CHAPTER 5

DISCUSSIONS

In this study, the PCR based assay was developed for the detection and serotype identification of *A. pleuropneumoniae* using both pure culture of bacterial colony and lung tissue. The assay was verified using 13 reference strains including subtype 5a and 5b of serotype 5 of *A. pleuropneumoniae*. Ten swine pleuropneumonic lung samples and forty-seven field isolates of *A. pleuropneumoniae* were successfully detected and identified. The results of PCR technique for serotype identification were similar to rapid slide agglutination test (SAT). Moreover, the minimum concentration of DNA templates in the inoculated-lung sample was 1.5 μg of DNA contents from bacterial concentration of 10^4 CFUs/ml could be detected by the nested PCR. Furthermore, the nested primers used in this developed PCR showed the negative results for other swine respiratory bacterial pathogens.

For the routine diagnosis, bacterial isolation in selective medium is a routine protocol for detection of *A. pleuropneumoniae* that promote their growth with either supplemented with NAD in the medium or streaking *S. aureus* as a nurse strain (Jacobsen and Nielsen, 1995, Møller and Kilian, 1990, Quin et al., 1999, Reinier, 1999, Taylor, 1999). Indeed, this is a time consuming protocol but it is a popular method because of its availability. Alternatively, there were several studies using the PCR detection of *A. pleuropneumoniae* from pure single colony (Blackall et al., 2002, Beck et al., 1994, Chiers et al., 2001, Cho and Chae, 2001a, 2001b, Gram et al., 1996,

1998, 2000a, Lo et al., 1998, Schaller et al., 1999, 2000, 2001). In this study, the nested PCR detection of *A. pleuropneumoniae* was performed directly from fresh swine pleuropneumonic lung samples without the need of bacterial isolation as reported previously (Cho and Chae, 2001a, 2001b, Schaller et al., 2001). The nested primers were designated from the 3' terminal region of the *apxIVA* gene of *A. pleuropneumoniae*. The 3' terminal of the *apxIVA* gene was shown to be species-specific characteristic of *A. pleuropneumoniae* (Schaller et al., 2001). All of the nested PCR products derived from ten pleuropneumonic lungs and forty-seven field samples showed similar nested PCR products as thirteen reference strains of *A. pleuropneumoniae*. In addition, the major advantage of this assay is to reduce turn around time required to isolate and identify *A. pleuropneumoniae* from more than two or three days to nine to ten hours (of the nested PCR detection) since no bacterial growth is required.

In this study, the multiplex PCR was developed for the serotyping of *A. pleuropneumoniae*. In the past, there were several techniques for serotyping of *A. pleuropneumoniae* such as the rapid slide agglutination test (SAT), coagglutination test, agar gel immunodiffusion test (AGID), and indirect hemagglutination test (IHA). However, these serological techniques are time consuming techniques and had some cross-reaction among each serotype (Blackall et al., 1999, Hommez et al., 1990, Sakpuaram, 1990, Taylor, 1999). The PCR typing system of *A. pleuropneumoniae* with the multiplex PCR have been reported and could differentiate serotype of *A. pleuropneumoniae* pure isolates from each other with the exception for serotype 1, 9 and 11, and serotype 2 and 8 (Gram et al., 2000a). The multiplex PCR developed in this study gave the results similar to that report (Gram et al., 2000a), however, our

multiplex PCR could differentiate serotype 1 from serotype 9 and serotype 11. Forty-seven field isolates of *A. pleuropneumoniae* can be serotyped and gave the similar results with rapid slide agglutination test. Furthermore, ten swine pleuropneumonic lungs could directly be used to serotype of *A. pleuropneumoniae* successfully. In this study, this PCR technique was developed on the basis of primers that belongs to the *apx* genes of *A. pleuropneumoniae* only and the number of primers using in this study (6 pairs) was two pairs of primers less than the previously report (8 pairs) (Gram et al., 2000a). Moreover, this serotype identification could be performed by using the fresh swine pleuropneumonic lung samples without the need for bacterial isolation as previously report. There were PCR based for the detection of *A. pleuropneumoniae* such as the *omlA* gene based PCR (Gram et al., 1998, 2000a), the *dsbE*-liked gene based PCR (Chiers et al., 2001) and the *apxIVA* gene based PCR (Schaller et al., 2001). These PCR assays were performed using pure single colony and consumed timing process in the bacterial isolation step. On the other hand, our developed PCR was performed using fresh swine pleuropneumonic lungs with the nested PCR, which could reduce the processing as described above.

Our PCR technique has been studied and showed high sensitivity and specificity for detection of *A. pleuropneumoniae*. The previously reported PCR assay could detect 10^4 CFUs/PCR tube of *A. pleuropneumoniae* similar to our study (Gram et al., 1996), however, it was performed using pure single colony of isolates for PCR but our developed PCR could be performed using the fresh swine pleuropneumonic lungs. The concentration of each serially ten-fold dilution gave the same nested PCR product as the inoculated pig lung samples with the same diluted concentration. The species-specific of characteristic from 3' terminal designated-primers of the *apxIVA*

gene when used the nested primers, the nested PCR could increased the sensitivity from 10 pg in the first reaction to 10 fg in the second reaction (Schaller et al., 2001). According to the results, calculated DNA contents of the dilution number 10^{-4} was 2.2 pg, which was less than the reported lower limit of detectable DNA contents by the first reaction of the nested PCR as reported previously (Schaller et al., 2001). Our result opposite to the theoretical nested PCR that will increase the sensitivity of the method when performed with the second reaction of nested PCR. The nested results could not amplify the higher serially dilution number than the dilution number 10^{-3}, which have 2.2 pg of DNA contents. Theoretically, this DNA content could be detected with the second reaction of nested PCR when compared with the lower limit of the detectable DNA contents (10 fg) by the second reaction of the nested PCR (Schaller et al., 2001). This result might be explained as followed. 1) There was any error in the preparation of serially dilution. 2) In comparison with the original report, there was different in the DNA extraction method. Then, in a routine application, because of the PCR method vary between laboratory to laboratory, the proper PCR reaction and PCR condition must be changed a little to increase the sensitivity of the nested PCR.

The nested primers used in this study showed the accuracy in the nested PCR assay with others swine respiratory bacterial infected-lung samples. This was also similar to the original study, which showed the accuracy of these primers in differentiating *A. pleuropneumoniae* from other related bacterial species in the family *Pasteurellaceae* (Schaller et al., 2001). Hence, these previous evaluations gave more confidants in both detectability level and accuracy of DNA templates in PCR tube if pig lung samples were contaminated with other swine respiratory bacterial organism.

In addition to the routine application, the DNA yields from the clinical lung samples might be less than serially bacterial dilution samples because of the difference in DNA extraction method. Since the amounts of DNA in each dilution may vary by different DNA extraction methods. In order to elucidate of this problem, the routine laboratory work should choose the best DNA extraction method, which can extract in a high yield of DNA.

In conclusion, PCR technique developed in this study was successfully used for the detection and the serotype identification of *A. pleuropneumoniae* in lung tissues. Moreover, *A. pleuropneumoniae* serotype 1, the most virulent serotype could be discriminated with this developed PCR. These results suggested that our PCR technique might be a useful tool for the routine diagnosis and the serotyping of *A. pleuropneumoniae* directly from the fresh pleuropneumonic lungs in laboratory work.