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Development and Application of a Polymerase Chain Reaction to Early Detect *Haemophilus parasuis*

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Abstract: *Haemophilus parasuis* mainly affect piglets during suckling period and the infected pigs showed clinical symptoms at 5-6 weeks old after weaning. To develop a specific PCR for early detection of *Haemophilus parasuis*, early treatment would effectively prevent phenotypic expression of the disease. A pair of specific primers were designed to amplify a 414 bp specific DNA fragment based on *H. parasuis* Outer Membrane Protein (*OMP*) gene published in GenBank also the PCR conditions were optimized. The established PCR test could detect a minimum of 425 cfu mL^{-1} (0.425 cfu/PCR reaction) pure cultures of *H. parasuis* and only *H. parasuis* could amplify a 414 bp specific DNA fragment. No amplification was observed while tested with the closely affiliated species and the mainly contaminating flora. From 2 days post infection (dpi) to 5 dpi, all of the piglets challenged with *H. parasuis* showed a fever above 41°C and all blood samples from those feverish piglets were positive for *H. parasuis*. Through blood PCR detection, 37 positive samples were picked out in 84 sucking pigs which were natural infected with *H. parasuis*, the result was verified by dissecting five positive piglets and isolating bacteria from lung tissue. The developed PCR was successfully applied for early diagnosis of *H. parasuis* infection, early treatment will reduce the incidence and mortality of disease.

Key words: *Haemophilus parasuis*, OMP, PCR, early detection, blood

INTRODUCTION

Haemophilus parasuis (*H. parasuis*) is a Gram-negative bacillus causes Glasser's disease which is characterized by fibrinous polyserositis, meningitis and arthritis (Amano *et al*., 1994). *H. parasuis* is a commensal bacterium of the upper respiratory tract of conventional pigs that could be isolated from upper respiratory tract of healthy pigs (Moller and Kilian, 1990). But under appropriate conditions, it can invade and cause severe systemic disease of pigs (Rapp-Gabrielson *et al*., 2006) and *H. parasuis* could be isolated from lung of the infected pigs. A recent survey in North American herds showed that those *H. parasuis* from the upper respiratory tract of healthy animals were genetically homogenous (and different from those isolated from systemic sites) and predominant of serotype 3 or non-typable (Oliveira *et al*., 2003).

Serovar has been commonly used as an indicator of *H. parasuis* virulence. Intraperitoneal inoculation of serovars 1, 5, 10, 12, 13 and 14 with SPF pigs, it would cause death or morbidity of the pigs within 4 days and these strains were classified as high virulent *H. parasuis* disease bacteria. Serovars 2, 4 and 15 could cause polyserositis of infected pigs, generally not death and were classified as moderately virulent strains. The remaining serovars (serovars 3, 6, 7, 8, 9 and 11) would not give rise to clinical signs and were considered to be non-virulent strains (Kielstein and Rapp-Gabrielson, 1992; Oliveira and Pijoan, 2004). Serovars 4, 5, 12 and 13 are the main epidemic strains in China (Cai *et al*., 2005).

H. parasuis is a fragile, slow-growing, NAD-dependent organism and antibiotic use could inhibit its growth, all these factors would impair accurate diagnosis of *H. parasuis* even if tissue lesions of pigs are very characteristic. When perfoming bacteria isolation and identification, overgrowth of contaminants often lead to misdiagnosis of *H. parasuis* infections, especially when samples are not collected and transported properly (Kielstein *et al*., 2001).

Oliveira *et al*. (2001) developed a PCR test to diagnose *H. parasuis* based on its 16S rDNA and the PCR Method was successfully used to detect *H. parasuis* in both pure culture and clinical samples which was more sensitive and accurate than bacteria cultivation (Oliveira *et al*., 2001). However, when the PCR test came to the closely affiliated species *A. indolicus*, nonspecific amplificartion appeared, making the interpretation of a

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positive reaction for *H. parasuis* difficult. Angen *et al.* (2007) developed an improved specific PCR test for *H. parasuis* detection, the improved PCR could detect a minimum of 0.5 CFU per PCR reaction and no band was amplified with *A. indolicus*, the method was suitable for detection of *H. parasuis* in clinical samples, regardless of the presence of contaminating flora and affiliated species (Angen *et al*., 2007). Both methods require tissues for diagnosis and don't concern about early detection.

Piglets were always infected with *H. parasuis* during period of suckling and protected by colostral immunity. The antibody level of colostral decreases after weaning at the age of 5-6 weeks, piglets are highly susceptible to the infection and usually show clinical symptoms (Nedbalcova *et al*., 2006). Acute infections are sporadic and the insidious onset usually results in delayed diagnosis and high mortality rate (Nicolet, 1992). In this study, a Polymerase Chain Reaction (PCR) test was developed to investigate a way to *H. parasuis* early diagnosis. PCR Method shows high sensitivity and accuracy in rapid diagnosis of clinical pathogens.

MATERIALS AND METHODS

Bacterial strains and culture conditions: The *H. parasuis* strain SC1101, a moderate virulent strain of serovar 4 was isolated from lung of a diseased pig and stored in the laboratory. The bacteria was routinely cultured on Tryptic Soy Agar (TSA) or in Tryptic Soy Broth (TSB) supplemented with 10 μg mL^{-1} Nicotinamide Adenine Dinucleotide (NAD) and 5% fetal calf serum (Gibco) at 37°C (del Rio *et al*., 2005).

Escherichia coli utilized during the plasmids construction were grown on Luria Bertani (LB) plates or in broth overnight at 37°C with or without ampicillin (50 μg mL^{-1}).

Microorganism used in the experiment *Pasteurella multocida*, *Streptococcus*, *Staphylococcus hyicus* were isolated and identified by the own laboratory, *Escherichia coli* C83684, *Salmonella* C78-2, *Actinobacillus pleuropneumonia*, *Actinobacillus indolicus*, *H. parasuis* serovar 1, 5, 6, 12 and 13 were purchased from China Institute of Veterinary Drugs Control.

DNA extractions: The pure *H. parasuis* strain SC1101 bacterial culture was harvested from TSA plates and suspended in 200 μL ddH_2O, the suspension was boiled for 10 min and centrifuged at 13,000 g for 5 min, the supernatant was collected as DNA template for PCR (Oliveira *et al*., 2001).

PCR primers and conditions: A pair of specific primers were designed to amplify a 414 bp DNA fragment based on the conserved sequence of *H. parasuis* Outer Membrane Protein (*OMP*) gene published in the GenBank [Accession number: CP001321] primers were as follows: F: 5'-CGTTATGAGACTCGCCTTG-3' and R: 5'-TTGAC TTTCCG CAATCTTAG -3'.

The PCR was performed in a total volume of 20 μL containing 1 μL of template DNA (about 10 ng of DNA), 20 pmol of each primer, 10 μL 2×Taq PCR Master Mix (TakaRa) and water was added to 20 μL. The condition of the PCR was as follows: 95°C for 5 min, 30 cycles at 95°C for 40 sec, 55°C for 40 sec, 72°C for 40 sec and final elongation at 72°C for 7 min. The PCR product was analyzed in 1% agarose in Tris-Borate-EDTA (TBE) buffer gel containing 0.5 mg mL^{-1} ethidium bromide.

PCR sensitivity and specificity: Serial 10 fold dilutions of *H. parasuis* (strain SC1101, serotype 4) broth culture (about 4×10^8 CFU mL^{-1}) were tested by PCR to determine the sensitivity. *Pasteurella multocida*, *Streptococcus, Staphylococcus hyicus, Actinobacillus indolicus, Escherichia coli* C83684, *Salmonella* C78-7, *Actinobacillus pleuropneumonia* as well as *H. parasuis* serovar 1, 4, 5, 6, 12 and 13 were used to test the PCR specificity. DNA was extracted as described.

PCR test of clinical samples from experimental artificial infection: Nine, 35 days old SPF piglets were divided into two groups, six piglets were challenged with 2 mL (10^8 Colony-Forming Unit (CFU) per milliliter) of *H. parasuis* strain SC1101 through nasal spray. The last three pigs were challenged with 2 mL of Phosphate Buffered Saline (PBS) pH 7.4 through nasal spray and served as negative control. The project was supervised and supported by China Animal Protection Association. After artificial infection, the pigs were maintained separately in two rooms and given food and water for 10 days. Nasal swabs and blood samples were collected at 2, 4, 6, 8, 10 days post infection. After 10 days, all animals were euthanized and lung samples were taken to detect *H. parasuis*. All samples were inoculated into TSB and cultured overnight at 37°C with supplemental NAD, the bacteria culture was collected and DNA was extracted as described. The optimized PCR was performed to test all samples.

PCR test of clinical samples from natural infection: Blood samples were collected from eighty four suckling pigs with cough through precaval vein in Sichuan Province, China and chromosomal DNA were prepared as method described in DNA extractions. Five positive piglets were dissected in the aseptic technique to collect lung tissues for further verifying the PCR detection of blood samples (Table 1) and the test was supported by China Animal Protection Association.

Table 1: *H. parasuis* isolation and PCR results for clinical samples from naturally infected animals

Samples	Age (days)	Region	Macroscopic findings	*H. parasuis* isolation	PCR detection
24 blood samples	16-21 days old piglets	Meishan (SC)	Cough, fever and running nose	Positive 8/24	Positive 18/24
35 blood samples	15-17 days	Ya'an (SC)	Cough, fever and running nose	Positive 7/35	Positive 8/35
25 blood samples	20-27 days	Deyang (SC)	Cough and fever	Positive 3/25	Positive 11/25
Blood (a*)	18 days	Meishan (SC)	Cough, fever and running nose	-	+
Lung (a)	18 days	Meishan (SC)		+	+
Blood (b)	20 days	Meishan (SC)	Cough, fever and fibrinous polyserositis	-	+
Lung (b)	20 days	Meishan (SC)		+	+
Blood (c)	16 days	Ya'an (SC)	Cough and fever	+	+
Lung (c)	16 days	Ya'an (SC)		+	+
Blood (d)	21 days	Deyang (SC)	Cough, fever and neurological deficit	+	+
Lung (d)	21 days	Deyang (SC)		+	+
Blood (e)	21 days	Deyang (SC)	Cough and fever	+	+
Lung (e)	21 days	Deyang (SC)		+	+

*Animal a, b, c, d and e; SC = Sichuan Province China; + = Positive; - = Negative

RESULTS AND DISCUSSION

Amplification and sequence alignment: A specific amplified band was observed when tested with *H. parasuis* strain SC1101 and the product was inserted into pMD18-T vector for sequencing. After comparing with sequences in the GenBank by BLAST, the amplicon had 99% similarity with *H. parasuis OMP P2* gene. The PCR test was successfully amplified a purposed fragment of 414 bp with *H. parasuis* strain SC1101.

PCR sensitivity and specificity: The PCR could detect a minimum 425 CFU mL^{-1} (1 μL per PCR reaction equal to 0.425 cfu/PCR reaction) pure cultures of *H. parasuis* organisms, the method was as sensitive as the improved PCR test developed by Angen (0.5 CFU/PCR reaction) (Angen *et al*., 2007). Specific bands were observed in the range of 425 CFU mL^{-1} *H. parasuis* strain SC1101 to above 4×10^6 CFU mL^{-1} (0.425 CFU to above 4000 CFU per reaction) (Fig. 1).

The worldwide epidemic strains *H. parasuis* serovars 1, 4, 5, 6, 12 and 13 the closely affiliated species as *Pasteurella multocida*, *Actinobacillus indolicus*, *Actinobacillus pleuropneumonia* and the mainly contaminating flora *Streptococcus*, *Staphylococcus hyicus*, *Escherichia coli* C83684, Salmonella C78-7 were selected to test the specificity of the new PCR Method. Every *H. parasuis* strain was observed a 414 bp band as predicted (Fig. 2) and no bands appeared in the seven other bacterial species that were tested (Fig. 3). The new PCR test successfully overcame the cross-reaction with closely affiliated species as specificity as the improved PCR test developed by Angen *et al*. (2007).

Application of clinical samples from experimental artificial infection: Most of the artificially infected piglets showed high fever 24 h post infection (pi) and all of the

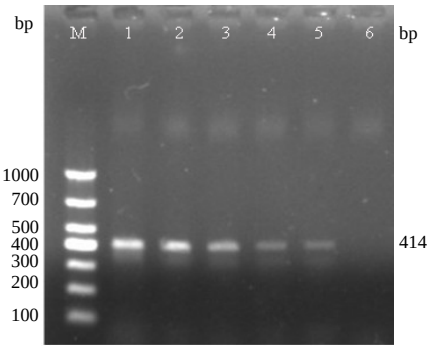

Fig. 1: Effect of template concentrations on PCR. M: DNA Marker DL2000; 1: 4×10^6 cfu mL^{-1}; 2: 4×10^5 cfu mL^{-1}; 3: 4×10^4 cfu mL^{-1}; 4: 4250 cfu mL^{-1}; 5: 425cfu mL^{-1}; 6: <50 cfu mL^{-1}

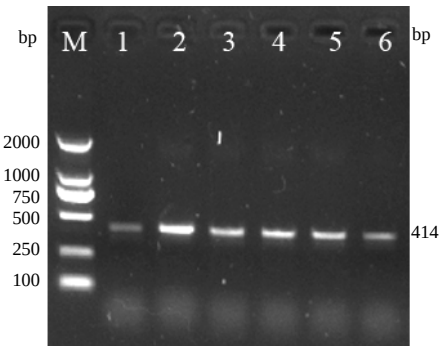

Fig. 2: The specificity of the new PCR Method

them had a fever above 41°C during 2-5 dpi (Fig. 4a), in addition, coughing, nose running and inappetency went along. On 6 dpi all the experimental pigs manifested a normal temperature.

The PCR was used to detect *H. parasuis* with nasal swabs and blood samples and the results revealed that *H. parasuis* could be detected from nasal swabs during 2-6 dpi (Fig. 5) and blood samples during 2-4 dpi

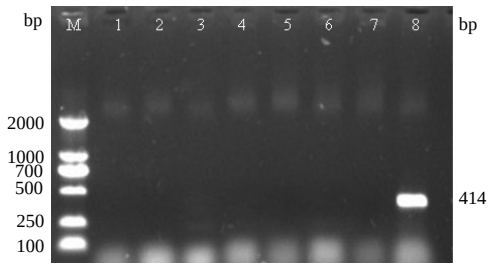

Fig. 3: Specificity test of the PCR test; 1: *Pasteurella multocida*; 2: *Hemolytic streptococcus*; 3: *Aureus staphylococcus*; 4: *Actinobacillus indolicus*; 5: *Escherichia coli* C83684; 6: Salmonella C78-7; 7: *actinobacillus pleuropneumoniae*; 8: *H. parasuis* serovar 4

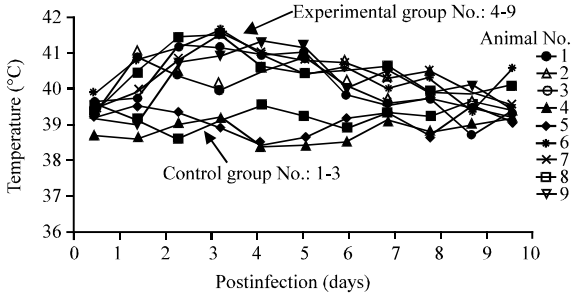

Fig. 4: Body temperature changes of animals

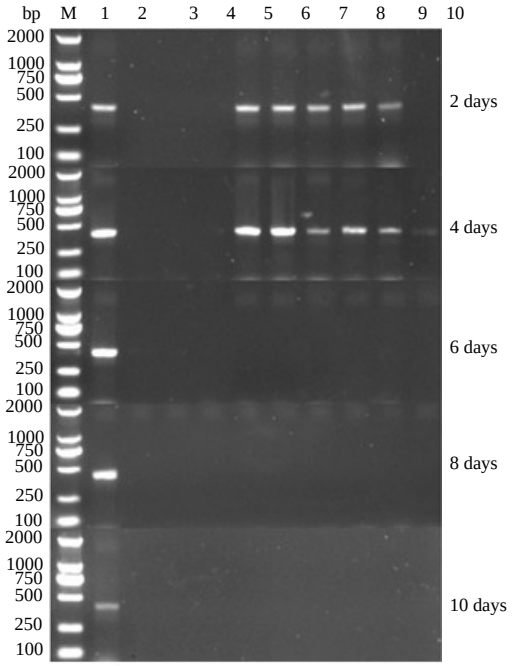

Fig. 5: Detection results of the nasal swab. M: DNA Marker DL2000; 1: Positive sample; 2-4: control group; 5-10: experimental group

(Fig. 6). But it was not stable in nasal swabs detection, many infected piglets could not be picked out. When infected piglets had a fever during 2-4 dpi, *H. parasuis* could be 100% picked out through PCR in blood samples. *H. parasuis* isolation was failed in nasal swabs during all the time but succeeded in some blood samples. Nasal swabs in spite of characteristic lesions, misdiagnosis of *H. parasuis* infections often occur in PCR test and bacteria isolation, this may mainly due to overgrowth of contaminants. The negative control had never showed any symptoms of *H. parasuis* all the way. At necropsy on 10 dpi, severe fibrinous polyserositisand meningitis was observed, arthritis was not distinct. *Haemophilus parasuis* isolation and PCR results were compatible positive to all of the lung samples obtained from the experimental artificial infected animals and negative to control group.

Application of clinical samples from natural infection: The PCR results for clinical samples are summarized in Table 1. All of the blood samples were collected from sucking pigs with cough and fever to early detect *H. parasuis*. Isolation of *H. parasuis* was successful on 18 out of 84 samples in blood and PCR was positive for 37 out of these 84 blood samples. Samples obtained from naturally infected animals showed higher variation regarding *H. parasuis* isolation and PCR results. Five

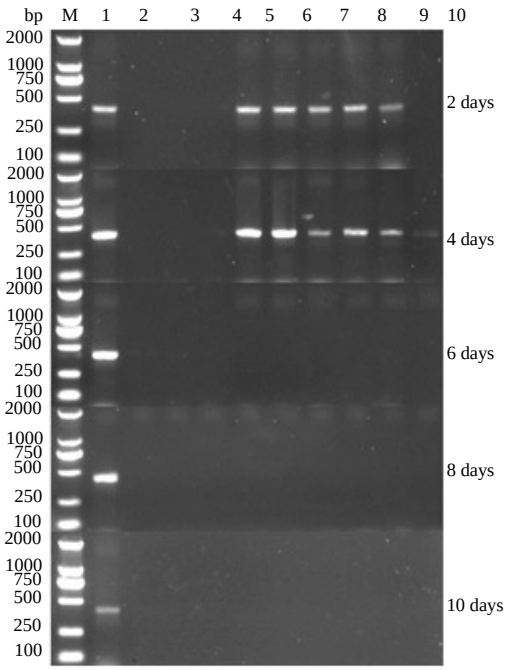

Fig. 6: Detection results of the blood samples. M: DNA Marker DL2000; 1: Positive sample; 2~4: control group; 5~10: experimental group

blood detection positive piglets, named a, b, c, d and e were dissected to collect lungs for bacteria cultivation and tissue PCR detection and *H. parasuis* was isolated from all of their lung tissues. The new method highly showed advantage on *H. parasuis* detection without killing animals, especially on early diagnosis. Combine early clinical symptom (cough and fever) with PCR test in blood, early detection of *H. parasuis* will come true. In this study, 16-27 days old infected suckling pigs were picked out without pathological injure, *H. parasuis* was isolated from their lung tissues which meant the pigs were infected with virulent *H. parasuis*, non-virulent *H. parasuis* strians could never copy in lung.

H. parasuis was the main co-infection pathogenic of Porcine Reproductive and Respiratory Syndrome (PRRS). They are the most economically important diseases that affect swine industry worldwide and causes great economic loss every year. PRRS virus replicates mainly in Porcine Alveolar Macrophages (PAMs) and characterized with interstitial pneumonia and immunosuppression (Xiao *et al*., 2010) which offers favorable conditions for *H. parasuis* to reproduce Initial symptom of *H. parasuis* infection is calorific and running nose which are easy to confused with epidemic colds. The pathological characteristic containing fibrinous polyserositis and arthritis appeared in the later period of this disease and then *H. parasuis* sequestered in biofilms to shield from being attacked by the host immune system and possess more obstinate properties to resist antibiotics compared to the planktonic counterparts. The insidious onset and slow progression of symptoms usually result in delayed diagnosis and high death rate. It is very important to develop a method for early detection of *H. parasuis*. Early treatment could improve the cure rate and greatly reduce the incidence and mortality of the disease.

Although, there were some PCR test for *H. parasuis* detection such as Oliveira *et al*. (2001) developed based on its 16S rDNA sequence and the improved PCR test developed by Angen *et al*. (2007), all of them did not concern about early detection and diagnosis without killing pigs. In this study, a set of primers were designed by the highly conservative outer membrane protein of *H. parasuis*. The PCR could detect a minimum concentration of 0.425CFU/PCR reaction of *H. parasuis* organisms which was as sensitive as the improved PCR test (0.5CFU/PCR reaction) developed by Angen *et al*. (2007) and without cross-reaction with the closely affiliated species of *H. parasuis* and the mainly contaminating flora in upper respiratory tract such as *Actinobacillus indolicus, Actinobacillus pleuropneumonia* and *Streptococcus suis*.

More importantly, the new PCR test was used to explore early diagnosis of *H. parasuis* infection. Kielstein *et al*. (2001) reported that samples obtained from systemic sites such as pleura, pericardium, peritoneum, meninges or joints is a better option for diagnosing *H. parasuis* infection (Kielstein *et al*., 2001). In this study, blood was selected as the proper sample for *H. parasuis* diagnosis without killing piglets. During 2-4 dpi, all the experimentally infected piglets could be 100% picked out through PCR test in blood which would due to a quick bacteremia after *H. parasuis* infection while pathogenic bacterium diffusing to different tissues of host. Combining with fever and cough in this period, the new method may succeed in early detection of *H. parasuis* though PCR test in blood.

Eighty four suckling pigs with cough and fever were selected to test the new method of early detection of *H. parasuis*. The 37 positive samples were successfully picked out from 84 blood samples and a part of selected results were verified by *H. parasuis* isolation from lung tissues. The new test could distinguish *H. parasuis* from swine flu within cough and fever piglets, it makes *H. parasuis* diagnosis earlier and more accurate, guiding early treatment before *H. parasuis* sequestered in biofilms to shield from being attacked by the host immune system and antibiotics.

Nasal swabs could also realize live diagnosis but it was not stable. Some of the real infected piglets failed to be picked out. Sharing the same results as Kielstein *et al*. (2001) and Angen *et al*. (2007) reported, samples obtained from the upper respiratory tract may not be the suitable ones to assess *H. parasuis* infections in swine herds. The main reason for failing detection in nasal swabs may be the existence of contaminants. Secondly, *H. parasuis* is a very fragile Gram-negative bacillus. What's more, *H. parasuis*, normally avirulent was found colonizing the nasal cavity of high-health animals which would make the interpretation of a positive reaction difficult.

CONCLUSION

The most important step for the PCR test was sample collection. In this study, samples were detected within 6 h after collection but it's hard to come true in practical research. Misdiagnosis of *H. parasuis* infections often occurs while samples were not properly collected and transported (Oliveira *et al*., 2001). *H. parasuis* could maintain vigorous for a period of time in tissue but it's not suitable for live detection. DNA extraction from blood will contain a large of genomic DNA of host and interfere *H. parasuis* diagnosis, so the new method strongly recommends that samples should be saved and transported in TSB that will keep *H. parasuis* alive and improve the accuracy of the test.

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