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RESEARCH ARTICLE

Detection of *Actinobacillus pleuropneumoniae* through Duplex PCR Based on ApxIA and ApxIVA Genes

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ABSTRACT

Actinobacillus pleuropneumoniae (APP) is the bacterial pathogen of pleuropneumoniae, a severe contagious and fatal respiratory disease of swine. For the purpose of setting up an easy way to detect this bacteria in swine, a duplex polymerase chain reaction (PCR) assay was built up for the detection of APP. Two pairs of primers were designed according to the APP ApxI and ApxIV gene sequences. Using these primers, a duplex PCR method was developed to fast identify APP in different bacteria samples, such as *Enterococcus faecalis, Escherichia coli* from cattle and swine, *Staphylococcus aureus* and *Streptococcus.* Our results have shown that duplex PCR could be a potential molecular method for the diagnosis of APP infection in swine.

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INTRODUCTION

Porcine pleuropneumoniae (PCP) is a highly fatal infectious respiratory disease of swine. Its main pathogenic bacteria are *Actinobacillus pleuropneumoniae* (APP) (To *et al.*, 2018). Acute PCP can cause serious economic losses and slow growth of infected swine (Gómez-Laguna *et al.*, 2014). It commonly occurs between age 8 and 16 weeks in pigs. Its clinical signs are characterized by increased respiratory rate, dyspnea, high fever, sneezing, coughing, and severe respiratory distress with cyanosis (Bosse *et al.*, 2002).

The Gram-negative bacterium *Actinobacillus pleuropneumoniae* is recognized as an obligate parasite in the respiratory tract of pigs (Bosse *et al.*, 2002; Antenucci *et al.*, 2017). Until now, 15 serotypes of *Actinobacillus pleuropneumonia* have been identified which secrete four types of RTX (repeats-in-toxin) toxins (ApxIA, ApxIIA, ApxIIIA, and ApxIVA) (Shin *et al.*, 2011; Gómez-Laguna *et al.*, 2014; To *et al.*, 2016; Yee *et al.*, 2018). RTX toxins are the main factors of *Actinobacillus pleuropneumoniae* virulence and the virulence level of each *Actinobacillus pleuropneumoniae* serotype may be directly related to the presence of Apx toxins (Park *et al.*, 2009; Hsu *et al.*, 2016; Hathroubi *et al.*, 2017). ApxIA is a strongly hemolytic and cytotoxic 105-kDa protein (Frey, 1995; Liu *et al.*, 2009; To *et al.*, 2016). It is commonly secreted by the most virulent strains of serotypes 1, 5, 9, 10 and 11 (Jansen *et al.*, 1993; Yee *et al.*, 2018). ApxIIA is also a 105-kDa moderately hemolytic and weakly cytotoxic protein secreted by all kinds of serotype strains except serotype 10 (Jansen *et al.*, 1993; Frey, 1995). ApxIIIA is a strongly cytotoxic but non-hemolytic 112.8-kDa protein secreted by the serotypes 2-4, 6, 8 and 15 with (Frey, 1995; Huang *et al.*, 2006; Liu *et al.*, 2009). ApxIVA is a 200-kDaweakly-hemolytic protein predicted from the protein sequence and secreted by all serotypes (Bosse *et al.*, 2002; Huang *et al.*, 2006).

However, due to the difference among the feeding environment, strain virulence, the immune status of animals and infecting dose, APP can cause infected pigs to develop a per-acute, acute, sub-acute or chronic form of the disease (Savoye *et al.*, 2000). The upper respiratory tract or the tonsils of the chronic form or asymptomatic

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carriage pig may harbor APP which can infect other pigs in the same pigsty (Savoye *et al*., 2000). Therefore, it is helpful to develop an effective way of the detection of virulent APP.

In this study, we intended to develop a duplex polymerase chain reaction (PCR) test for the detection and screening of the virulent APP based on the ApxIA and ApxIVA gene segments. We compared the difference in annealing temperature, concentration of primers and DNA template between the single PCR and the duplex PCR. We evaluated the specificity of this duplex PCR using strains of commonly-used clinical bacteria to ensure the validity of this assay.

MATERIALS AND METHODS

Bacterial strains, media and growth conditions: In this study, *Actinobacillus pleuropneumoniae* was purchased from China Veterinary Culture Collection Center. The other strains such as *Enterococcus faecalis* and *Escherichia coli* isolated from pig intestinal tract, *Staphylococcus aureus* and Streptococcus isolated from pig liver were provided by the Laboratory of Clinical Veterinary Medicine, Guangxi University. The strains of *Actinobacillus pleuropneumoniae* were cultured in 3% Tryptic soy broth (TBS) complemented with 10μg/mL of NAD. The other strains were grown in LB Broth.

DNA extraction and PCR amplification: The whole bacterial DNA for PCR amplification was extracted by DNA extraction kit. The detection primers of APXI A-F: 5'-TCGGTCGTAGCATTAGCG-3' and APXI A-R: 5'-GACATCCCAACGCTGTTG-3'weredesigned according to the gene of *Actinobacillus pleuropneumoniae* ApxI (PCR product size: 507; GenBank accession no. AF240779.1). The detection primers of ApxIV A-F: 5'-CTTTTTTGTTATAGAAGAATCA-3' and ApxIV A-R: 5'-TCGTCAATAGGCGTAACAGTT-3' were designed according to the gene of *Actinobacillus pleuropneumoniae* ApxIV (PCR product size: 1092; GenBank accession no. FJ 848574.1). The single PCR reactions were set up into 20μL volume containing 10μL 2×Taq Master Mix, 1μL of each primer (2μmol/L of each primer), and 1μL of the extracted DNA template, and the 7μL of the ddH_2O. The configuration was 95℃ for 3 min, then denaturation at 94℃ for 30 seconds, annealing for 30 seconds, extension at 72℃ for 1 min for 35 cycles, and then a final extension step 72℃ for 10min in a thermocycler. Meanwhile, the optional annealing temperature, concentration of DNA and primers were carried out. Briefly, 6 temperatures at an interval of 2℃ between 50℃ to 60℃ were used as the annealing temperatures for the ApxIA and ApxIVA amplification; 10 different concentrations of primer at an interval of 0.2ng/mL from 0.2ng/mL to 2.0ng/mL were used to select the best concentration of primers for target gene amplification; 10 different concentrations of DNA at an interval of 15μg/mL from 15μg/mL to 150μg/mL were used to examine the sensibility of the PCR reaction. The results of PCR reactions were examined by electrophoresis on 1% agarose and visualized by the GelDoc XR System (Bio-Rad, USA).

Development of a duplex PCR assay for the detection of *Actinobacillus pleuropneumoniae*: Duplex PCR amplification conditions were 95℃ for 3 min, then denaturation at 94℃ for 30seconds, annealing for 30sec, extension at 72℃ for 1 min for 35cycles, and then a final extension step 72℃ for 10min in a thermocycler. Meanwhile, the optional annealing temperature, concentration of DNA and primer, PCR reaction system were carried out. Briefly, 6 different temperatures at an interval of 2℃ from 50℃ to 60℃ were used as the annealing temperature; 9 different volume of PCR reaction system at an interval of 5μL from 10μL to 50μL, were used to this study; 10 different concentration of primer at an interval of 0.2ng/mL from 0.2ng/mL to were used to choose the best concentration of primer for target gene amplification; 10 different concentration of DNA at an interval of 24μg/mL from 24μg/mL to 240μg/mL were used to examine the sensibility of the ApxIA gene detection; 10 different concentration of DNA at an interval of 15μg/mL from 15μg/mL to 150μg/mL were used to examine the sensibility of the ApxIVA gene detection. The results of PCR reactions were examined by electrophoresis on 1% agarose and visualized by the GelDoc XR System (Bio-Rad, USA).

Specificity of the duplex PCR assay: The specificity of the duplex PCR was evaluated by comparing the results obtained using DNA extracts from the strains of *Enterococcus faecalis*, *Escherichia coli*, *Staphylococcus aureus* and *Streptococcus*. The duplex PCR products were purified by commercially available kit (TaKaRa, Otsu, Japan), and then sequencing analysis was conducted (Sangon Biotech, Shanghai, China).

RESULTS

Single PCR results: Different annealing temperatures had various effects on ApxIA gene amplification, whereas there was no obvious visualization difference in the ApxIVA gene products (Fig.1A and 1B). The results showed that the annealing temperature 52℃, 54℃, 56℃, 58℃ were the best temperature for the ApxIA gene amplification (Fig. 1A). The results of different concentrations of primers showed that the higher the primer concentration was, the brighter the strap of PCR was (Fig. 2A and 2B). The best concentration of primer was determined to be 2.0ng/mL. The results of different concentrations of DNA showed that the higher the DNA concentration was, the brighter the strap of PCR product was in our study (Fig. 3A and B).

Duplex PCR results: The results of six different annealing temperatures showed that 58℃ and 60℃ are the best temperatures for the ApxIA and ApxIVA gene amplification (Fig. 4A). The results of 9 different volumes of PCR reaction systems showed that 30μL is the best for the ApxIA and ApxIVA gene amplification (Fig.4B). Under the 10 different ApxIA primer concentrations, the results showed that the concentrations between 0.6ng/mL and 2.0ng/mL are conductive to the ApxIA and ApxIVA gene amplification (Fig. 5A). In the 10 different concentrations of ApxIVA primer, the results showed that the concentration 1.0ng/mL is the best for the ApxIA and ApxIVA gene amplification (Fig. 5A). The PCR sensitivity examination showed that the DNA concentration 15μg/mL is the best concentration for the duplex PCR detection.

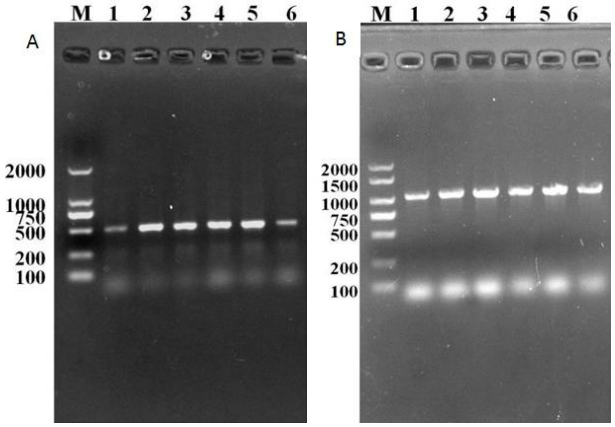

Fig. 1: Effects of 6 different annealing temperatures in ApxIA (A) and ApxIVA (B) gene amplification. M: DNA Marker 2000; 1-6: Annealing temperature 50, 52, 54, 56, 58 and 60°C.

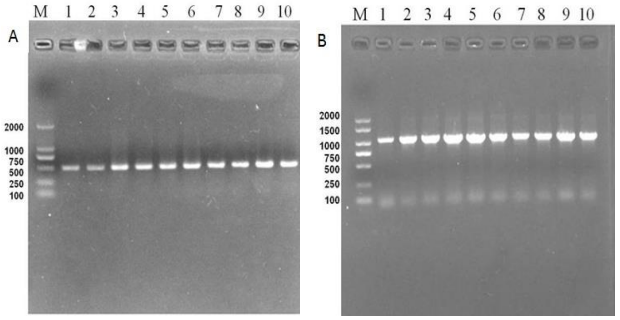

Fig. 2: Results of 10 different concentrations of ApxI A (A) ApxIV A (B) primer primer in PCR amplification. M: DNA Marker 2000; 1-10: primer concentration 0.2ng/m, 0.4ng/mL, 0.6ng/mL, 0.8ng/mL, 1.0ng/m, 1.2ng/mL, 1.4ng/mL, 1.6ng/mL, 1.8ng/mL, 2.0ng/mL.

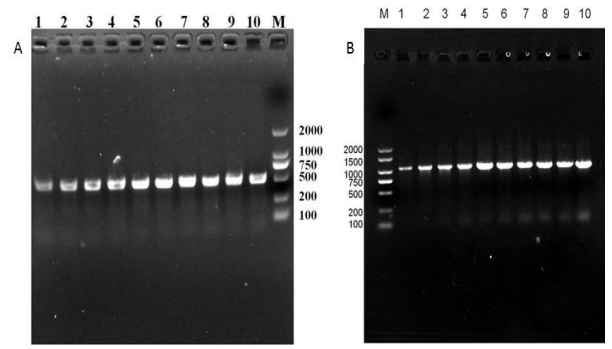

Fig. 3. A: Results of sensibility detection in ApxIA gene. M: DNA Marker 2000; 1-10: DNA concentration 24, 48, 72, 96, 120, 144, 168, 192, 216 and 240 μg/mL. **B:** Results of sensibility detection in ApxIVA gene.M: DNA Marker 2000; 1-10: DNA concentration 15, 30, 45, 60 75, 90, 105, 120, 135 and 150 μg/mL.

Specificity of the duplex PCR assay: To test whether the duplex PCR assay can specifically amplify the ApxIA and ApxIVA gene of *Actinobacillus pleuropneumoniae,* it was used to test the strains of *Enterococcus faecalis* and *Escherichia coli, Staphylococcus aureus* and *Streptococcus*. The results showed that the duplex PCR assay could specifically amplify the ApxIA and ApxIVA gene of *Actinobacillus pleuropneumoniae* (Fig. 5B). The sequencing and NCBI blast analysis of the products of PCR confirmed that these sequences belong to the ApxIA and ApxIVA gene of *Actinobacillus pleuropneumoniae* (Fig. 6-7).

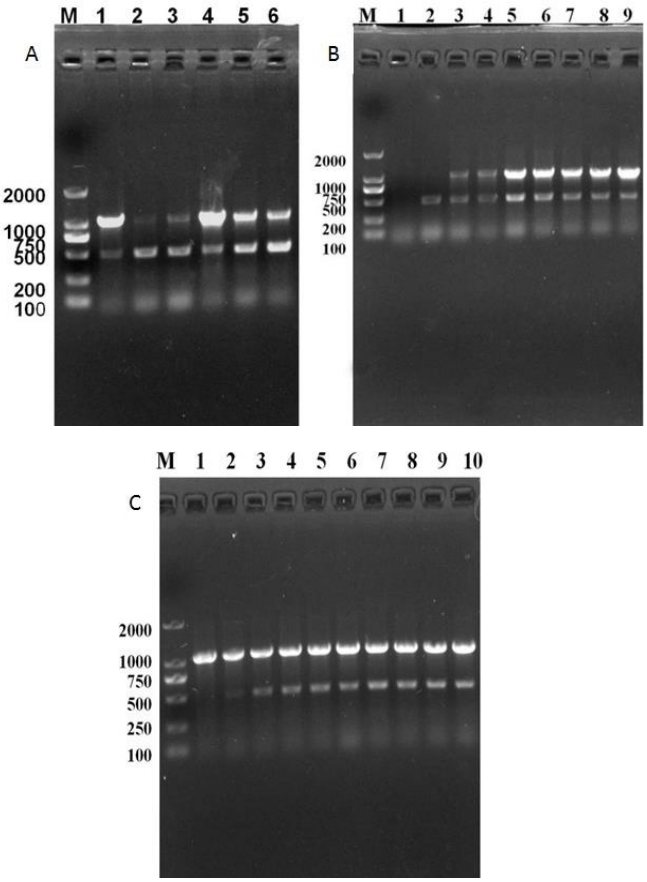

Fig. 4. A: Effects of 6 different annealing temperatures in duplex PCR amplification. M: DNA Marker 2000; 1-10: annealing temperature 50°C, 52°C, 54°C, 56°C, 58°C, 60°C. **B:** Effects of 9 different volumes of PCR reaction system in duplex PCR detection. M: DNA Marker 2000; 1-10: PCR reaction system volume 10μl, 15μl, 20μl, 25μl, 30μl, 35μl, 40μl, 45μl, 50μl. **C:** Effects of 10 different ApxI A primer concentrations in duplex PCR detection. M: DNA Marker 2000; 1-10: ApxI A primer concentration 0.2ng/mL, 0.4ng/mL, 0.6ng/mL, 0.8ng/mL, 1.0ng/mL, 1.2ng/mL, 1.4ng/mL, 1.6ng/mL, 1.8ng/mL, 2.0ng/mL.

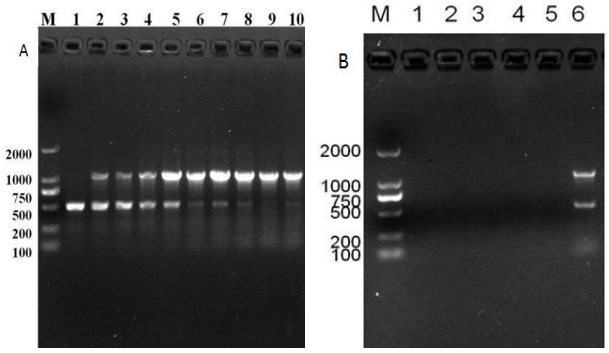

Fig. 5. A: Effects of 10 different ApxIVA primer concentrations in duplex PCR detection. M: DNA Marker 2000; 1-10: ApxIV A primer concentration 0.2ng/mL, 0.4ng/mL, 0.6ng/mL, 0.8ng/mL, 1.0ng/mL, 1.2ng/mL, 1.4ng/mL, 1.6ng/mL, 1.8ng/mL, 2.0ng/mL. **B:** Results of specificity detection in duplex PCR. M: DNA Marker 2000; 1-6: *Staphylococcus aureus*, *Escherichia coli* from cattle, *Enterococcus faecalis, Escherichia coli* from swine, *Streptococcus*, *Actinobacillus pleuropneumoniae*.

DISCUSSION

Previous studies have reported several techniques for the identification of *Actinobacillus pleuropneumoniae* such as ribotyping and restriction analysis of PCR(PCR-REA) (Jaglic *et al.*, 2004), multiple PCR based on the omlA gene

(Angen *et al.*, 2008) and enzyme-linked immunosorbent assay (ELISA) using the recombinant ApxIA, ApxIIA, ApxIIIA, ApxIVA proteins and lipopolysaccharide (Shin *et al.*, 2011; Sassu *et al.*, 2017; Teshima *et al.*, 2017). Tremblay *et al.* (2017) developed an ApxIV probe which could specifically detect APP in the swine lung tissue.

Apx exotoxins secreted by *Actinobacillus pleuropneumoniae* are toxic factors that deeply impact the pathogenesis of porcine pleuropneumonia. Former studies reported that ApxIVA is produced by all the 15 serotypes of APP and is highly specific to APP (Wei *et al.*, 2012; Giménez-Lirola *et al.*, 2014). Due to the specificity of the ApxIVA gene to the *Actinobacillus pleuropneumoniae* strains, the detection assays based on this gene have been widely explored for the identification of the *Actinobacillus pleuropneumoniae* strains (Schaller *et al.*, 2001; Dreyfus *et al.*, 2004; Turni and Blackall, 2007). Previous studies have reported that ApxI A may be the most virulent protein with the product in the strains could increase 100-fold of bacterial toxicity (Liu *et al.*, 2009; Hsu *et al.*, 2016; To *et al.*, 2016). Shin *et al.* (2011) studies have developed a useful ELISA for the detection of *Actinobacillus pleuropneumoniae* based on the Apx exotoxins (ApxIA, ApxII and ApxIIIA). But its major use was the detection of serum antibodies to *Actinobacillus pleuropneumoniae* and this assay was difficult to develop and apply for clinical test. Tremblay *et al.* (2017) developed a effective fluorescence in situ hybridization way using an ApxIV probe for the detection of *Actinobacillus pleuropneumoniae.* This way could specifically localize *Actinobacillus pleuropneumoniae* cells within the lung tissue by using species-specific oligonucleotide probe. But this assay was complicated and also difficult to apply for *Actinobacillus pleuropneumoniae* clinical test. In this study, the duplex PCR assay was developed for the detection of *Actinobacillus pleuropneumoniae* strains that could simultaneously produce ApxIA and ApxIVA toxins. According to the results of single PCR (Fig.1-3), we found that the primers we designed could specifically amplify the ApxIA and ApxIVA gene segments of the *Actinobacillus pleuropneumoniae* strains. According to the results of duplex PCR, we found that an increase in ApxIVA primer concentration could induce a decrease in the ApxIA product (Fig.4B). It means that the same best reaction conditions optimized for the *single* PCR could be inappropriate to set up the *duplex* PCR. But it could help us to examine whether the primers could specifically amplify the target gene. In the duplex PCR, it is important to design the fully-optimized primers for the target gene to avoid non-specific binding among the primers. Meanwhile, the optimal template concentration, optimal annealing temperature and optimal reaction volume were also playing an important role in effectively setting up the useful duplex PCR. In this study, we also used this duplex PCR to detect other common veterinary clinical bacteria to confirm the specificity of this duplex PCR in the *Actinobacillus pleuropneumoniae* strains detection. In conclusion, the results obtained in this research show that the duplex PCR test is a specific, sensitive, and highly effective diagnostic way for simultaneous identification of *Actinobacillus pleuropneumoniae*. It could be a useful way to quickly diagnose and control the infection of *Actinobacillus pleuropneumoniae* in pigs.

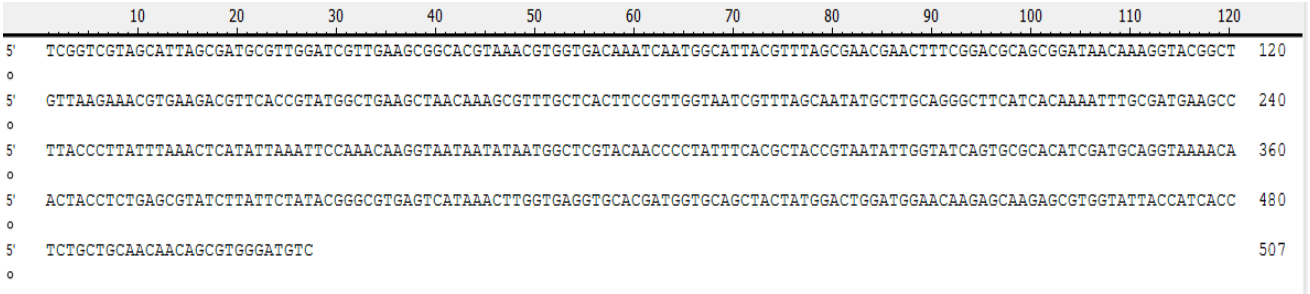

Fig. 6: The sequence of ApxIA gene.

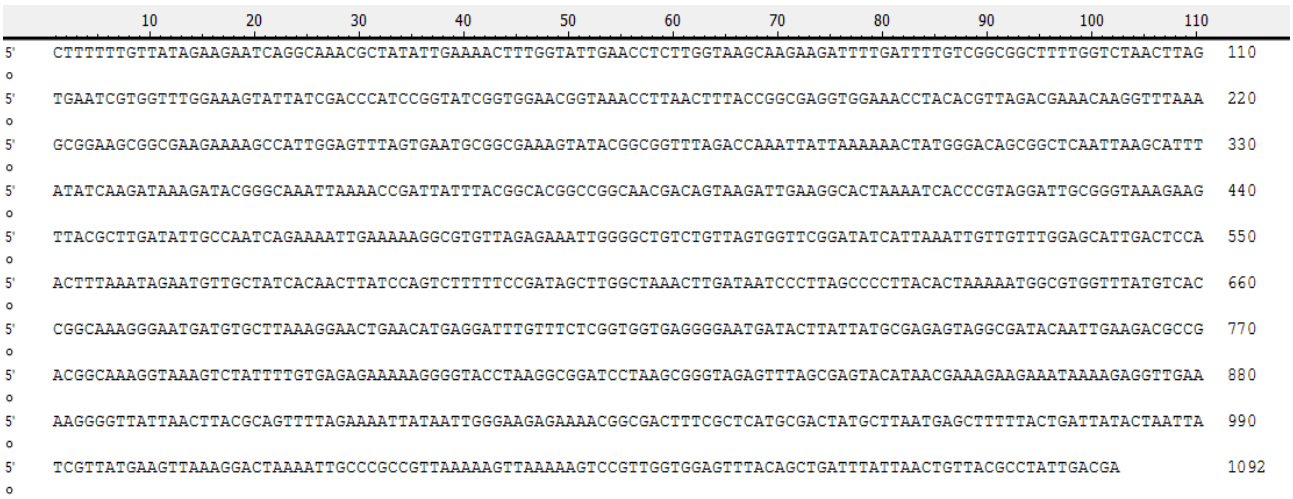

Fig. 7: The sequence of ApxIVA gene.

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Authors contribution: PL, GH and XG conceived and designed the experiment. HL, HM, SC MY, HC, YH, CW, ZX, YR, LL and ZX executed the experiment of bacteria sample collection, bacteria DNA extraction, PCR amplification and gene sequence. PL and HL analyzed the data and wrote the paper; All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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