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Establishment and application of a multiplex PCR method for the simultaneous detection of PRV, PCV2, PRRSV, CSFV, and SIV associated with porcine respiratory disease

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ABSTRACT: Swine respiratory tract disease is a significant issue in pig farming, causing substantial harm and loss. The main pathogens involved in porcine viral respiratory diseases are porcine pseudorabies virus (PRV), porcine circovirus type 2 (PCV2), porcine respiratory and reproductive syndrome virus (PRRSV), swine fever virus (CSFV), and swine influenza virus (SIV). These pathogens often co-infect pigs, leading to increased morbidity and mortality, complicating disease diagnosis and control. This study focused on designing specific primers for the gene sequences of PRV gB, PCV2 ORF2, PRRSV ORF6, CSFV C, and SIV M genes to enable rapid diagnosis of multi-pathogen infections. The optimized single and multiplex polymerase chain reaction (PCR) systems demonstrated high specificity, sensitivity, and repeatability. A single PCR/RT-PCR method was successfully established for the rapid diagnosis of all tested pathogens, laying the groundwork for a five-plex PCR method. The established five-plex PCR showed a minimum detection limit at the picogram level with optimal annealing temperature at 56.5 °C. Specificity tests confirmed detection of only the target bands with no amplification of other viruses. Clinical sample analysis demonstrated quick and accurate detection using this method.

KEYWORDS: PRV, PCV2, PRRSV, CSFV, SIV, multiplex PCR

INTRODUCTION

With the industrialization and rapid development of intensification of swine industry in China, the mixed virus infection and secondary infection of pig population become more and more common [1]. Porcine pseudorabies virus (PRV), porcine circovirus type 2 (PCV2), porcine respiratory and reproductive syndrome virus (PRRSV), swine fever virus (CSFV), and swine influenza virus (SIV) also cause different degrees of damage to the body immune system, reducing the body resistance to environmental challenges and causing secondary mixed infection that leads to disease complications and increased pathology [2], and these viruses cause porcine respiratory diseases characterized by fever, cough, asthma, difficulty in breathing, increased nasal discharge, loss of appetite, and rapid weight loss [3]. Some also cause pregnant sows to abort, stillbirth, mummified fetuses, nondynamic weak progeny, deformed fetuses, piglet and infertile pigs, and breeding disorders [4]. At present, there is no good treatment for PRV, PCV2, PRRSV, CSFV and SIV. Prevention is the main method, including keeping indoor ventilation, keeping the environment clean and tidy, and giving pigs with good nutritional ingredients. It can reduce the risk of PRV, PCV2, PRRSV, CSFV and SIV in pigs [5].

Since the birth of polymerase chain reaction (PCR) technology in 1985, gene diagnosis technology has entered a new stage of unprecedented development [6]. It is widely used in molecular biology and related fields and has also become the most valuable tool in the detection and diagnosis of animal diseases [7]. With the continuous development of this technology, the use of multiplex PCR (mPCR) to detect pathogens in different animals is becoming more common. The mPCR has the advantages of strong specificity, high sensitivity, short detection time, and the cost of instruments and reagents is low, and it can detect multiplex pathogens simultaneously, which is useful for the rapid, accurate diagnosis of diseases and the rapid detection of pathogens in a large number of clinical diseases [8, 9]. Zeng et al [10] established a duplex PCR method for the simultaneous detection of PRV and PCV2. Chen et al [11] established a six-plex PCR method for simultaneously detecting the viral etiological agents of porcine reproductive disorders, PCV2, PRV, porcine parvovirus (PPV), CSFV, PRRSV, and japanese encephalitis vaccine (JEV). In this study, we designed a pair of specific primers for the conserved gene sequences of these 5 viruses, using the positive genomic nucleic acid of each virus as a template and established an mPCR method that can simultaneously identify PRV, PCV2, PRRSV, CSFV and SIV infections in a reaction system by optimizing their conditions to provide technical help for rapid, accurate detection of clinical disease materials, epidemiological investigation, prevention and purification of disease [12].

MATERIALS AND METHODS

Viruses and strains

PRRSV, PCV2, CSFV, and PRV were obtained from provincial institute of animal disease isolation, identification, and preservation. SIV was obtained from a clinically diseased animal from the Guizhou Provincial Institute of Animal Disease Isolation, Identification, and Preservation.

Reagents and instruments

The TaKaRa MiniBEST Universal Genomic DNA Extraction Kit Ver.5.0, TaKaRa MiniBEST Viral RNA/DNA Extraction Kit Ver.4.0, DL2000 DNA Marker, and pMD19-T Vector were purchased from TaKaRa Company (Dalian, China). The Gel Extraction Kit and E.z.N.a.MIT Plasmid Mini Kit are products of OMEGA Company (Madison, USA). IPTG and X-gal were purchased from Sigma Company (Livonia, Michigan, USA). GoldView is a product of Hyclone Company (Logan, USA). Agarose was purchased from Biowest Company (Cholet, France). The Gradient PCR amplification instrument, Thermo Forma −80 °C cryogenic refrigerator, and Gel DOC XR Gel Imaging system are from BIO-RAD Company (California, USA).

Primer and probe design

The steps to perform mPCR reactions and obtain primers are as follows: referring to NCBI PRV gB (AF257079), PCV2 ORF2 (NC_005148), PRRSV ORF6 (NC_001961), CSFV C (AY805221), PPV NS1 (NC_001718), SIV M (GU086140) and other gene sequences. Canadian Primer design software "Primer 5.0" was used to design specific primers for the target genes of the above viruses. The primer sequences are shown in Table S1.

Viral nucleic acid extraction

Viral DNA Extraction Kit was extracted according to the TaKaRa MiniBEST Universal Genomic DNA Extraction Kit Ver.5.0. Viral RNA Extraction was performed according to the TaKaRa MiniBEST Viral RNA/DNA Extraction Kit Ver.4.0 extraction kit instructions.

PCR and reverse transcription (RT)-PCR amplification of individual DNA and RNA viruses

Simplex PCRs or RT-PCRs were established for PRRSV, SIV, PCV2, CSFV, and PRV.

RT-PCR reaction of single RNA virus: the reaction volume was 25 μl; 12.5 μl of 2 × 1 step Buffer, 1 μl of upstream and downstream primers, 1 μl of template, 1 μl of PrimeScript One Step Enzyme Mix, and 8.5 μl of RNase free H_2O. Reaction procedure: 40 min at 50 °C; 95 °C for 5 min; 35 cycles of 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 45 s; and 72 °C for 10 min.

Simplex DNA virus PCR reaction system: the reaction volume was 50 μl; specifically: 5 μl of 10 × *Taq* PCR buffer, 1 μl of 10 mmol/l dNTP, 1 μl of 10 μmol/l upstream and downstream primers, 1 μl of template, 1 μl of *rTaq* polymerase, and sterile ultrapure water added to make 50 μl.

Reaction procedure: predenaturation at 95 °C for 5 min; 35 cycles of 95 °C 30 s, 56 °C 30 s, and 72 °C 30 s; and 72 °C for 10 min.

After the reaction, 5 μl of PCR/RT-PCR products were respectively taken for electrophoresis, and the results were observed and analyzed.

Identification of the target genes of PRV, PCV 2, PRRSV, CSFV, and SIV

All the remaining PCR/RT-PCR products with target bands in the results of PRV, PCV2, PRRSV, CSFV and SIV electrophoresis were electrophoresed, and the target DNA fragments were recovered and purified by Gel Extraction Kit. The PCR products of the 5 viruses were ligated into the pMD19-T vector at 16 °C overnight. TOP10 competent cells were transformed with the ligated products, and the cells were screened with ampicillin (Amp), isopropyl β-D-1-thiogalactopyranoside (IPTG), and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). White colonies were expanded in culture, and positive bacterial samples were sent to Shanghai Ying Chun Biotechnology Co., Ltd. (China) for sequencing.

Establishment of the best reaction conditions for single PCRs/RT-PCRs

Optimization of single PCRs/RT-PCRs

Based on the amplification results for the PRRSV, SIV, PCV2, CSFV, and PRV genes, 5 viruses were detected by upstream and downstream primers with different concentrations (0.5, 1, 1.5, and 2 μM) to determine the best reaction system.

The optimum annealing temperature was determined by testing various temperatures (51, 53, 55, 56, 57, 58, and 60 °C) for the individual PCRs/RT-PCRs.

Testing the sensitivity of the single PCRs/RT-PCRs

The sensitivity of the 5 virus single PCRs/RT-PCRs was determined with the method from Shi et al [13] and Elnnifro et al [14]. The optimal concentrations of the PRV, PCV2, PRRSV, CSFV, and SIV nucleic acids were determined with UV absorbance quantification at 260 nm using Nanodrop, Qubit fluorimetry, etc., and then serially diluted with sterile distilled water (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, or 10^{-5}-fold). An aliquot (1 μl) of each dilution of the viral nucleic acid template was tested with the PCRs/RT-PCRs.

Establishment of optimal reaction conditions for multiplex PCR

Optimization of the five-plex PCR method

Based on the amplification results for the PRV, PCV2, PRRSV, CSFV, and SIV genes, various concentrations of the 5 pairs of viral upstream and downstream primers (0.5, 1, 1.5, or 2 µM) were tested individually to determine the best multiplex reaction system.

The optimum annealing temperature was determined by testing various annealing temperatures (51, 51.6, 52.7, 54.5, 56.5, 58.2, 59.3, and 60 °C) in the five-plex PCR.

Testing the specificity of the five-plex PCR method

In order to detect the specificity of five-plex PCR, nucleic acids of PRV, PCV2, PRRSV, CSFV, SIV, porcine epidemic diarrhea virus (PEDV), and PPV were extracted and detected by mPCR.

Five-plex PCR method for the sensitivity test

The sensitivity of the five-plex PCR reaction was determined with the method of Shi et al [13] and Elnnifro et al [14]. The purified PRV, PCV2, PRRSV, CSFV, and the nucleic acid of SIV virus were determined by protein-nucleic acid assay and serially diluted 10-fold with sterile distilled water (10^0, 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}). Each dilution of the viral templates was PCR amplified to detect the sensitivity of detection.

Five-plex PCR analysis of clinical samples

In total, 48 clinical samples were collected from Guizhou Province to test the established five-plex PCR method. The mixed samples from dead pigs were repeatedly freeze-thawed 3 times, then thoroughly ground with a tissue homogenizer, and centrifuged at 12,000 rpm/min for 5 min. An aliquot (200 µl) of each sample was subjected to viral DNA or RNA extraction and analyzed with the method described above.

RESULTS

Single PCR/RT-PCR amplification results

Two-hundred µl of PRV, PCV2, PRRSV, CSFV, and SIV positive disease venom were taken for nucleic acid extraction according to the instructions of the RNA/DNA extraction kit, and PCR/RT-PCR detection was performed to determine the effectiveness of primer amplification. As a result, specific bands of PRV, PCV2, PRRSV, CSFV, and SIV appeared. The cloning and sequencing results of each amplified fragment showed that the amplified fragment was the same size as the expected result and belonged to the target gene sequence. The amplification results were shown in Fig. 1. The strip sizes of PRV, PCV2, PRRSV, CSFV, and SIV are 192, 255, 364, 530, and 981 base pair (bp), respectively, which are consistent with the result in Fig. 1.

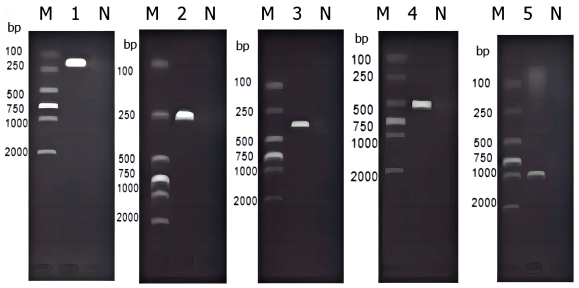

Fig. 1 Results of the PCR detection of PRV, PCV2, PRRSV, CSFV, and SIV. M: DL2000 Marker; N: negative sample; 1–5: PCR products of PRV, PCV2, PRRSV, CSFV, and SIV, respectively.

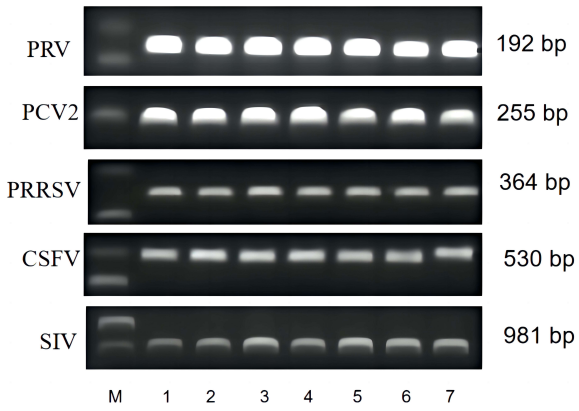

Fig. 2 Optimization of annealing temperatures for single PCR of PRV, PCV2, PRRSV, CSFV, and SIV. M: DL2000 Marker; 1: 51 °C; 2: 53 °C; 3: 55 °C; 4: 56 °C; 5: 57 °C; 6: 58 °C; and 7: 60 °C.

Identification of the PCR products

The single PCR/RT-PCR products of PRV, PCV2, PRRSV, CSFV, and SIV were purified, cloned, and sequenced. The results showed that the amplified fragments were the specific gene fragments of each virus.

Establishment of a single PCR/RT-PCR diagnostic method

Optimized conditions for single PCR/RT-PCR

By fixing the concentration of the positive sample template, the synthesized primer was diluted with sterile water to determine the optimal concentration of the primer. The results showed that for the 5 primers, the optimal concentrations of PRV and PRRSV were 1 µM each, and PCV2, CSFV, and SIV were 1.5 µM each

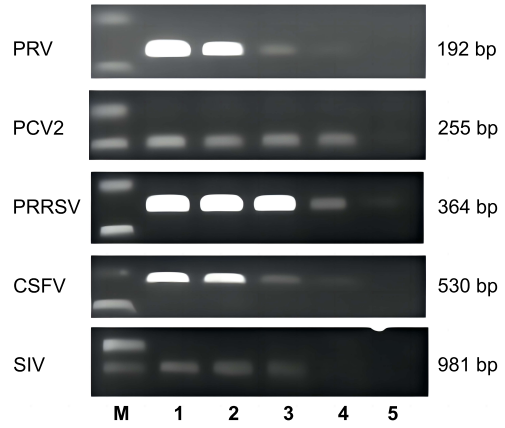

Fig. 3 Sensitivity of the single PCRs/RT-PCRs for PRV, PCV2, PRRSV, CSFV, and SIV. M: DL2000 Marker; 1–5: 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} dilution, respectively.

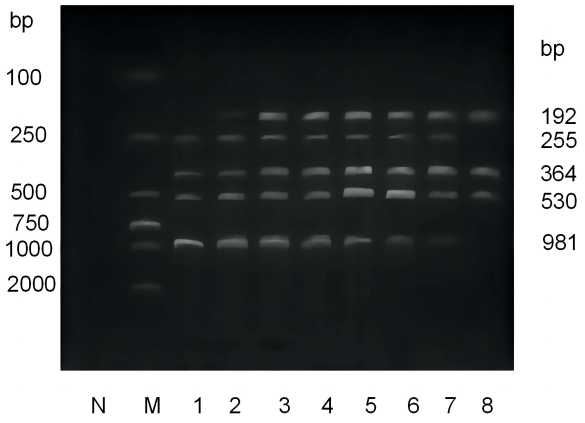

Fig. 4 Optimization of the annealing temperature for mPCR of PRV, PCV2, PRRSV, CSFV, and SIV. M: DL2000 Marker; N: negative control; 1: 51.0 °C; 2: 51.6 °C; 3: 52.7 °C; 4: 54.5 °C; 5: 56.5 °C; 6: 58.2 °C; 7: 59.3 °C; and 8: 60.0 °C.

when the upstream and downstream primers were the most effective for PCR/RT-PCR detection. The optimal reaction system for PRV and PCV2 was 50 µl in total, including 5 µl of 10 × PCR buffer, 1 µl of 50 µM dNTPs, 1 µM PRV/1.5 µM PCV2 upstream and downstream primers, 1 µl of template DNA, 2 µl of *rTaq*, and 39 µl and 38 µl of ddH_2O, respectively. The best reaction system for PRRSV, CSFV, and SIV is 25 µl in total, including 12.5 µl of 2 × 1 Step Buffer, 1 µl of PrimeScript One Step Enzyme Mix, 1 µM PRRSV/1.5 µM CSFV or SIV upstream and downstream primers, 1 µl of each template, 9 µl and 8 µl of RNase Free H_2O, respectively.

The annealing temperatures of the individual PCR were tested to determine the optimum reaction conditions. PRRSV showed best amplification at an annealing temperature of 55.0 °C; PRV, PCV2, and CSFV at 56.0 °C; and SIV at 57.0 °C. (1) The optimal conditions for PRV and PCV2 were: 95 °C for 5 min; 35 cycles of 95 °C for 30 s, 56.0 °C for 30 s, and 72 °C for 30 s; and 72 °C 10 min. (2) The optimal conditions for PRRSV, CSFV, and SIV were 50 °C 40 min; 94 °C 5 min; 35 cycles of 94 °C 30 s, 55.0 °C (PRRSV)/56.0 °C (CSFV)/57 °C (SIV) 30 s, and 72 °C 30 s; and 72 °C 10 min. The optimization of amplification is shown in Fig. 2. The PCR amplified products of PRV gB, PCV2 ORF2, PRRSV ORF6, CSFV C, and SIV M genes were 192, 255, 364, 530, and 981 bp, respectively, as expected.

Testing the sensitivity of single PCRs/RT-PCRs

The target fragment was cloned into PMD19-T vector, and the plasmid was extracted. The 5 extracted plasmids, PRV, PCV2, PRRSV, CSFV, and SIV, were determined by protein-nucleic acid assay. After that, the concentrations of the extracted plasmids were diluted 10-fold with ultrapure water under similar conditions, and then the virus template of each dilution was respectively used for PCR reaction.

In the PCRs for single viruses, the minimum amounts of each virus detected were 2.5 pg (PRV), 2.2 pg (PCV2), 3.2 pg (PRRSV), 8.3 pg (CSFV), and 3.7 pg (SIV) (Fig. 3).

Establishment of the five-plex PCR method

Optimization of the five-plex PCR method

The optimal primer concentrations were determined by varying the concentrations of the primers used to bind the sample templates. The result of the mPCR was the best when the number of reaction cycles was 35 and the annealing temperature was 56.5 °C. The optimal reaction system was a 50-µl reaction volume containing 25 µl of 2 × One Step Buffer, 2 µl of PrimeScript One Step Enzyme Mix, 0.5 µM (PRV/PCV2), 1.5 µM (PRRSV/CSFV), or 1 µM (SIV) of each forward and reverse primer, 1 µM of each template (PRV, PCV2, PRRSV, CSFV, and SIV), and 9 µl of RNase-free H2O. The optimal cycling parameters were 50 °C for 40 min; 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 56.5 °C for 30 s, and 72 °C for 30 s; and 72 °C for 10 min. The optimization of the amplification results is shown in Fig. 4.

Specificity of the five-plex PCR method

No bands were amplified from the PEDV or PPV template with the five-plex PCR, whereas when the PRV, PCV2, PRRSV, CSFV, and SIV templates were amplified, the target fragments were the sizes of the specific bands expected. The results are shown in Fig. 5.

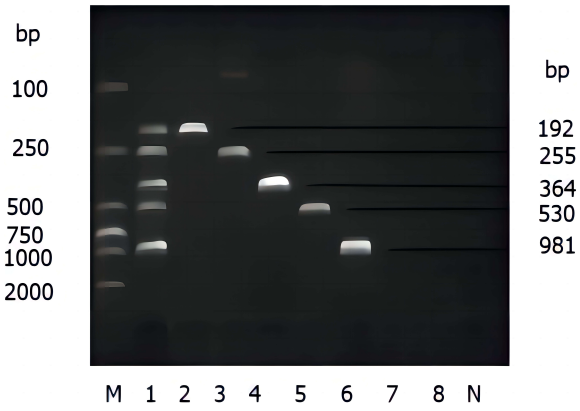

Fig. 5 Specificity test of the mPCR for PRV, PCV2, PRRSV, CSFV, and SIV. M: DL2000 Marker; 1: PCR products of PRV, PCV2, PRRSV, CSFV, and SIV; 2–8: PCR products of PRV, PCV2, PRRSV, CSFV, SIV, PPV, and PEDV, respectively; N: negative control.

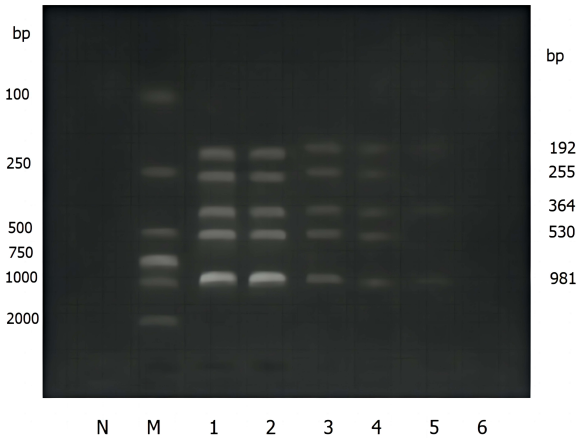

Fig. 6 Sensitivity of the mPCR for PRV, PCV2, PRRSV, CSFV, and SIV. N: negative control; M: DL2000 Marker; 1–6: 10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} dilution, respectively.

Sensitivity of the five-plex PCR method

The target fragment was cloned into the PMD19-T vector, and the plasmids were extracted and diluted with double distilled water at a multiple ratio of 10 times, respectively. The plasmids with each dilution degree were used as the template for PCR reaction, and their sensitivity was detected.

In the mPCR, the minimum amounts of the various viruses detected were 18 pg (PRV), 22 pg (PCV), 26 pg (PRRSV), 48 pg (CSFV), and 32 pg (SIV). The results are shown in Fig. 6.

Five-plex PCR method used for clinical samples

The mPCR was used to test 48 clinical samples, showing positive results. The positive rates for PRV, PCV2, PRRSV, CSFV, and SIV were 13% (6/48), 42% (20/48), 38% (18/48), 21% (10/48), and 40% (19/48), respectively. The rates of double, triple, quadruple, and quintuple infections were 31% (15/48), 17% (8/48), 6% (3/48), and 0, respectively. The correspondence between the test results and the single RT-PCR results was 100%.

DISCUSSION

In recent years, porcine viral diseases have seriously threatened the world's large-scale pig industry, and many large-scale pig farms in China have been affected by porcine viral respiratory diseases to varying degrees [15]. PRV, PCV2, PRRSV, CSFV, and SIV are major pathogenic agents of viral respiratory diseases, and the clinical symptoms and pathological changes that occur after infection with these viruses are similar. Therefore, they are difficult to distinguish intuitively [16]. These 5 viruses are also applicable in mixed infection, especially of 2–3 kinds of viruses. Consequently, the resulting animal diseases are more complex, leading to increased morbidity and mortality in pigs. These swine diseases entail huge economic losses to the pig industry [17]. A rapid, accurate diagnosis is a prerequisite for the effective prevention and treatment of these diseases [5]. Cao et al [18] have shown that SIV/PRRSV and SIV/PCV-2 double infections and SIV/PRRSV/PRV and SIV/PRRSV/CSFV triple infection are widespread. Hu et al [19] showed that there were PRRSV/CSFV and PCV2/PRV double infection, or even triple or quadruple mixed infection in some pig farms in Shanxi Province. The clinical results of this study demonstrate that there are also many mixed viral infections on pig farms in Guizhou Province, so the phenomenon of multiple-virus mixed infections is widespread on pig farms in this country [1]. The rapid, accurate diagnosis of these diseases is essential to ensure a timely and targeted response with drug treatments or immunoprophylactic measures to reduce the risk of disease in pig production, which has important practical significance [20]. Our mPCR method might be applicable for this purpose and is suitable for the analysis of large numbers of mixed infections in clinical samples.

The mPCR is a kind of amplification technology improved on the basis of conventional PCR, which is adding more than 2 pairs of primers in the same reaction system, so as to amplify multiple nucleic acid fragments, making up for the shortcomings of conventional PCR that can only use a pair of primers to amplify a nucleic acid fragment [21]. Based on this advantage, mPCR technology can detect a single pathogen or multiple pathogens at the same time. Compared with conventional PCR technology, mPCR technology has the advantages of strong specificity, simplicity and timesaving; however, it is inevitable to

be susceptible to contamination, improper selection of amplification products, and poor control of experimental conditions, resulting in unsatisfactory experimental results [22]. To establish an mPCR reaction system, a series of optimization of its main components and reaction conditions as well as a more comprehensive understanding of PCR techniques are required. Viral nucleic acids (DNA and RNA) can be extracted at the same time, and the same PCR procedure can be used to rapidly detect both DNA and RNA viruses [23]. The present study has shown that the optimum annealing temperature for these templates and primers in the mPCR is 56.5 °C.

In the present study, specific primers were designed to bind the genomes of PRV, PCV2, PRRSV, CSFV, and SIV. The five-plex PCR detection method was established by optimizing the conditions for the amplification of the target genes of PRV, PCV2, PRRSV, CSFV and SIV in order to identify the clinical disease quickly and accurately. This is a practical and rapid detection method with broad application prospects [24]. In recent years, more and more mPCR methods for different virus detection have been successfully established, and the methods are becoming more and more perfect [25]. The mPCR method was originally established to detect either DNA or RNA viruses. However, the detection of clinical disease materials with mixed infection of DNA and RNA viruses often cannot be carried out in one step, the disease materials to be tested should be divided into 2 parts to extract DNA and RNA, and the 2 procedures should be applied for detection. Using the newly developed approach, total viral nucleic acids (DNA and RNA) can be extracted at once. Then, the rapid detection using the mPCR has shortened the total processed time compared to the formerly separate assays.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at https://dx.doi.org/10.2306/scienceasia1513-1874.2024.105.

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Appendix A. Supplementary data

Table S1 Specific primers used for amplification of target genes.

Virus	Target gene	Primer sequence (5′–3′)	Product length (nucleotide)
PRV	gB	R1: 5′-ATGGTGGAGGTGCCCG-3′ F1: 5′-GACCACGCGGTCAATGTCG-3′	192
PCV2	ORF2	R2: 5′-GGCGGTGGACATGCTGAGAT-3′ F2: 5′-TGGGGGATTGTATGGCGGG-3′	255
PRRSV	ORF6	R3: 5′-GGCCGACTGCTAGGGCTTTT-3′ F3: 5′-TTCTGCCACCCAACACGAGG-3′	364
CSFV	C	R4: 5′-GTCAGTAGTTCGACGTGAGCAG-3′ F4: 5′-ACCTCGCAGAACTGCACTT-3′	530
SIV	M	R6: 5′-TTAAAGATGAGCCTTCTGACCGAGG-3′ F6: 5′-AGCGCTATGTTGACAAAATGACC-3′	981