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RESEARCH ARTICLE

The Development of a SYBR Green I Multiple Real-time Fluorescence PCR Assay for Detection of *Actinobacillus pleuropneumoniae, Haemophilus parasuis* and *Pasteurella multocida*

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Abstract

Actinobacillus pleuropneumoniae, Haemophilus parasuis, and *Pasteurelle multocida* are common pathogens of respiratory diseases in the pig industry, and they may cause secondary infections and serious economic losses to the pig industry. The clinical symptoms caused by these three pathogens are difficult to distinguish with the naked eye, and mix infections bring difficulties to the diagnosis of diseases. In this study, specific primers were designed on the basis of *A. pleuropneumoniae Apx IV, H. parasuis Omp P2* and *P. multocida PlpE* gene. The expected amplified products of *A. pleuropneumoniae, H. parasuis,* and *P. multocida* were 157, 120 and 305 bp, respectively. After the amplified fragment was cloned into a vector, a standard plasmid was constructed. By using the standard plasmid as template, a fluorescence quantitative PCR method for simultaneous detection of *A. pleuropneumoniae, H. parasuis,* and *P. multocida* multiple SYBR Green I was established. Combined with melting curve analysis, the sensitivity, specificity, and repeatability were also evaluated. The results showed that the sensitivity of the method for detecting the three pathogens were 147, 145, and 61 copies/μL. On the same melting curve that produced three specific Tm peaks, no cross reaction with other bacteria was observed, and the method demonstrated good specificity and repeatability. This method could be used for the simultaneous detection of the three pathogens, thus providing an effective detection tool for disease prevention and treatment.

Keywords: *Actinobacillus pleuropneumoniae, Haemophilus parasuis, Pasteurelle multocida, SYBR Green I, Multiplex PCR*

Actinobacillus pleuropneumoniae, Haemophilus parasuis ve *Pasteurella multocida*'nın Saptanmasında SYBR Green I Multiple Gerçek Zamanlı Floresan PCR Yönteminin Geliştirilmesi

Öz

Actinobacillus pleuropneumoniae, Haemophilus parasuis ve *Pasteurelle multocida,* domuz yetiştiriciliğinde yaygın solunum sistemi hastalıkları patojenleridir ve domuz endüstrisinde sekonder enfeksiyonlara ve ciddi ekonomik kayıplara neden olabilirler. Bu üç patojenin neden olduğu klinik semptomların çıplak gözle ayırt edilmesi güçtür ve miks enfeksiyonlar hastalıkların tanısını zorlaştırır. Bu çalışmada, *A. pleuropneumoniae Apx IV, H. parasuis Omp P2* ve *P. multocida PlpE* gen bazında spesifik primerler tasarlandı. *A. pleuropneumoniae, H. parasuis* ve *P. multocida* için sırasıyla 157, 120 ve 305 bp amplifiye ürünler beklendi. Amplifiye fragment bir vektöre klonlanarak standart bir plazmit oluşturuldu. Kalıp olarak standart plazmit kullanılarak, *A. pleuropneumoniae, H. parasuis* ve *P. multocida* multipl SYBR Green I'in eşzamanlı tespiti için kantitatif bir floresan PCR yöntemi geliştirildi. Erime eğrisi analizi ile birlikte duyarlılık, özgüllük ve tekrarlanabilirlik de değerlendirildi. Sonuçlar, bu yöntemin, bu üç patojeni saptamada duyarlılığının 147, 145 ve 61 bakteri/μL olduğunu gösterdi. Üç spesifik Tm piki veren aynı erime eğrisinde diğer bakterilerle çapraz reaksiyon gözlenmedi ve yöntem iyi bir özgüllük ve tekrarlanabilirlik sergiledi. Bu yöntem, üç patojenin eşzamanlı tespiti için kullanılabilir ve böylelikle hastalıkların önlemesi ve tedavisi için etkili bir teşhis aracı niteliğindedir.

Anahtar sözcükler: *Actinobacillus pleuropneumoniae, Haemophilus parasuis, Pasteurella multocida, SYBR Green I, Multipleks PCR*

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Introduction

Actinobacillus pleuropneumoniae is one of the pathogens causing porcine contagious pleuropneumonia, which is distributed all over the world [1]. This pathogen has many different serotypes, no cross-reaction exists between different serotypes; the serotypes prevalent in different countries and regions are not exactly the same, making it difficult to accurately diagnose *A. pleuropneumoniae* infection [2,3]. When pigs are infected with *A. pleuropneumoniae*, they are mainly characterized by acute hemorrhage and chronic fibrinous necrotizing pleuropneumonia. They have a high mortality rate and cause huge losses in the pig industry.

Haemophilus parasuis is a pathogenic bacterium that can cause infections in pigs. *H. parasuis* is a Gram-negative bacterium belonging to the *Pasteurellaceae* family; it exists in the upper respiratory tract of healthy pigs [4]. When tolerance to the environment is weakened, it can invade various organs of the body and cause disease. In pigs, the clinical symptoms are fibrinous serositis, meningitis, and arthritis, and the disease is also known as pig Glasser's disease [5]. The disease is prevalent worldwide, and it mainly harms nursery pigs and weaned piglets, with a high incidence and fatality rate. It is one of the major swine diseases affecting the development of the pig industry [6,7].

Pasteurella multocida is an important Gram-negative pathogen that usually exists in the nasal cavity, peach body, lung, and other parts of pigs; it can cause diseases, such as swine pneumonitis and swine infectious atrophic rhinitis [8]. Clinically, the most acute type is mainly manifested as sepsis. The acute type is the most common, with inflammation and swelling of the throat, unsmooth breathing, late weakness, and death from suffocation. The chronic type is rare, and the symptoms are not obvious. The disease is generally not affected by the season, it is endemic, and pigs of any age could be infected with it [9]. It also has caused huge economic losses to the pig industry [10].

Clinically, the symptoms of *A. pleuropneumoniae*, *H. parasuis*, and *P. multocida* are difficult to distinguish as they are all common and important pathogenic bacteria in the respiratory tract of pigs, and they may cause mixed infections, which are also difficult to distinguish [11]. How to quickly and accurately distinguish and identify these three kinds of bacteria has attracted extensive attention. At present, the traditional methods of pathogen isolation and identification and serological diagnosis are still widely used in veterinary clinical diagnosis of these three diseases, but the methods of pathogen isolation and serological diagnosis are time-consuming, with low sensitivity and specificity, thus not meeting the needs of rapid clinical diagnosis. Although conventional PCR detection methods could be used to detect the three pathogens, they need to be tested separately, thus time-consuming, laborious, and cumbersome to operate, resulting in great inconvenience. Therefore, establishing a rapid, efficient, and accurate detection method is considerably important [12].

Multiplex PCR technology is a method to simultaneously amplify nucleic acid fragments of multiple purposes by adding two or more pairs of primers into the same PCR amplification system, and simultaneously identify and detect various pathogens [13,14]. It has the advantages of strong specificity, high sensitivity and fast diagnosis [17]. Real-time fluorescence quantitative PCR is a new technique developed in the 1990s to detect nucleic acid molecules. Fluorescent dyes are added in the PCR system, and through the change in fluorescence intensity, this system could be used for real-time PCR process inspection; finally, through the standard curve of nucleic acids, it could be used in the accurate quantitative analysis of unknown samples and qualitative analysis through the dissolution curve of unknown samples; it has high sensitivity, short reaction time, observational results, and many other advantages [15]. Multiplex real-time fluorescence quantitative PCR is a detection method that uses multiple specific primers to simultaneously amplify multiple target nucleic acid fragments in the same PCR reaction system. In accordance with different Tm values, multiple specific Tm peaks could be generated on the same melting curve to achieve the purpose of simultaneous detection.

In this study, a multiple SYBR Green I fluorescence quantitative PCR method for simultaneous detection of *A. pleuropneumoniae*, *H. parasuis*, and *P. multocida* was established. Through the melting curve, this method could simultaneously detect one, two, or three kinds of pathogens, thus providing technical support for the rapid and accurate detection of diseases in clinical practice.

Material and Methods

Strains

A. pleuropneumoniae, *H. parasuis*, *P. multocida*, *Staphylococcus aureus*, *Staphylococcus suis* and *Escherichia coli* are all preserved in the laboratory of Henan Institute of Science and Technology.

Primers

According to the conserved sequences of *A. pleuropneumoniae Apx IV*, *H. parasuis Omp P2,* and *P. multocida PlpE* by GenBank, three pairs of specific primers were designed by using the software Premier 5, and the modified primers were synthesized by Shanghai Shenggong Bioengineering Co., Ltd. The sequence of the three pairs of primers is shown in *Table 1*.

Standard Recombinant Plasmid Construction

Bacterial genomic DNA was extracted in accordance with the operating instructions of the TAKARA DNA extraction kit. It was stored at -20ºC for later use. The extracted *A.*

Table 1. *Primers used in the FQ-PCR of A. pleuropneumoniae, H. parasuis and P. multocida gene*

Gene	Primer	Sequence (5′-3′)	Tm/°C	Length/bp
A. pleuropneumoniae	Forward	GCAGCTACGGTGCGGACA	61.8	157
	Reverse	TCATTATCTACTCGTCGGAATTTCACT	54.8	
H. parasuis	Forward	AAAAGATACCAAAGGCAAGG	49.9	120
	Reverse	ACCACAGTAATAGTTTCACCGA	52.9	
P. multocida	Forward	TGGCTACCTTGTTACGACTTC	54.3	305
	Reverse	CATGAGGGCAGGAGAGGAG	54.8	

pleuropneumoniae, *H. parasuis*, and *P. multocida* were used as templates to amplify the target gene. DNA STAR enzyme, primers, and template were added to 20 μL reaction system in sequence, finally making up to 20 μL with double-distilled water. The EP tube was placed in the PCR machine, and amplification was performed according to the following procedure: after pre-denaturation at 95°C for 5 min, cycle at 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min; after 30 cycles, 72°C for 10 min; and save at 4°C. Then, the amplified product was electrophorized on agarose gel, and the target fragment was recovered according to the TAKARA gel recovery kit. The recovered product was linked with pMD-19T vector, transformed into DH-5a competent cells, and spread on LB plate containing ampicin antibiotics. White colonies were selected and identified by PCR. Plasmids were extracted using the TAKARA plasmid extraction kit and sent to Beijing Genomics Institution in Beijing for sequencing. The correct plasmid verified by sequencing was used as the standard substance to establish the standard curve.

Simplex SYBR Green I Fluorescence Quantitative PCR Assay

Single SYBR Green I fluorescence quantitative PCR was established for *A. pleuropneumoniae*, *H. parasuis* and *P. multocida*, respectively. OD26 was measured by the recombinant plasmid of *A. pleuropneumoniae*, *H. parasuis* and *P. multocida*. Its copies/uL was calculated by substituting the value into the following formula (copies/μL = (6.02 x 10^{23}) x (ng/μL x 10^{-9})/(DNA length x 660). Then, a gradient dilution was established, with dilution of concentrations of 10^{10}, 10^{9}, 10^{8}, 10^{7}, 10^{6}, 10^{5}, 10^{4}, 10^{3}, 10^{2}, 10^{1}, 10^{0} copies/μL, making the standard curve as a reference standard. The total reaction system of SYBR Green I was 10 μL, 5 μL SYBR PreMix enzyme with 0.5 μL each for F and R; 1 μL template; and 3 μL double-distilled water were added. Fluorescent quantitative PCR instrument was used for reaction amplification. The program was set as follows: 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, with a total of 40 cycles.

Duplex SYBR Green I Fluorescence Real-time PCR Assay

On the basis of the constructed simplex fluorescence real-time PCR method, duplex SYBR Green I fluorescence quantitative PCR was established for *A. pleuropneumoniae* and *H. parasuis*, *A. pleuropneumoniae* and *P. multocida*, and *H. parasuis* and *P. multocida*. The SYBR Green I real-time fluorescent PCR 20 μL reaction system was as follows: SYBR Premix enzyme 10 μL, with 0.5 μL for the F and R of each two pathogenic bacterial primers, 1 μL each of the two pathogenic bacteria templates; and 6 μL double-distilled water. After instantaneous centrifugation, the EP tube was placed on a fluorescence quantitative PCR instrument. After pre-denaturation at 95°C for 5 min, then at 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min, 40 cycles were performed for PCR reaction, and a negative control without template was set up.

Multiple SYBR Green I Fluorescence Real-time PCR Assay

Based on the constructed single and double fluorescence real-time PCR methods, multiple SYBR Green I fluorescence quantitative PCR was established. The 20 μL system of SYBR Green I real-time fluorescent PCR reaction was as follows: SYBR Pre Mix enzyme 10 μL, *A. pleuropneumoniae*, *H. parasuis* and *P. multocida* primer F, R each 0.5 μL, template 1 μL each, and 4 μL double-distilled water. After instantaneous centrifugation, the EP tube was placed on the fluorescence quantitative PCR instrument for pre-denaturation at 95°C for 5 min, then for 30 s at 95°C; 55°C for 30 s; 72°C for 1 min, 40 cycles were performed for PCR reaction, and a negative control without template was set up.

Sensitivity, Specificity and Repeatability Analysis

By using 1 μL DNA of *A. pleuropneumoniae*, *H. parasuis*, *P. multocida*, *S. aureus*, *S. suis,* and *E. coli* as templates and double-distilled water as negative control, fluorescence quantitative PCR was performed to verify the specificity.

Plasmid standard samples with the same concentration of *A. pleuropneumoniae*, *H. parasuis*, and *P. multocida* were selected for multiple SYBR Green I real-time PCR repeatability test, and the reaction was repeated three times. The stability of *A. pleuropneumoniae*, *H. parasuis*, and *P. multocida* multiple SYBR Green I real-time PCR method was verified by analyzing the Tm values and melting curves of each bacterium.

Plasmid standard samples with the same concentration

of *A. pleuropneumoniae*, *H. parasuis* and *P. multocida* were selected for multiple SYBR Green I real-time fluorescent PCR reaction repeatability test, and the reaction was repeated three times. The stability of *A. pleuropneumoniae*, *H. parasuis* and *P. multocida* multiple SYBR Green I real-time PCR method was verified by analyzing the Tm values and melting curves of each bacterium.

The OD260 value of recombinant plasmids *A. pleuropneumoniae*, *H. parasuis*, and *P. multocida* was measured, and the copy number per microliter was calculated. Then, the 10 times gradient dilution was performed. The *A. pleuropneumoniae*, *H. parasuis*, and *P. multocida* standard plasmids after gradient dilution were used as templates for PCR amplification by using the proposed method, and the sensitivity of fluorescence quantitative PCR reaction for the detection of bacteria was determined.

Results

Construction and Identification of Standard Recombinant Plasmid

Specific primers were used to amplify *A. pleuropneumoniae*, *H. parasuis*, and *P. multocida*, and the results showed that the sizes of their target bands were 157, 120, and 305 bp, respectively *(Fig. 1)*. After enzymatic digestion was identified, the product was found to be consistent with the expected band.

Establishment of Standard Curve of Simplex Fluorescence Quantitative PCR

The concentration of the extracted positive plasmid was determined using a protein nucleic acid quantifier. The concentrations of *A. pleuropneumoniae*, *H. parasuis*, and *P. multocida* were 254.6, 191.1, and 205.7 ng/μL respectively. By using the above formula, the actual concentration of copies could be calculated. The *A. pleuropneumoniae*, *H. parasuis*, and *P. multocida* plasmid template copy number were 1.47 x 10^{12}, 1.45 x 10^{12}, and 6.05 x 10^{11} copies/μL respectively. According to the results obtained, the plasmid was diluted 10 times to the gradient concentration and used as a standard sample to make a standard curve. From *Fig. 2-A,B, Fig. 3-A,B,* and *Fig. 4-A,B,* the amplification curves were smooth and evenly spaced; the Ct values of the repeated samples of each dilution gradient were the same; and the dissolution temperatures of *A. pleuropneumoniae*, *H. parasuis*, and *P. multocida* standard at each dilution degree were approximately 76.9°C, 80.0°C and 83.8°C, respectively. The height of the peak was positively correlated with the concentration of DNA, and the CT value gradually increased with the increase in the dilution degree of the standard substance. *A. pleuropneumoniae* standard curve: Y = -3.449X + 39.682, E = 95.0%, R^2 = 0.9968; *H. parasuis* standard curve: Y = -3.5805X + 41.26, E = 90.2%, R^2 = 0.996;

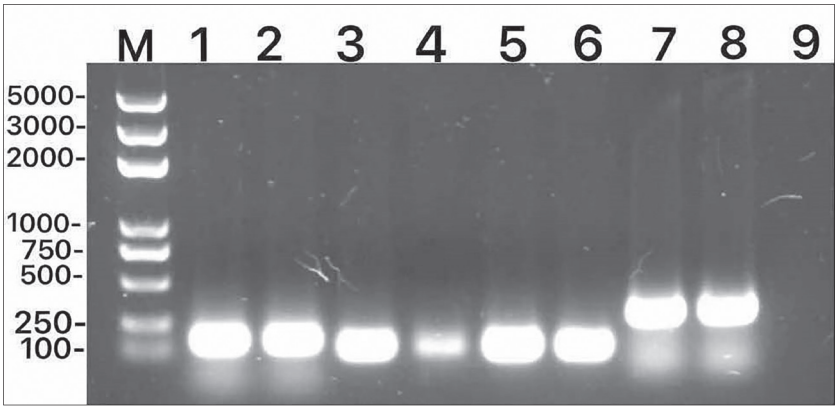

Fig 1. PCR amplification of *A. pleuropneumoniae*, *H. parasuis* and *P. multocida* plasmid. M:DL 5000 Marker; 1,2: amplification product of *A. pleuropneumoniae* plasmid; 3-6: amplification product of *H. parasuis* plasmid; 7,8: amplification product of *P. multocida* plasmid; 9: Blank control

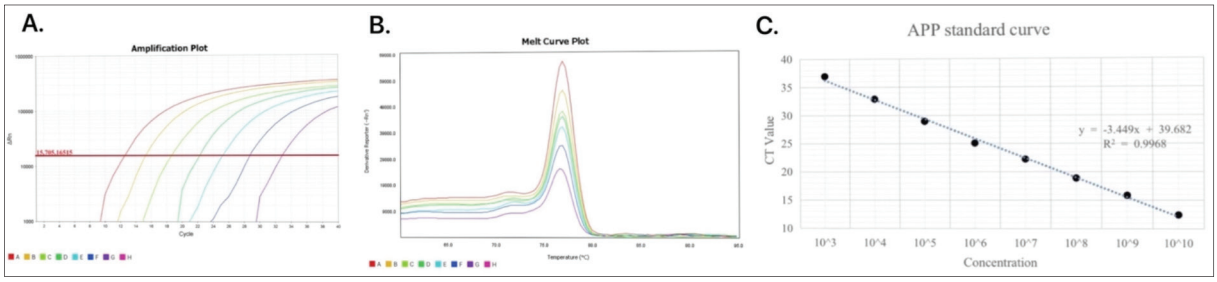

Fig 2. Simplex real-time PCR assay of *A. pleuropneumoniae*. **(A)** The amplification curves of different plasmid concentrations of *A. pleuropneumoniae*; **(B)** The melting curves of different plasmid concentrations of *A. pleuropneumoniae*; **(C)** The standard curve of *A. pleuropneumoniae*

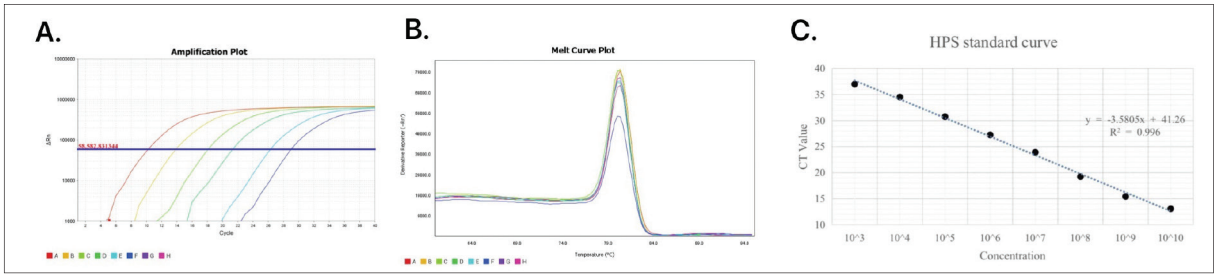

Fig 3. Simplex real-time PCR assay of *H. parasuis.* **(A)** The amplification curves of different plasmid concentrations of *H. parasuis*; **(B)** The melting curves of different plasmid concentrations of *H. parasuis*; **(C)** The standard curve of *H. parasuis*

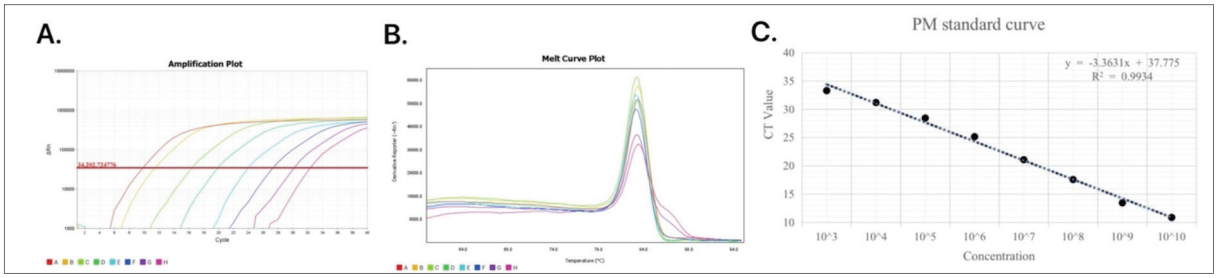

Fig 4. Simplex real-time PCR assay of *P. multocida.* **(A)** The amplification curves of different plasmid concentrations of *P. multocida*; **(B)** The melting curves of different plasmid concentrations of *P. multocida*; **(C)** The standard curve of *P. multocida*

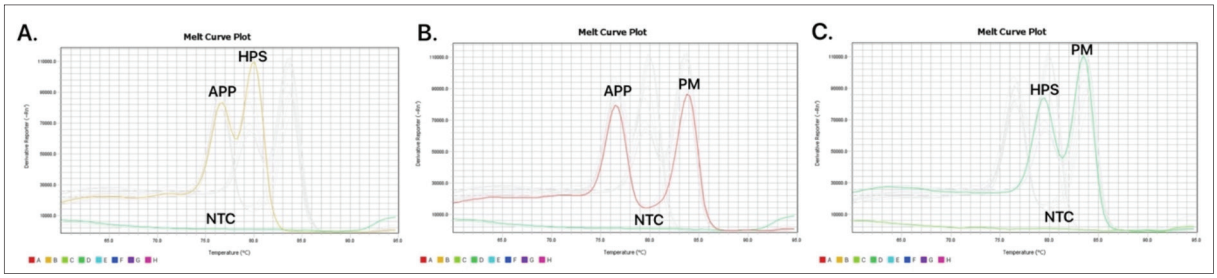

Fig 5. Double real-time PCR results. **(A)** *A. pleuropneumoniae* and *H. parasuis* double SYBR Green I real-time fluorescent quantitative PCR results; Targets are indicated above each peak along with non-target negative controls (NTC). **(B)** *A. pleuropneumoniae* and *P. multocida* double SYBR Green I real-time fluorescent quantitative PCR results; Targets are indicated above each peak along with non-target negative controls (NTC). **(C)** *H. parasuis* and *P. multocida* double SYBR Green I real-time fluorescent quantitative PCR results: Targets are indicated above each peak along with non-target negative controls (NTC)

and *P. multocida* standard curve: Y = -3.3631X + 37.775, E = 98.3%, R^2 = 0.9934. The results showed a good linear relationship among the three plasmids.

According to the fluorescence quantitative PCR test, the melting curve showed that the temperature corresponding to the two specific peaks is the Tm value of the pathogen. The Tm values of *A. pleuropneumoniae* and *H. parasuis* in *Fig. 5-A,* Tm values of *A. pleuropneumoniae* and *P. multocida* in *Fig. 5-B,* Tm values of *H. parasuis* and *P. multocida* in *Fig. 5-C,* that displayed a same degree in the single fluorescence quantitative PCR test. No specific peak was found in the negative control.

Establishment of Multiplex Fluorescence Real-Time PCR Assay

The recombinant plasmid of *A. pleuropneumoniae*, *H. parasuis*, and *P. multocida* was used as the template for PCR reaction, and the dissolution curve was obtained using the fluorescence quantitative PCR instrument. From *Fig. 6-A,* shows three specific Tm peaks on the same melting curve, and these peaks were the Tm values of *A. pleuropneumoniae*, *H. parasuis*, and *P. multocida*. The Tm values of *A. pleuropneumoniae*, *H. parasuis*, and *P. multocida* were 76.47-76.93°C, 79.77-80.10°C, and 83.67-83.81°C, respectively, whereas the negative control had no peak value.

Specificity, Repeatability, and Sensitivity Analysis

The results of multiple SYBR Green I real-time fluorescence PCR specificity test on *A. pleuropneumoniae*, *H. parasuis* and *P. multocida* in *Fig. 6-B* showed that in the control group, *S. aureus*, *S. suis, E. coli* and the negative control had no specific peaks. Only the *A. pleuropneumoniae*, *H. parasuis*, and *P. multocida* multiple real-time PCR in the test group produced three specific peak values, and no cross-

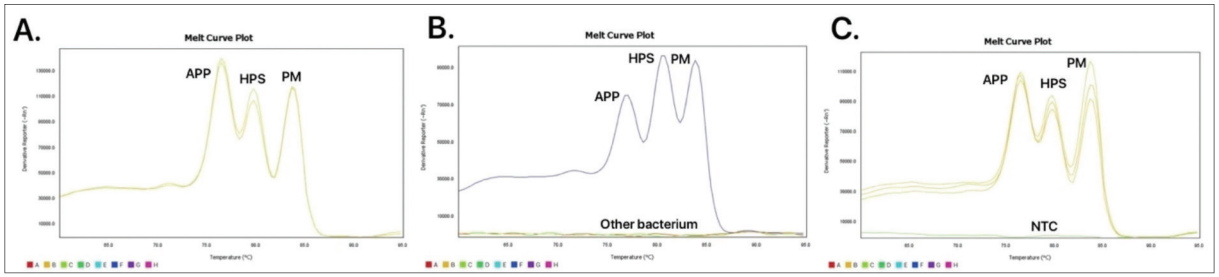

Fig 6. *A. pleuropneumoniae*, *H. parasuis* and *P. multocida* multiple SYBR Green I real-time fluorescent PCR results. **(A)** The determination melting curve; Targets are indicated above each peak along with non-target negative controls (NTC). **(B)** The specific test results; N = 3 **(C)** The repeatability detection results of different concentration gradients; Targets are indicated above each peak along with non-target negative controls (NTC)

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

Pathogenic Species	The Tm/°C Values of 3 Tests			Means	S	CV(%)
	1st	2nd	3rd			
A. pleuropneumoniae	76.47	76.47	76.47	76.63	0	0
H. parasuis	79.77	79.77	79.77	79.77	0	0
P. multocida	83.66	83.81	83.81	83.76	0.07	0.0836

reaction occurred in the other test groups. The Tm values of *A. pleuropneumoniae*, *H. parasuis*, and *P. multocida* were 76.9°C, 79.8°C, and 83.7°C respectively. These results showed that the method had strong specificity.

In the three repeated experiments, the Tm value of each pathogen was relatively stable. The results are shown in *Fig. 6-C* and *Table 2*. The melting curves showed a high degree of overlap and the corresponding Tm values were relatively stable. The standard deviations were all less than 0.1, and the coefficients of variation were less than 0.1%, indicating that the real-time fluorescence PCR reaction of *A. pleuropneumoniae*, *H. parasuis*, and *P. multocida* multiple SYBR Green I under the same concentration had good stability and repeatability.

The established *A. pleuropneumoniae*, *H. parasuis*, and *P. multocida* multiple fluorescent quantitative PCR method was used to detect the standard plasmids diluted in multiple ratios. The sensitivity of the *A. pleuropneumoniae*, *H. parasuis*, and *P. multocida* recombinant plasmids could reach 147, 145, and 61 copies/μL, respectively.

Discussion

Respiratory infectious diseases are one of the three syndromes that harm the pig industry. Especially with the development of large-scale pig industry in recent years, the respiratory diseases show an increasing trend. *A. pleuropneumoniae*, *H. parasuis*, and *P. multocida* are important common pathogenic bacteria in pig respiratory tract, and distinguishing the clinical symptoms caused by them is difficult [16]. They separately cause pig disease and often infect each other or combine with other pathogen infections [11]. These three bacteria are all Gram-negative bacilli of the Pasteurella family. They also require two-stage concentrated staining, and they are pleomorphic, thus difficult to complete in clinical isolation and identification [17]. Therefore, obtaining a convenient and effective detection method is particularly important.

SYBR Green I is a non-sequence-specific fluorescent dye that can bind to double-stranded DNA heterotectically [18]. At present, it is widely used in quantitative PCR. SYBR Green I real-time PCR not only can accurately quantify the nucleic acid of the target but also carry out multiple PCR reactions in the same PCR tube by analyzing the dissolution curve and using different Tm values of the target fragment, thus providing a new method for simultaneous diagnosis of multiple diseases.

A real-time fluorescent quantitative PCR method based on SYBR Green I was developed for rapid identification of multiple bacteria or viruses. The experiment was highly specific, and it did not cross-react with other common bacteria. This method has been used in many articles, and the detection efficacy is very good [19-21].

In this study, specific primers for *A. pleuropneumoniae*, *H. parasuis*, and *P. multocida* were designed, and multiple SYBR Green I real-time fluorescence PCR technology was used to amplify the three pathogens. A multiple SYBR Green I real-time quantitative PCR method that could simultaneously detect the three bacteria was also established. In the experiment, different Tm values were used to distinguish nucleic acid fragments. The Tm value of nucleic acid fragments is mainly related to sequence length and sequence structure. Three specific Tm peaks were produced on the same melting curve. Therefore, the purpose of differential diagnosis could be achieved by

monitoring the location of the peak Tm of the fusion curve of the target product.

A. pleuropneumoniae, *H. parasuis*, and *P. multocida* single SYBR Green I real-time quantitative PCR assays; *A. pleuropneumoniae* and *H. parasuis*, *A. pleuropneumoniae* and *P. multocida*, and *H. parasuis* and *P. multocida* double SYBR Green I real-time quantitative PCR assays; and *A. pleuropneumoniae*, *H. parasuis*, and *P. multocida* multiple SYBR Green I real-time quantitative PCR assays were successfully established. The specificity test results of this study were good, with only three specific peaks appearing at Tm values of 76.9°C, 79.8°C and 83.7°C respectively. The repeatability test had good stability. The sensitivity test showed that the minimum detection limits of multiple quantitative PCR for *A. pleuropneumoniae*, *H. parasuis*, and *P. multocida* plasmid standard were 147, 145, and 61 copies/μL, respectively. The greatest advantage of this study is that *A. pleuropneumoniae*, *H. parasuis*, and *P. multocida* could be detected simultaneously, which is conducive to the identification and diagnosis of pig respiratory diseases.

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 Interest

The authors declare no conflict of interest.

Authors' Contribution

YZ performed experiments and wrote the manuscript. YZ, YD, YX, ZW, NY performed experiments, HL and LW wrote the article and conceived the experiments.

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