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RESEARCH ARTICLE

Development of Multiple Real-time Fluorescent PCR for Detection of *Porcine parvovirus* (PPV), *Porcine circovirus* Type 2 (PCV2) and *Haemophilus parasuis* (HPS)

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ABSTRACT

Porcine Parvovirus (PPV) and *porcine circovirus type 2* (PCV2) often have mixed infection in the process of clinical breeding, and PCV2 infection will cause immunosuppression in pigs, which is easy to stimulate or complicated with other infectious pathogens. *Haemophilus parasuis* (HPS) is a typical "opportunistic" pathogen, which often leads to mixed infection with PCV2 as a secondary pathogen. In order to establish a rapid and simultaneous detection of three pathogens of PPV, PCV2 and HPS, referring to the relevant genome sequence of GenBank, specific primers were designed according to the conserved region of *VP2* gene of PPV, *Cap* gene of PCV2 and *infB* gene of HPS, and the amplified fragments were cloned into the vector to construct plasmid standard. Using standard samples with different dilutions as templates, adjusting primer concentration, annealing temperature and other conditions, a real-time fluorescence quantitative PCR method for PPV, PCV2 and HPS triple SYBR Green I was established. Three specific Tm peaks could be generated on the same melting curve without cross-reaction with other pathogens. The minimum detection limits of this method were 153 copies/μL, 128 copies/μL and 91 copies/μL, with good specificity and repeatability, which provided technical support for rapid diagnosis of these three diseases and could be used for clinical tissue material detection.

Keywords: *Differential diagnosis, Haemophilus parasuis, Multiple RT-PCR, Porcine circovirus type 2, Porcine parvovirus*

INTRODUCTION

Porcine parvovirus (PPV) is a member of parvovirus genus of *Parvoviridae* [1]. It is the smallest single stranded linear autonomous replication virus in animal DNA virus. It is a disease causing reproductive disorder of sows and causing huge losses to the global pig industry [2]. PPV is mainly harmful to sows. The common clinical symptoms of the disease are abortion, stillbirth, mummification and other phenomena in pregnant sows, but the sows themselves have no obvious symptoms [3]. PPV is also one of the main causes of reproductive disorders in sows and post weaning multisystem failure syndrome in piglets infected with *porcine circovirus type 2* (PCV2) [4]. Once the pig farm is infected with PPV, it is difficult to eliminate it. Therefore, the main measure to control the epidemic of the disease is vaccination.

Porcine circovirus is a single negative strand circular DNA virus without capsule, belonging to *circovirus* genus of *circovirus* family, which is the smallest known animal virus [5]. *Porcine circovirus* is classified into three serotypes, PCV1, PCV2, and PCV3. The diseases caused by PCV2 infection are collectively known as *porcine circovirus* associated paraplegics. Clinical manifestations include weight loss, dyspnea, pale skin, emaciation, and jaundice [6]. In addition, PCV2 infection can lead to immunosuppression in pigs, and a variety of concurrent or secondary diseases, resulting in a large number of deaths in pigs and huge economic losses [7].

Haemophilus parasuis (HPS) belongs to *haemophilus* pasteurelaceae, which is a gram-negative *Bacillus* with capsule structure, no spores and flagella. HPS infection can cause polycellulose serositis, arthritis and meningitis

in pigs, which is also known as porcine Glasser's disease [8]. HPS can achieve a dynamic equilibrium symbiosis with the immune system in the upper respiratory tract of pigs [9]. However, when the body is stimulated by stress or mixed infection, especially when secondary infection or mixed infection occurs with immunosuppressive pathogens, it has a high morbidity and mortality [10]. With the development of intensive pig farming, the prevalence of HPS has become increasingly serious, causing huge economic losses to the pig industry [11].

Under the conditions of high density and intensive breeding, the phenomenon of mixed infection or secondary infection of multiple pathogens often occurs in pigs, which increases the difficulty of prevention and control and causes serious losses. PPV and PCV2 are common reproductive disorders in the breeding process. PCV2 infection will lead to immunosuppression, which is easy to stimulate or complicated with other infectious diseases, and HPS is often a secondary pathogen infection. It is difficult to determine which pathogen is caused by the disease only by clinical symptoms [12]. Therefore, the establishment of rapid and specific diagnostic methods is of great practical significance for the control and epidemiological investigation of these three diseases.

In clinical detection methods, the differential diagnosis of multiple viruses or bacteria is more common, but the differential diagnosis of mixed infection of viruses and bacteria is less, and has not received due attention [13]. At present, the existing diagnostic methods mainly include pathogen isolation and identification, serological detection, molecular biology detection and so on. Multiplex real-time quantitative PCR technology is a detection method that can simultaneously amplify multiple target nucleic acid fragments in the same PCR reaction system using multiple specific primers. In this study, SYBR Green dye was used to establish multiplex real-time fluorescent quantitative PCR technology that could make some specific diagnosis of these three pathogens [14].

Material and Methods

Ethical Statement

Experimentation with animals was approved by the Experimental Animal Management Methods of Xinxiang Medical University (Approval number: 201206078) and followed Henan Authority's Experimental Animal Regulations. Written informed consent was obtained from all the participants prior for the publication of this study.

Strains

PK-15 cells, PPV, PCV2, *Classical swine fever virus, Pseudorabies virus, Porcine epidemic diarrhea virus, Swine Japanese encephalitis virus, Actinobacillus pleuropneumonia, Streptococcus, Pasteurella multocida,* HPS, *Porcine reproductive* and *Respiratory syndrome virus* were kept by Henan institute of science and technology laboratory. Disease materials from Henan province animal prevention and control center gift.

Primers

According to the *NS1* gene sequence of PPV (M38367.1), *Cap* gene sequence of PCV2 (JX912914.1) and *infB* gene sequence of HPS (CP001321.1) logged in GenBank, Primer 5.0 biological software was used to design specific primers for the conserved regions of these three pathogens, and the modified primers were synthesized in Shanghai Bioengineering Co., LTD.

Standard Recombinant Plasmid Construction

According to the instructions of the DNA extraction kit, the viral and bacterial genomes were extracted respectively, and the target genes were amplified by PCR. The amplification conditions were as follows: A total of 40 cycles were carried out, including pre-denaturation at 95°C for 5 min, denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 60 s. Agarose gel electrophoresis was used to retrieve the target fragment, and the target gene fragment was linked with PMD-19T vector and transformed into DH5a escherichia coli susceptible cells. After enzymatic digestion, the positive plasmid was sent to Wuhan GeneCreate Biological Engineering Co., LTD for sequencing. The copy number of extracted plasmid was calculated according to the formula: copies/μL = (6.02 x 10^{23}) x (plasmid concentration ng/μL x 10^{-9})/(DNA length x 660). The plasmid concentration was measured by NanoDrop 2000 spectrophotometer.

Simplex SYBR Green I Fluorescence Quantitative PCR Assay

The positive plasmids correctly sequenced were named PMD-PPV, PMD-PCV2 and PMD-HPS, respectively, and were diluted by 10 times ratio. The standard sample of the diluted plasmids was used as the template for fluorescence quantitative PCR amplification test with a total volume of 10 μL. The specific reaction system was SYBR PreMix enzyme with 5 μL. Primer and primer 0.5 μL, template 1 μL, DEPC water 3 μL; The setup procedure of fluorescence quantitative PCR was as follows: 95°C for 5 min, 95°C for 30 s, and 72°C for 60 s, with a total of 40 cycles. Meanwhile, DEPC hydraulic negative control without template was set, and standard curves of PPV, PCV2 and HPS were drawn according to the results.

Duplex SYBR Green I Fluorescence Real-time PCR Assay

Gradient diluted standard plasmids of PPV, PCV2 and HPS were used as templates to conduct fluorescence

quantitative PCR amplification test of the 20 μL reaction system. Two standard plasmids of pathogens were used as templates in each test. The specific reaction system was as follows: SYBR PreMix enzyme was 10 μL, the upper and lower primers of the two pathogens were 0.5 μL each, the standard plasmid template of the two pathogens was 1 μL each, and DEPC water was 6 μL. The setup procedure of fluorescence quantitative PCR was as follows: 95°C for 5 min, 95°C for 30 s, and 72°C for 60 s, with a total of 40 cycles. Meanwhile, DEPC water without template was set as negative control [15].

Multiple SYBR Green I Fluorescence Real-time PCR Assay

The following ingredients were added into the 20 μL reaction system: SYBR PreMix enzyme 10 μL, PPV DNA template 1 μL, PCV2 DNA template 1 μL, HPS DNA template 1 μL, then PPV, PCV2 and HPS upstream and downstream primers were added to 0.5 μL respectively, supplemented with DEPC water, and then centrifuged and mixed. The procedure of fluorescence quantitative PCR instrument was set as follows: 95°C for 5 min, 95°C for 30 s, and 72°C for 60 s, with a total of 40 cycles, and negative control without template DEPC water was set [16].

Sensitivity, Specificity and Repeatability Analysis

DNA/CDNA 1 μL of PPV, PCV2, PRRSV, CSFV, PRV, PEDV, JEV, HPS, APP, SS and PM positive samples were used as templates, and DEPC water negative control was set up for amplification according to triple fluorescence quantitative PCR reaction system. Verify the specificity of the method.

Three standard plasmids with the same concentration gradient of PPV, PCV2 and HPS were randomly selected as templates, and the established triple SYBR Green I real-time fluorescence quantitative PCR method was used for continuous anti-proliferation for 3 times. The stability of the method was verified by analyzing CT value, Tm value and other data.

Gradient diluted PPV, PCV2 and HPS standard plasmids were used as templates for PCR amplification using the established method. The dilution concentration and CT value of fluorescence signal could not be detected by fluorescence quantitative PCR instrument, and the lower limit of sensitivity detected by the method was calculated.

Clinical Sample Testing

Thirty samples of suspected PPV, PCV2 and HPS mixed infection tissues were detected by the established triple SYBR Green I fluorescent quantitative PCR method and conventional PCR method. The detection rates of the two methods were compared and their clinical practicability was evaluated.

Results

Construction and Identification of Standard Recombinant Plasmid

The constructed standard plasmids of PPV, PCV2 and HPS were amplified by conventional PCR. The size of PPV was 352 bp, PCV2 was 168 bp and HPS was 183 bp. From *Fig. 1* that the amplified product fragment was consistent with the expected size. After enzyme digestion identification, the product was consistent with the expected band.

Establishment of Standard Curve of Simplex Fluorescence Quantitative PCR

From *Fig. 2-A*, *Fig. 3-A*, and *Fig. 4-A*, the kinetic amplification curves of the plasmids with different concentrations of the three pathogens were smooth and evenly separated, and the CT values of repeated samples at each dilution gradient were the same. With the increase of the dilution degree of the standard substance, the CT values gradually increased. By observing the Tm values of PPV, PCV2 and HPS plasmid standards with different concentration gradients when amplified, from *Fig. 2-B*, *Fig. 3-B*, and *Fig. 4-B* that the melting curves of the three pathogens all have only one melting peak, and the Tm values of PPV plasmid standards with different dilution concentrations are about 77.07°C. The Tm values of PCV2 plasmid standard were 79.83°C, and those of HPS plasmid

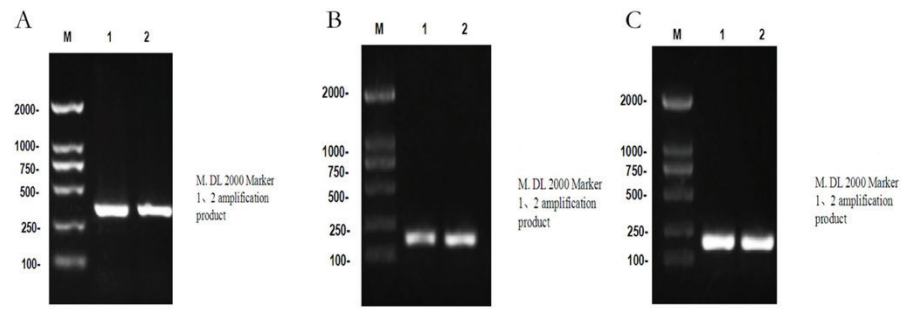

Fig 1. PCR amplification of PPV, PCV2 and HPS plasmid. (A) Amplification product of PPV plasmid; (B) Amplification product of PCV2 plasmid; (C) Amplification product of HPS plasmid

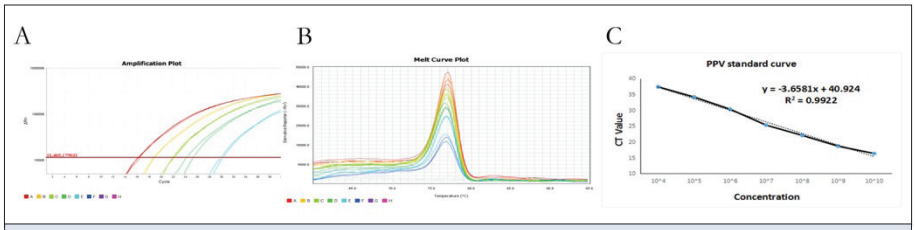

Fig 2. Simplex real time PCR assay of PPV. (A) The amplification curves of different plasmid concentrations of PPV; (B) The melting curves of different plasmid concentrations of PPV; (C) The standard curve of PPV

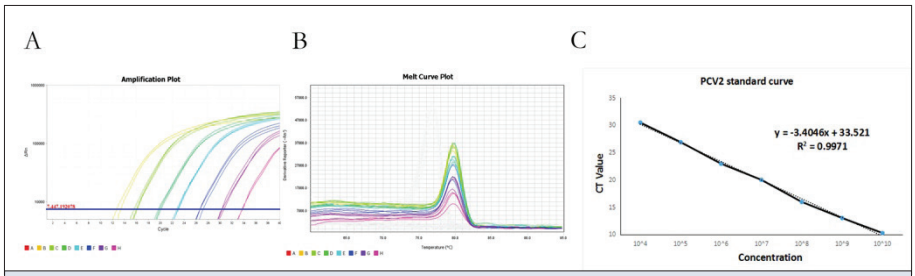

Fig 3. Simplex real time PCR assay of PCV2. (A) The amplification curves of different plasmid concentrations of PCV2; (B) The melting curves of different plasmid concentrations of PCV2; (C) The standard curve of PCV2

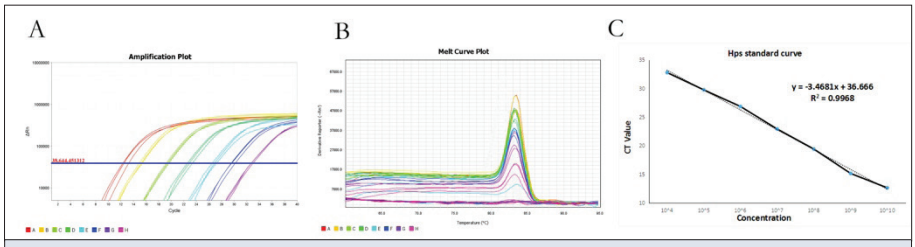

Fig 4. Simplex real time PCR assay of HPS. (A) The amplification curves of different plasmid concentrations of HPS; (B) The melting curves of different plasmid concentrations of HPS; (C) The standard curve of HPS

standard were 83.27°C. According to the correlation diagram of standard plasmid concentration and CT value, three standard curves were obtained. The equation is as follows: PPV standard curve: Y = -3.6581X + 40.924, E = 87.7%, R^2 = 0.9922; PCV2 standard curve: Y = -3.4046X + 33.521, E = 96.7%, R^2 = 0.9971; and HPS standard curve: Y = -3.4681X + 36.666, E = 94.2%, R^2 = 0.9968.

Determination of Dissolution Curve and Tm Value by Double Fluorescence Quantitative PCR

From *Fig. 5-A*, the method established in this study generates two specific peak values on a smooth melting line, namely, Tm values of PPV and PCV2. Tm values of PPV are between 77.03-77.16°C, and Tm values of PCV2 are between 79.80-80.27°C. The results were consistent with the fusion line Tm values of PPV and PCV2 single SYBR Green I real-time fluorescence PCR. From *Fig. 5-B*, two specific peaks are generated on a smooth melting curve, namely, Tm values of PPV and HPS. Tm values of PPV and HPS are between 77.03-77.15°C and 83.27-83.58°C, respectively. It was consistent with the Tm value of the fusion curve in PPV and HPS single SYBR Green I real-time fluorescence PCR detection method. From *Fig. 5-C*, a smooth melting produces two specific peak values on the line, which are respectively the Tm values of PCV2 and HPS. The Tm values of PCV2 are 79.93-80.12°C and the Tm values of HPS are 83.28-83.54°C. The results were consistent with the Tm values of the fusion curves of PCV2 and HPS single SYBR Green L real-time fluorescence PCR.

Establishment of Multiplex Fluorescence Real-time PCR Assay

From *Fig. 6-A*, the method established in this study produces three specific peaks on A smooth melting curve, namely, Tm values of PPV, PCV2 and HPS. Tm values of PPV are between 76.67-76.93°C, and Tm values of PCV2 are between 79.74-80.12°C. The Tm values of HPS ranged from 83.13°C to 83.58°C, which was consistent with the Tm values of the fusion curves of PPV, PCV2 and HPS single SYBR Green I real-time fluorescence PCR.

Specificity, Repeatability, and Sensitivity Analysis

From *Fig. 6-B*, the detection method for fluorescence

quantitative PCR amplification, in a melting curve produced three specific Tm peak, only group a specific amplification, do not cross reaction with other patients, other pathogens are not present amplification curve, judged to be negative, the detection method has good specificity.

From *Fig. 6-C*, *Table 2*, the error between each repeated test is less than one cycle, the fusion curve has a high degree of coincidence, the corresponding Tm value is relatively stable, the standard deviation is less than 0.2, and the coefficient of variation is less than 0.2%, indicating that the method has good repeatability.

The established triple fluorescence quantitative PCR method of PPV, PCV2 and HPS was used to detect the standard plasmid with gradient multiplication dilution. The lower limit of PPV detection was 153 copies/μL, the lower limit of PCV2 detection was 128 copies/μL and the lower limit of HPS detection was 91 copies/μL.

Clinical Sample Testing

Ordinary PCR and triple SYBR Green I real-time fluorescence PCR were respectively used to detect 30 samples of suspected PPV, PCV2 and HPS mixed infection tissue samples. The detection results are shown

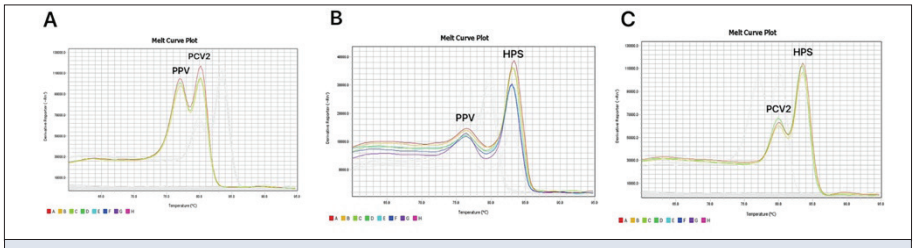

Fig 5. Double real time PCR results. (A) PPV and PCV2 double SYBR Green I real-time fluorescent quantitative PCR results; (B) PPV and HPS double SYBR Green I real-time fluorescent quantitative PCR results; (C) PCV2 and HPS double SYBR Green I real-time fluorescent quantitative PCR results

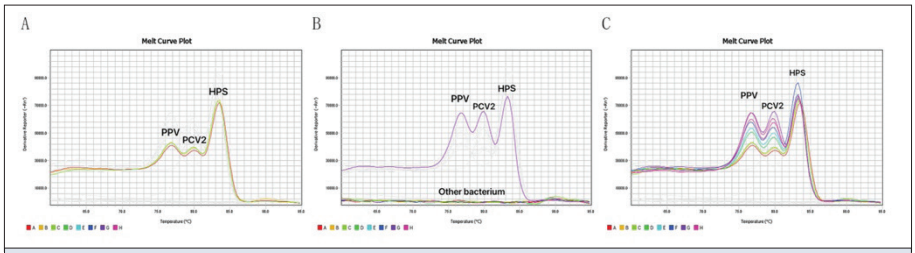

Fig 6. PPV, PCV2 and HPS multiple SYBR Green I real-time fluorescent PCR results. (A) The determination melting curve; (B) The specific test results; N = 8 (C) The repeatability detection results of different concentration gradients

Table 1. Primers used in the FQ-PCR of PPV, PCV2 and HPS gene

Gene	Primer	Sequence (5'-3')	Length/bp
PPV	Forward	GATGGCTCAAACCGGAGGAG	352
	Reverse	TGGAAAGTTCACATTGGCTGC	
PCV2	Forward	TGCCCTAACCTATGACCC	168
	Reverse	TGTAGTTTGTAGTCTCAGCCAG	
HPS	Forward	CACCCTTATCCTTTGTTGCC	183
	Reverse	CACTTTCTGAGATTCACTCCACC	

Table 2. The Tm analysis of repeatability for the multiplex SYRR Green I real time PCR intra-assay

Pathogens	The Tm/°C Values of 3 Tests			Means	S	CV(%)
	1st	2nd	3rd			
PPV	76.80	76.73	76.67	76.73	0.05	0.069
PCV2	80.12	80.05	79.99	80.05	0.05	0.066
HPS	83.58	83.43	83.43	83.48	0.14	0.169

Table 3. *Triple SYBR Green* I *real-time fluorescent PCR detection method in clinical samples*

Method	PPV	PCV2	HPS	Control
Conventional PCR method	7	9	13	0
Real-time PCR	9	17	23	0

in *Table 3*. CT values of all positive samples were between 16.24 and 35.68. No more than 38 cycles, neither method can detect negative samples. When using conventional PCR, the positive rate of PPV, PCV2 and HPS was 23%, 30% and 43% respectively. When using triple real-time fluorescence PCR, the positive detection rate of PPV was 30%, 7% higher than that of conventional PCR; the positive detection rate of PCV2 was 56.7%, 23.7 higher than that of conventional PCR: The positive rate of HPS was 76.7%, 33.7% higher than that of conventional PCR, and the method could detect all the positive samples detected by conventional PCR.

Discussion

In recent years, respiratory and reproductive diseases occur frequently in the process of pig disease prevention and control, which poses a serious threat to the development of pig industry. PPV and PCV2 are common reproductive disorders in the process of breeding. In the process of breeding, these two pathogens often have mixed infection [17]. HPS often appears as a secondary pathogen of porcine parvovirus disease, leading to secondary infection or other infectious diseases, resulting in a large number of deaths of pigs [18]. When respiratory diseases occur in pigs, it is difficult to accurately judge the pathogen only by clinical symptoms. Therefore, it is of great practical significance to establish a rapid and specific diagnosis method for disease control and epidemiological investigation.

In the process of differential diagnosis of swine diseases, the differential diagnosis between viruses or bacteria is more common, especially in the daily detection of swine diseases, the single detection and verification of pathogens is more common. However, the identification of mixed infection or secondary infection between viruses and bacteria has not been paid enough attention and ignored. In the daily detection of these pathogens, it is found that the mixed infection between viruses and bacteria is increasing day by day, especially the mixed infection between viral pathogens such as PPV and PCV2 and bacterial pathogens such as HPS is more frequent [19, 20]. Therefore, rapid and accurate diagnostic detection of these three pathogens is essential for early detection, surveillance and prevention of swine diseases.

Due to the establishment of differential diagnosis methods for viral and bacterial pathogens has not been paid enough attention, so this study established a real-time fluorescence PCR method for simultaneous detection of these three pathogens [21]. The method can not only bind the double-stranded DNA with SYBR Green fluorescent dye, but also accurately quantify the target nucleic acid. Furthermore, multiple PCR reactions for the diagnosis of multiple diseases can be performed simultaneously in the same reaction system, and differential diagnosis can be achieved by monitoring the Tm peak position of the fusion curve of the target product [22].

In this study, the sensitivity, specificity and repeatability analysis of the method showed that the lower limit of PPV detection was 148 copies/μL, the lower limit of PCV2 detection was 153 copies/μL, and the lower limit of HPS detection was 91 copies/μL. There were three specific peaks in the melting curve. The Tm values of 77.07°C, 79.96°C and 83.27°C showed good specificity and repeatability, indicating that the established SYBR Green I real-time fluorescence PCR technology could achieve the purpose of differential diagnosis of these three pathogens, and provide a more convenient and reliable monitoring means for the routine diagnosis of clinical pig mixed infection. It also provides a basis for the revision of immunization procedures for this disease.

In conclusion, through the optimization of test conditions, a triple SYBR Green I real-time fluorescent PCR method was established for simultaneous identification and diagnosis of PPV, PCV2 and Hps without cross-reaction with other pathogens, indicating that a triple real-time quantitative fluorescent PCR method for simultaneous detection of PPV, PCV2 and Hps was successfully established. To provide technical support for rapid diagnosis of mixed infection between these viruses and bacteria.

Ethical Statement

Experimentation with animals was approved by the Experimental Animal Management Methods of Xinxiang Medical University (Approval number: 201206078) and followed Henan Authority's Experimental Animal Regulations. Written informed consent was obtained from all the participants prior for the publication of this study.

Availability of Data and Materials

The data sets during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Competing Interests

The authors declare no conflict of interest.

Authors' Contributions

Y. Z. performed experiments and wrote the manuscript. Y. Z., N. Y., L. W. performed experiments, Y. D. and H. X. wrote the article and conceived the experiments.

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