was extracted from each supernatant using the QIAamp MinElute virus spin kit (Qiagen, Netherland) referring to the manufacturer's instruction. DNA was quantified using SYBR Green, and then stored at –80 °C for the subsequent tests.

2.2. Primers and probe design
The full-length genome of reference strains of BPV was obtained from the GenBank database (Accession: JN191349). One pair of universal primers (BPV-F/BPV-R), one pair of specific primers and one pair of specific TaqMan probe (BPV-probe) from 5′-untranslated region (5′-UTR) of BPV VP2 was designed using Primer 5.0 software. The 5' end and 3' end of the BPV-probe were labeled with CY5 and BHQ-2, respectively. The specificity of the designed primers and probes was verified with Primer-BLAST at NCBI online. All primers and probes were synthesized by the Takara Bio INC (Beijing, China) (Table 1).

2.3. Standard PCR
For standard PCR, primer pairs (BPV-F and BPV-R) were used to detect BPV. PCR reaction system contained 0.5 µL Takara LA Taq 0.5 µL, PCR Buffer (Mg^{2+} plus 8 µL, dNTP Mixture8 µL, BPV-F 2 µL, BPV-R 2 µL, template cDNAs 2 µL and 30.5 µL of diethyl pyrocarbonate (DEPC) water). PCR was performed at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 56 °C for 45 s, and 72 °C for 45 s, with a final extension at 72 °C for 10 min.

2.4. Construction of standard plasmids and copies
The DNA was amplified by PCR using universal primers. Then, 5 µL reaction supernatants were electrophoresed on 1% agarose gel to identify the amplified products. Electrophoresis-verified PCR products were harvested utilizing EasyPure® Plasmid MiniPrep kit (Transgen Biotech Company, Beijing, China) and ligated into pMD18-T vector (Takara, Shanghai, China) for 2 h at 16 °C. Then, BL-21competent cells were transferred with the recombinant plasmids of DNA and pMD18-T at 37 °C overnight.

The sequence homology of the recombinant plasmids was compared with those of NCBI Genbank after they were sequenced (Shanghai Sangon Biotec, Shanghai, China). The concentrations of plasmids were determined using Ultramicro nucleic acid protein analyzer (Thermo Fisher, USA). Copies per µL were calculated.

2.5. Optimization of qRT-PCR conditions and the establishment of standard curve
TaqMan probe qRT-PCR was performed in a 20 µL reaction system consisting of SYBR Select Master Mix 10 µL, forward primer (BPV-qF, 10 µmol/L) 0.4 µL, reverse Primer (BPV-qR, 10 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 under the conditions of 95 °C for 30 s, 95 °C for 5 s, 60 °C for 40 s.

TaqMan qRT-PCR were optimized on the basis of the different primer concentrations, probe concentrations in the gradient of 0.5 µmol/L and annealing temperatures in 43.0–53.0 °C. Subsequently, recombinant plasmids were amplified with the optimized qRT-PCR at different concentrations (6.90×10^7 to 6.90×10^3 copies/µL) to acquire the melting curve, standard curve, and the regression equation.

2.6. Specificity tests
In order to evaluate its specificity, TaqMan probe qRT-PCR was utilized to synchronously detect Haden strain of BPV and viruses, such as Japanese encephalitis virus (JEV), classical swine fever virus (CSF), rabies virus (RABV), bovine rotavirus (BRV), bovine viral diarrhea virus (BVDV), and bovine foot and mouth disease virus (bFMDV). All these viruses were provided or gifted by the State key 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 RNA or DNA was extracted using commercial RNA or DNA kit, respectively.

2.7. Sensitivity verifications
Sensitivity of TaqMan probe qRT-PCR was assessed using 10-fold dilutions (5.26×10^{10} copies/µL to 5.26×10^0

Table 1. Primers for Bovine parvovirus (BPV) amplification.

Primers	Primer sequences (5′→3′)	Products/bp
BPV-F	GCGAAAACACGACTTTGGCT	554
BPV-R	GAGCCGTGTCACCAGTGTTA	
BPV-qF	CAAGCACATCCAATCAAC	198
BPV-qR	CCACAATGTTCTCGCTAA	
BPV-probe	CY5-CGTCCATCCGCCAGTTGAGTA-BHQ-2	198

copies/µL) of the recombinant plasmids according to the optimized conditions with RT-PCR instrument (ABI 7900, USA). The standard curves were used to set for respective absolute quantifications [17]. The results were compared with those of universal PCR to determine the minimum detection limit. The tests were performed in triplicate.

2.8. Repeatability evaluations

In order to evaluate the stability of TaqMan probe qRT-PCR, the standard plasmids of 5.26×10^8, 5.26×10^6, and 5.26×10^4 copies/µL were detected with the optimized reaction conditions at least three times. The intraassay and interassay coefficients of variation (CVs) were calculated.

2.9. Clinical applications

A total of 308 clinical feces samples were detected with both TaqMan probe qRT-PCR and universal PCR to testify the repeatability of qRT-PCR assay.

3. Results

3.1. Construction of standard plasmids

After the BPV DNA was amplified, the bands of agarose gel electrophoresis showed the PCR product was 554 bp, which was consistent with the expected product sizes (Figure 1). The coincidence rate of the sequence of positive plasmids was 99% with those described in GenBank.

3.2. Optimization of qRT-PCR

Based on the screening of the different concentrations of specific primers and probes as well as annealing temperatures, the optimum reaction conditions for TaqMan probe qRT-PCR were 0.8 µL probes (18.0 µmol/L), 0.4 µL primers (10.0 µmol/L), and annealing temperature of 43.2 °C for 40 s.

3.3. Establishment of the standard curve and dynamic curves

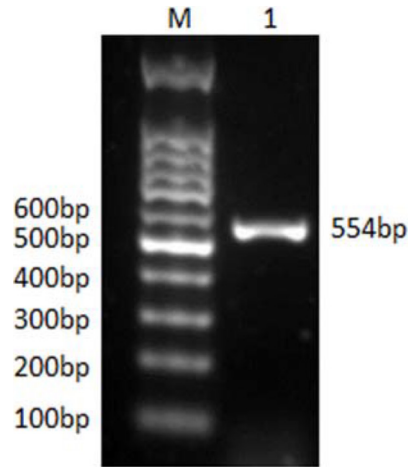The standard curve and dynamic curves were acquired after the different concentrations of recombinant plasmids

Figure 1. Electrophoresis image of PCR amplification of BPV. A: M, 100bp DNA Marker; 1, BPV F/R PCR product.

(6.90×10^7 to 6.90×10^3 copies/µL) were amplified using the optimized qRT-PCR (Figure 2). The results showed a good linearity.

3.4. Specificity assesses

As shown in Figure 3, the probe qRT-PCR could clearly detect BPV. However, other six viruses displayed no signal bands, including JEV, CSF, RABV, BRV, BVDV, and bFMDV. The findings indicated that the established TaqMan probe qRT-PCR had a strong specificity.

3.5. Sensitivity

After the standard plasmids of 5.26×10^{10} copies/µL to 5.26×10^1 copies/µL concentrations were detected with this qRT-PCR assay. The plasmids displayed positive amplification signals. However, common PCR assay only detected 5.26×10^5 copies/µL. The results demonstrated that the least detection limit of the established TaqMan
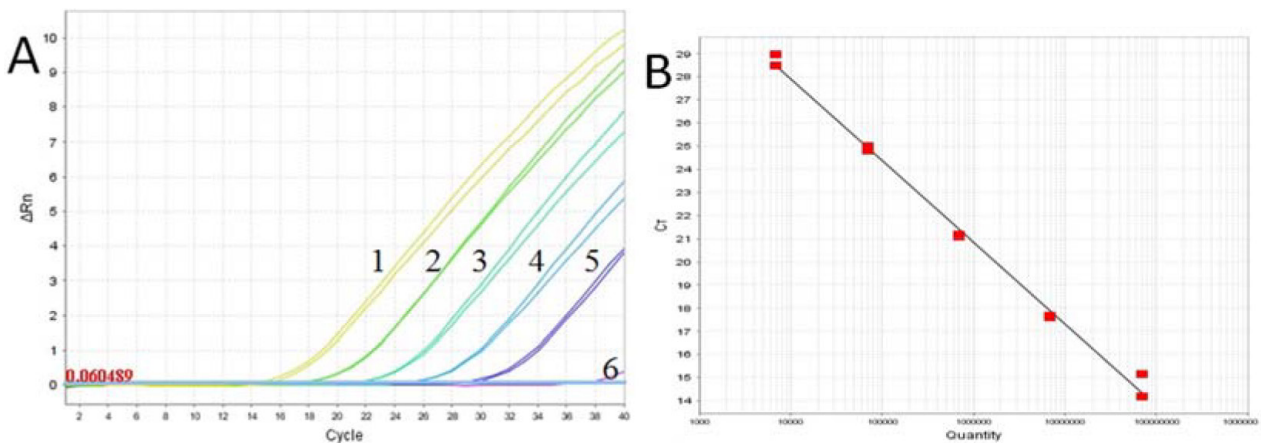

Figure 2. Dynamic curves (A) and standard curves (B). 1–5: 6.90×10^7 to 6.90×10^3 copies/µL, respectively; 6: Negative control

qRT-PCR was 5.26×10^1 copies/μL, or 5.26 copies/μL (Figure 4). That was 10,000-fold higher than conventional PCR.

3.6. Repeatability
The results of repeatability tests were presented in Table 2. It could be found that the intraassay and interassay variation of the coefficient (VC) was 0.04% to 0.25% and 1.02% to 1.64%, respectively. This indicated that qRT-PCR possessed an excellent repeatability, or stability.

3.7. Detection of feces samples
Three hundred and eight feces samples collected from six cattle farms were detected using TaqMan probe qRT-PCR for the presence of BPV of the diarrhea animals to find the exact etiology. The results showed 36 positive feces samples for TaqMan probe qRT-PCR compared to 32 positive samples for universal PCR. Sensitivity and specificity of qRT-PCR were preferable to the universal PCR. The coincidence rate of both qRT-PCR and universal PCR was 88.89% for feces samples.

4. Discussion
Bovine parvovirus (BPV) can cause a contagious disease of cattle herds. The rate of occurrence of BPV infections is about 83% to 100% across the world [18, 19]. BPV can adhere erythrocyte, so it is also known as adsorption-blood cells enteritis virus [20]. Currently, the majority of the data available for these viruses are epidemiological. Few effective measures are offered for the treatment or prevention of *Bocaparvovirus* infections [6,21].
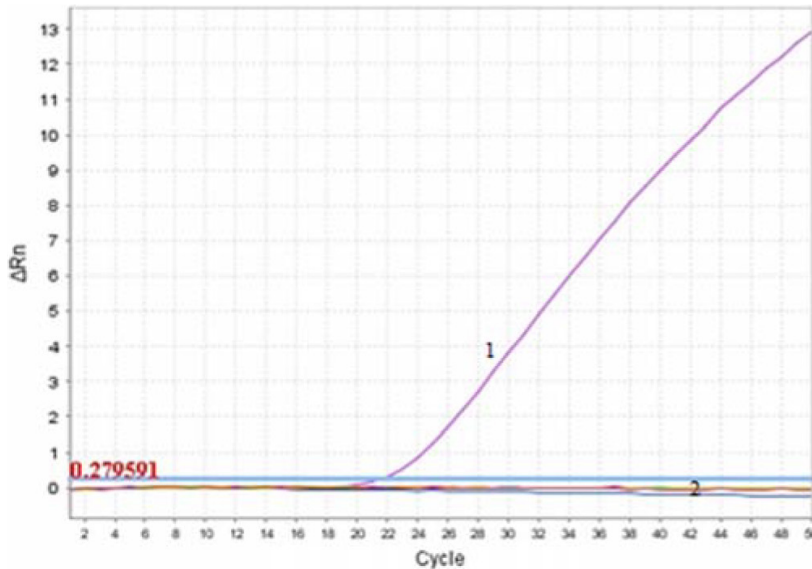

Figure 3. Specificity results of BPV single qRT-PCR. 1, BPV; 2, Control group (JEV, CSF, RABV, BRV, BVDV, bFMDV) and Negative control
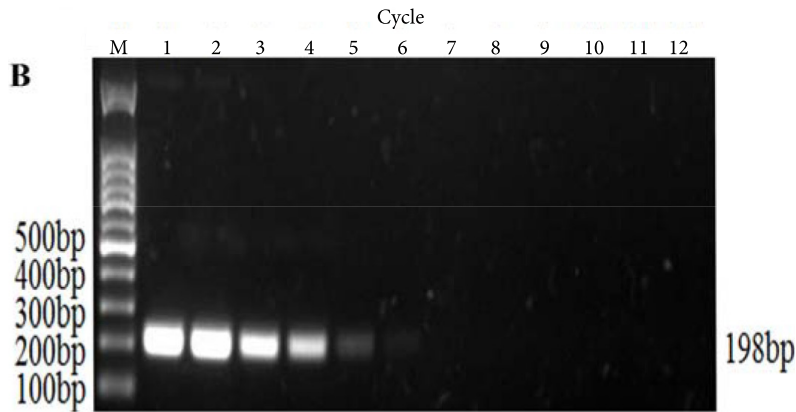

Figure 4. Sensitivity results of TaqMan qRT-PCR. 1–11: 5.26×10^{10} to 5.26×10^0 copies/μL, respectively; 12: Negative control

Table 2. Reproducibility results of TaqMan qRT-PCR assay.

Concentration (copies/μL)	Intraassay Ct valves			Interassay Ct valves		
	Mean	SD	VC (%)	Mean	SD	VC (%)
5.26×10^8	20.01	0.12	0.61	19.78	0.16	0.82
5.26×10^6	27.56	0.08	0.30	27.20	0.26	0.95
5.26×10^4	34.86	0.10	0.28	34.36	0.40	1.18

The normalization is crucial to control the variation introduced by various steps of qRT-PCR assay. The quantity and quality of RNA/DNA samples and fluorescence fluctuations affect normalization control of the signal [22]. In order to avoid these faults, we designed one pair specific primer (BPV-qF/BPV-qR) and a specific TaqMan probe (BPV-probe) targeting the highly conserved 5′-untranslated region (5′ UTR) [10]. An internally controlled TaqMan probe qRT-PCR was developed and evaluated for detection and quantification of Haden strain of BPV VP2.

The specificity of primers and probes was validated with Primer-BLAST online. Specificity of TaqMan probe qRT-PCR was further validated by the negative control and other six viruses. The outcomes testified that TaqMan probe qRT-PCR could only detect BPV and had an excellent specificity and ideal reproducibility. The minimum detection of the probe qRT-PCR was 5.26×10^1 copies/μL. The sensitivity was increased by 10,000-fold over universal PCR (5.26×10^5 copies/μL). Special emphasis was given to the suitability of the probe qRT-PCR for rapid and reliable detection of viral infections in the field. A total of 308 feces samples of diarrhea calves were detected using the qRT-PCR. The results demonstrated that the sensitivity and specificity of TaqMan probe qRT-PCR were better than the universal PCR. Our findings were in agreement with earlier studies [23]. Therefore, the established TaqMan probe qRT-PCR could be implemented to diagnose BPV infections in dairy farms.

In conclusion, it was determined that the optimum reaction conditions for TaqMan probe qRT-PCR were 0.8 μL probes (18.0 μmol/L), 0.4 μL primers (10.0 μmol/L), and annealing temperature of 43.2 °C for 40 s in the present study. Minimum detection level of TaqMan probe qRT-PCR was 5.26 copies/μL. Its sensitivity was 10,000-fold over the conventional PCR. The novel TaqMan probe qRT-PCR had excellent sensitivity, specificity and stability. This accurate detection method is beneficial for enhancing accuracy and reliability of BPV detection in cattle farms, which will improve pertinence of disease therapy and reduce the cost and increase the economic benefits.

Conflict of Interests
None of the authors has any conflict of interest.

Author contributions
Professor Zhuandi GONG took and detected the samples. Miss Haoqin LIANG designed the probe and assessed the specificity and sensitivity. Miss Jinjing GENG optimized TaqMan probe qRT-PCR. Dr. Xiaoyun SHEN did the data statistics analyses. Professor Suocheng WEI was responsible for the experimental designs and writing the manuscript. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved for the final version.

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