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Original article

SYBR Green-based real-time polymerase chain reaction assay for detection of porcine parvovirus 6 in pigs

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Abstract

In this study, a SYBR Green-based real-time quantitative polymerase chain reaction (qPCR) assay was developed for rapid detection of porcine parvovirus (PPV) 6. Primer pairs targeting the conserved regions of PPV6 *Capsid* gene were designed. Sensitivity analyses revealed the lowest detection limit of the SYBR Green-based real-time PCR assay to be 47.8 copies/μL, which indicated it was 1000 times higher than that found in the conventional PCR investigations. This assay was specific and showed no cross-species amplification with other six porcine viruses. The assay demonstrated high repeatability and reproducibility; the intra- and inter-assay coefficients of variation were 0.79% and 0.42%, respectively. The positive detection rates of 180 clinical samples with SYBR Green-based real-time PCR and conventional PCR were 12.22% (22/180) and 4.44% (8/180), respectively. Our method is sensitive, specific, and reproducible. The use of SYBR Green-based real-time PCR may be suitable for the clinical detection and epidemiological investigation of PPV6.

Key words: porcine parvovirus 6, real-time polymerase chain reaction, SYBR Green

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Introduction

The parvovirus is a non-enveloped single-stranded DNA virus with a genome size of 4 to 6.3 kb (Ren et al. 2013). It belongs to the *Parvoviridae* family, which consists of two subfamilies – *Parvovirinae* and *Densovirinae* (Xiao et al. 2013). The genomic organization of porcine parvovirus (PPV) 6 is similar to that of other parvoviruses; it contains a linear genome, with two major gene cassettes from the 5'-end to 3'-end, and the open reading frames (ORFs), ORF1 and ORF2. ORF1 encodes a non-structural protein required for transcription and DNA replication. ORF2 encodes a structural capsid protein (Streck et al. 2013). An additional ORF, ORF3, was identified in some parvoviruses, such as PPV4 and bocavirus (Cheng et al. 2010, Cheung et al. 2010).

Over the past few decades, seven parvovirus species, including PPV1, PPV2, PPV3, PPV4, PPV5, PPV6, and PPV7, have been identified in pigs (Palinski et al. 2016, Cui et al. 2017a). Among these, PPV1 is the main pathogenic agent in breeding herds and was first isolated in Germany in 1965. PPV1 infection results in early embryonic death, infertility, stillbirths, and delayed recovery to estrus phase (Mayr et al. 1968, Mayr and Mahnel 1964, Xu et al. 2013). Unlike PPV1, pathogenic involvement in reproductive failure of the other porcine parvovirus serotypes remains to be identified. The PPV6 serotype was first discovered in 2014 in China (Ni et al. 2014) and was subsequently found in North America and Poland (Cui et al. 2017b). Presently, its ability to cause disease remains unclear.

Till date, no studies have assessed the potential of SYBR Green-based real-time PCR approach to identify PPV6. The present study aimed to develop a rapid and reliable detection method for PPV6 infections in pigs using a SYBR Green-based real-time PCR assay, which targeted the highly conserved region of PPV6 capsid gene. This method could be useful in the epidemiological investigations and laboratory research of PPV6.

Materials and Methods

Viruses

PPV6 (GenBank: MK825573), PPV7 (GenBank: MK484102), porcine circovirus 2 (PCV2, GenBank: MK426833), porcine epidemic diarrhea virus (PEDV, GenBank: MF462814), porcine reproductive and respiratory syndrome virus (PRRSV, SCH07 strain, GenBank: FJ716695.1) were isolated and preserved in our laboratory. PCV3 (GenBank: KY075995.1) and porcine pseudorabies virus (PRV, HB-98 vaccine strain) was received as a gift from Professor Jianzhong Wei of the Anhui Agricultural University, China. PPV6, PPV7, PCV2, PCV3, and PRV were cultured and propagated using PK-15 cells. PEDV and PRRSV were cultured and propagated using Vero cells and Marc-145 cells, respectively. All viruses were cultured in DMEM containing 10% fetal bovine serum (FBS, Gibco, Waltham, MA, USA).

Primer design

Using the Primer Premier software (Premier Biosoft International, Palo Alto, U.S.A.), primer pairs, PPV6-F: 5'-GAAAAAGAACAGGCGCAGAC-3' and PPV6-R: 5'-GGGATTATGAAGCCAGACG-3', were designed complementary to the target gene, PPV6 partial Capsid (GenBank accession no. MK378405.1). The primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd.

Nucleic acid extraction

After three rounds of continuous freeze-thawing, all virus-infected tissue samples were homogenized in 1 mL phosphate-buffered saline and centrifuged at 12,000 r/min for 10 min. The supernatant was stored at -80°C. Viral DNA and RNA from all samples were extracted from tissue homogenates using a commercial kit, TIANamp Virus DNA/RNA kit (Tiangen, Beijing, China), according to the manufacturer's instructions. The total RNA was reverse transcribed using the FastQuant RT Kit (with gDNase) (Tiangen). Viral genomic DNA/cDNA was immediately used for real-time PCR or stored at -80°C until further use.

Construction of standard recombinant plasmids

The extracted DNA samples, which were positive for PPV6, were used as template for routine PCR with PPV6-F/R primers. The PCR protocol was performed in a 20 µL reaction tube; 10 µL r*Taq* (Takara, Dalian, Japan), 10 µM of each primer, 1 µL DNA template, and RNase-free H_2O were mixed to raise the final volume to 20 µL. The PCR parameters were as follows: 95°C for 5 min, followed by 30 cycles at 95°C for 30 s, 56°C for 30 s, and 72°C for 20 s. After the last cycle, the reaction mixture was maintained at 72°C for 10 min.

The PCR products were purified using a TIANgel Midi Purification Kit (Tiangen) according to the manufacturer's instructions. The purified amplicons were cloned into the pMD19-T vector (Takara) at 16°C, overnight, and transformed into chemically competent *Escherichia coli* DH5α cells (Tiangen), according to the manufacturer's instructions. The recombinant plasmid DNA were extracted using the AxyPrep Genomic DNA Miniprep Kit (Axygen, Silicon Valley, U.S.A.), identi-

fied through sequencing using Genscript (Genscript Biotech Corporation, Nanjing, China), and verified using restriction enzyme digestion and PCR. The positive plasmids were submitted to Sangon Biotech (Shanghai) Co., Ltd for sequencing. After exact matching of the sequences with the GenBank sequences, the recombinant plasmid was quantified using a ND-2000c spectrophotometer (Thermo Fisher, Wilmington, U.S.A.), and the recombinant plasmid copy number was calculated using the following formula: Amount (copies/μL) = $(6.02 \times 10^{23}) \times (ng/\mu L \times 10^{-9})$ / (DNA length × 660). The extracted standard plasmid templates were used in a 10-fold serial dilution to represent the range from 4.78×10^8 to 4.78×10^1 copies and stored at -20°C until further use. The diluted plasmids were subsequently used in conventional and real-time PCR with the PPV6-qPCR-F and PPV6-qPCR-R primers.

Standard curve construction

A 10-fold serial dilution of standard plasmids was used for SYBR Green-based real-time PCR amplification reactions, and as the negative control. All reactions were performed with the CFX96™ Real-Time System (Bio-Rad, CA, U.S.A). Each reaction was carried out using 10 μL of 2× SuperReal PreMix Plus (Tiangen), 10 μM each of the forward and reverse primers (PPV6-qPCR-F and PPV6-qPCR-R), 1.0 μL DNA template, and 7.8 μL RNase-free ddH_2O to make up the final volume to 20 μL. The reaction parameters consisted of an initial step at 95°C for 15 min, followed by 40 cycles at 95°C for 10 s and 65°C for 30 s. Melting curve analysis was performed by assessing the fluorescence of SYBR Green signal from 65°C to 95°C. All reactions were performed in triplicates.

Analytical sensitivity of real-time PCR

To evaluate the sensitivity of SYBR Green real-time PCR and conventional PCR analysis methods, we used 10-fold serial dilutions of the recombinant plasmid ranging from 4.78×10^8 to 4.78×10^1 copies/μL as template for the amplification.

Analytical reproducibility of real-time PCR

For assessing the intra- and inter-assay repeatability and stability of the SYBR Green-based real-time PCR assay, three different concentrations of standard plasmid containing PPV6 DNA were used (4.78×10^7, 4.78×10^5, and 4.78×10^3 copies/μL) and were detected by SYBR Green-based real-time PCR. All reactions were performed in triplicates, simultaneously, with same reaction conditions. The average values of Ct and coefficient of variation (CV) were calculated based on the test results, and the stability of the assay was evaluated by assessing the CV.

Analytical specificity of real-time PCR

The specificity of our SYBR Green-based real-time PCR assay was assessed by real-time PCR analysis of the DNA or cDNA of six porcine viruses (PPV6, PPV7, PCV2, PCV3, PEDV, PRRSV, and PRV) as templates and ddH_2O as the negative control, and the detection of PPV6 was assessed.

Detection in clinical samples

To assess the clinical performance of SYBR Green-based real-time PCR for the detection of PPV6, we tested clinical samples obtained from 180 pigs with respiratory symptoms; these specimens were collected from four pig farms of the Anhui province, China. All specimens were tested using both conventional and real-time PCR techniques.

Results

Standard Curve

The 10-fold dilutions of DNA used generated a standard curve with a correlation coefficient (R^2) of 0.997, and amplification efficiency of 96.2%. The slope of the standard curve was -3.417, and the Y-intercept was 36.255 (Fig. 1B). The correlation coefficient of the standard curve of SYBR Green-based real-time PCR exhibited good linear relationship in the investigated concentration range. The melting curve showed specific amplification product at 85°C ± 0.50°C, without the presence of non-specific products, such as primer dimers (Fig. 1C), which suggested high specificity of the SYBR Green-based real-time PCR assay.

Sensitivity of real-time PCR

Sensitivity of the SYBR Green-based real-time PCR assay was tested using conventional and real-time PCR with 10-fold serial dilutions of plasmid reference materials. The limit of detection of conventional PCR was 4.78×10^4 copies, while that of the real-time PCR method was 47.8 copies (Fig. 1A, Fig. 2). The results indicated that the established method was 1,000 times more sensitive than conventional PCR and had high sensitivity.

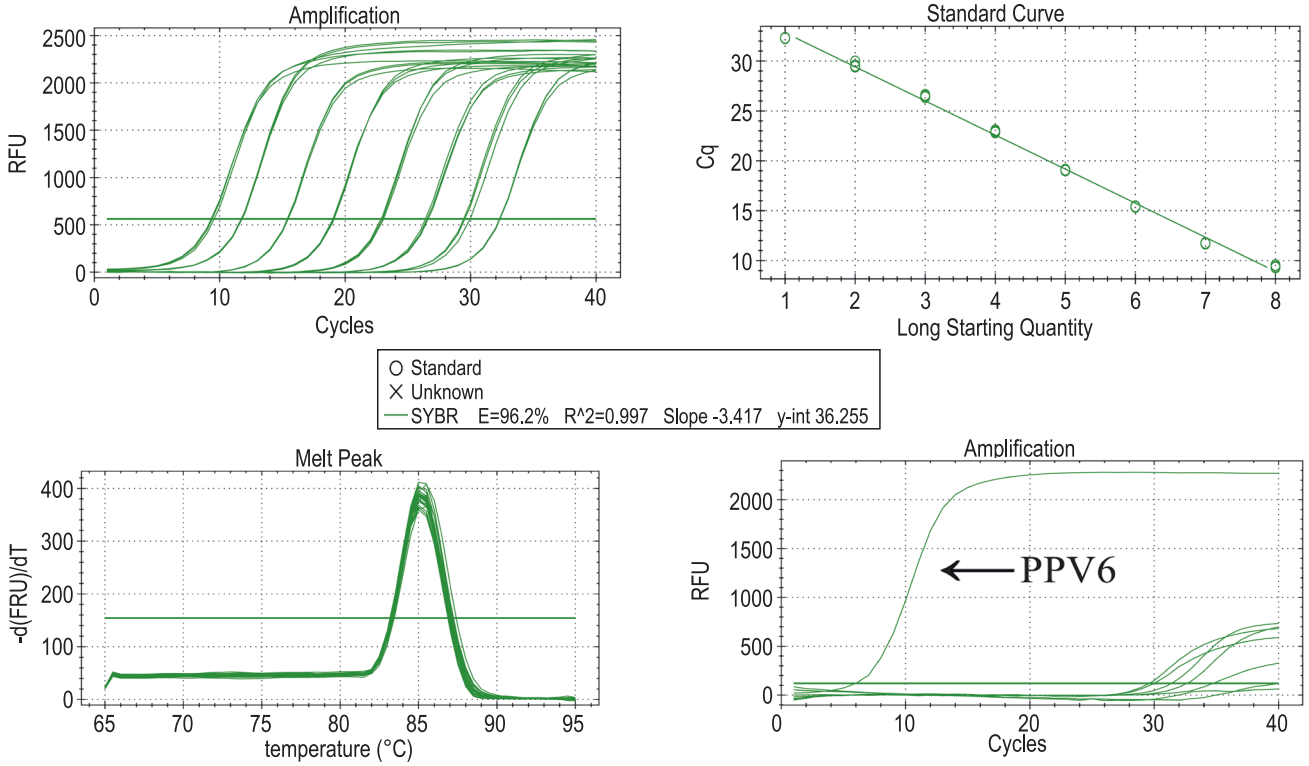

Fig. 1. (A) Amplification curve showing serial dilution of porcine parvovirus 6 (PPV6) clone genomic DNA templates ranging from 4.78×10^8 to 4.78×10^1 copies/µl. (B) Standard curve of SYBR Green-based real-time polymerase chain reaction (PCR) ranging from 4.78 × 108 to 4.78 × 101 copies per reaction (Correlation coefficient: $R^2 = 0.997$; reaction efficiency: 96.2%) (C) Melting curve analysis. (D) Specificity of SYBR Green-based real-time PCR directed against the PPV6 *Cap* gene. Amplification plot representing PPV6, porcine circovirus type 3 (PCV3), PCV2, porcine epidemic diarrhea virus, pseudorabies virus, PPV7, Porcine reproductive and respiratory syndrome virus, and a negative control (Cq, cycle quantity; RFU, relative fluorescence units).

Reproducibility of real-time PCR

The SYBR Green-based real-time PCR assay demonstrated high repeatability with coefficients of variation within runs (intra-assay variability) and between runs (inter-assay variability), and ranged from 0.36% to 0.79% and 0.21% to 0.42%, respectively, indicating high accuracy and reproducibility of the newly developed test.

Specificity of real-time PCR

The SYBR Green-based real-time PCR amplification plot showed strong fluorescent signals from reactions with PPV6 DNA. The Ct values of negative control and six other six porcine viruses were greater than 35 (Fig. 1D), which further confirmed the specificity of the assay.

PPV6 detection in clinical samples

In this experiment, a total of 180 clinical samples were tested simultaneously for the detection of PPV6 DNA using conventional and SYBR Green-based real-time PCR. The positive detection rate determined with SYBR Green-based real-time PCR assay was 12.22% (22/180), while conventional PCR had a positive rate of only 4.44% (8/180). The positive coincidence rate was 100%. The detection rate of PPV6 with SYBR Green-based real-time PCR was about 7.78% higher than that with conventional PCR, which is further proof of higher sensitivity of the former method.

Discussion

In this study, we successfully developed a SYBR Green I-based real-time PCR assay to detect PPV6 infection in pigs. This method is rapid, sensitive, and specific. Because real-time PCRs can detect the presence of viruses in clinical samples, this method enables more rapid quantification of PPV6 than conventional PCR. Moreover, the assay can be monitored real-time and interpreted correctly. Many studies have indicated SYBR Green-based real-time PCR to be accurate and effective in the quantification of viral DNA (Espy et al. 2000, Lo and Chao 2004, Liu et al. 2013, Mohamed et al. 2013). Moreover, real-time PCR results can be obtained in only approximately 1.5 h. It is particularly suitable for high-throughput detection

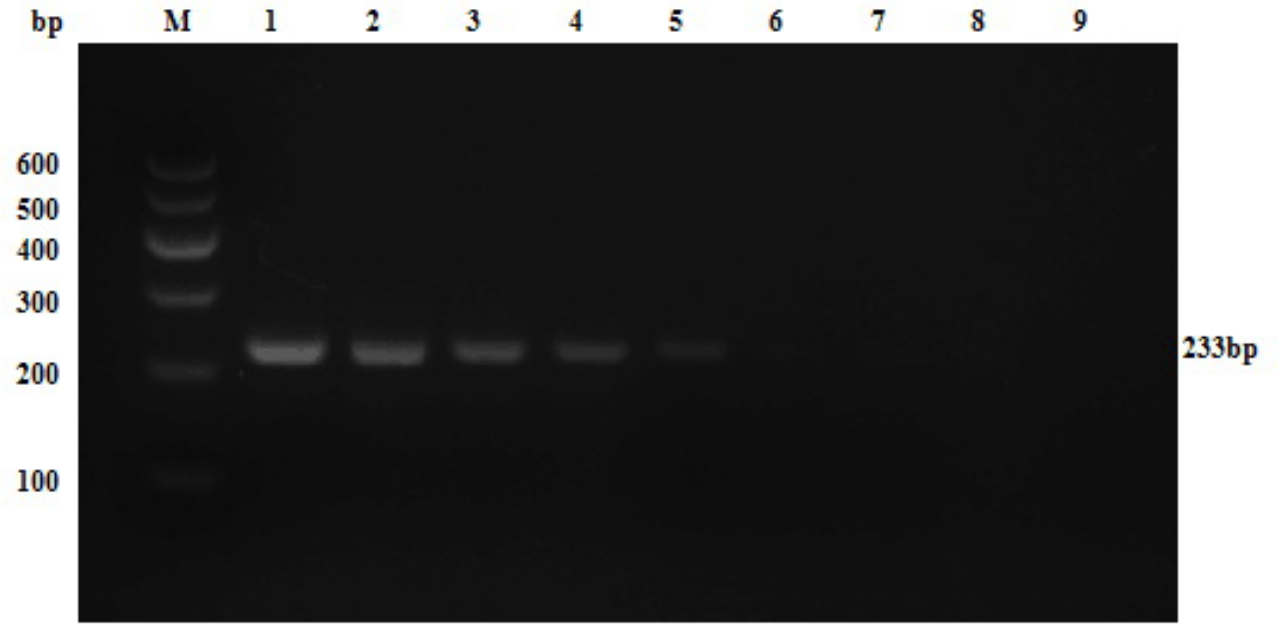

Fig. 2. Sensitivity of conventional polymerase chain reaction on 2% agarose gel electrophoresis M: DL 600 marker; lanes 1–8: standard DNA ($4.78 \times 10^8 - 4.78 \times 10^1$ copies/µl); lane 9: negative control.

of PPV6 in clinical samples. Compared to the TaqMan probe-based method, SYBR Green-based real-time PCR does not require separate probes, which generally adds to the complexity and cost (Song et al. 2010, Gava et al. 2015). The double-stranded DNA polymerase intercalating agent, SYBR® GREEN, has been used to diagnose other diseases, demonstrating the sensitivity and rapidity in disease diagnosis (Ma et al. 2018, Zhang et al. 2018, Han et al. 2019, Ma et al. 2019).

The standardized assay in this study demonstrated an efficiency of 96.2% and R^2 value of 0.997. This novel method can detect as few as 48.7 copies of viral DNA, and the sensitivity of the assay is 1,000 times higher than that of conventional PCR. The CV values of inter- and intra-assay comparison ranges from 0.21% to 0.42% and from 0.36% to 0.79%, respectively, which demonstrates high stability and good repeatability of the established method. In the assessment of specificity of the established real-time PCR, in under 30 cycles, the established method could detect PPV6 well. Moreover, among other common porcine viruses, only PPV6 showed positive results. Moreover, the melting curve data enables the verification of product amplification and obviates the need for post-PCR manipulation, thus diminishing the risk of potential contamination of samples.

In this study, we accurately detected PPV6 from the lung and spleen of infected pigs from Anhui province, China. PPV6 infection could reportedly result in viremia, which indicates the probability of viral DNA being present in the serum of the animal (Schirtzinger et al. 2015). The prevalence of PPV6 DNA is similar to PPV1 (14.7%) and PPV3 (19.2%) in sera, and that of PPV4 (5.9%) and PPV5 (7%) in the sera and tissues (9.3%) (Zhou et al. 2017). Hence, the established PCR method was used to detect PPV6 DNA from serum samples. This method will increase the efficiency of PPV detection in serum samples, and eliminate the need for harvesting the infected organs and tissues. The results of detection of PPV6 in clinical samples show that PPV6 prevalence is a noteworthy issue and requires further investigation.

In summary, to our knowledge, SYBR Green I-based real-time PCR assay is the first sensitive and rapid molecular assay that can be used for the detection of PPV6 in field samples. It could potentially be used in epidemiological surveillance and laboratory research.

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