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TaqMan real-time RT-PCR assay with an internal amplification control for rapid detection of Bluetongue virus*

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A TaqMan real-time RT-PCR assay with an internal amplification control for rapid detection of Bluetongue virus

Summary

Bluetongue (BT) is an arbovirus disease caused by Bluetongue virus (BTV), which has spread all over the world. In this study, a qRT-PCR assay with an internal amplification control was established for preventing false-negative results in Bluetongue virus (BTV) detection. The primers and probes for BTV NS3 were based on the qRT-PCR method for the detection of BTV in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (2022) of the World Organization for Animal Health (WOAH). The GAPDH gene was selected as the internal amplification control, and the primers and probes for the GAPDH gene were designed based on the analysis of conserved sequences in the genome sequences of cattle, sheep and goats. Optimization of the reaction conditions, specificity, sensitivity and repeatability tests were conducted, and a qRT-PCR method was established for the simultaneous detection of BTV and the reference gene GAPDH. The results showed that the optimal primers and probe concentrations of BTV-NS3 were 0.30 μmol/L and 0.25 μmol/L, and were 0.15 μmol/L and 0.20 μmol/L for the reference gene GAPDH. The established method has no cross-reaction with other viruses, which showed good specificity. The minimum detection of BTV- and GAPDH-positive plasmid standard copies was 6.685 copies/μL and 209 copies/μL, respectively. The intra-assay and interassay CVs of the Ct values measured by BTV were less than 1.2%, and the intra-assay and interassay CVs of the Ct values measured by GAPDH were less than 0.7%, which suggested a high degree of repeatability and stability for this assay. The established BTV qRT-PCR method was used to detect BTV in 88 sheep serum samples stored in the laboratory, revealing 4 BTV-positive samples with a positive rate of 4.55%, which was consistent with the result obtained by nested PCR for detecting BTV recommended by WOAH. The qRT-PCR method established in this study for simultaneously detecting BTV and the internal reference gene GAPDH has high sensitivity, strong specificity and high repeatability. The addition of an internal reference gene can effectively control the detection accuracy and enable false-negative results to be avoided. It can provide a reference for the standardization of qRT-PCR methods for Bluetongue disease.

Keywords: Bluetongue virus, real-time quantitative RT-PCR, GAPDH, NS3 gene, internal reference gene

Bluetongue (BT) is an arbovirus disease caused by Bluetongue virus (BTV) and transmitted by hematophagous midges to which ruminants such as cattle and sheep are susceptible (13). At present, the control strategies against BT outbreaks are being explored (1). BT is defined by the World Organization for Animal Health (WOAH) as an animal infectious disease that must be reported in a timely manner, and it is listed as a class I animal epidemic disease in China. The disease mainly infects sheep, and its clinical symptoms

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# Chen C. and Han D. contributed equally to this study.

are characterized by a febrile response, inflammation and edema, accompanied by mucosal ulceration. Some infected pregnant animals can vertically infect the fetus through the placenta, causing abortion, stillbirth, and so on. The morbidity is generally 30%~40%, and the fatality rate is 3%~30%, which causes serious economic losses to the cattle and sheep breeding industries (4). The epidemic and outbreak of BT have seriously affected the normal international trade of livestock products. From 2006 to 2008, the African-origin BTV-8 strain spread across most of Europe, resulting in the worst BT outbreak ever recorded in Europe. At the end of 2007, the BTV-8 strain caused the largest and most economically devastating outbreak ever recorded (9, 16). BTV is a double-stranded RNA (dsRNA) virus with a genome size of approximately 20 kb. The BTV genome consists of 10 linear double-stranded RNA segments encoding seven structural proteins (VP1 to VP7) and four nonstructural proteins (NS1, NS2, NS3/NS3a, and NS4) (21).

Currently, the commonly used laboratory diagnostic methods for BTV mainly include molecular biology and serological detection. The pathogens that cause BT were identified in international trade operations by WOAH using designated methods, which mainly included chicken embryo or cell isolation and culture identification, nested PCR and qRT-PCR. qRT-PCR has low biosafety requirements and can enable rapid detection of all serotypes of BTV in one step. It has been widely used in BT detection. Mulhollandet al. (11) developed and validated a qRT-PCR assay, and the Primers and probes were based on genome segment 10 of the virus, called the NS3 gene, which could be used as part of a panel of diagnostic assays for the detection of all serotypes of BTV. The qRT-PCR technique requires standardized policies to ensure data reliability (6, 18). A common strategy relies on the comparison of target genes with endogenous controls, also called reference genes. The function of the internal reference gene is to reduce the error caused by sample addition and reduce the difference caused by the quality of RNA of different samples and by reverse transcription to make the results more accurate (7). Therefore, internal reference genes play an important role in relative quantitative data analysis, and the selection of reliable internal reference genes under specific conditions is the key to quantitative accuracy. Common reference genes include glyceraldehyde-3-phosphate dehydrogenase (GAPDH), glucose 6-phosphate dehydrogenase (G6PDH), tyrosine 3-monoxygenase (YWHAZ), actin beta (ACTB), and beta-2 microglobulin (B2M) (22). Several studies found that GAPDH was the most stable reference gene (10, 14, 23, 24).

In this study, a TaqMan qRT-PCR assay with an internal amplification control was established for preventing the false-negative results and improving the accuracy in BTV detection. The optimization of the reaction conditions, specificity, sensitivity and repeatability tests were explored, and a qRT-PCR method was established for the simultaneous detection of BTV and the reference gene GAPDH. The reference gene GAPDH enabled monitoring of the whole reaction process of qRT-PCR to effectively prevent false-negative results and promote the standardization of the detection method of Bluetongue disease.

Material and methods

Virus and samples. The 88 sheep serum samples, epizootic hemorrhagic disease virus (EHDV), bovine viral diarrhea virus (BVDV), foot-and-mouth disease virus (FMDV), peste des petits ruminants virus (PPRV), orf virus (ORFV), goat pox virus (GPV), vesicular stomatitis virus (VSV), and Bluetongue viruses (BTV1, BTV2, BTV3, BTV5, BTV8, BTV11, BTV17, BTV18, BTV24) were kept by the Animal Quarantine Laboratory of Kunming Customs Technology Center, and nucleic acid was extracted from strains by a viral DNA/RNA extraction kit (Tianlong, Xi'an, China).

Primer and probe design. The primers and probes for BTV NS3 were based on the qRT-PCR method for the detection of BTV in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (2022) of the WOAH. The primers and probes named BTV-NS3-F, BTV-NS3-R and BTV-NS3-P are shown in Table 1. The internal reference GAPDH gene sequence was downloaded from the NCBI GenBank database, and the gene sequences of cattle, sheep and goats were downloaded for comparison. A pair of primers and probes, GAPDH-F, GAPDH-R and GAPDH-P, were designed by selecting conserved regions (Tab. 1), and the primers of the nested PCR method for detecting BTV were according to the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (2022) of the WOAH (Tab. 1). All of them were synthesized by Tsingke Biotechnology Co., Ltd.

Tab. 1. Primer and probe sequences of qRT-PCR and nested PCR

Primer name	Primer sequence
BTV_IVI_-F	5'-TGG-AYA-AAG-CRA-TGT-CAA-A-3'
BTV_IVI_R	5'-ACR-TCA-TCA-CGA-AAC-GCT-TC-3'
BTV_IVI_P	5'FAM-ARG-CTG-CAT-TCG-CAT-CGT-ACG-C-3' BHQ1
GAPDH-F	GGT CAC CA GGG CTGC TTTT A
GAPDH-R	CCCGTTCTCAGCCATGTAGT
GAPDH-P	VIC-5'-TGCCATCAATGACCCCTTCATTGACC-3'-BHQ
FW	5'-GTTCTCTAGTTGGCAACCACC-3'
RV	5'-AAGCCAGACTGTTTCCCGAT-3'
nFW	5'-GCAGCATTTTGAGAGAGCGA-3'
nRV	5'-CCCGATCATACATTGCTTCCT-3'

Preparation of standard RNA and standard curve production. To obtain TaqMan assay standards, a 290 bp RT-PCR product containing the BTV NS3 gene and a 91 bp RT-PCR product containing the GAPDH gene were amplified using the primer pairs BTV-NS3-F/BTV-NS3-R and GAPDH-F/GAPDH-R. The RT-PCR products were cloned into the pUC57 vector system (Tsingke Biotechnology Co., Ltd.). One hundred microliters of the bacterial solution con-

taining the positive plasmid was absorbed and added to 5 mL of LB liquid medium and 5 µL of 100 mg/mL ampicillin. Overnight culture occurred at 37°C. The DNA standard was extracted, and the DNA concentration was determined by a NanoDrop 1000 ultra micro spectrophotometer (Thermo, American Nanodrop Corporation, ND-1000) at $OD_{260/280}$. The number of DNA copies in the sample was estimated by Formula (1) based on the molecular weight of the DNA standard and the DNA concentration. Tenfold serial dilutions of DNA stock were prepared in DNase/RNase-free water. Dilutions of standard DNA were tested by qRT-PCR, and a standard curve was generated with Excel software.

$$\text{copies}/\mu\text{L} = \frac{6.02\times10^{23}\ (\text{copies/mol})\times(\text{ng}/\mu\text{L}\times10^{-9})}{\text{DNA length}\times\text{average molecular mass}} \quad (1)$$

Optimization of reaction conditions. The reaction system and procedure recommended in the HiScript II U^+ One Step qRT-PCR Probe Kit (Vazyme, Nanjing, China) were used for real-time amplification and detection of the BTV NS3 gene and reference gene GAPDH in this study, and the concentrations of primers and probes were optimized. The optimal primer concentration and probe concentration were determined by the reaction product reaching the minimum sample threshold cycle number (Ct value), the highest fluorescence value. The upstream and downstream primers of BTV were set as 0.4 µmol/L, 0.35 µmol/L, 0.3 µmol/L, 0.25 µmol/L and 0.2 µmol/L, and the probe concentrations were as follows: 0.3 µmol/L, 0.25 µmol/L, 0.2 µmol/L, 0.15 µmol/L, and 0.1 µmol/L. The concentrations of GAPDH primer and probe were 0.3 µmol/L, 0.25 µmol/L, 0.2 µmol/L, 0.15 µmol/L and 0.1 µmol/L. The reaction procedure was as follows: 55°C for 15 min; 95°C for 30 s; 95°C for 10 s, 60°C for 30 s, 50 cycles.

Specificity analysis. Epidemic hemorrhagic disease virus (EHDV), bovine viral diarrhea virus (BVDV), foot-and-mouth disease virus (FMDV), Peste des petits ruminants virus (PPRV), Orf virus (ORFV), goat pox virus (GPV), and vesicular stomatitis virus (VSV) can infect both cattle and sheep. Mixed or cross-infection of these pathogens may occur in clinical diagnosis. Therefore, to evaluate the specificity, in this study, nucleic acids were extracted from strains of EHDV, BVDV, FMDV, PPRV, ORFV, GPV, VSV and Bluetongue virus (BTV1, BTV2, BTV3, BTV5, BTV8, BTV11, BTV17, BTV18 and BTV24) and used as templates. The established qRT-PCR method was used for amplification, and the specificity of the method was identified.

Sensitivity analysis. To determine the limit of detection, 10-fold serial dilutions of plasmid standards were prepared as templates as follows: pUC57-BTV-NS3 ranging from 6.685×10^0 to 6.685×10^9 copies/µL and pUC57-GAPDH plasmid ranging from 2.09×10^0~2.09×10^9 copies/µL. qRT-PCR was performed to detect sensitivity.

Reproducibility analysis. The reproducibility of this assay was detected by calculating the intra- and interassay coefficients of variation (CVs) according to the mean Ct value deviation. Four different dilutions, from 10^2 to 10^5, of two positive plasmids were used for intra- and interassay reproducibility tests. Triplicate samples were prepared, and each reaction was independently repeated three times. SPSS 19.0 software was used to analyze the test results.

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Application of TaqMan qRT-PCR in clinical sample detection. In this study, the established BTV qRT-PCR method containing an internal reference gene (GAPDH) and the nested PCR method for detecting BTV recommended by WOAH were used to detect BTV in 88 sheep serum samples stored in the laboratory. The first round of nested PCR was performed according to the PrimeScript™ One Step RT-PCR Kit Ver.2 (Takara, Beijing, China) instructions. Reaction system: PrimeScript 1 step enzyme mix 1.0 µL, 2 × 1 step buffer 12.5 µL, FW primer (25 pmol/µL) 1.0 µL, RV primer (25 pmol/µL) 1.0 µL, template 1.0 µL, supplement the system to 25 µL using RNase free H_2O. Reaction procedure: 30 min at 50°C; 94°C for 2 min; 94°C 30 sec, 60°C 30 sec, 72°C 1 min, 35 cycles. Next, the second round of nested PCR was performed according to Taq™ Version 2.0 plus dye (Takara, Beijing, China) instructions. Reaction system: Premix Taq 25 µL, nFW primer (25 pmol/µL) 1.0 µL, nRV primer (25 pmol/µL) 1.0 µL, DNA template from first round of nested PCR 1.0 µL, supplement the system to 50 µL using RNase free H_2O. Reaction procedure: 94°C 30 sec, 60°C 30 sec, 72°C 1 min, 35 cycles. After the reaction, 5 µL PCR solution was used for agarose gel electrophoresis.

Results and discussion

Construction of standard plasmids. The BTV-NS3 gene amplification products were sent to Tsingke Biotechnology Co., Ltd. for sequencing. The size of the amplification products was 290 bp, and the cloned plasmid was obtained. The plasmid standard concentration was determined by ultramicro nucleic acid protein detector to be 219.13 ng/µL, and the copy number was 6.685×10^{10} copies/µL by Formula (1). The size of the GAPDH amplification product was 91 bp. The plasmid standard substance concentration was 64.1 ng/µL by ultramicro nucleic acid protein analyzer, and the copy number was 2.09×10^{10} copies/µL by formula calculation.

Optimization of reaction procedures. The results of primers optimization for BTV showed that when the primer concentration of the FAM channel was 0.30 µmol/L, the Ct value was the minimum, and the fluorescence value was the maximum (Fig. 1, Tab. 2), with a good amplification curve. Therefore, the optimal primer concentration for the BTV qRT-PCR method was 0.30 µmol/L. The results of probe optimization for BTV showed that, when the concentration of probe in the FAM channel was 0.25 µmol/L, the Ct value was the minimum, and the fluorescence value was the maxi-

Tab. 2. Optimization results of BTV primer and probe concentration in qRT-PCR

Primer concentration (μmol/L)	Ct value
0.40	10.449
0.35	10.824
0.30	10.105
0.25	10.426
0.20	12.379
Probe concentration (μmol/L)	**Ct value**
0.30	9.535
0.25	9.480
0.20	10.066
0.15	9.684
0.10	9.840

Tab. 3. Optimization results of GAPDH primer and probe concentration in RT-qPCR

Primer concentration (μmol/L)	Ct value
0.30	27.309
0.25	26.668
0.20	26.504
0.15	26.324
0.10	26.457
Probe concentration (μmol/L)	**Ct value**
0.30	26.566
0.25	26.410
0.20	25.746
0.15	26.082
0.10	26.410

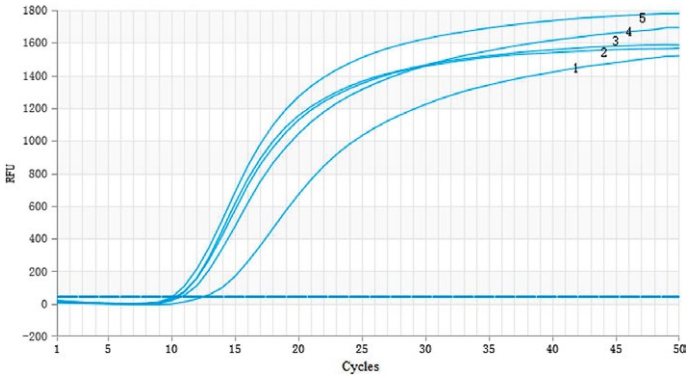

Fig. 1. Optimization results of BTV qRT-PCR primer concentration
Explanations: 1~5 – the primer concentrations were 0.20 μmol/L, 0.25 μmol/L, 0.40 μmol/L, 0.35 μmol/L and 0.30 μmol/L, respectively

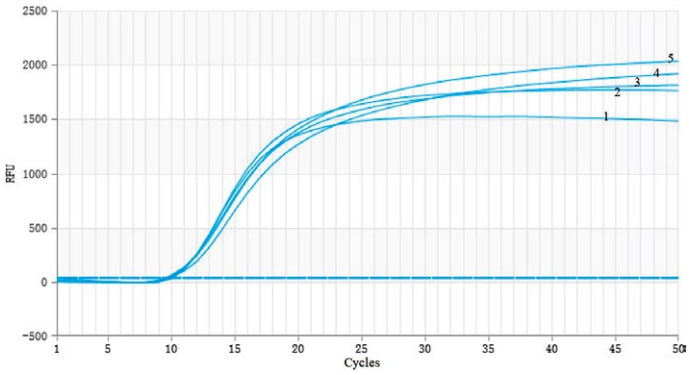

Fig. 2. Optimization results of BTV qRT-PCR probe concentration
Explanations: 1~5 – the probe concentrations were 0.10 μmol/L, 0.20 μmol/L, 0.15 μmol/L, 0.30 μmol/L and 0.25 μmol/L, respectively

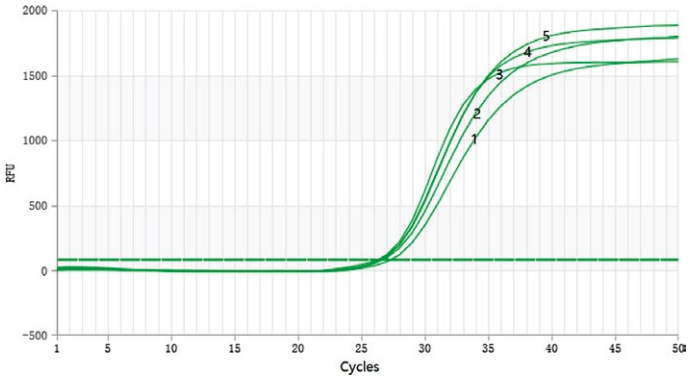

Fig. 3. Optimization results of GAPDH qRT-PCR primer concentration
Explanations: 1~5 – the primer concentrations were 0.30 μmol/L, 0.25 μmol/L, 0.10 μmol/L, 0.20 μmol/L and 0.15 μmol/L, respectively

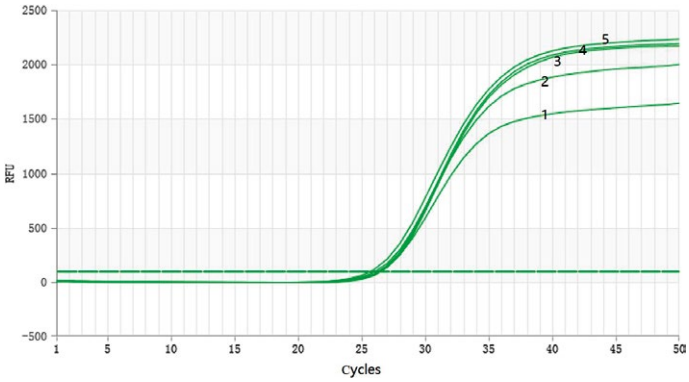

Fig. 4. Optimization results of GAPDH qRT-PCR probe concentration
Explanations: 1~5 – The probe concentrations were 0.10 μmol/L, 0.15 μmol/L, 0.30 μmol/L, 0.25 μmol/L and 0.20 μmol/L, respectively

mum (Fig. 2 and Tab. 2), showing a good amplification curve. Therefore, the optimal probe concentration for the BTV qRT-PCR method was 0.25 μmol/L.

The results of primers optimization for GAPDH showed that when the primer concentration of the FAM channel was 0.20 μmol/L, both Ct values and fluorescence values were the minimum (Fig. 3, Tab. 3), and a good amplification curve was presented. Therefore, the optimal primer concentration for GAPDH qRT-PCR was 0.20 μmol/L. The results of probe optimization for GAPDH showed that when the concentration of probe in the FAM channel was 0.20 μmol/L, the Ct value was the minimum, and the fluorescence value was the maximum (Fig. 4, Tab. 3), showing

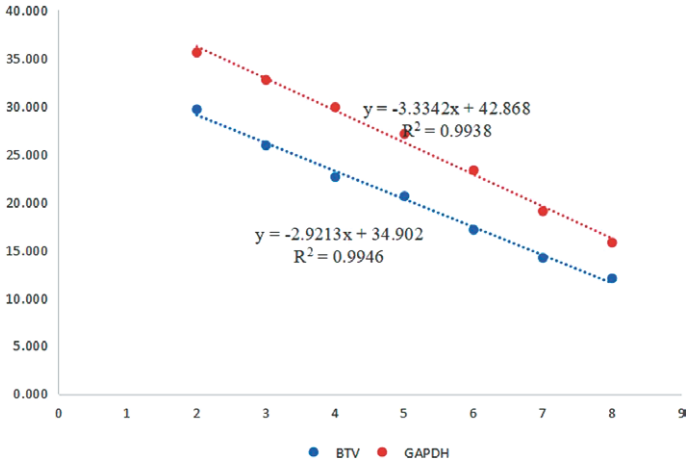

Fig. 5. Standard curve of TaqManqRT-PCR assay against BTV and GAPDH
Explanations: X-axis is the copies number of serial dilutions of the plasmid standard (copies/µL), Y-axis represents the threshold (Ct) values

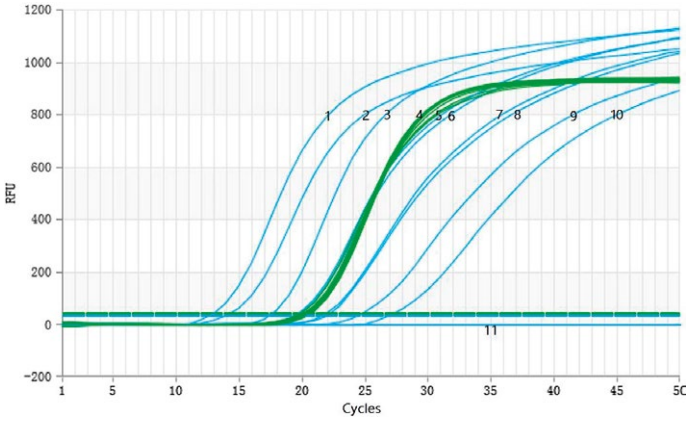

Fig. 6. Specificity of TaqManqRT-PCR assay against BTV
Explanations: 1~11: 1 – BTV3, 2 – BTV2, 3 – BTV1, 4 – GAPDH, 5 – BTV5, 6 – BTV11, 7 – BTV17, 8 – BTV24, 9 – BTV8, 10 – BTV18, 11 – EHDV, BVDV, FMDV, PPRV, ORFV, GPV, and VSV. The blue amplification curve shows the BTV plasmid amplification curve in the FAM channel, and the green amplification curve shows the GAPDH plasmid amplification curve in the HEX channel.

a good amplification curve. Therefore, the optimal probe concentration for GAPDH qRT-PCR was 0.20 µmol/L.

Standard curve of TaqMan qRT-PCR. The standard curve of the generated pUC57-BTV-NS3 plasmid (Fig. 5) shows that the dynamic range of copy number is $6.685 \times 10^2 \sim 6.685 \times 10^8$ copies/µL. The slope of the standard curve of this reaction is –2.9213, the correlation coefficient (R^2) is 0.9946, the intercept is 34.902, and the equation of the standard curve of the Ct value and copy number is y = –2.9213x + 34.902.

The standard curve of the generated pUC57-GAPDH plasmid (Fig. 5) shows that the dynamic range of copy number is $2.09 \times 10^2 \sim 2.09 \times 10^8$ copies/µL. The slope of the standard curve of this reaction is –3.3342, the correlation coefficient (R^2) is 0.9938, and the intercept is 42.868. The standard curve equation of the Ct value and copy number is y = –3.3342x + 42.868.

Specificity of TaqMan qRT-PCR. The nucleic acids of EHDV, BVDV, FMDV, PPRV, ORFV, GPV, VSV and Bluetongue virus (BTV1, BTV2, BTV3, BTV5, BTV8, BTV11, BTV17, BTV18, BTV24) that were stored in the laboratory and the DNA extracted from the pUC57-GAPDH plasmid were used as templates. The PCR products were amplified by the established qRT-PCR method, and the negative control of free water was set up. The results showed that the established method only produced fluorescence amplification curves for each BTV serotype and GAPDH and did not show characteristic amplification curves for other viruses and negative controls, indicating that the method had good specificity (Fig. 6).

Sensitivity assay. The plasmid standards pUC57-BTV-NS3, ranging from 6.685×10^0 to 6.685×10^9 copies/µL, and pUC57-GAPDH, ranging from 2.09×10^0 to 2.09×10^9 copies/µL, were used as templates. The sensitivity test showed that the minimum detectable limit of pUC57-BTV-NS3 was 6.685 copies/µL (Fig. 7), and the minimum detectable limit of pUC57-GAPDH was 2.09×10^2 copies/µL (Fig. 8), which

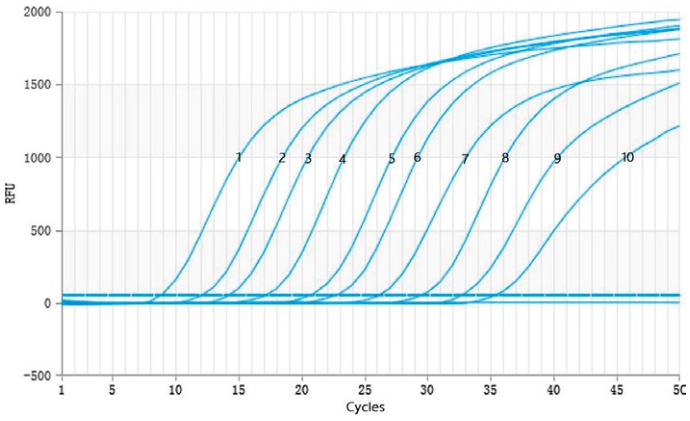

Fig. 7. Sensitivity of TaqMan qRT-PCR assay against BTV
Explanations: 1~10 – ten-fold serial dilutions of plasmid standard, with the concentration from 6.685×10^9 copies/µL to 6.685 copies/µL

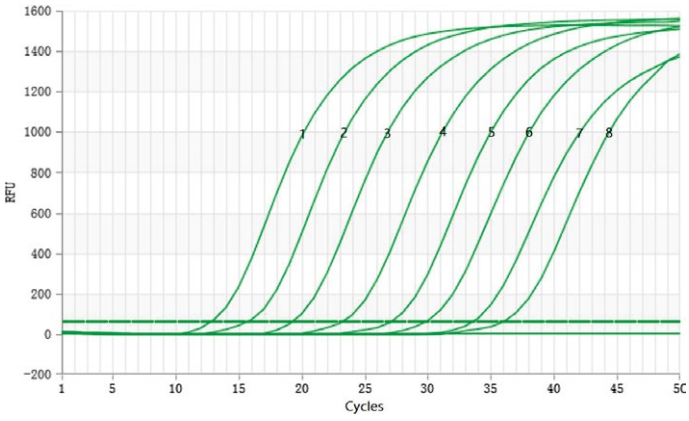

Fig. 8. Sensitivity of TaqMan qRT-PCR assay against GAPDH
Explanations: 1~8 – ten-fold serial dilutions of plasmid standard, with the concentration from 2.09×10^9 copies/µL to 2.09×10^2 copies/µL

Tab. 4. Intra- and interassay repeatability for qRT-PCR assay against BTV

Multiple of dilution	Intra-assay repetition threshold			Interassay repetition threshold		
	Mean value	Standard deviation	Coefficient of variation (%)	Mean value	Standard deviation	Coefficient of variation (%)
10^2	15.990	0.069	0.433	15.966	0.080	0.498
10^3	19.717	0.217	1.102	19.684	0.028	0.142
10^4	24.017	0.190	0.791	23.782	0.146	0.615
10^5	27.533	0.127	0.460	27.670	0.220	0.794

Tab. 5. Intra- and interassay repeatability for qRT-PCR assay against GAPDH

Multiple of dilution	Intra-assay repetition threshold			Interassay repetition threshold		
	Mean value	Standard deviation	Coefficient of variation (%)	Mean value	Standard deviation	Coefficient of variation (%)
10^2	15.950	0.0781	0.490	15.987	0.0404	0.253
10^3	19.547	0.0981	0.502	19.563	0.0289	0.148
10^4	22.807	0.1484	0.651	22.846	0.0732	0.320
10^5	26.440	0.0608	0.230	26.374	0.0914	0.347

indicated that the BTV qRT-PCR method established in this study had good sensitivity.

Repeatability assay. The concentrations of 6.685 × 10^{10} copies/μL pUC57-BTV-NS3 and 2.09 × 10^{10} copies/μL pUC57-GAPDH were diluted 10^2, 10^3, 10^4 and 10^5 times and used as templates. The intra- and interassay reproducibility of the qRT-PCR method was tested. As listed in Table 4 and Table 5, the intra-assay and interassay CVs of the Ct values measured by BTV were less than 1.2%, and the intra-assay and interassay CVs of the Ct values measured by GAPDH were less than 0.7%, which suggested a high degree of repeatability and stability for this assay, indicating that the established qRT-PCR had good repeatability.

Preliminary application of qRT-PCR assay in clinical samples. The nested PCR method for detection of BTV recommended by WOAH and the qRT-PCR method for simultaneous detection of BTV and the internal reference gene GAPDH established in this study were used to detect the sheep serum samples stored in the laboratory. The results (Fig. 9 and Fig. 10) showed that among the 88 samples, the qRT-PCR positive rate was 4.55% (4/88), the nested PCR positive rate was 4.55% (4/88), the positive coincidence rate between the two methods was 100%, the negative coincidence rate was 100%, and the total coincidence rate was 100%.

BT disease has spread all over the world. Although there are no outbreak reports in China, multiple serotypes of BTV are prevalent in China. It has been reported that 16 serotypes of BTV were isolated in China, including BTV-1, 2, 3, 4, 5, 7, 9, 11, 12, 14, 15,

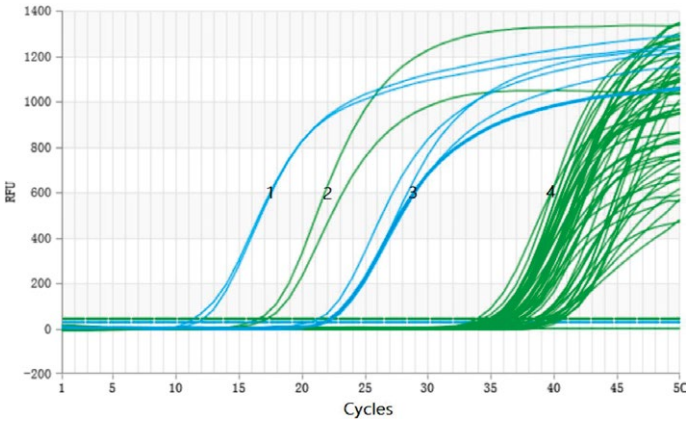

Fig. 9. Clinical test results of qRT-PCR method
Explanations: 1 – BTV positive control; 2 – GAPDH positive control; 3 – BTV positive serum sample; 4 – GAPDH amplification curve

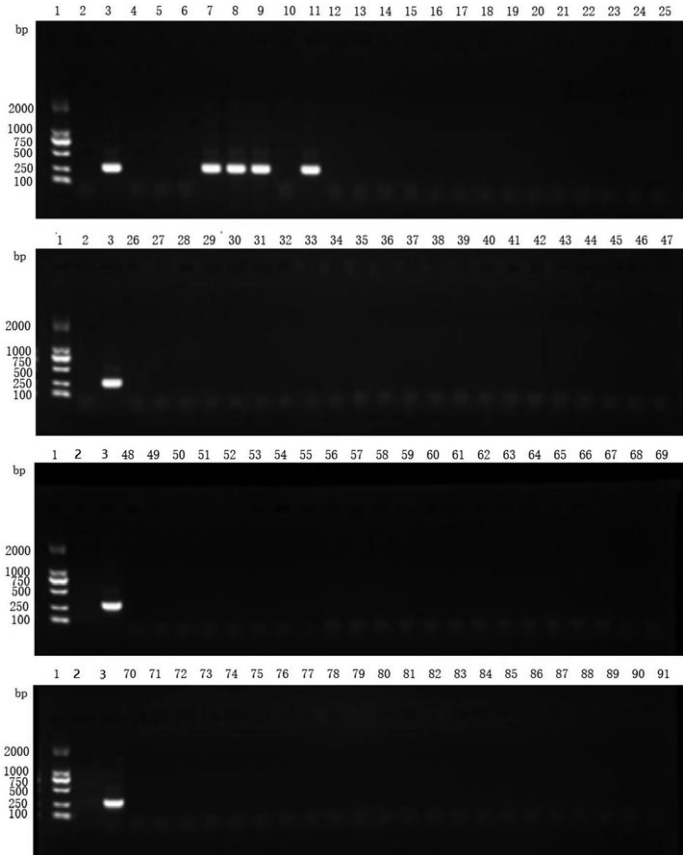

Fig. 10. Clinical test results of nested PCR method
Explanations: 1 – marker; 2 – negative control; 3 – positive control; 7/8/9/11 – positive serum sample; 4~6/10/12~91 – negative serum sample

16, 17, 21, 24 and 29 (21). Due to no cross-protection among the serotypes, it poses a great challenge for the prevention and control of BT disease. In view of the huge economic losses caused by BT to the livestock industry in the Libyan Peninsula from 1956~1957, BT was listed as a category animal disease by WOAH in the 1960s. To date, 36 serotypes of BTV have been identified worldwide (15). Because the BTV genome is a segmented dsRNA, recombination and variation among virus strains are prone to occur, which poses a risk of natural recombinant virulent BTV strains emerging. Therefore, it is of great significance to carry out basic research on BTV for the prevention and control of BT disease.

At present, the most powerful methods for BT disease prevention were the early detection and treatment. This requires the rapid diagnosis and detection of BTV. The qRT-PCR method can be used for rapid qualitative and quantitative detection of pathogens and has the characteristics of high accuracy, strong specificity and simple operation, and has been widely used in the detection of epidemic diseases (2, 5). However, factors such as RNA integrity, cDNA quality, primer specificity and amplification efficiency of the sample will affect the qRT-PCR results (12). Adding one or more reference genes with stable expression to correct and standardize the target gene can improve the accuracy of qRT-PCR results (25). Many housekeeping genes, such as the mammalian GAPDH gene, can be used as internal controls for sample viability in molecular assays (17, 19). Su et al. (20) showed that GAPDH can be stably expressed in different tissues and under different temperature conditions. Cássia-Pires et al. (3) developed a multiplex RT-PCR method to simultaneously detect the GAPDH gene and Leishmania genus kDNA in tissue samples from different wild mammalian species. Amplification of the mammalian GAPDH gene in the same reaction ensured the quality and viability of the DNA in the sample, thereby eliminating the possibility of false-negative results. In this study, primer probes for GAPDH were designed to amplify smaller fragments of the GAPDH gene that could be used in combination with primer pairs for BTV without affecting qRT-PCR conditions.

In this study, a qRT-PCR assay containing two pairs of primer probes was developed to detect the GAPDH gene and BTV. The constructed pUC57-BTV-NS3 was used to prepare the standard curve, and the correlation coefficient (R^2) was 0.9946, which could be used for real-time monitoring of virus passage content and virus titer. The standard curve prepared by pUC57-GAPDH with the correlation coefficient (R^2) was 0.9946. The established BTV qRT-PCR method was able to detect the minimum copy number of BTV copies at 6.685 copies/μL, and the minimum copy number of GAPDH was 2.09×10^2 copies/μL. In Mulholland's study for the detection of BTV full serotypes, the analytical sensitivity of the rRT-PCR assay was 200 copies of RNA per reaction (11). No specific curve was detected for EHDV, BVDV, FMDV, PPRV, ORFV, CaPV and VSV, indicating that the established method had a strong specificity for BTV. The intra-assay and interassay CVs of the Ct values measured by BTV were less than 1.2%, and the intra-assay and interassay CVs of the Ct values measured by GAPDH were less than 0.7%. The coefficient of variation of the real-time TaqMan qRT-PCR method with an internal amplification control for rapid detection of Muscovy duck reoviruswas less than 1.5% (26). The method established in this study has relatively better repeatability. The results showed that in 88 samples, 4 positive samples were measured both by qRT-PCR and nested RT-PCR. The positive rate was 4.55%, and the total coincidence rate was 100% between the two methods. In the investigation by Li et al. (8), the seropositive rate of BT disease in Tibetan yaks in China was 2~4.89%. Compared with nested PCR, which is complicated and time-consuming, qRT-PCR is simple, sensitive and less time-consuming and is more suitable for the detection of a large number of samples. Compared with traditional PCR, the qRT-PCR method established in this study added a primer probe for the reference gene GAPDH, which could detect both BTV and mammalian glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the samples simultaneously. It enabled avoidance of qRT-PCR failures caused by poor sample quality, improper nucleic acid extraction and/or PCR inhibition and effectively prevented false-negatives.

The qRT-PCR method established in this study for simultaneously detecting BTV and the internal reference gene GAPDH has high sensitivity, strong specificity and high repeatability. The addition of an internal reference gene can effectively control the detection accuracy and enable false-negative results to be avoided. The method is simple to operate and suitable for rapid detection of Bluetongue disease with a large sample size. It can be applied to epidemiological investigation and epidemic monitoring, and it would provide a reference for the standardization of qRT-PCR methods for Bluetongue disease.

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