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Development of SYBR Green I-based quantitative PCR assay for identification of porcine circovirus 1, 2 and 3

Chang Liu[1], Yao Tian [1,2], Li-mei Zheng[2], Ying-Ru Ma[2], Jian-Li Shi[1], Chen Li[1], Shao-Jian Xu[1], Xiao-Yan Wu[1], Hong Han[1], Jun Li[1,2]* and Xian-Jie Han[2]*

[1]Shandong Provincial Key Laboratory of Animal Disease Control & Breeding, Institute of Animal Science and Veterinary Medicine Shandong Academy of Agricultural Sciences, China
[2]College of Veterinary Medicine, Qingdao Agricultural University, China.

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ABSTRACT

Porcine Circovirus (PCV) includes Porcine Circovirus 1(PCV1), Porcine Circovirus 2 (PCV2) and Porcine Circovirus 3 (PCV3). In recent years, co-infection exists between PCV1, PCV2 and PCV3 serotypes. Therefore, it is particularly necessary to establish a fast, specific and sensitive SYBR Green I real-time quantitative PCR detection method for PCV1, PCV2 and PCV3. In this experiment, specific primers were selected and the reaction conditions were optimized. A real-time quantitative PCR identification method was established. The results showed the detection limits of this assay were 40.3 copies/μl for PCV1, 25.2 copies/μl for PCV2 and22.4 copies/ μl for PCV3. There was no cross-reactivity with swine fever virus (CSFV), porcine reproductive and respiratory syndrome virus (PRRSV), porcine pseudorabies virus (PRV) and porcine parvovirus (PPV). The intra-assay and inter-assay coefficients of variation were less than 1%. The test results of 100 PCV suspected positive samples revealed that the PCV1, PCV2 and PCV3 singular infection rate was 10% (10/100), 64% (64/100) and 52% (52/100), respectively. The PCV1 and PCV2 co-infection rate was 8% (8/100), the PCV1 and PCV3 co-infection rate was 7% (7/100), the PCV2 and PCV3 co-infection rate was 26% (26/100), and the PCV1, PCV2 and PCV3 co-infection rate was 7% (7/100). This method has good specificity, sensitivity and stability. It provides a promising tool for rapid differential detection of PCV1, PCV2 and PCV3.

Key words: porcine circovirus; specificity; sensitivity; SYBR Green I based quantitative PCR; single identification method

*Corresponding author:
Jun Li, Shandong Provincial Key Laboratory of Animal Disease Control & Breeding, Institute of Animal Science and Veterinary Medicine Shandong Academy of Agricultural Sciences, No.202, Industrial north Rd., Jinan 250100, China, e-mail: junli79@163.com.
Xian-Jie Han, College of Veterinary Medicine, Qingdao Agricultural University, China, e-mail: hanxianjie2006@163.com.

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Introduction

Porcine circovirus is a non-enveloped, circular, single-stranded DNA virus of the genus Circovirus, and it is also the smallest animal virus with the ability to replicate autonomously that we know so far (HANNA et al., 2022). Before 2016, only two serotypes of PCV1 and PCV2 had been found, and only PCV2 was considered to be pathogenic (HAN et al., 2019), and it is the main cause of multi-system failure syndrome in weaned piglets (MOROZOV et al., 1998). It is related to pig dermatitis symptoms (OPRIESSNIG et al., 2007), causing huge losses to the world's pig industry. In 2015, scientists at Kansas State University Veterinary Diagnostic Laboratory detected PCV3 from pigs with dermatitis nephrotic syndrome and reproductive disorders (PALINSKI et al., 2017). PCV3 infection has been reported to be associated with cardiac and multisystem inflammation, porcine dermatitis and nephropathy syndrome (PDNS), and reproductive failure (ZHAO et al., 2019; LI et al., 2018; PHAN et al., 2016). Subsequently, it was detected in the United Kingdom, Poland, Korean, China, Brazil, Thailand, Denmark, Italy and Spain (COLLINS et al., 2017; STADEJEK et al., 2017; KWON et al., 2017; FAN et al., 2017; ZHAI et al., 2017; ZHENG et al., 2017; TOCHETTO et al., 2018; KEDKOVID et al., 2018; FRANZO et al., 2018). Several PCV differential detection methods (both conventional PCR and real-time PCR) have been reported (LI et al., 2013; LI et al., 2018; Zhao et al., 2019). However, no real-time quantitative PCR assay suitable for differential detection of three species of PCVs (PCV1, PCV2, PCV3) has been established yet.

In this study, a SYBR Green-real-time quantitative PCR assay was developed for differential detection of PCV1, PCV2 and PCV3 strains. The specificity, sensitivity and repeatability of the assay were evaluated. In addition, the new real-time PCR assay was validated for the detection of 100 clinical samples collected in Shandong province.

Materials and methods

Virus strains and samples. All viruses used in this study were stored in the Shandong Provincial Key Laboratory of Animal Disease Control and Breeding, including CSFV, PRRSV, PRV, PPV, PCV1, PCV2,and plasmid T-PCV1, pEASY-Blunt-PCV2, pEASY-Blunt-PCV3.

A total of 100 tissue samples (including livers, spleens, kidneys, lungs, lymph nodes) were collected from 100 different dead pigs on 10 different pig farms in 5 cities in Shandong province of China, between July 2019 and June 2021.

Design of primers. On the basis of the ORF1 gene sequences of PCV1 (AY193712.1), PCV2 (KT719404.1) and PCV3 (MG947596.1) published by GenBank, a pair of specific primers were designed using Primer Premier 5 software (Premier, Canada) (Table 1).

Table 1. Primers used for the differential detection of PCV1-3 strains

Primer	Primer sequence (5'→3')	Positions*	Amplicons
PCV-F	TKGATGATYTTTATGGSTGG	(PCV1:678-697;PCV2:691-710;PCV3:845-864)	
PCV1-R	GTCAGCAGTTGAGGACTACCATT	819-841	164 bp
PCV2-R	AGTAATCCGCCGATAAAGAGC	868-887	196 bp
PCV3-R	CTCCTAAACAAGGCCTCCAACT	1017-1037	193 bp

Viral nucleic acid extraction. The viral nucleic acid was extracted using the Simply P virus DNA / RNA extraction Kit (Bori, Hangzhou, China), according to the manufacturer's instructions. First-strand cDNA was synthesized using the extracted viral RNA and the Prime Script 1st Strand cDNA Synthesis kit (TaKaRa, Dalian, China). The DNA and cDNA were stored at -80 °C until use.

Preparation of the standard template. Recombinant plasmids were extracted using a plasmid mini-extraction kit (OMEGA, USA) according to the manufacturer's instructions, and quantified by measuring OD260 with the ultra-micro spectrophotometer Nanodrop-2000 (Thermo, USA). The recombinant plasmids were identified as positive by PCR and then sequenced. The concentration was converted into copy numbers using the following formula:

$y(copies/\mu l) = (6.02\times10^{23}) \times(x(ng/\mu l)\times10^{-9}DNA)$ /(DNA length×660) (Chen, et al., 2021).The recombinant plasmids were diluted to the copy number of 10^{10}, then 10-fold dilution was performed, and 10^{1-9} dilutions of recombinant plasmids taken as the standard template.

Establishment of the real-time PCR method. The triplex real-time PCR assay containing three pairs of primers was performed using 2 × SYBR® Green Pro Taq HS Premix (Saience, Shandong, China) and the LightCycle® 480II real-time PCR System (Roche, USA). Then, amplification conditions were further optimized to yield the highest fluorescence with the lowest threshold cycle (LI et al., 2013). The viral genomes of PCV1, PCV2 and PCV3 were differentiated with the specific melting peaks. After optimization, the 25μl reaction mixture, including 12.5 μl 2 × SYBR® Green Pro Taq HS Premix, 3μl upstream primers (10μM) and 1 μl (10μM) each of the downstream primers, 1.5 μl of the template, and ultrapure water added to 25μl. The negative controls contained the PCR reaction mix and molecular grade water, substituting the volume of genetic material added to the other experimental tubes. The amplifications were performed under the following conditions: pre-denaturation at 95°C for 30s; denaturation at 95°C for 5s; annealing at 62°C for 30s, extension at 72°C for 10s, and 40 cycles of amplification.

Establishing the standard curve and dissolution curve for absolute quantification. A 10^{1-9} dilution of the recombinant plasmid was used as a standard template for quantitative PCR amplification and analysis using LightCycler480 Software 1.5 (Roche, USA) to establish the amplification standard curves and lysis curves.

Specificity test. To estimate the specificity of the new real-time PCR assay, DNA from three species of PCV (PCV1, PCV2 and PCV3) positive samples and cDNA/DNA from the other viruses (CSFV, PRRSV, PRV and PPV) were tested.

Sensitivity test. The recombinant plasmid standard was diluted 10-fold and used as a template for amplification. The lowest copy number of the template that can be detected by the institute. Simultaneous routine PCR was performed to compare the sensitivity differences between the two methods.

Repeatability test. A 10^{3-5} dilution of plasmid standard was selected for the repeatable amplification test. Each template in the group was repeated 3 times at the same time, and the test between groups was repeated 3 times in succession.

Clinical sample testing. 100 samples of suspected PCV were taken for quantitative real-time PCR and conventional PCR to compare the sensitivity of the two methods. Statistical analysis was performed using the SPSS version 21.0 package (SPSS, USA).

Results

Establishment of the real-time PCR standard curve and dissolution curve. 10^{1-9} copies / μl of recombinant plasmid were selected for SYBR Green I fluorescence quantitative PCR amplification, and standard curves (Fig. 1, Fig. 2 and Fig. 3) and lysis curves (Fig. 4) were automatically generated by the LightCycler480 Software 1.5. It was found that the linear relationship curve expressions between PCV1, PCV2, PCV3 copies (x) and cycle threshold (Ct) were $Ct = -3.096lgx + 38.846$ $R^2 = 0.9992$; $Ct = -3.356lgx + 39.513$ $R^2 = 0.9999$; $Ct = -3.611lgx + 40.783$ $R^2 = 0.9998$. The melting temperatures of PCV1, PCV2 and PCV3 are respectively about 84°C, 85°C, and 82°C. There was no melting point

peak in the negative control, while the amplification of the standard samples was a single wave peak with high coincidence degree, indicating that no pollution occurred in this test and the obtained data were highly reliable.

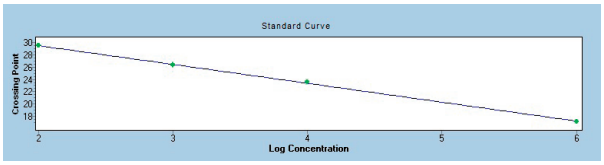

Fig. 1. Standard curve of PCV1

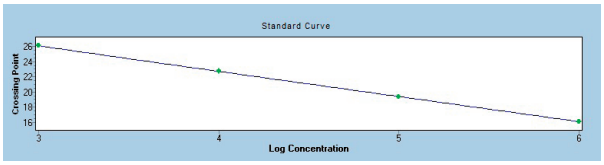

Fig. 2. Standard curve of PCV2

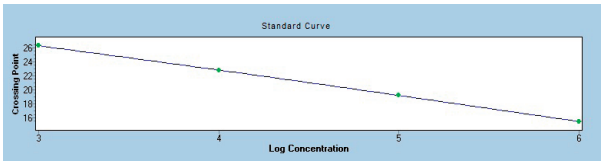

Fig. 3. Standard curve of PCV3

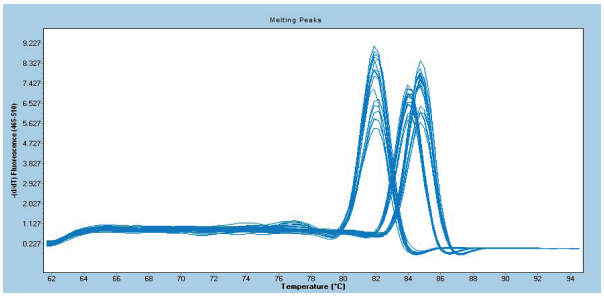

Fig. 4. PCV1, PCV2 and PCV3 melting curve
1.PCV3; 2.PCV1; 3.PCV2

Sensitivity test. The sensitivity of 9 standard dilutions (10^1 to 10^9) of the standard template was tested, and the results showed that the lower limit of detection by the fluorescent quantitative PCR method was PCV1: 40.3 copies / μl (Fig. 5) and PCV2: 19.7 copies / μl (Fig. 6). PCV3: 22.4 copies / μl (Fig. 7), while the detection limits of conventional PCR methods are PCV1: 1.11×10^3 copies / μl (Fig. 8), PCV2: 1×10^3 copies / μl (Fig. 9), PCV3: 9.98×10^2 copies / μl (Fig. 10), which shows that the real-time quantitative PCR method established in this study is more sensitive than conventional PCR, and the test results are more reliable.

Specificity test. Fluorescent quantitative PCR amplification results showed that only PCV1, PCV2 and PCV3 had fluorescent signals. Others, such as PRV, PPV, PRRSV, CSFV, and the negative controls, did not produce fluorescent signals, indicating that the method has good specificity (Fig. 11).

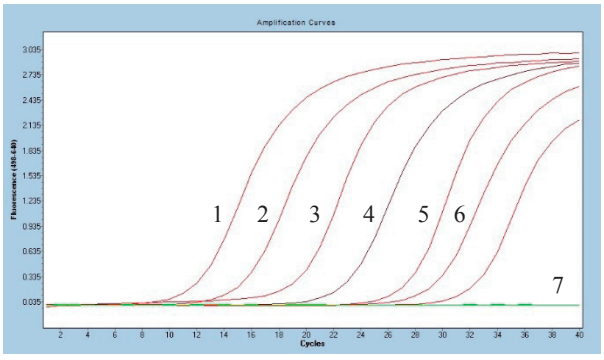

Fig. 5. PCV1 sensitivity test
1~7: 1.00×10^7、1.05×10^6、8.58×10^4、1.11×10^3、1.00×10^2、4.04×10^1copies/μl、negative control

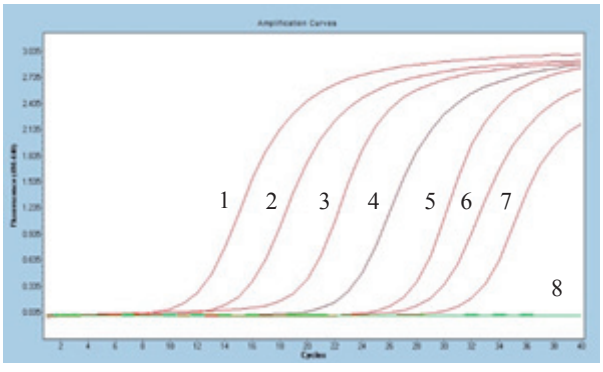

Fig. 6. PCV2 sensitivity test
1~8: 1.50×107、1.78×10^6、1.02×10^5、9.75×10^3、7.75×10^2、3.02×10^2、1.97×10^1copies/μl、negative control

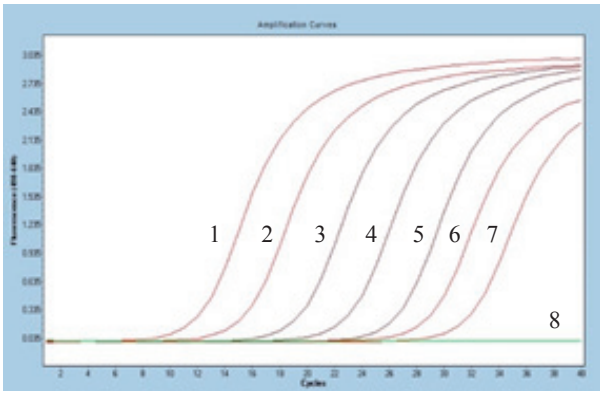

Fig. 7. PCV3 sensitivity test
1~8: 9.30×10^6、1.18×10^6、1.00×10^5、1.01×10^4、9.98×10^2、1.87×10^2、2.24×10^1copies/μl、negative control

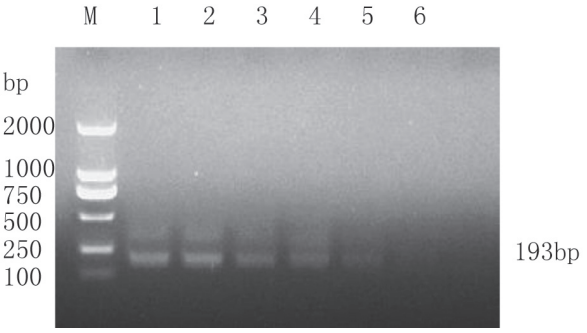

Fig. 10. PCV3 sensitivity test
M: maker 2000; 1~6: 9.30×10^6、1.18×10^6、1.00×10^5、1.01×10^4、9.98×10^2 copies/μl、negative control

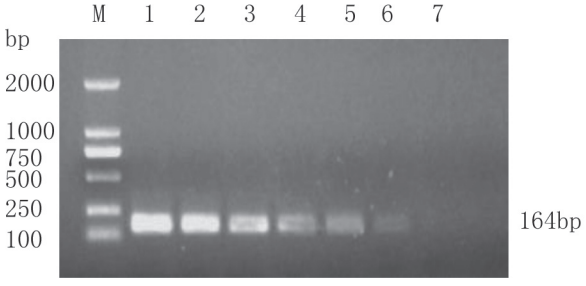

Fig. 8. PCV1 sensitivity test
M: maker 2000; 1~7: 1.26×10^8、1.00×10^7、1.05×10^6、7.02×10^4、8.58×10^3、1.11×10^3copies/μl、negative control

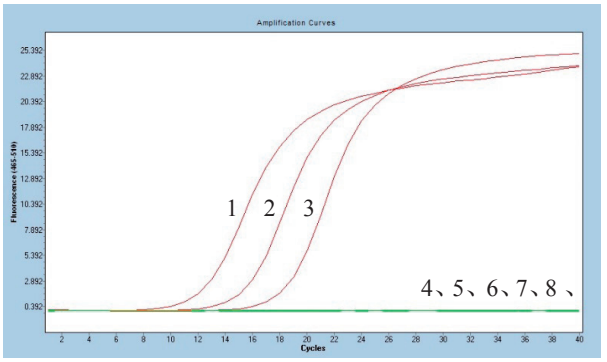

Fig. 11. PCV1 PCV2 and PCV3 specificity tests
1.PCV1sample、2.PCV2sample、3.PCV3sample、4.CSFV、5.PRRSV、6.PPV、7.PRV、8.negative control

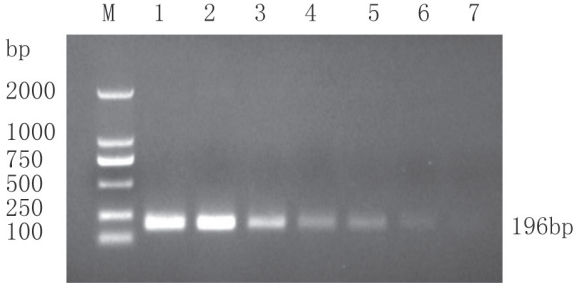

Fig. 9. PCV2 sensitivity test
M: maker 2000; 1~7: $9.84\times10^7$1.50×10^7、1.78×10^6、1.02×10^5、9.75×10^3、7.75×10^2copies/μl、negative control

Repeatability test. PCV1, PCV2, and PCV3 standards with copy numbers of 10^5, 10^4 and 10^3 copies/μl were repeated in 3 intra-groups and 3 inter-groups. The average value, standard deviation and coefficient of variation of the obtained Ct were analyzed (Table 2, Table 3, Table 4, Table 5, Table 6, and Table 7). The results showed that the coefficients of variation of the amplification standards of the three dilutions were all less than 1.00%, and the established quantitative PCR detection method had high reproducibility.

Table 2. Intra-group statistics of PCV1 detected by fluorescence quantitative PCR

Standard template (copies / μl)	Ct value of 3 replicates in the same batch			Mean ± standard deviation (X ± S)	Coefficient of variation CV (%)
	1	2	3		
1×10^5	20.89	20.71	20.78	20.79±0.07	0.33
1×10^4	23.58	23.65	23.63	23.62±0.03	0.13
1×10^3	26.87	26.91	26.93	26.90±0.02	0.07

Table 3. Inter-group statistics of PCV1 detected by fluorescence quantitative PCR

Standard template (copies / μl)	Ct value of three batches between groups			Mean ± standard deviation (X ± S)	Coefficient of variation CV (%)
	1	2	3		
1×10^5	20.71	20.50	20.89	20.70±0.16	0.77
1×10^4	23.65	23.75	24.07	23.82±0.18	0.75
1×10^3	26.93	27.34	27.00	27.09±0.18	0.66

Table 4. Intra-group statistics of PCV2 detected by fluorescence quantitative PCR

Standard template (copies / μl)	Ct value of 3 replicates in the same batch			Mean ± standard deviation (X ± S)	Coefficient of variation CV (%)
	1	2	3		
1×10^5	20.02	20.11	20.06	20.06±0.03	0.15
1×10^4	23.60	23.46	23.52	23.52±0.06	0.25
1×10^3	26.32	26.44	26.25	26.33±0.08	0.30

Table 5. Inter-group statistics of PCV2 detection by fluorescence quantitative PCR

Standard template (copies / μl)	Ct value of three batches between groups			Mean ± standard deviation (X ± S)	Coefficient of variation CV (%)
	1	2	3		
1×10^5	20.06	19.90	20.19	20.05±0.11	0.55
1×10^4	23.52	23.66	23.46	23.54±0.05	0.08
1×10^3	26.25	26.23	26.42	26.30±0.12	0.09

Table 6. Intra-group statistics of PCV3 detected by fluorescence quantitative PCR

Standard template (copies / μl)	Ct value of 3 replicates in the same batch			Mean ± standard deviation (X ± S)	Coefficient of variation CV (%)
	1	2	3		
1×10^5	17.60	17.79	17.58	17.65±0.09	0.51
1×10^4	20.71	20.84	20.61	20.72±0.09	0.43
1×10^3	23.83	23.93	23.89	23.88±0.04	0.17

Clinical sample detection test. A total 100 clinical samples of suspected PCV were tested by SYBR Green I fluorescence quantitative PCR. The results were as follows: The PCV1-positive, PCV2-positive and PCV3-positive rate at the farm level was 10% (10/100), 64% (64/100), and 52% (52/100), respectively. The positive rates of PCV1, PCV2 and PCV3 were 7% (7/100), 58% (58/100), and 43% (43/100), respectively (Table 8). The clinical examination results show that the quantitative PCR is more sensitive than conventional PCR.

Additionally, the PCV1 and PCV2 co-infection rate was 8% (8/100), the PCV1 and PCV3 co-infection rate was 7% (7/100), the PCV2 and PCV3 coinfection rate was 26% (26/100), and the PCV1, PCV2 and PCV3 co-infection rate was 7% (7/100), respectively, in the samples from ten pig farms (Table 9).

Table 7. Inter-group statistics of PCV3 detection by fluorescence quantitative PCR

Standard template (copies / μl)	Ct value of three batches between groups			Mean ± standard deviation (X ± S)	Coefficient of variation CV (%)
	1	2	3		
1×10^5	17.79	17.69	17.72	17.73±0.04	0.23
1×10^4	20.84	20.78	20.96	20.86±0.07	0.34
1×10^3	23.83	23.81	23.73	23.79±0.04	0.17

Table 8. Statistics of clinical detection results of real-time PCR

Types	PCV1+	PCV2+	PCV3+
Real-time PCR	10/100(10%)	64/100(64%)	52/100(52%)
conventional PCR	7/100(7%)	58/100(58%)	43/100(43%)

Table 9. Detection of the co-infection of clinical specimens by real-time PCR

Types	PCV1+PCV2+	PCV1+PCV3+	PCV2+PCV3+	PCV1+PCV2+PCV3+
Real-time PCR	8/100(8%)	7/100(7%)	26/100(26%)	7/100 (7%)
conventional PCR	6(6%)	5(5%)	13(5%)	5/100(5%)

Discussion

In order to meet the requirements for the rapid detection of PCV1, PCV2 and PCV3 in veterinary clinics, this test was the first in China to establish a SYBR Green I fluorescent quantitative PCR detection method for PCV1, PCV2 and PCV3. SYBR Green I is a fluorescent dye that binds to double-stranded DNA. It can be combined with double-stranded PCR products to release a fluorescent signal during the real-time PCR reaction, which can be monitored by the instrument in real time. Compared with ordinary PCR, this method has high sensitivity, specificity, reproducibility, high stability, and a small coefficient of variation. The porcine circovirus 2 SYBR Green I-based quantitative PCR detection method established is better than the conventional PCR (YANG et al. 2007). The SYBR Green I fluorescent quantitative PCR detection method established in this experiment is 100 times more sensitive than conventional PCR; the minimum copy number of PCV3 SYBR Green I -based real-time quantitative PCR method is 1.73×10^2 copies / μl (CHEN et al. 2021). The minimum copy numbers of the PCV2 and PCV3 Taq Man real-time PCR method are 2.9 copies/ μl for PCV2 plasmid and 22.5 copies / μl for PCV3, respectively (LI et al., 2018). The minimum copy numbers of the PCV1 and PCV2 fluorescent quantitative detection methods established in our laboratory are 10 copies / μ for PCV1and 100 copies / μl for PCV 2 (LI et al., 2013). The minimum copy number of the SYBR Green I fluorescent quantitative PCR detection method established in this experiment is 22.4 copies / μl. Compared with probe-based fluorescent quantitative PCR, for SYBR Green I it is not necessary to design probes, only primers are used, which simplifies analysis and design, and saves costs. This research method requires a short time, it only takes about 2h from sample collection to result evaluation, and this method implements a completely closed-tube operation, which fundamentally eliminates the problems of contamination and false positives of amplification products in conventional PCR methods. The use of a common upstream primer for PCV1, PCV2 and PCV3 makes the experimental operation more simple and fast. In recent years, the incidence of circovirus in China has been on the rise, and infections have occurred in most areas, which has caused large economic losses to the pig industry. Clinical test results show that mixed infections exist among different serotypes of porcine circovirus. This problem should therefore be addressed.

Conclusions

In conclusion, the SYBR Green I real-time quantitative PCR method established in this study has high sensitivity, specificity and reproducibility, and can be used for epidemiological investigation of PCV1, PCV2 and PCV3 infections, rapid detection of clinical samples, early detection, quantitative detection and later research.

Conflict of Interest

The authors declare that there is no conflict of interest.

Author's contribution

Chang Liu, Yao Tian and Li-mei Zheng contributed equally to this work

Acknowledgments

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SAŽETAK

Među cirkovirusima svinja razlikujemo cirkovirus svinja tipa 1 (PCV1), cirkovirus svinja tipa 2 (PCV2) i cirkovirus svinja tipa 3 (PCV3). Posljednjih se godina pojavljuje koinfekcija ovim trima serotipovima, stoga je potrebno uspostaviti brzu, specifičnu i osjetljivu metodu kako bi se kvantitativnim PCR testom temeljenom na SYBR Green I mogli identificirati PCV1, PCV2 i PCV3. U ovom su istraživanju upotrijebljene specifične početnice te su optimizirani uvjeti rekacije za uspostavljanje kvantitativnog PCR-a u stvarnom vremenu. Rezultati su pokazali da su granice detekcije ovog testa 40,3 kopije/μL za PCV1, 25,2 kopije/μL za PCV2 i 22,4 kopije/μL za PCV3. Nije bilo križne reaktivnosti s virusom svinjske kuge (CSFV), virusom reproduktivnog i respiratornog sindroma svinja (PRRSV), virusom pseudobjesnoće svinja (PRV) i parvovirusom svinja (PPV). Koeficijenti varijacije unutar testa i među testovima bili su manji od 1 %. Rezultati analize 100 uzoraka sa sumnjom na PCV pokazali su da je stopa infekcije serotipom PCV1 bila 10% (10/100), PCV2 64% (64/100), a PCV3 52% (52/100). Stopa koinfekcije serotipovima PCV1 i PCV2 bila je 8% (8/100), PCV1 i PCV3 7% (7/100), a PCV2 i PCV3 26% (26/100). Koinfekcija svim trima serotipovima, PCV1, PCV2 i PCV3, bila je 7% (7/100). Metoda primjenjena u ovom istraživanju ima dobru specifičnost, osjetljivost i postojanost te je obećavajući alat za brzo otkrivanje serotipova PCV1, PCV2 i PCV3.

Ključne riječi: cirkovirus svinja; specifičnost; osjetljivost; kvantitativni PCR test temeljen na SYBR Green I; metoda jednostruke identifikacije