Establishment of a Dual SYBR Green I Fluorescence PCR Assay for African Swine Fever Virus and Porcine Epidemic Diarrhea Virus

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Abstract: The present study envisaged the development of a fluorescence PCR test for the simultaneous detection of the African swine fever virus (ASFV) and the porcine epidemic diarrhea virus (PEDV) by designing specific primers based on the sequences of the p72 gene of ASFV and the N gene of PEDV in GenBank. Subsequently, the sample loading system and the PCR program was optimized to establish a dual SYBR Green I fluorescence PCR assay for ASFV and PEDV. Furthermore, the specificity, sensitivity and repeatability of the established assay were evaluated. Finally, the established PCR assay was tested using clinical samples. The results demonstrated that the optimal loading amount of each primer in a 20 μL reaction system was: F1 0.5 μL, R1 0.8 μL, F2 1.5 μL and R2 1.2 μL; the optimal PCR program was: reverse transcription at 42 ℃ for 5 min; pre-denaturation at 95°C for 2 min; and 40 cycles of (denaturation at 94°C for 5 s followed by annealing at 53°C for 25 s, where the fluorescence was collected). The established assay exhibited a good specificity and did not cross-react with other common swine viruses. The sensitivity of detecting the ASFV and PEDV was 8.8 copies/μL and 3.7 copies/μL, respectively, and the within-run and between-run coefficients of variation of T_m values were not more than 1.0%. The test results of 162 clinical samples using the established PCR assay were consistent with the reference methods. The dual SYBR Green I fluorescence PCR assay established in this study for detecting ASFV and PEDV showed high sensitivity and good specificity, and it can be used for the rapid detection of these two clinical diseases.

1 INTRODUCTION

African swine fever virus (ASFV) can cause African swine fever (ASF), a severe and highly contagious infectious disease in swine (Yu and Li, 2018). The clinical signs include high fever, vomiting, petechiation of the skin, and bloody diarrhea in pigs (Jin et al., 2020). Extensive hemorrhage of the various internal organs was observed in the swine autopsy (Zhang et al., 2019). ASFV was first detected in a pig farm in Shenyang, China in August 2018 (Chen et al., 2018), and it has been prevalent in pig farms in China for more than 3 years till now. As the clinical variants emerged, the virulence of the virus diminished, and the pig farm epidemic eased. The mortality rate of the infected swine was low in some pig farms (Zhang et al., 2021). Porcine epidemic diarrhea virus 2 (PEDV) is a member of the genus *Alphacoronavirus* in the family *Coronaviridae* (Dong et al., 2021). It can cause porcine epidemic diarrhea (PED) in swine and is widespread in pig herds worldwide. The clinical signs include vomiting, watery diarrhea, and dehydration. The disease has an acute onset, rapid spread, and a high mortality rate. It can occur throughout the year, but occurs more often in the winter and spring (Geng et al., 2021).

Although there are kits for detection of single pathogen, the operations are cumbersome, and the cost is high. There is an urgent need to develop a rapid on-site screening kit for simultaneous detection of both ASFV and PEDV. Fluorescence polymerase chain reaction (PCR) has been widely used as a fast, sensitive and cheap detection method. Compared to traditional PCR, it has a higher sensitivity and does not require detection of PCR products by electrophoresis, reducing the risk of aerosol

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contamination in the laboratory. To date, there have been no reports on the dual SYBR Green I fluorescence PCR method for the simultaneous detection of ASFV and PEDV. In this pursuit, the present study aimed to establish a dual SYBR Green I fluorescence PCR assay to improve the detection efficiency of ASFV and PEDV.

2 MATERIALS AND METHODS

2.1 Plasmids, Strains, and Field Samples

The plasmids pMD-p72 and pMD-N containing the ASFV and PEDV-specific gene fragments, respectively, were synthesized by General Biosystems (Anhui) Co., Ltd. The Classical swine fever virus (CSFV), Porcine reproductive and respiratory syndrome virus (PRRSV), Porcine circovirus type 2 (PCV2), Porcine transmissible gastroenteritis virus (TGEV), Porcine rotavirus (RV), Porcine pseudorabies virus (PRV) and Porcine deltacoronavirus (PDCoV) were employed from our laboratory in the study. A total of 162 pig nasal swab samples were collected from the pig farms in northern Shandong from January 2021 to December 2021.

2.2 Primers, Reagents, and Recombinant Standard Plasmid Construction

The sequences of the ASFV p72 gene and PEDV nucleocapsid (N) gene were downloaded from the GenBank. Specific primers were designed based on the conserved parts, and further synthesized by General Biosystems (Anhui) Co., Ltd. The primers are shown in Table 1. The One-Step TB Green PrimeScript RT-PCR Kit (Cat. No: RR096A) was purchased from Takara Bio Inc. (Dalian). The plasmid miniprep kit was procured from BioTeke Corporation Co. Ltd. The MyGo Pro quantitative fluorescence PCR instrument was procured from Qingdao Buffett Biological Company. The full length of p72 gene (1941 base pairs) of ASFV (Accession no. MK554698.1) and N gene (1326 base pairs) of PEDV (Accession no. MW122505.1) were synthetized and cloned into the pMD18 vector by Sangon (Shanghai, China), respectively. The concentration of these recombinant standard plasmids was determined by NanoDrop One (ThermoFisher Scientific). The copy numbers of pMD-p72 and pMD-N plasmids were 2.9×10 9 copies/μL and 1.5×10 9 copies/μL, respectively. All these plasmids were stored in −20°C before use.

Table 1: Primers used in the duplex SYBR Green I Fluorescence PCR assay.

Virus	Gene	Name	Primer Sequence (5'→3')	Position	GenBank No.
ASFV	p72	F1	CATGGGCAGCTTCAAACGT	391-409	MK554698.1
		R1	CAATGGGTCTTCCAAAAG	479-496	
PEDV	N	F2	TAAGGACCAGCAAATTGGA	135-153	MW122505.1
		R2	GTTGTTGCCATTACCACGA	453-471	

2.3 Viral Nucleic Acid Extraction

Viral nucleic acid was extracted from each sample using Simply P Virus RNA/DNA Extraction Kit (Cat.No:BSC67M1; Hangzhou Bori Technology Co., Ltd) according to the manufacturer's instructions. The viral DNA/cDNA were stored at − 20 °C for further study.

2.4 Establishment and Optimization of Dual Fluorescence PCR Assay

The lyophilized primers F1 and R1 of ASFV, and F2 and R2 of PEDV, respectively, were prepared to reach a concentration of 15 μmol/L using ultrapure sterile water. The reaction system was prepared according to the instructions of the One-Step TB Green PrimeScript RT-PCR Kit. A total volume of 20 μL was employed for the analysis. This included 10 μL of 2× One-Step TB Green RT-PCR Buffer III, 0.5 μL of PrimeScript RT Enzyme Mix II, and 0.5 μL of *Ex Taq* HS, 1 μL of plasmid template pMD-p72, 1 μL of plasmid template pMD-N, adjusted amounts of primers for optimization, and adjusted amount of ultrapure sterile water to make a final volume of 20 μL. The negative control was set up using ultrapure water to replace the template. After thorough mixing, the reaction systems were placed in the MyGo Pro quantitative fluorescence PCR instrument. The PCR program was set and conducted using various annealing temperatures such as 51°C, 52°C, 53°C, 54°C, 55°C, 56°C, 57°C, and 58°C.

2.5 Evaluation of Specificity, Specificity and Repeatability

To evaluate the specificity of the primer and probe sets, the synthesized plasmids pMD-p72 and pMD-N were diluted 10-fold serially, added to the fluorescence PCR reaction system as templates, and amplified using the established dual fluorescence PCR method. For the sensitivity evaluation, the nucleic acids of CSFV, PPRSV, PPrV, PCV2, TGEV, PRV, and PDCoV were used as templates to test the specificity of the established dual fluorescence PCR assay.

For the evaluation of the repeatability of this method, the plasmids pMD-p72 and pMD-N samples of three selected dilution gradients from section 1.6 were used as templates, with three replicates for each dilution, to perform dual fluorescence PCR. The T_m values of the melting curves were analyzed to calculate the within-run and between-run coefficients of variation (CV).

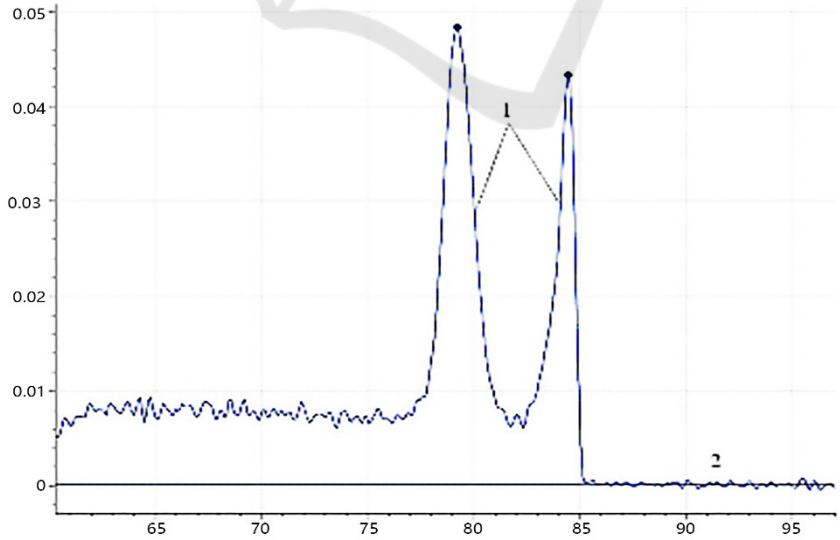
2.6 Detection of Clinical Samples

The test samples were collected from various pig farms in northern Shandong from January 2021 to December 2021, including healthy hogs and diseased hogs. In the operation of pig nasal swab collection, a sterile cotton swab was inserted into the pig's nasal cavity and rotated. Then, the swab was withdrawn from the nostril and placed in a sterilized centrifuge tube containing 2 mL of normal saline. The tube was securely closed, put in an ice incubator, and transported to the laboratory within 24 hours. The samples were tested using the established ASFV and PEDV dual SYBR Green I fluorescence PCR. At the same time, they were tested using the ASFV fluorescence PCR assay following the national standard GB/T 18648-2020 and the PEDV fluorescence PCR assay following the local standard DB33/T 2254-2020. The test results from our established PCR were compared to those obtained from the standard PCR.

3 RESULTS

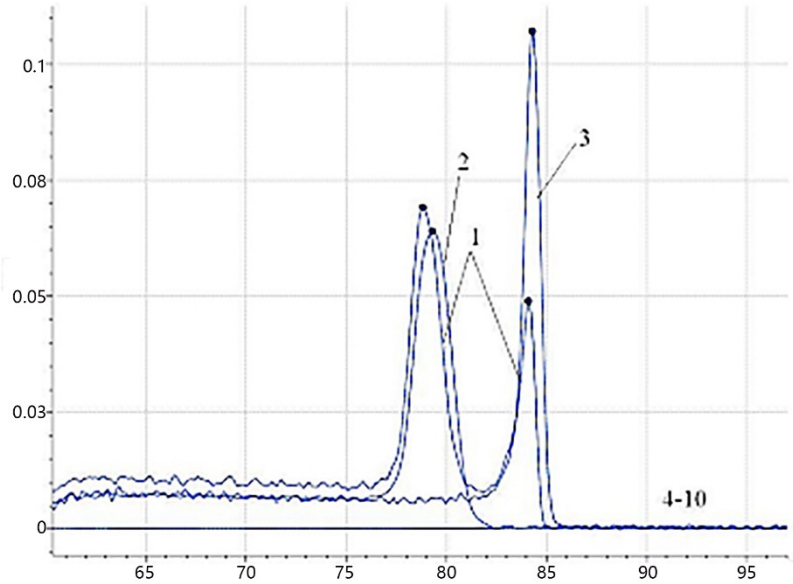
3.1 Establishment and Optimization of the Dual Fluorescence PCR Assay

The established optimal loading amounts of primers were: F1 0.5 μL, R1 0.8 μL, F2 1.5 μL and R2 1.2 μL in the 20 μL reaction system. The optimal annealing temperature was 53 °C. The optimal established fluorescence PCR reaction program was found to be: reverse transcription at 42 °C for 5 min, pre-denaturation at 95 °C for 2 min; a total of 40 cycles of denaturation at 94 °C for 5 s and annealing at 53 °C for 25 s (where the fluorescence was collected). After the amplification, the melting curves were drawn and analyzed, with the increase in temperature to 97 °C at a rate of 0.1 °C/s and the fluorescence was measured (Figure 1).



Notes: 1. Plasmid standard template, 2. Negative control.

Figure 1: Melting curves of the dual fluorescent PCR.



Notes: 1. Plasmid pMD-p72/ORF1, 2. Plasmid pMD-p72, 3. Plasmid pMD-ORF1, 4~10. SFV, PPRSV, PPrV, PCV2, PTGEV, PRV, and PDCoV.

Figure 2: Amplification specificity of the dual fluorescent PCR assay.

3.2 Evaluation of Sensitivity

The concentration of the plasmid pMD-p72 was determined to be 8.8×10^9 copies/μL. The fluorescence PCR using 10-fold gradient diluted plasmid showed that the detection limit of plasmid pMD-p72 was 8.8 copies/μL. The concentration of plasmid pMD-N was determined to be 3.7×10^9 copies/μL, and the fluorescence PCR using 10-fold gradient diluted plasmid showed that the detection limit of plasmid pMD-N was 3.7 copies/μL.

3.3 Evaluation of Specificity

The established dual SYBR Green I fluorescence PCR was subjected to the specificity test. The results showed that the plasmid pMD-p72 had a specific peak with a T_m value of (79.21 ± 0.5) ℃, the plasmid pMD-N had a specific peak with a T_m value of (84.11 ± 0.5) ℃, the plasmid pMD-p72/N had double peaks with T_m values of (79.21 ± 0.5) ℃ and (84.11 ± 0.5) ℃. While the other tested pathogens, SFV, PPRSV, PPrV, PCV2, PTGEV, PRV, and PDCoV, did not demonstrate specific T_m values at (79.21 ± 0.5) ℃ or (84.11 ± 0.5) ℃ (Figure 2). The results indicated that the established method offered a good specificity and no cross-amplification reactions with other pathogenic genes.

3.4 Evaluation of Repeatability

Three different concentrations of 8.8×10^5, 8.8×10^3, and 8.8×10^1 copies/μL of the plasmid pMD-p72 were selected from the serial dilution, with three repeats at each concentration, were subjected to the dual fluorescence PCR. Similarly, three concentrations of 3.7×10^5, 3.7×10^3, and 3.7×10^1 copies/μL of plasmid pMD-N were selected with three repeats for each concentration, were subjected to the dual fluorescence PCR. The T_m values of the tested samples were recorded, and the within-run and between-run coefficients of variation were calculated, respectively. The results showed that the within-run and between-run coefficients of variation of the two plasmids were not more than 1%, indicating a good repeatability of the proposed assay (Table 2).

3.5 Diagnostic Performance

The results of the ASFV and PEDV dual SYBR Green I fluorescence PCR on162 pig nasal swab samples revealed 0 ASFV positive and 18 PEDV positive, with no cases of dual infections. Single fluorescence PCR for the ASFV and PEDV following related standards, demonstrated consistent results with the developed dual SYBR Green I fluorescence PCR assay.

Table 2: Within-run and between-run repeatability test results of dual fluorescence PCR.

Virus	Plasmid Concentration	N	Within-run CV		Between-run CV	
			Mean ± standard deviation	CV/%	Mean ± standard deviation	CV/%
ASFV	8.8×10^4	3	79.150±0.254	0.321	78.880±0.165	0.209
	8.8×10^3	3	79.520±0.148	0.186	79.220±0.425	0.536
	8.8×10^2	3	78.920±0.632	0.801	79.320±0.612	0.772
PEDV	3.7×10^4	3	84.110±0.280	0.333	83.960±0.341	0.406
	3.7×10^3	3	84.220±0.172	0.204	84.130±0.655	0.779
	3.7×10^2	3	83.870±0.625	0.745	84.350±0.351	0.416

4 DISCUSSION

China has a huge market for the pork production and consumption, and the outbreak of ASF and PED have dealt a severe blow to the swine industry in China. The accurate and rapid diagnosis of ASF and PED is an important step in the control and prevention of these diseases. The fluorescence PCR method has the advantages of being rapid, high sensitivity and ease of operation, and it has been widely applied in the detection of various pathogens. Fluorescence PCR methods can be divided into two classes: dye-based and probe-based. Compared with the probe-based fluorescence PCR, the dye-based assays do not require the synthesis of expensive probes, thereby reducing the cost in research and development. However, the dye-based assays have a higher requirement for primers, especially for dual PCR assay, which is the key in the assay establishment. Another difference from the probe-based method is, the dye-based fluorescence PCR has an extra step of melting curve analysis after amplification. With the increase in the temperature, the melting temperature at which half of the amplified products dissociate is the T_m peak. Each peak represents a specific product. By analyzing the difference in T_m peaks, multi-pathogen detection can be realized. Additionally, it has no special requirements for fluorescent PCR instruments, and a good versatility. At present, there is no dual SYBR Green I fluorescence PCR assay available for detecting ASFV and PEDV.

The ASFV genome consists of a double-stranded DNA sized 170-190 kb. The p72 gene is highly conserved, and often serves as a target gene for the detection of ASFV (Jia et al., 2020). The nucleocapsid (N) gene encodes the PEDV nucleocapsid protein, and it is highly conserved and often used as a target gene for the detection of PEDV (Pan et al., 2019). The dual SYBR Green I fluorescence PCR assay proposed in this study for the simultaneous detection of ASFV and PEDV can screen the two pathogens rapidly in just 50 minutes. The key to the successful establishment of the dual fluorescence PCR assay is the design of primers. In this study, four primers were found after the screening of a large number of primers. They exhibited close annealing temperatures, but did not interfere with each other. We further optimized the loading amount of each primer in the reaction system.

The proposed method can reduce costs and simplify the tedious sample loading steps. The specific T_m peaks of ASFV and PEDV were found to be at (79.21 ± 0.5) ℃ and (84.11 ± 0.5) ℃, respectively. Our assay exhibited a good specificity and did not cross-react with common porcine pathogens. The limits for detecting ASFV and PEDV were found to be 8.8 copies/μL and 3.7 copies/μL, respectively. Tests on different concentrations of ASFV and PEDV plasmid samples exhibited good repeatability of the established assay. Neither of the within-run CV nor between-run CV of T_m of the two plasmids was greater than 1%. Furthermore, among the clinically obtained 162 pig nasal swabs tested with the dual fluorescence PCR assay, 0 positive ASFV cases and 18 positive PEDV cases were found, and no dual infection cases were found. The 162 samples were subjected to single-plex ASFV and PEDV PCR assays following related standard and the results were consistent with the dual SYBR Green I fluorescence PCR assay. In this study, the T_m values of the two viruses were obtained using the existing kits and instruments, but the difference between the two T_m values remained unchanged. Although the T_m values of the two target products fluctuated, the amplitudes were not more than 1 °C. In conclusion, a

dual SYBR Green I fluorescence PCR assay was established in the present study, which was found to be time-saving with high sensitivity, high efficiency, and good repeat-ability. The results indicate that the proposed assay has an excellent potential to become a useful laboratory diagnostic tool in the detection of ASFV and PEDV in clinical samples.

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