

The Establishment of SYBR Green I Real-Time PCR Assay For the Detection of Bovine Parainfluenza Virus 3

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Abstract

Original Research Article

Bovine parainfluenza virus type 3 (BPIV3) causes epidemic diseases in worldwide. A SYBR Green real-time reverse transcription-polymerase chain reaction (rRT-PCR) assay was developed for the rapid detection and quantitation of BPIV3 in this study. Methods: A pair of specific primers was designed in the HN gene of this virus. Results: When comparing this assay with conventional RT-PCR, the rRT-PCR assay was 10 times more sensitive and could detect levels as low as 5.13×10^1 DNA copies of the BPIV3 DQ strain. All the standard shows a sharp and narrow dissolution peak at 78.8°C. The specificity of this technique was evaluated in three other bovine pathogens. Conclusion: This high sensitivity, specificity and simplicity of the SYBR Green rRT-PCR approach can be a more effective method than the conventional one for BPIV3 diagnosis and surveillance.

Keywords: BPIV3; SYBR Green I real-time quantitative PCR; detection.

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INTRODUCTION

Bovine Parainfluenza virus type 3 (BPIV3) is a major pathogen affecting cattle and is widely distributed throughout the world. In 1959, Reisinger *et al.* isolated BPIV3[1] for the first time in a "transport-hot" bovine nose swab in the United States, and subsequently reported BPIV3 infection in the United Kingdom, France, Australia and Canada [2, 3]. BPIV3 is a respiratory virus of the paramyxoviridae family; a single-stranded negative stranded RNA virus with a capsule. BPIV3 is in natural condition only infection cow, main infection source is disease cow and take poison cow, main transmission way is to pass air droplet to pass respiratory tract to infect, also can produce inside uterus infection. Serological studies have found that the presence of antibodies to the virus in healthy bovine serum is very common, so the virus is thought to be widely distributed around the world. 12 provinces in our country for BPIV3 epidemiological investigation, found that in addition to the individual provinces such as Jiangxi, BPIV3 serum positive rate is very high in the other provinces, especially cattle large province of Heilongjiang, Inner Mongolia and Shandong and other places, serological positive rate were above 80%, these results suggest that BPIV3 wide popular in our country, high infection rates [4, 5]. Gulliksen's serological survey on Norwegian cattle

farms found that the serological positive rate of BPIV3 was as high as 50.2% [6]. Calderon conducted a serological survey of BPIV3 in 54 cattle farms on Mexico's Yucatan peninsula and found that the positive rate was 88.9% [7]. BPIV3 infection poses a serious threat to the global cattle industry, yet there is still no effective vaccine or treatment for BPIV3 infection [8]. Therefore, the effective prevention and treatment of BPIV3 infection occurred and spread of important means is to take early diagnosis and prevention.

Real-time fluorescent quantitative PCR detection method is developed in recent years to a detection method, SYBR Green I principle of real-time fluorescent quantitative PCR is add fluorescent groups in ordinary PCR reaction system, using PCR amplification reaction, the steady accumulation of fluorescent signal which can be used for real-time detection of the PCR reaction process [9]. Through detection of known template construction of standard curve, quantitative analysis was carried out on the unknown template, SYBR Green I real-time fluorescent quantitative PCR has the advantages of high specificity, high sensitivity, high repeatability. In recent years, in the diagnosis of viral disease SYBR Green I real-time fluorescent quantitative PCR method widely used [10]. Zhu tong [11] designed primer according

conservative region of BEV 3D gene, established the detection BEV SYBR Green I real-time fluorescent quantitative PCR method. TI[12] use the conservative gene of the Tembusu virus gene E as detecting target, established Temple Sue virus SYBR Green I real-time fluorescence quantitative PCR (qRT-PCR), the virus in tissues and cells can be relatively quantitative. BPIV3 SYBR Green I real-time fluorescent quantitative PCR method established in the literature has reported. BPIV3 viral genome approximately 15 KB, coding 6 structural protein coding and three kinds of the nonstructural proteins, including capsule membrane protein hemagglutinin neuraminidase protein (HN) and fusion protein (F), as well as the nucleoprotein (NP), matrix protein (M) protein, phosphorus (P), large (L) polymerase subunits and attachment protein C, D, V. HN protein is located on the surface of virion and is highly conserved. It is the main protein that produces virus-neutralizing antibodies and is involved in target cell adsorption, penetration and membrane fusion of viruses during viral infection [13]. In this study, HN gene as detecting target, BPIV3 SYBR Green I qRT-PCR method was established, and provided the laboratory tests and clinical diagnosis of BPIV3 detection means.

MATERIALS AND METHODS

Material

Bovine parainfluenza type 3 DQ strain (BPIV3) was isolated and identified by the laboratory of preventive veterinary medicine of Heilongjiang Bayi agricultural university. The standard positive plasmid is the PET-30-HN plasmid constructed in our laboratory.

Reagents and instruments

DNA extraction kit purchased from Corning life sciences LTD. Common agarose gel DNA recovery kit is the product of Bao biological engineering company. DNA SYBR®Premix ExTaq™MII and high purity plasmid extraction kit were purchased from TaKaRa. ABI7500 fluorescence quantitative PCR instrument was purchased from American LIFE company; ABI general PCR instrument purchased from Genomic; Gel Doc2000 UV imaging system was purchased from Bio-rad, USA. NanoDrop 2000 UV-visible spectrophotometer purchased from Thermo.

Primers

According to the HN gene sequence of the published BPIV3 (registration no. AF178654.1) genome, a pair of specific primers were designed by Premier5.0 software and synthesized by Shanghai Sangon Bioengineering.

Detection of standard positive plasmids

The engineered bacteria containing PET-30-HN recombinant plasmid were cultured, and the PET-30-HN plasmid was extracted for PCR identification. The concentration of the standard positive plasmid was

determined by NanoDrop, and the copy concentration of the plasmid was calculated according to the calculation formula. The copy concentration of the template (copies/μl) = (recombinant plasmid concentration mg/l × 10⁻⁹) × 6.02 × 10²³ / (660 × recombinant plasmid base number). Dilute the original solution with ultra-pure water, dilute it with 10 times gradient as standard, and store it at -80 °C for later use.

Optimization of SYBR Green I real-time fluorescent quantitative PCR reaction conditions

Optimize the optimal primer concentration

SYBR®Premix Ex 10 μl DNA, template 2 μl (5.13 × 10⁵ copies /μl), upper and lower primers 0.5 μl, 0.6 μl, 0.7 μl, 0.8 μl, 0.9 μl, 1.0 μl, 6 gradients were selected, and ultrapure water was added to 20 μl. Reaction condition: 94 °C, 10 min; 94 °C, 30 s; Annealing at 60 °C, 30 s, a total of 35 cycles, according to the amplification effect to determine the optimal primer concentration.

2.5.2 Optimal DNA template concentration 20 μl system: 10 μl SYBR® Premix Ex, 1 μl of upstream and downstream primers, 5.13 × 10⁷ ~ 5.13 × 10¹ copies/μl gradient was set as 2 μl. Fill with super pure water to 20 μl. Reaction condition: 94 °C, 10 min; 94 °C, 30 s; Annealing at 60 °C, 30 s, a total of 35 cycles. The optimal concentration of DNA template was determined based on the amplification effect.

Draw the standard curve

A standard template with a concentration of 5.13 × 10⁷ ~ 5.13 × 10⁴ copies/μl was obtained by gradient dilution of the PET-30-HN plasmid at a ratio of 10 times. The standard curve was drawn as the standard with known concentration. Meanwhile, the negative control was set with ultra-pure water.

Repetitive test

In-and inter-batch repeatability tests were performed on the standards with different concentrations (5.13 × 10⁷ copies/μl, 5.13 × 10⁶ copies/μl, 5.13 × 10⁵ copies/μl), and negative controls were set up. Finally, Excel software was used to calculate the mean, standard deviation (SD) and coefficient of variation (CV) of the circulating threshold (Ct).

Specificity test

The established real-time fluorescence quantitative PCR method was used to detect bovine parainfluenza virus type 3 (BPIV3), bovine respiratory syncytial virus (BRSV) and bovine infectious rhinotracheitis virus (IBRV) nucleic acid samples and analyze the specificity of the established method.

RESULTS

Design of HN gene-specific primers for bovine parainfluenza virus type 3

After homology comparison analysis of HN gene sequence, the specific conserved sequence of

bovine parainfluenza virus type 3 was selected as the candidate region of primers, and then the optimal specific detection primer was obtained according to the basic principles of primer design and selection. The final specific detection primer used in this experiment, the upstream primer: 5' -ctctctgtgtttgccagga-3'; The downstream primer was 5' -tccatcagtaggaccaggaa-3', the length of the amplified target fragment was 125 bp, and the annealing temperature was 58 °C.

Preparation of bovine parainfluenza virus type 3 standard positive plasmids

The engineered bacteria containing the PET-30-HN recombinant plasmid were cultured, and the PET-30-HN plasmid was extracted. After PCR identification, the agarose gel electrophoresis amplification results were shown in Fig.1. The target band of about 125 bp was obtained, which was consistent with the expected amplification results. The concentration of the recombinant plasmid was 33.9mg/μl, and the copy number of the recombinant plasmid was calculated by referring to the calculation formula 5.13×10^9 copies/μl.

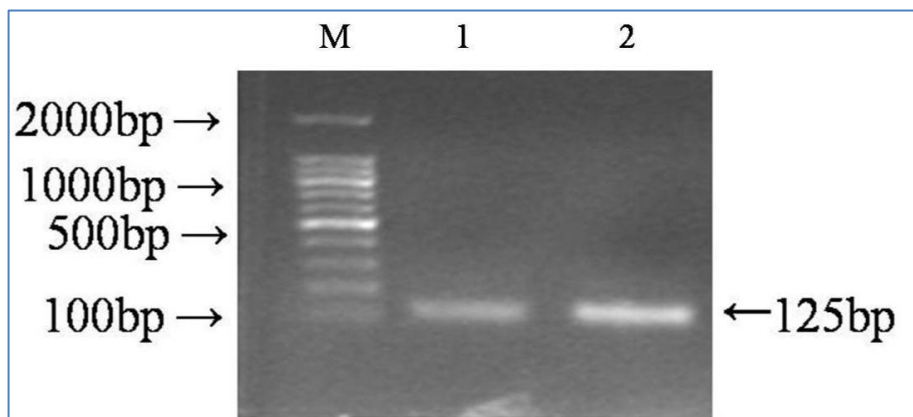


Fig-1: PCR amplification detection of fragment of HN gene of BPIV3 M: DL2000Marker 1,2: Target gene

Real-time fluorescent quantitative PCR reaction system and optimization results of reaction conditions

SYBR Green I real-time fluorescent quantitative PCR reaction system was established after 20 μl optimization by screening the optimal primer concentration and template concentration: SYBR® Premix Ex 10 μl, DNA template 2 μl, upstream and downstream primers 1 μl, ultrapure water 6 μl to 20 μl. 94 °C, 10 min; 94 °C, 30 s; Annealing at 60 °C, 30 s, a total of 40 cycles, the end of each cycle after collecting the fluorescence signal. Finally, the reaction was carried out with the melting curve from 60 °C to 95 °C. The concentration of 5.13×10^7 copies/μl standard positive plasmids was diluted by a gradient of 10 times. After the experiment, the amplification curve and melting curve were as shown in Fig. 2 and Fig.3. The analysis of the amplification results showed that the analysis was as follows: the melting curve had a single specific peak, and all the standard plasmids showed specific dissolution peak at 78.8 °C, indicating that there was no primer dimer, indicating specific amplification.

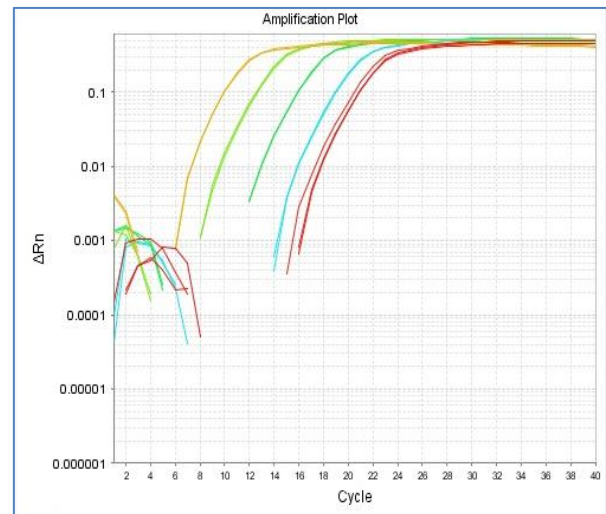


Fig-2: Amplification curve of real-time PCR for BPIV3 detection

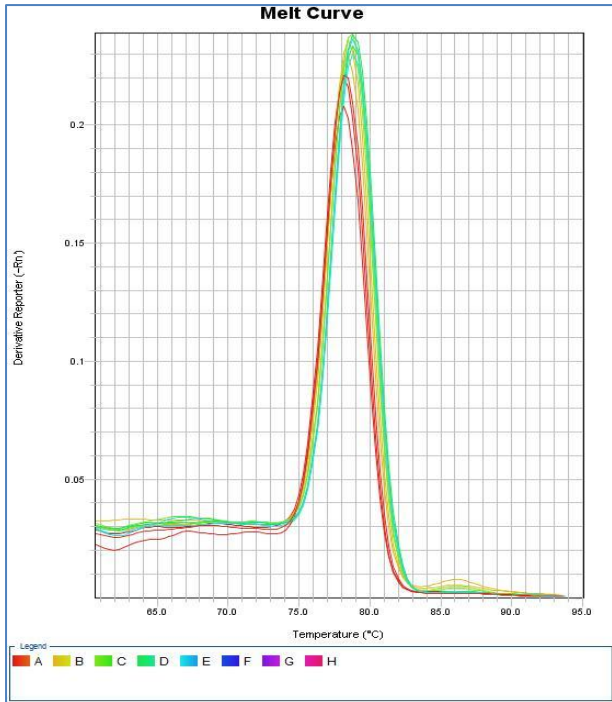


Fig-3: Melting curve of real-time PCR for BPIV3

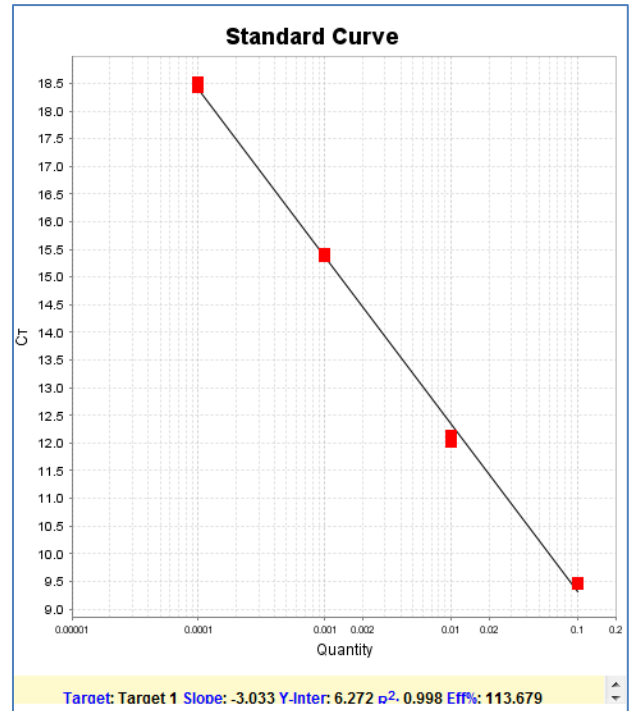


Fig-4: Standard curve of real-time PCR for BPIV3

Drawing results of standard curves

Standard recombinant positive plasmids with concentrations of 5.13×10^7 copies/ μ l, 5.13×10^6 copies/ μ l, 5.13×10^5 copies/ μ l and 5.13×10^4 copies/ μ l were used as templates, and the above methods were used for quantitative fluorescence PCR reaction to draw the standard curve, as shown in Fig.4. Through curve observation, the linear relationship of real-time fluorescence quantitative PCR established in this experiment was good, with correlation coefficient $R_2=0.998$, amplification efficiency $E=113.679$, and slope of -3.033 , all of which were within the normal range.

Sensitivity test results

Standard positive plasmids with concentrations ranging from 5.13×10^7 copies/ μ l to 5.13×10^1 copies/ μ l were detected by fluorescence quantitative PCR and normal PCR. The sensitivity of quantitative fluorescence PCR was 5.13×10^1 copies/ copies 1-1 (Fig.2), while the sensitivity of conventional PCR was 5.13×10^2 copies/ μ l (Fig.5).

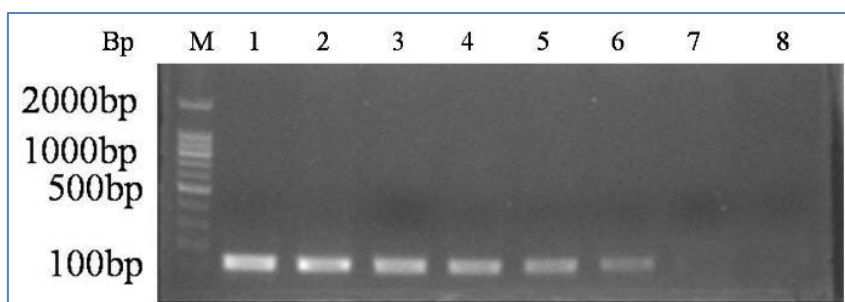


Fig-5: Results of electrophoresis after PCR for BPIV3

M:DL2000Marker; 1: 5.13×10^7 copies· μ l⁻¹; 2: 5.13×10^6 copies· μ l⁻¹; 3: 5.13×10^5 copies· μ l⁻¹; 4: 5.13×10^4 copies· μ l⁻¹; 5: 5.13×10^3 copies· μ l⁻¹; 6: 5.13×10^2 copies· μ l⁻¹; 7: 5.13×10^1 copies· μ l⁻¹; 8:阴性对照;

Repetitive experiment results

Three plasmid standards with different concentrations of 5.13×10^7 copies/ μ l, 5.13×10^6 copies/ μ l and 5.13×10^5 copies/ μ l were used for in-batch and inter-batch repeatability experiments. Finally, the mean value, standard deviation (SD) and coefficient of

variation (CV) of circulating threshold (Ct) were calculated by Excel software. The results showed that the maximum variation coefficient was 0.64%, both less than 1%, as shown in table 1, indicating that the established detection method had high repeatability and stability.

Table-1: Reproducibility of real-time PCR for BPIV3 (n=3)

Standard dilution (copies/ μ l) .	Intra batch CV%		Inter batch CV	
	X \pm SD	CV (%)	X \pm SD	CV (%)
5.13 \times 10 ⁷	9.47 \pm 0.017	0.17	10.39 \pm 0.038	0.36
5.13 \times 10 ⁶	12.07 \pm 0.078	0.64	13.16 \pm 0.096	0.72
5.13 \times 10 ⁵	15.39 \pm 0.018	0.11	18.47 \pm 0.066	0.35

Specific experimental results

The established real-time fluorescence quantitative PCR method was used to detect the nucleic acid samples of BPIV3, BRSV and IBRV. Only the samples of BPIV3 showed positive fluorescence signal, while the other virus samples did not show specific amplification, indicating that this test method has good specificity for bovine parainfluenza virus type 3 virus.

DISCUSSION

In this study based on BPIV3HN gene SYBR Green I real-time fluorescent quantitative PCR method, according to a report in the literature Horwood and Dong[14-15] based on cattle parainfluenza M gene primer design, established strains and BPIV3SD BPIV3 BN-1 strain of TaqMan fluorescence quantitative polymerase chain reaction (PCR), specificity and repeatability are strong. The BPIV3DQ strain used in this study was isolated and identified for the first time in 2009 by the laboratory of preventive veterinary medicine of Bayi agricultural university in Heilongjiang province. After sequence comparison, the similarity between BPIV3DQ strain and BN-1 strain was 99.4%, B genotype, and C genotype SD strain was significantly different^[3]. This is the first time for genotype B BPIV3 strains of SYBR Green I real-time fluorescent quantitative PCR method.

The expression product of BPIV3 HN gene is an important membrane protein, which is an important component of BPIV3 membrane. Studies have demonstrated that HN recombinant protein expression products can not only be used as diagnostic antigens for detection of BPIV3 specific serum antibodies by ELISA and immunofluorescence, but also can be used in the study of anti-BPIV3 nucleic acid and subunit vaccine [16]. HN proteins was studied more and relatively clear, HN gene was chose as testing target in this study, establishing the SYBR Green I real-time fluorescent quantitative PCR detection method. Yang plasmid testing standards, with built method can detect the minimum template copy number, copy of 5.13 x 10¹ copies/ μ l, ordinary RT-PCR method of detecting the minimum template copy number, copy of 5.13 x 10² copies/ μ l, after completion of the PCR reaction and the need for agarose gel electrophoresis detection, brought difficulties for bulk sample screening, easy to pollution in the process of operating a false positive result. In addition, after ruling out any possibility of contamination, the amplification product was detected in the agarose gel and only a single band of amplification was observed, indicating that the amplification was specific. Specific analysis of the primers showed that the designed primers could not

amplify the DNA of other viruses, such as BRSV and IBRV. SYBR Green I real-time fluorescent quantitative PCR method compared with TaqMan fluorescence quantitative polymerase chain reaction (PCR), TaqMan the sensitivity of the method is higher, but this method to the design of probe and primer and use requirement is very high.

Many studies have shown that the prevalence of BPIV3 was high. Some detection methods were established, such as traditional PCR, immunofluorescence and immunohistochemistry. These methods are less sensitive than real-time quantitative PCR, indicating that the actual infection rate of BPIV3 may be higher, and conventional PCR and other techniques could not be detected. A sensitive, efficient and convenient test can help researchers get a clear picture of the actual rate of BPIV3 infection and also help detect BPIV3 infection in different trials. About BPIV3 SYBR Green I real-time fluorescent quantitative PCR detection method has not been reported in the literature at home and abroad. This study is established for detecting the BPIV3 SYBR Green I real-time fluorescent quantitative PCR method, on the specificity, sensitivity and repeatability has more obvious advantages than other traditional methods, for the laboratory and veterinary clinical detection of BPIV3 provide rapid diagnostic methods, has practical application value.

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