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Original article

Development of a SYBR Green I real-time PCR assay for detection of novel porcine parvovirus 7

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Abstract

In this study, we developed a SYBR Green I real-time PCR method for the rapid and sensitive detection of novel porcine parvovirus 7 (PPV7). Specific primers were designed based on the highly conserved region within the *Capsid* gene of PPV7. The established method was 1,000 times more sensitive than the conventional PCR method and had a detection limit of 35.6 copies. This method was specific and had no cross-reactions with PCV2, PCV3, PRV, PEDV, PPV1, and PPV6. Experiments testing the intra and interassay precision demonstrated a high reproducibility. Testing the newly established method with 200 clinical samples revealed a detection rate up to 17.5% higher than that of the conventional PCR assay. The established method could provide technical support for clinical diagnosis and epidemiological investigation of PPV7.

Key words: *Capsid* gene, PPV7, SYBR Green I real-time PCR

Introduction

The parvovirus is a small, non-enveloped virus with a single-stranded linear DNA genome of approximately 4–6.3 kb. It belongs to the family *Parvoviridae*, which consists of two subfamilies that infect either vertebrates (*Parvovirinae*) or arthropods (*Densovirinae*) (Cui et al. 2017). To date, seven porcine parvovirus genotypes (PPV1-PPV7) have been described: a) porcine parvovirus 1 (PPV1), which is a major pathogenic factor leading to porcine reproductive failure (first isolated in Germany in 1965) (Mayr et al.1964, Mayr et al. 1968); b) porcine parvovirus 2 (PPV2), which was first identified in Myanmar in 2001 (association of PPV2 with

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clinically relevant diseases is still under debate) (Hijikata et al. 2001); c) porcine parvovirus 3 (PPV3), also known as porcine hokovirus or porcine PARV4, which was detected in Hong Kong in 2008 (Lau et al. 2008); d) porcine parvovirus 4 (PPV4), which was diagnosed in the USA in 2010, coinfected with porcine circovirus 2(PCV2)-associated disease (Huang et al. 2010); e) porcine parvovirus 5 (PPV5), which is similar to BPV2 and was discovered in the USA (Xiao et al. 2013); f) porcine parvovirus 6 (PPV6), which was first identified in China in aborted fetuses of swine (Ni et al. 2014); g) porcine parvovirus 7 (PPV7), a new porcine parvovirus genotype, which was discovered in the USA in 2016 (Palinskib et al. 2016). The clinical signs of PPV7 infections have yet to be determined.

PPV7 was first discovered in the USA in rectal swabs of healthy pigs and was subsequently also detected in China, Switzerland, and Poland (Palinski et al. 2016, Blomstrom et al. 2018, Milek et al. 2018, Xing et al. 2018). There is an urgent need to establish a rapid, sensitive, and specific detection method to understand the prevalence of PPV7. A probe-based real-time quantitative polymerase chain reaction (qPCR) for detecting PPV7 has been established by amplifying a 119-bp fragment targeting the *VP* gene (Poojari et al. 2016). However, detection based on the SYBR real-time qPCR method only needs primers, does not require an expensive probe, is easier to use, and has a low cost compared with probe-based RT-qPCR (Jiang et al. 2014, Poojari et al. 2016). Therefore, in the present study, we established a quantitative SYBR Green I fluorescence detection method targeting a 165-bp fragment of the PPV7 *Cap* gene that can reliably detect PPV7 in pigs and is also effective for detection via conventional PCR.

Materials and Methods

Virus and tissue samples

A new Porcine parvovirus 7 strain, PPV7/China/ /AHbz (accession number:MK484100), was isolated from an infected pig in Anhui Province, China. Porcine circovirus 2 (PCV2), porcine circovirus 3 (PCV3), porcine pseudorabies virus (PRV), porcine epidemic diarrhea virus (PEDV), porcine parvovirus 1 (PPV1) and porcine parvovirus 6 (PPV6) were stored in our lab. A total of 200 clinical samples were collected from 20 commercial pig farms in nine cities of the Anhui Province, China, from March 2017 to September 2018.

Primer design

This study designed a pair of specific primers based on the PPV7 42 strain (GenBank accession number: KU563733.1) as a template; the primer pair F1/R1 (5′ to 3′: GCGACCAGTCGAAAGTCTTC, TTGGTG TTGCCCATTCTGTA) targets the conserved Cap*sid* gene and was used for real-time PCR and conventional PCR, respectively (product length=165 bp). The genomic sequences with the accession numbers KU563733.1, MG696112.1, MG696111.1, KY996758.1, KY996757.1, KY996756.1, and MG914435.1 were aligned using MegAlign 7.1.0 (DNAstar, Madison, WI, USA). All primers were designed for conserved regions and analyzed using GenBank's BLAST search (http:// //www.ncbi.nlm.nih.gov/).

Extraction of nucleic acid from sample

Viral DNA and Viral RNA were extracted from the samples using the TIANamp Virus DNA/RNA kit (Tiangen, Beijing, China) according to the manufacturer's instructions. Reverse transcription of 100 ng viral RNA sample was conducted using the FastQuant cDNA Kit (Tiangen) according to the manufacturer's instructions. The extracted DNA and synthesized cDNA were stored at -20°C.

Construction of standard recombinant plasmids

The DNA genome of PPV7 was extracted, and PCR amplification performed using the primer PPV7-F1/R1. The composition for the conventional PCR system in a total of 20 μL was as follows: 10 μL r*Taq* (Takara, Dalian, Japan), 10 μM of each primer, 1 μL DNA template, and RNase-free H_2O to the final volume. The PCR conditions were as follows: pre-denaturation for 5 min at 95°C, followed by 30 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 20 s, with a final extension at 72°C for 10 min. The amplified product size was165 bp, as analyzed by 2% agarose gel electrophoresis. The 165-bp target fragment was amplified and cloned into the pMD18-T vector according to the manufacturer's protocol (TaKaRa, Beijing, China) and transformed into *Escherichia coli* DH5α cells (Tiangen). The recombinant plasmids were extracted using the AXGEN Plasmid Mini Kit (AXGEN, China) and sequenced by Genscript (Genscript Biotech Corporation, Nanjing, China). The concentration of the extracted plasmid was determined using an ND-2000c spectrophotometer (NanoDrop, Wilmington, DE, USA), and the copy number was calculated according to the following formula: (concentration in ng $\times 6.02\times10^{23}$) / / (genome length $\times$ $10^9\times660$ Da/bp).

Standard curve construction

A serial 10-fold dilution of standard plasmids (ranging from 3.56×10^1 to 3.56×10^8 copies/μL) for PPV7

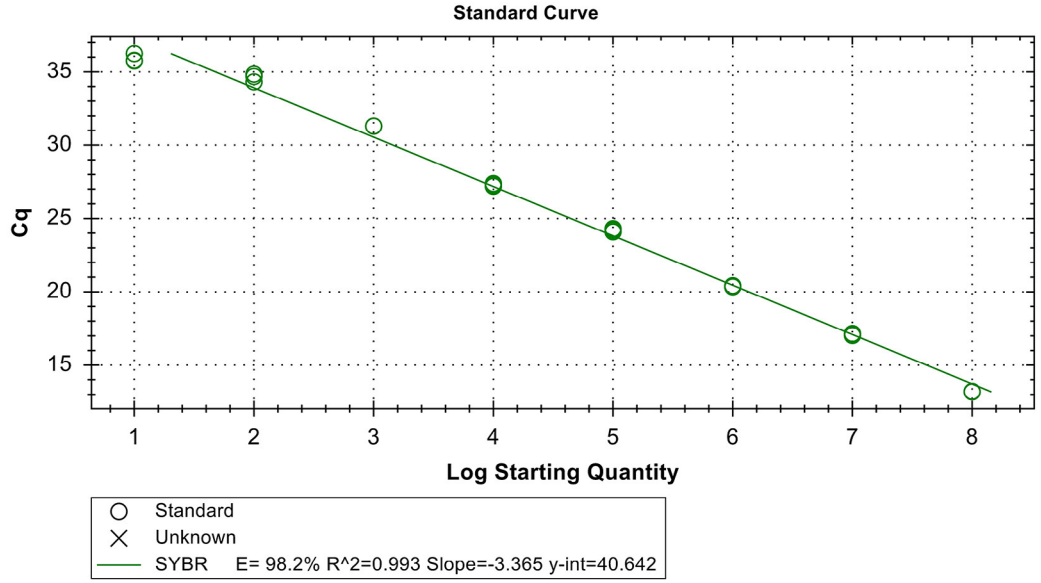

Fig. 1. Standard curve. There is a good linear correlation between the threshold and the plasmid concentration between 3.56×10^{1} and 3.56×10^{8} copies/µL. Experiments were conducted in triplicate.

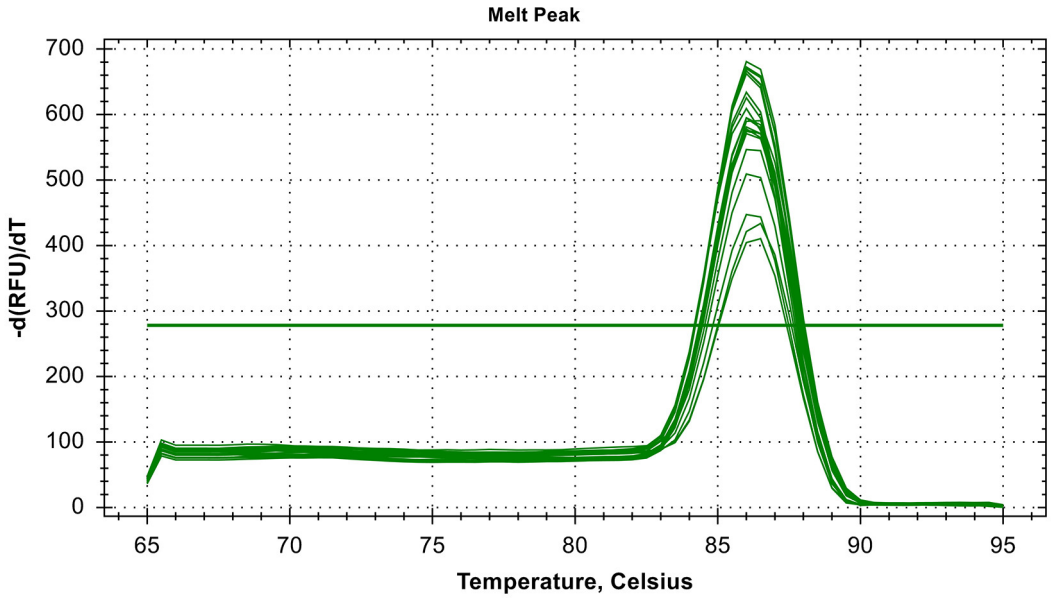

Fig. 2. Melting curve. Melting peaks of standard plasmid DNA 10-fold dilutions. The result shows that the Tm value at the single peak position is 86°C.

was used for SYBR Green real-time PCR amplification, and real-time standard curves were generated using the CFX96™ Real-Time System (Bio-Rad, Hercules, CA, USA). Real-time PCR was carried out in a 20 µL reaction volume containing 10 µL 2× SuperReal PreMix Plus (Tiangen), 0.6 µL (10 µM) each of primers F2 and R2, 1.0 µL DNA template, and 7.8 µL RNase-free ddH_2O. This system was used with the following reaction conditions: pre-denaturation at 95°C for 15 min, followed by 40 cycles of 95°C for 10 s and 65°C for 30 s. A melting curve analysis was performed by monitoring the fluorescence of the SYBR Green signal from 65°C to 95°C. All reactions, including those involving negative controls, were repeated three times.

Sensitivity and specificity of the real-time PCR assay

Ten-fold serial dilutions (ranging from 3.56×10^{1} to 3.56×10^{8} copies/µL) of plasmids were used as a template for conventional PCR and real-time PCR to detect the sensitivity of the real-time PCR assay. To determine the specificity of the real-time PCR, the assay was examined using PCV2, PCV3, PRV, PEDV, PPV1, and PPV6. Using conventional PCR, all viruses were positive when their specific primers were used and negative for the PPV7 primer.

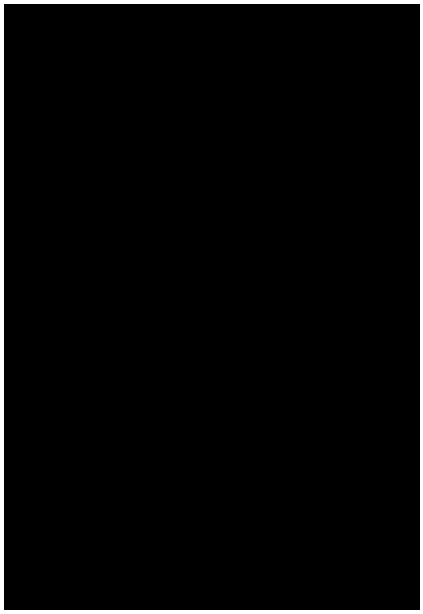

Fig. 3. Amplification curve. Lanes 1-8: serial dilutions of PPV7 standard plasmid DNA (3.56×108 to 3.56×101 copies/μL).

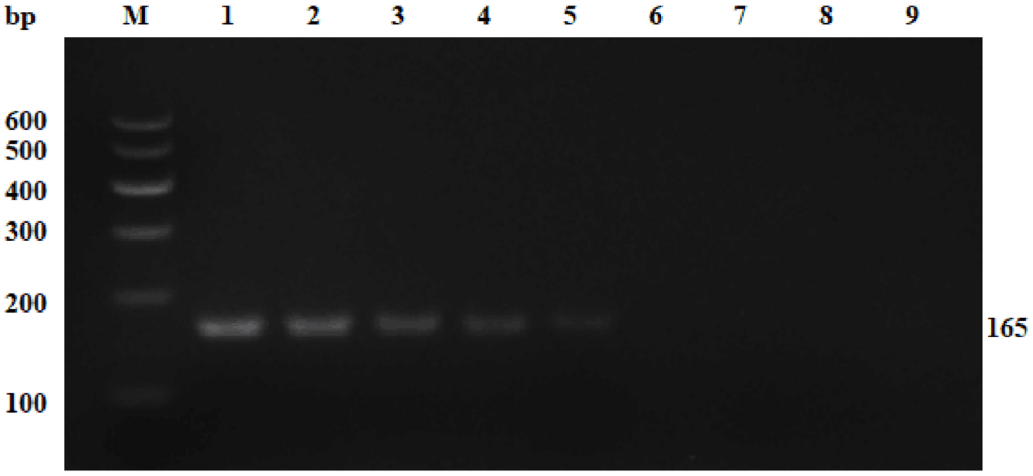

Fig. 4. Sensitivity of conventional PCR. Marker: 600-bp DNA ladder, Lanes 1-8: serial dilutions of PPV7 standard plasmid DNA (3.56×108 to 3.56×101 copies/μL), Lane 9: negative control.

Reproducibility of real-time PCR

To evaluate the repeatability and stability of the real--time PCR assay, three dilutions (3.56×10^7, 3.56×10^5, and 3.56×10^3 copies/μL) of the standard plasmid of PPV7 were selected as templates and amplified under the same reaction conditions. Three parallel experiments were performed on each diluted standard plasmid. The average mean cycle threshold (Ct) value and the coefficient of variation (CV) were calculated based on the test results, and the stability of the assay was evaluated using the CV.

Detection of clinical samples in the clinical samples

The 200 clinical samples were tested using conventional PCR and Real-time PCR assays, and the positive detection rates were simultaneously recorded.

Results

Standard Curve

Ten-fold dilutions of the standard recombinant plasmid for PPV7 (ranging from 3.56×10^1 to 3.56×10^8 copies/μL) were used to generate a standard curve. The standard curve was determined as $y=-3.365x+40.642$. The logarithm of the copy numbers showed a strong linear correlation with the Cq value ($R^2=0.993$), and the correlation coefficient was calculated to be 98.2% (Fig. 1). Furthermore, the melting curve showed only a single peak, indicating that no primer dimers or non--specific amplifications were detected (Fig. 2).

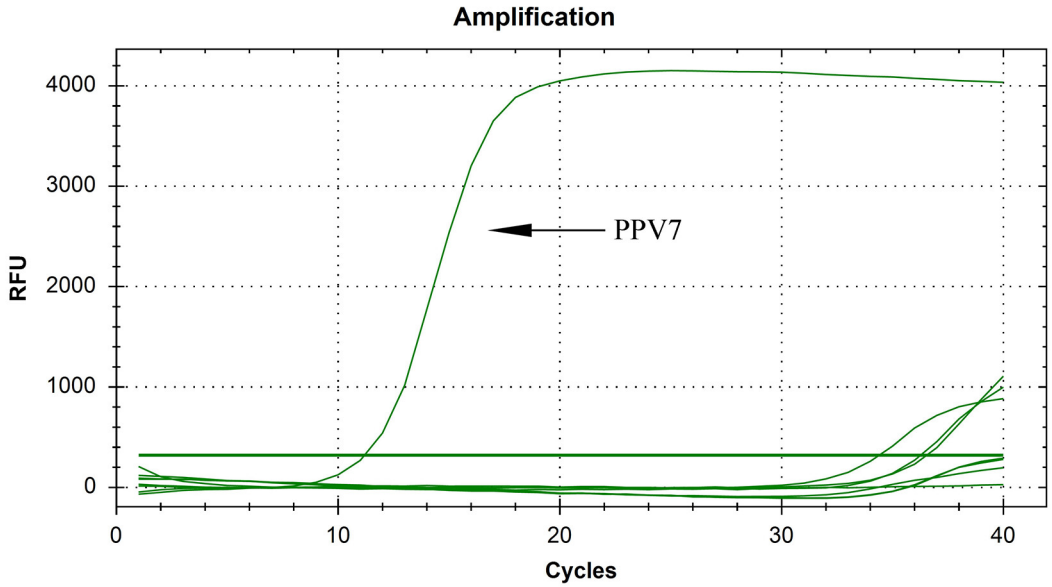

Fig. 5. Specificity of the SYBR Green I real-time PCR for different viruses. The samples include porcine parvovirus 7 (PPV7), porcine circovirus 2 (PCV2), porcine circovirus 3 (PCV3), porcine pseudorabies virus (PRV), porcine epidemic diarrhea virus (PEDV), porcine parvovirus 1 (PPV1), porcine parvovirus 6 (PPV6), and ddH_2O (negative control).

Table 1. Intra-and inter-assay reproducibility of the real-time PCR assay.

DNA standard (copies/μL)	n	Intra-assay			Inter-assay		
		Mean Ct	SD	CV/%	Mean Ct	SD	CV/%
1×10^7	3	17.1	0.05	0.29	17.2	0.07	0.41
1×10^5	3	24.2	0.09	0.37	24.2	0.11	0.45
1×10^3	3	31.3	0.04	0.13	31.2	0.06	0.19

Ct = cycle threshold; CV = coefficient of variation; PCR = polymerase chain reaction; SD = standard deviation.

Sensitivity and specificity of real-time PCR

The standard recombinant plasmid (ranging from 3.56×10^1 to 3.56×10^8 copies/μL) was used as template to determine the minimum detection limit, and the sensitivities of conventional PCR and real-time PCR methods were compared. The results showed that the detection limit of real-time PCR was 3.56×10^1 copies/μL (Fig. 3), while the lower detection limit for conventional PCR was 3.56×10^4 copies/μL (Fig. 4).

When several viruses (PPV7, PCV2, PCV3, PRV, PEDV, PPV1, and PPV6) were screened using real-time PCR amplification, the real-time PCR was able to detect PPV7. However, as shown in (Fig. 5), the Ct values of negative control and other six viruses were greater than 35, which further confirmed the specificity of the assay.

Reproducibility of real-time PCR

The standard recombinant plasmid content of 3.56×10^7, 3.56×10^5, and 3.56×10^3 copies/μL was detected using the established real-time PCR method, and the content of each plasmid was repeated three times to evaluate the intra- and inter-assay reproducibility. The Ct, standard deviation (SD), and CV were calculated (Table 1).

Evaluation of the real-time-PCR assay using clinical samples

Two hundred samples collected from nine cities of the Anhui Province of China were detected by conventional PCR and real-time PCR, respectively. The results demonstrated that the positive rate of real-time PCR was 74%, whereas that of the conventional PCR was 56.5% (Table 2), further demonstrating the higher sensitivity of the real-time PCR assay.

Discussion

PPV7 was first reported in the USA in 2016 (Palinski et al. 2016). At present, the clinical signs, distribution, pathogenicity, and pathological mechanism of PPV7 are not clear. To understand the infection situation of PPV7 in Chinese pigs, a rapid, accurate, and sensitive detection assay is urgently needed.

Table 2. Comparison of detection rates of porcine parvovirus 7 in clinical samples using conventional PCR and SYBR Green I real-time PCR.

Regions of the Anhui province	Number of samples	Positive (%) in conventional PCR	Positive (%) in real-time PCR
Bozhou	35	34.3 (12/35)	65.7 (23/35)
Hefei	22	50.0 (11/22)	72.7 (16/22)
Maanshan	14	64.3 (9/14)	100 (14/14)
Anqing	17	58.8 (10/17)	82.4 (14/17)
Huaibei	21	76.2 (16/21)	85.7 (18/21)
Suzhou	20	75.0 (15/20)	80.0 (16/20)
Chaohu	13	84.6 (11/13)	92.3 (12/13)
Luan	26	53.8 (14/26)	69.2 (18/26)
Huainan	32	46.9 (15/32)	53.1 (17/32)
All regions	200	56.5 (113/200)	74.0 (148/200)

PCR = polymerase chain reaction.

SYBR Green I Real-time PCR is a common detection method in molecular epidemiology, which is rapid, accurate, and sensitive (Patel et al. 2016). Compared with the TaqMan real-time qPCR assay, it does not require a complicated probe design and is economic (Schirtzingernet al. 2015, Su et al. 2018). Moreover, compared with conventional PCR, it can not only improve sensitivity and accuracy and avoid sample contamination, but can also be used for quantitative analysis. Further, in the SYBR Green I Real-time PCR, real-time dynamic continuous monitoring can be carried out for monitoring to obtain test results in a timely manner, save time, and complete the detection task quickly, and thus the SYBR Green I Real-time PCR has been widely used in the detection of clinical pathogens (Jiang et al. 2014, Han et al. 2019, Zhao et al. 2019).

In this study, we successfully developed an SYBR Green I based real-time PCR assay to detect PPV7 in pigs. In spite of the problems of primer dimer and non-specific binding, the results could be determined by a melting curve. The low nucleic acid detection limit under the novel method is 35.6 copies/μL, and the sensitivity is 1,000-fold higher than that of conventional PCR. The CV values of the inter- and intra-assay comparison ranged from 0.13 to 0.37 and from 0.19 to 0.45, respectively, demonstrating that the established method has stable repeatability.

In summary, in the present study, a novel, sensitive, and rapid and accurate SYBR Green I real-time PCR assay for PPV7 detection was established. It could be used in epidemiological investigation and clinical diagnosis of PPV7. This study provides technical support for the comprehensive control of PPV7 in China.

Acknowledgements

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Accession Number

A new Porcine parvovirus 7 strain, PPV7/China/ /AHbz (accession number: MK484100), was uploaded to the GenBanK database.

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