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Research Article

Development of a SYBR Green Real-Time PCR Assay with Melting Curve Analysis for Simultaneous Detection of *Actinobacillus pleuropneumoniae* and *Haemophilus parasuis*

Bin HU [1,a,‡] Shouping ZHANG [1,b,‡] Yanhua XU [1,c] Zhichen WANG [1,d]
Qiuxuan REN [1,e] Jingfei XU [1,f] Yongjun DONG [1,g] Lirong WANG [1,h]

[‡] *These authors contributed equally to this study*

[1] College of Animal Science and Veterinary Medicine, Henan Institute of Science and Technology, Xinxiang 453003, CHINA
ORCIDs: [a] 0000-0003-3305-5473; [b] 0000-0003-1774-1924; [c] 0000-0003-1452-9485; [d] 0000-0003-4455-8615; [e] 0000-0002-1289-6065
[f] 0000-0002-4404-9530; [g] 0000-0001-9596-7908; [h] 0000-0003-3833-4703

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Abstract

In the present study, a duplex SYBR Green real-time PCR assay was developed in order to indentify *Actinobacillus pleuropneumoniae* and *Haemophilus parasuis* infection in one reaction, through a melting curve analysis. This method utilized two pairs of specific primers that allowed the amplification of highly conserved regions of *A. pleuropneumoniae* Apx IV and *H. parasuis* omp P2 gene. Reconstitution experiments were conducted by using PMD - 19T plasmid in order to determine the sensitivity of the assay. The results showed that the Tm values of the melting curves of *A. pleuropneumoniae* and *H. parasuis* were 83.36±0.09ºC and 76.48±0.17ºC, respectively that could accurately distinguish these two pathogens. And no cross reaction were observed between other respiratory pathogens, which suggested a high specificity of two primers. The detection sensitivity of the assay was 127 and 96 copies/µL which was higher than that of the ordinary PCR detection methods. This rapid technique may present a simple, useful option for simultaneous detection of *A. pleuropneumoniae* and *H. parasuis*, which would be feasible and attractive for clinical samples diagnosis and epidemiological investigations.

Keywords: *Actinobacillus pleuropneumoniae, Haemophilus parasuis, SYBR Green I, Real-time PCR, Melting curve*

Actinobacillus pleuropneumoniae ve *Haemophilus parasuis*'in Eşzamanlı Saptanması Amacıyla Erime Eğrisi Analizi İle SYBR Green Real-Time PCR Testinin Geliştirilmesi

Öz

Bu çalışmada, bir erime eğrisi analizi yoluyla tek bir reaksiyonda *Actinobacillus pleuropneumoniae* ve *Haemophilus parasuis* enfeksiyonunu tanımlamak için bir dubleks SYBR Green gerçek zamanlı PCR analizi geliştirildi. Bu yöntemde, *A. pleuropneumoniae* Apx IV ve *H. parasuis* omp P2 geninin yüksek oranda korunmuş bölgelerinin amplifikasyonuna izin veren iki çift spesifik primer kullanıldı. Analizin duyarlılığını belirlemek için PMD - 19T plazmid kullanılarak sulandırma deneyleri gerçekleştirildi. Sonuçlar, *A. pleuropneumoniae* ve *H. parasuis*'in erime eğrilerinin Tm değerlerinin sırasıyla bu iki patojeni doğru şekilde ayırt edebilen 83.36±0.09ºC ve 76.48±0.17ºC olduğunu gösterdi. Diğer solunum sistemi patojenleri ile aralarında çapraz reaksiyon gözlenmedi, bu da iki primerin yüksek özgüllüğünü ortaya koydu. Testin saptama hassasiyeti, sıradan PCR yöntemlerinden daha yüksek olan 127 ve 96 kopya/µL idi. Bu hızlı teknik, *A. pleuropneumoniae* ve *H. parasuis*'in eşzamanlı tespiti için basit ve kullanışlı bir seçenek sunabilir;i söz konusu seçenek klinik örneklerin teşhisi ve epidemiyolojik araştırmalar için uygulanabilir ve cazip olacaktır.

Anahtar sözcükler: *Actinobacillus pleuropneumoniae, Haemophilus parasuis, SYBR Green I, Real-Time PCR, Erime eğrisi*

INTRODUCTION

Actinobaccillus pleuropneumoniae is the etiologic agent of porcine pleuropneumonia, which is characterized by serious bleeding and fibrinous pleuropneumonia [1]. The disease is susceptible to various pigs and accompanies

Correspondence
+86-0373-3040718 (Y. Dong), +86-0373-3040718 (L. Wang)
yaoli205@sina.com (Y. Dong), vet_lirong@sina.com (L. Wang)

with high morbidity and mortality. To date, 15 serovars of *A. pleuropneumoniae* have been described. Some *Streptococcus pleuropneumoniae* strains can also cause recessive infections in local herds. Most of them are secondary infections in swine respiratory disease syndrome, which increase the herd mortality under the combined action of other viruses or bacteria [2]. Rapid and accurate determination of the involved pathogen is important for both limiting the severity of an outbreak and tracing the source of the infectious agent. Molecular methods based on genetic differences of *A. pleuropneumoniae*, such as PCR, MPCR have been developed to detect *A. pleuropneumoniae* [3,4]. Furthermore, two real-time PCR assays were constructed to detect *A. pleuropneumoniae* serovars 1-9-11 and serovar 2, which are useful for the serotyping of *A. pleuropneumoniae* in diagnostic laboratories to control porcine pleuropneumonia [5].

Haemophilus parasuis, the causative agent of Glasser's disease, which is characterized by fibrinous polyserositis and arthritis. In addition to Glasser's disease, *H. parasuis* produces other clinical outcomes, such as pneumonia, and colonizes the upper respiratory tract of healthy animals in herds in which acute infections occur only sporadically [6]. In recent years, the immunosuppression caused by various reasons has worsened the occurrence of *H. parasuis* infection, which also easly develops secondary to *A. pleuropneumoniae* and aggravates the disease [7]. The isolation of *H. parasuis* is not as simple as general techniques due to the fragility and fastidious growth requirements of the bacterium. Coupled with classical diagnostic methods, various molecular methods have been developed recently to characterize *H. parasuis* strains [8,9]. PCR was shown to be more sensitive than cultivation.

A. pleuropneumoniae and *H. parasuis* are important pathogens in the respiratory tract of pigs, and the clinical symptoms they cause are difficult to distinguish [10]. Multiple infections also cause difficulties in traditional bacteriological diagnosis. It is necessary to establish a rapid diagnosis and identification method to distinguish the two bacteria. "Repeats in toxin" (RTX) and some toxins (ApxI, ApxII, ApxIII and ApxIV) are involved in the virulence of many organism including *A. pleuropneumoniae*. Unlike other three toxins, ApxIV toxin has been demonstrated to be a specific marker of *A. pleuropneumoniae* infection [11]. Outer membrane protein (OMP) P2 is the most abundant protein in the outer membrane and is a crucial virulent factor of *H. parasuis*. It has been found that OMP P2 belonged to 2 genotypes and is highly conserved in all of the published sequences of *H. parasuis* [12]. A real-time PCR assay based on OMP P2 gene was developed, and performed similar to previously described other diagnostic tool [8].

In this study, according to the each conservative area of *A. pleuropneumoniae* Apx IV and *H. parasuis* omp P2 gene, two pairs of primers were designed, and after the specificity and sensitivity test, a duplex fluorescent quantitative PCR detection method was developed to identify *A. pleuropneumoniae* and *H. parasuis*.

MATERIAL and METHODS

Strains

A. pleuropneumoniae (ATCC27088), *Escherichia coli (ATCC25922)*, *Salmonellae choleraesuis (ATCC10708D-5)*, *Pasteurella multocida (ATCC 31610)*, *Streptococcus aureus (ATCC25923)* and *H. parasuis (ATCC 19417)* were maintained in our laboratory (Microbiology Laboratory of College of Animal Science and Veterinary Medicine, Henan Institute of Science and Technology). *E. coli*, *S. choleraesuis* and *Streptococcus aureus* were cultivated in nutrition agar medium and broth medium at 37°C for 12 h. *A. pleuropneumoniae, P. multocida* and *H. parasuis* were cultivated in tryptic soy broth (TSB, Oxoid) accompany with 1% nicotinamide adenine dinucleotide (NAD, Sigma-Aldrich)and 10% fetal bovine serum (FBS, Sigma-Aldrich) at 37°C for 18 h.

Primers

According to the conserved sequences of *A. pleuropneumoniae* Apx IV gene and *H. parasuis* ompP2 gene published in GenBank database, two pairs of primers were designed for quantitative fluorescence detection through the Primer 5.0 software. The primers were synthesized by Sangon (Shanghai, China). The sequence of two pairs of primers is shown in *Table 1*.

DNA Extraction

Bacterial DNA extraction from organ and cell supernatants was performed using a MiniBEST Universal Genomic DNA Extraction Kit (TaKaRa, Japan) according to the manufacturer's instructions. The purity and concentration of the extracted DNA samples were determined by NanoDrop 2000 spectro-photometer (NanoDrop 2000, Thermo Scientific, Unite state) and stored at -80°C.

Table 1. *Primers for real-time PCR assay used in this study*

Genes	Primer	Sequence (5'-3')	Tm/°C	Length
Apx IV	Forward	GCAACAACGTCGCACAAT	55.4	90bp
	Reverse	GAAGCAGCCAACTCCTCAG	55.3	
Omp P2	Forward	AATCGGTGATAGCATTGGTC	54.8	218bp
	Reverse	ACACTTCTCCCTTATTATTACGC	55.1	

Standard Recombinant Plasmid Construction

The PCR were performed in a thermocycler with 100ng DNA as template in a 20 µL reaction volume containing 1 µM of each primer and 1 U Taq DNA polymerase (Promega, Unite State). The amplification reactions were performed according to the following protocol: 95°C for 5 min, followed by 30 cycles of 95°C for 45 s, 53°C for 45 s, 72°C for 2 min, and a final elongation step of 10 min at 72°C. The equal volume of deionized water was added (replace the template DNA) as a negative template control (NTC) reactions. PCR products were examined by electrophoresis on a 1.5% (w/v) agarose gel and visualized after ethidium bromide (EB). PCR products of the expected length were purified with a Gel Extraction kit (OMEGA, USA), then cloned into the PMD19-T vector (TaKaRa, Japan) according to the manufacturer's instructions and sequenced at BGI (Beijing, China). After the plasmid identification was correct, the plasmid was extracted using a Plasmid mini Kit (OMEGA, USA) followed the manufacturer's procedure. The copy number of extracted plasmid was calculated according to the formula: copies/µL = (6.02×10^{23}) x (plasmid concentration ng/µL$\times 10^{-9}$)/(DNA lengthx660). The plasmid concentration was measured by NanoDrop 2000 spectrophotometer.

Simplex and Duplex SYBR Green Real-Time PCR Assays

Each simplex PCR reaction was carried out in a total reaction volume of 10 µL containing 5 µL 2×SYBR green PCR master mix (ABI, US), 1 µM of each primer and 100ng of DNA template and then adjusted to final volume with double-distilled water. PCR conditions for simplex PCR were: an initial denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 10 s, 55°C 15 s and 72°C for 20 s. The amplified DNA was detected by fluorescence quantification of the double-stranded DNA binding dye SYBR Green with melting curve analysis. The melting curves were acquired at 72°C for 15 s in 1 cycle and then an increase in temperature to 95°C at 0.1°C/s.

The duplex PCR reaction was carried out according to previously described [13]. Briefly, in a total volume of 20 µL in one tube containing 10 µL 2×SYBR green PCR master mix (ABI, US), 1 µM forward and reverse primer of *A. pleuropneumoniae* and 1 µM forward and reverse primer of *H. parasuis*, and 100ng DNA template of *A. pleuropneumoniae*, 100ng DNA template of *H. parasuis* and the final volume was adjusted with deionized water. NTC reactions were carried out simultaneously. The melting curve and PCR condition were similar to simplex PCR. Data were analyzed using ABI QuantStudio 5 Gene software version.

Standard Curve and Limit of Detection

The simplex real-time PCR standard curve was generated for both *A. pleuropneumoniae* and *H. parasuis* by serial 10-fold dilutions of the two recombinant plasmids with a known copy number (from 1×10^{10} to 1×10^{1} copies/µL). These dilutions were tested in triplicate and used as quantification standards to construct the standard curve by plotting the plasmid copy number against the corresponding threshold cycle values (Ct value). The PCR efficiency (E) was calculated using the standard curve slope according to the following formula: $E=(10^{(-1/slope)} -1)$. The correlation coefficient (R^2) was also calculated. A melting curve analysis of the obtained amplification products was carried out.

Sensitivity, Specificity and Repeatability Analysis

The analytical sensitivity of real-time PCR is the lowest amount of sample (the highest dilution of plasmid) which can be detected. Genomic DNA of *E. coli*, *S. choleraesuis*, *P. multocida*, *Str. aureus*, *A. pleuropneumoniae* and *H. parasuis* stored in our laboratory was extracted and employed as a template for specific detection of duplex SYBR green real-time PCR assay. 100ng DNA of each of the above-mentioned bacteria was mixed and used as template to evaluate the specificity of the dual fluorescence quantitative PCR method. All amplification reactions were performed in duplicate in three independent experiments.

The repeatability of the melting temperature analysis was examined by comparing the results obtained from the replicates of three dilutions (10^4, 10^5, 10^6 copies/µL) of both *A. pleuropneumoniae* and *H. parasuis* recombinant plasmids during a single PCR reaction and comparing the mean values obtained from the same plasmids on three different days.

Clinical Sample Testing

Clinical samples of lung materials from 37 pigs with respiratory system infection symptoms were collected, detected and compared respectively by conventional PCR and fluorescent-based quantitative PCR.

RESULTS

Simplex Real-Time PCR Assay

We cloned the expected PCR amplicons (90bp and 218bp) into PMD-19T to constructed *A. pleuropneumoniae* and *H. parasuis* recombinant plasmid respectively. The linearity and efficiency of the SYBR Green real-time PCR were determined by generating a standard curve for each of the two pathogens *(A. pleuropneumoniae* and *H. parasuis)* in which serial 10-fold dilutions of recombinant plasmids were tested. The standard curve was generated by plotting the real-time PCR threshold cycle numbers (Ct) of each dilution against the known copy numbers of recombinant plasmid. The resulting slope showed a linear relationship over 10 orders of magnitude ranging from 1×10^{1} to 1×10^{10} copies/µL for both *A. pleuropneumoniae* and *H. parasuis*

standard plasmids. From the *Fig. 1-A,* the slope was -3.4951 with a coefficient of determination (R2) >0.99 and reaction efficiency (E) of 0.932 for *A. pleuropneumoniae* and -3.7076 with a R2 >0.99 and an E of 0.861 for *H. parasuis*. The LOD determined on the *H. parasuis* standard curves was found to be 127 and 96 copies/µL respectively, thus showing a high sensitivity of the assay.

The optimal concentration of primers for each of these two pathogens was found to be 1 µM. Amplicon melting curve showing a single peak for each set of primers suggests that non-specific amplification products or primer dimmers (results not shown) for each of the two pathogens confirms the specificity of the reaction. The melting temperature analysis showed a variation of Tm values between samples of 83.36°C±0.09 and 76.48°C±0.17 for *A. pleuropneumoniae* and *H. parasuis*, respectively *(Fig. 1-B)*.

These two plasmids with three serial dilutions were tested at different times and under the same conditions. Each sample was repeated three times, and the coefficient of variation of the Ct value was calculated. The results in *Table 2* showed that the intra-assay coefficient of variation and inter-assay coefficient of variation of the three samples were between 0.16% and 0.32%; both less than 10%, indicating that the method was reproducible.

Duplex Real-Time PCR Assays

Duplex assay was developed using two recombinant plasmids in one tube. Optimal primer concentrations for the duplex assays were 1 µM of each primer. The melting curve showed two melting peaks corresponding to the expected Tm values of the simplex PCR assay of *A. pleuropneumoniae* and *H. parasuis (Fig. 2-A)*. The primers did not cross react with other pathogens *(Fig. 2-B)*. This duplex real-time assay enabled the simultaneous detection of *A. pleuropneumoniae* and *H. parasuis* in co-infected samples in a single tube PCR assays.

Validation of Real-Time PCR Assays Using Field Samples

To evaluate the practical utility and accuracy of the duplex real-time PCR assay, a total of 37 susceptive lung samples were tested for the presence of these two bacteria species. Based on the melting curve, 20 samples displayed a peak with a Tm value of 83.4, and 9 samples displayed a peak with a Tm value of 76.6, and 6 samples displayed two peaks with Tm value of 83.4 and 76.6. In contrast, only 4 samples were positive for two bacteria when diagnosed using conventional PCR detection method *(Table 3)*. These results indicate that the duplex real-time PCR assay is more sensitive than bacterial isolate for the detection of co-infection from field samples.

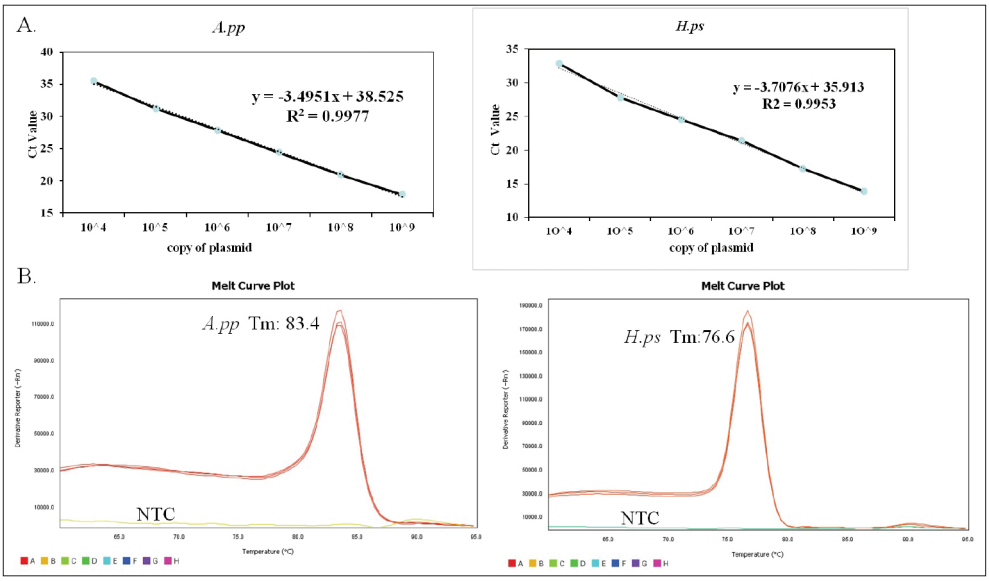

Fig 1. Simplex real time PCR assay of *A. pleuropneumoniae* and *H. parasuis*. **(A)** Standard curves for *A. pleuropneumoniae* and *H. parasuis* using recombinant plasmid template DNA, **(B)** Melting curve analysis showing the melting temperature peaks (Tm) of *A. pleuropneumoniae* and *H. parasuis* and negative template controls (NTC); N=3

Table 2. *Intra- and inter-assay variations of Tm values (°C) obtained from real-time PCR with melting curve analysis*

Pathogens	Intra-assay Variation			Inter-assay Variation		
	Mean	SD	CV (%)	Mean	SD	CV (%)
A. pleuropneumoniae	76.63	0.16	0.22	76.64	0.24	0.32
H. parasuis	83.48	0.12	0.16	83.47	0.17	0.23

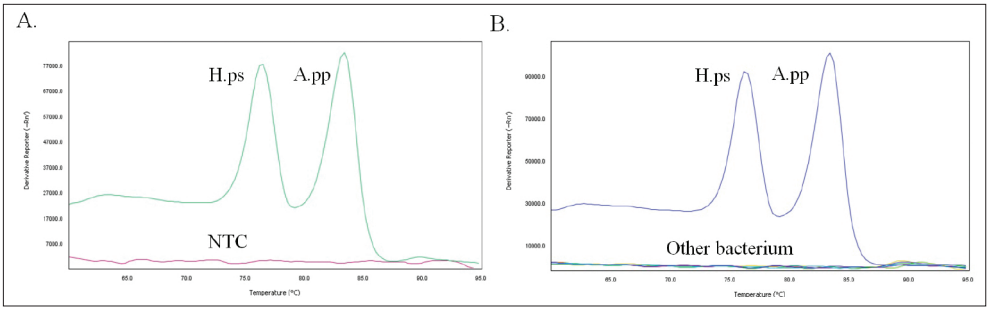

Fig 2. Duplex real time PCR assay. **(A)** Separation of amplicon melting peaks in SYBR® Green duplex real-time quantitative reverse transcription PCR assays. Targets are indicated above each peak along with non-target negative controls (NTC), **(B)** Specificity of duplex real time PCR assay with other respiratory bacterium; N=3

Table 3. *A. pleuropneumoniae and H. parasuis duplex real-time PCR methods were compared with conventional PCR methods*

Method	***A. pleuropneumoniae***	***H. parasuis***	***A. pleuropneumoniae* + *H. parasuis***
Conventional PCR method	19	9	4
Real-time PCR	20	9	6

DISCUSSION

Although several end points PCR have been described previously for the detection and differentiation of *A. pleuropneumoniae* and *H. parasuis*, they usually involve two separate reactions for each bacterium or further procedures (such as DNA sequencing) are needed for species-specific identification [14]. These additional procedures cause consumption of additional time and cost more, thus delay the results. In our research, simplex and duplex SYBR Green based real-time were optimized for the detection of two pathogens. In contrast, TaqMan-based assays are more highly target gene specific than dyes of SYBR® Green in real time PCR assay. However, considering the cost, the assays developed in this study provide a rapid and cost-effective alternative to TaqMan qPCR with similar efficiency and accuracy, particularly useful when large numbers of samples are to be analyzed such as epidemiological investigation.

SYBR® Green in combination with melting curve analysis (MCA) has been successfully used to detect the co-infection of Canine adenovirus type 1 (CAdV-1) and canine adenovirus type 2 (CAdV-2) [15]. In our study, the two primers employed in this research yielded a Tm difference of nearly 7°C (83.4°C, 76.6°C) between the reference strains of *A. pleuropneumoniae* and *H. parasuis* which were most suitable for identifying the two pathogens. This obvious difference in Tm values for *A. pleuropneumoniae* and *H. parasuis* makes it easy to simultaneous identification of these two pathogens in one reaction. This reliable and rapid technique may represent a simple, useful and economic option for simultaneous detection of *A. pleuropneumoniae* and *H. parasuis*.

Compared to Real-time PCR, the conventional PCR is still widely used since it is cheap and easy to operate. The conventional duplex PCR with agarose gels can easily identify two different amplicons in one reaction [16]. Furthermore, these specific two primers involved in our real-time PCR assay can also be used in conventional duplex PCR assay with detection of amplicons by agarose gels for detection and differentiation of *A. pleuropneumoniae* and *H. parasuis* for resource-limited mycology laboratories that do not have a real-time PCR machine. These data suggest that these two primers can be widely used.

In conclusion, the real-time PCR method based on melting point analysis with SYBR Green dye described here is a simple and rapid molecular tool for accurate detection and differentiation of strains of *A. pleuropneumoniae* and *H. parasuis*. In case of multiple infections, the assays will provide valuable information on epidemiological investigation and diagnosis.

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Author Contributions

Conceived and designed the experiments: Y. DONG, L. WANG, S. ZHANG. Performed the experiments: B. Hu, Y. XU, Z. WANG, Q. REN, J. XU. Analyzed the data: B. HU, Y. XU. Wrote the paper: B. HU, S. ZHANG.

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