Application of PCR technique in diagnosis of four respiratory pathogenic bacteria in pigs at the slaughterhouse

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ABSTRACT

The polymerase chain reaction (PCR) for *Actinobacillus pleuropneumonia* (*App*), *Haemophilus parasuis* (*Hps*), *Pasteurella multocida* (*Pm*) and *Bordetella bronchiseptica* (*Bb*) were performed in pure colonies isolated from 114 lung specimens with lesions collected from the Vissan slaughterhouse in Ho Chi Minh City from July 2018 to May 2019. The aim of the experiment was to identify the four respiratory pathogenic bacteria in pigs at slaughterhouse by using PCR technique. The criteria for evaluating the results included the proportion of positive samples with multiplex PCR and percentage of samples co-infected with 2, 3, and 4 bacteria. Among a total of 114 injured lung samples, 21% of the samples was positive to at least one of the four bacteria, 3 samples (2.63%) were positive for *App*, 2 samples (1.75%) were positive for *Hps*, 7 samples (6.14%) were for *Pm*, and 12 lungs (10.53%) were positive for *Bb*. One sample (0.88%) was found co-infected with *Pm* and *Hps*.

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1. Introduction

Respiratory disease in pigs is one of the leading concerns in the livestock industry. The major direct loss effects on the farmer's economy due to respiratory illnesses include increased mortality and morbidity rate, reduced weight gain, long finishing time, and high consumption. expenses for treatment (de Jong et al., 2014). Usually, viral respiratory diseases (PRRS, CSFV, PCV-2, etc.) or some important bacteria such as *Actinobacillus pleuropneumoniae* (*App*), *Bordetella bronchiseptica* (*Bb*) are the primary factors causing diseases. However, the immunodeficiency of infected pigs creates favorable conditions for the arising secondary infections of *Haemophillus parasuis* (*Hps*), and *Pasteurella multocida* (*Pm*) that normally reside in the upper respiratory tract of the animals. The most important respiratory pathogen is *P. multocida* (de Jong et al., 2014). The App causes severe acute pleuropneumonia with very high mortality rates of up to 80%. Infectious rhinitis caused by Bb and Pm is common in commercial pig herds. The *Hps* causes acute infection with characteristic of causing multi-serous inflammation. When these infectious pathogens co-infect, they increase the severity of the disease.

While isolation is time-consuming and requires good laboratory skills, diagnosis by PCR method helps to provide accurate results, high reliability

while saving test time and giving faster results. Thus, the objective of this study was to detect the presence of four respiratory pathogenic bacteria in pigs at the slaughterhouse by using the PCR technique.

2. Materials and Methods

The experiment was conducted from July 2018 to May 2019 at the laboratory of Department of Veterinary Biosciences and the Veterinary Hospital, Faculty of Animal Science and Veterinary Medicine, Nong Lam University. Four bacteria that have significant impact on respiratory diseases in pigs, including *App*, *Hps*, *Pm* and *Bb* were analyzed from 114 swine lung specimens.

2.1. Sample collection

Sample collection was performed at the slaughterhouse of Vissan company in Ho Chi Minh City. Injured lungs with lesions, such as congestion, haemorrhage, and inflammation were separated from the carcass and stored in separate zip bags to avoid contamination and transported to the laboratory for culture.

2.2. Isolation method

Tryptone Soybean Agar (TSA) (Merck Group, Germany) with 5% bovine serum (Gibco, New Zealand) and Nicotinamide adenine dinucleotide (NAD) (Merck Group, Germany) were used to optimize the growth of four bacteria. Before culture, surface of samples and equipment were disinfected by using an alcohol swab to clean the surface of the lung until surface was dry. The scissors and forceps were heated using an alcohol-burner and allowed to cool down before use. To obtain an uncontaminated tissue, lung samples were cut deeply in small tissues. Direct smear of the newly cut tissue was performed into a Petri dish containing the culture medium and a sterile loop to streak the sample was used. Plates were incubated at 37^0C for 24 h in bacteriological incubator (Memmert, Germany). If bacteria growth was seen, the colonies were selected based on colony morphology, catalase reaction (Table 1) and Gram stain (the target bacteria have negative Gram stain). The suitable colonies were transferred into the new TSA medium for pure isolation for the next 24 h.

2.3. Preparation of samples for PCR

Bacterial DNA samples were extracted from whole cells by using thermal shock. Pure colonies were placed into an eppendorf containing 50 μL of Tris EDTA buffer solution (TBR, Vietnam) and went through heat cycles (10 min, 100^0C; 1 min, -20^0C). Cell debris was removed by centrifugation at 12000 rpm in 2.5 min. The supernatant was used directly for PCR process or stored at -20^0C.

The total volume for m-PCR of App, *Pm* and *Hps* was 20 μL. The mixture contained 10 μL of Gotaq G2 Green MasterMix, 2 μL of Nulease-Free water (Promega, USA), 1 μL per each primer x 6 primers (AP-IV (Xiao et al., 2006), KMT1 (Townsend et al., 1998), HPS (Oliveira et al., 2001)) (Table 2) and 3 μL of DNA samples. Bacterial DNA samples were isolated directly from pure colonies by thermal shock. The heat cycle was adapted from Hričínová (2010) research: (1) the initial phase lasted for 5 min at 95^0C, then the denaturation was performed at 94^0C for 30 s. The priming phase lasted for 30 s at 58^0C, followed by the extended phase (72^0C, 45 s) and finally the last 10-min process at 72^0C.

The reaction mixture for s-PCR of Bb was 20 μL including 10 µL of Gotaq G2 Green Master-Mix, 1 μL per each primer (*Bb-fla* (Hozbor et al., 1999)) (Table 2), 6 μL Nulease-free water and 2 μL DNA extracted from the sample. The initial phase lasted for 5 min at 95^0C, after which the denaturation took place at 95^0C for 30 s. The priming phase lasted for 30 s at 58^0C, followed by the extended phase (72^0C, 55 s) and finally the last 10-min process at 72^0C (Xue et al., 2009). There were 30 cycles performed for each reaction by the peqSTAR thermal cyclers (peqLAB Biotechnologie GmbH, Germany).

2.4. Electrophoresis

After completing the PCR reaction, 5 μL of each PCR products used for electrophoresis. Seven μL of 100 bp DNA ladder (Promega) was used to identify the approximate size of the PCR products. The steps were performed in 1% agarose (Promega) at U = 150 V, I = 144 mA for 20 min (Xue et al., 2009). *Actinobacillus pleuropneumonia* ATCC 27090 and *Pasteurella multocida* ATCC 12945 were used as positive controls for these two bacteria. Meanwhile, *Haemophilus parasuis* and *Bordetella bronchiseptica* isolated from the field were used as positive control af-

Table 1. Colony morphology of four bacteria on TSA medium after 24-h incubation

Name	Colony morphology	Catalase reaction
Actinobacillus pleuropneumoniae	Circular, raised, smooth, cloudy white, 1-1.5 mm in diameter	Negative
Haemophillus parasuis	Circular, raised, smooth, transparent white, the smallest size in 4 bacteria (< 1 mm)	Positive
Pasteurella multocida	Circular, raised, smooth, opaque white, 3-3.5 mm in diameter	Positive
Bordetella bronchiseptica	Circular, raised, smooth, greyish white, 1-2 mm in diameter	Positive

ter being analyzed by PCR and genotyed at Nam Khoa Biotek Company Limited. The PCR products were observed under Biorad UV2000 (Finetech, Taiwan).

3. Results and Discussion

There were 24 total objective bacteria strains isolated from 114 injured lungs (21.05%) collected at the slaughterhouse of Vissan Limited Company from July 2018 to May 2019. Three isolates of *App* (2.63%), 2 isolates of *Hps* (1.75%), 7 isolates of *Pm* (6.14%) and 12 isolates of Bb (10.53%) were found (Table 3 and Figure 1).

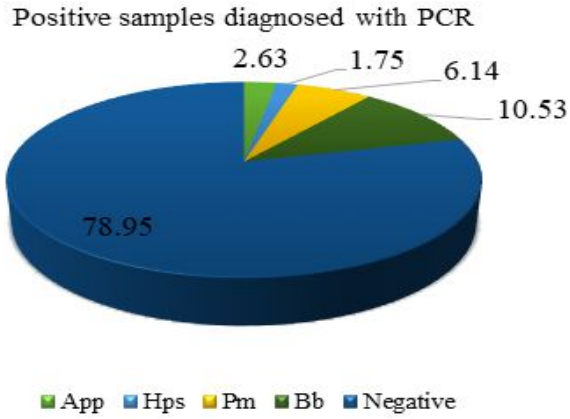

Figure 1. Proportion of positive samples diagnosed with PCR.

3.1. Proportion of positive samples diagnosed with PCR

The results of this study were different from those of other previous ones in different areas. *Bb* caused atrophic rhinitis when co-infecting with *Pm* and resulted in the severity of respiratory in pigs. In this study, Bb had the highest incidence with 10.53% (Figure 1). Zhao et al. (2011) found that 652/3506 lung samples were positive with *Bb* (18.6%). In North India, 8.2% of nasal swabs were positive with Bb by using PCR technique (Kumar et al., 2014). The gel electrophoresis after amplification of Bb is illustrated in Figure 2.

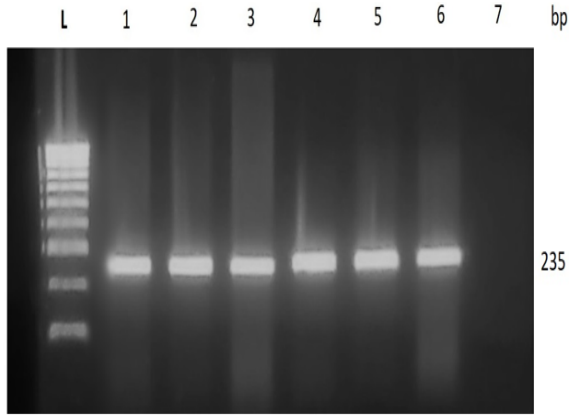

Figure 2. PCR product of Bb - *fla* gene for detection of *Bb* after electrophoresis process L: Ladder (1000 bps); Well: 1 - 5: DNA purified from field samples after cultivation; Well 6: positive control (235bps); Well 7: negative control.

In this study, Pm infection had the second highest proportion of positive samples diagnosed with PCR method (6.14%); however, this figure was lower than those reported by other researchers. In 2017, 296/3212 samples (9.2%) were positive with *Pm* in China (Liu et al., 2017). In other studies, the presence of *Pm* was found in 74.9% of lung samples collected from a slaughterhouse by using m-PCR technique (Hričínová et al., 2010). In Vietnam, Le et al. (2012) found that in Bac Giang, the percentage of Bb was 17.14% in the cases of 245 samples that were confirmed

positive with porcine reproductive and respiratory syndrome virus (PRRSV). In North of Cao Bang and Bac Giang in 2010, it was found that 5% of the pig herd had Pm*text* (Le et al., 2012). The gel electrophoresis after amplification of Pm is illustrated in Figure 3.

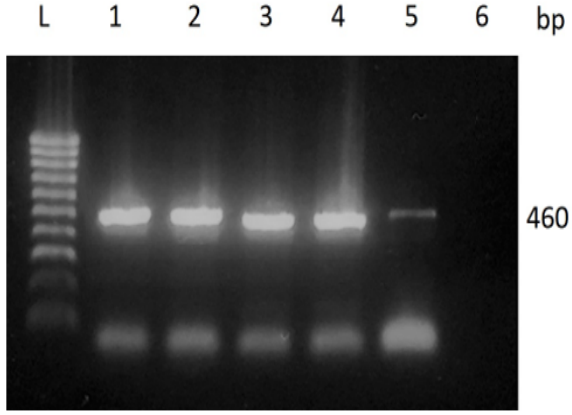

Figure 3. PCR product of KMT1 gene PMT gene after electrophoresis processL: Ladder (1000 bps); Well 1-4: DNA purified from field samples after cultivation; Well 5: positive control (460bps); Well 6: negative control.

App is the causative pathogen of pleuropneumonia in pigs. This bacterium can cause severe lung injuries. The results of this study showed that 2.63% of the samples were positive with this bacterium. This percentage was much lower as compared with those of other studies. In Ben Tre province, the prevalence of App was 24.62% (Thanh et al., 2018) while in Can Tho province, this percentage was 25.9% (Giang et al., 2015) and in Kien Giang, the proportion was 27.69% (Thanh et al., 2017). In some Northen provinces such as Bac Giang, 19.59% of samples positive with PRRSV were also positive with App. According to Hričínová et al. (2010), there was 20.5% of lungs from pigs in slaughterhouse positive to App. The gel electrophoresis after amplification of App is illustrated in Figure 4.

Hps is known as the bacteria causing Glasser's disease and an important agent in the porcine respiratory disease complex. In this study, it was found that only 2/114 lung samples (1.75%) were positive with Hps. Hričínová et al. (2010) found that 1,83% of lung samples from slaughterhouse were positive with Hps. In Thanh Hoa, Hung Yen and Ha Nam, 20/205 samples (9.7%) including nasal swab, tracheal fluid, heart and lungs of

Table 2. Primer sequences used for PCR

Gene name	Primer name	Sequence (5'⟶ 3')	Size (bp)	Preference
AP-IV	AP-IVF	ATA CGG TTA ATG GCG GTA ATG G	346	Xiao et al. (2006)
	AP-IVR	ACC TGA GTG CTC ACC AAC G		
KMT1	KMT1 T7	ATC CGC TAT TTA CCC AGT GG	460	Townsend et al. (1998)
	KMT1 SP6	GCT GTA AAC GAA CTC GCC AC		
	AP-IVR	ACC TGA GTG CTC ACC AAC G		
HPS	HPS-F	GTG ATG AGG AAG GGT GGT GT	821	Oliveira et al. (2001)
	HPS-R	GGC TTC GTC ACC CTC TGT		
Bb-*fla*	Bb-*fla*-F	GCT CCC AAG AGA GAA AGG CT	235	Hozbor et al. (1999)
	Bb-*fla*-R	GGT GGC GCC T GC CCT ATC		

Table 3. Positive samples diagnosed with PCR

	Total sample	Total positive sample	*App*	*Hps*	*Pm*	*Bb*
Number of samples	114	23	3	2	7	12
Percentage	100%	21.05%	2.63%	1.75%	6.14%	10.53%

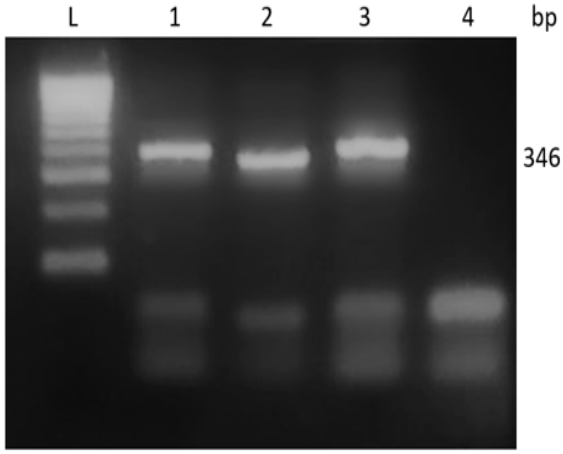

Figure 4. PCR product of the gene AP-IV for detection of App after electrophoresis process. L: Ladder (1000 bps); Well 1-2: DNA purified from field samples after cultivation; Well 3: positive control (346 bps); Well 4: negative control.

Glasser suspected pigs were found positive with Hps (Truong et al., 2018). In China, Zhao et al. (2011) reported that 26.7% samples were found positive with Hps. The gel electrophoresis after amplification of *Hps* is illustrated in Figure 5.

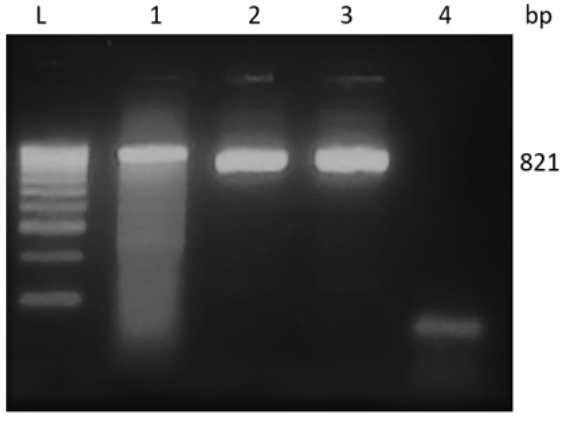

Figure 5. The PCR product of *Hps* gene, *Hps* bacteria after electrophoresis process L: Ladder (1000bps); Well 1, 2: DNA purified from field samples after cultivation; Well 3: positive control (821 bps); Well 4: negative control.

The differences in the percentage of positive samples of the four bacteria in different studies may be associated several factors such as husbandry conditions, weather, and disease pressure in various areas. The method of collecting samples may also affect the results as the bacteria are frequently isolated in the upper respiratory tract of pigs, but they would cause diseases when invading the lower respiratory tract. Another factor that should be considered is pig sources. In previous studies, samples were collected from clinically infected pigs, whereas in this study lungs were taken from pigs in the slaughterhouse with no clinical signs.

3.2. Proportion of samples with co-infection of 2, 3, and 4 bacteria

There was only 1 lung with co-infection of *Hps* and *Pm* (0.88%). Zhao et al. (2011) found the co-infection of *Pm* and *Bb* in all 63 pigs with the atrophy of turbinate bones. So far, the co-infection of those four bacteria has been rarely found in previous studies.

4. Conclusions

The prevalence of the investigated pathogens and their co-infection were not high because pigs at the slaughterhouse were relatively healthy and had no obvious clinical signs. However, it indicates that there is a potential risk for not only naïve herds when they are exposed to the healthy carriers but also the farms which currently have the presence of the pathogens without awareness of the farmers.

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