Evaluation of Lung Scoring System and Serological Analysis of Actinobacillus pleuropneumoniae Infection in Pigs

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ABSTRACT

Actinobacillus pleuropneumoniae (A. pleuropneumoniae) is a respiratory pathogen that causes a great economic loss every year in the swine industry worldwide. The objective of this study was to investigate the prevalence of pleuritis in central Taiwan using the slaughterhouse pleurisy evaluation system (SPES) and to evaluate the correlations among SPES, serum antibody (ApxI/Tbp2) positivity, and the presence of apxIVA in lung tissue caused by A. pleuropneumoniae. Lung and blood samples were collected randomly from the slaughterhouse. The pleuritis lesions were morphologically evaluated for a SPES score and then examined the positive rate of apxIVA by PCR, and the blood samples were analyzed by ELISA. The positive rate of the samples we collected from slaughterhouse indicated that the prevalence of A. pleuropneumoniae in central Taiwan measured by SPES, ELISA, and PCR was 21.2, 40.6 and 23.7%, respectively. Generally, the positive rate of serum antibody and apxIVA detection increased when SPES values rose. However, the lungs with SPES 4 presented a low ApxI/Tbp2 antibody titer in the sera, and that would be considered as a secondary infection of A. pleuropneumoniae because the lesion is usually accompanied by extensive polyserositis. In conclusion, according to cross-comparison and statistical analysis of our data, the serum antibody levels were strongly correlated with SPES, which promises a fast and useful evaluation tool for clinical investigation of A. pleuropneumoniae infection.

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INTRODUCTION

Actinobacillus pleuropneumoniae (A. pleuropneumoniae) is a gram-negative respiratory pathogen that causes swine pleuropneumonia worldwide and leads to severe economic losses (Choi et al., 2001; Wang et al., 2015; Kim et al., 2016; Wallgren et al., 2016). Biofilms protect A. pleuropneumoniae from killing by antimicrobial agents and host immune system (Li et al., 2016). However, the antimicrobial agents were still the indispensable strategy to prevent the spread of A. pleuropneumoniae because of the limited protection efficiency of vaccination (Bosse et al., 2015). In acute cases, swine show hemorrhagic, fibrinous, and necrotic pleuropneumonia, especially located on the diaphragmatic lobes, and sudden death (Fablet et al., 2012; Sarkozy et al., 2015). In chronic or subclinical cases, however, A. pleuropneumoniae causes minor pleuritis and little clinical signs, such as intermittent cough and exercise intolerance, which are not easy to identify the primary pathogen until it is found during slaughtering.

Pleuritis is one of the most common respiratory lesions found in slaughtered pigs (Grest et al., 1997; Enoe et al., 2002; Martinez et al., 2007). Pleuritis causes
intermittent cough and growth retardation, decreasing the daily average weight gain and feed conversion rate (Cleveland-Nielsen et al., 2002). In clinical, such losses usually result in a decrease in the quality of the carcass, postpone the production line speed, cause extra trimming time, and increase the risk of pathogen spread (Hurd et al., 2008; Jager et al., 2012). In order to estimate the severity of lung lesions, the slaughterhouse pleurisy evaluation system (SPES) was developed as a tool to value the severity of pleuritis based on the lesion location and extension range (Dottori, 2007). In particular, lesions located on the diaphragmatic lobe were considered to have strong association with A. pleuropneumoniae infection (Meraldi et al., 2012).

Although many reports have confirmed the relationship between pleuritis and A. pleuropneumoniae infection by molecular techniques and serological analysis, these examination processes take a long time to make sure it is the principal organism that caused the pleuritis, since other pathogens might also cause severe pleuropneumonia (Lun et al., 2007). In consideration of the difficulty of clinical detection, the aim of this study was to ascertain the prevalence of A. pleuropneumoniae in slaughtered pigs in central Taiwan, and to assess the relationship between different diagnostic assays for the clinical investigation of A. pleuropneumoniae infection.

MATERIALS AND METHODS

Ethical statement: This study did not involve in killing pigs. All samples were collected from carcass of swine after routine slaughter.

Slaughterhouse and sample selection: This study was conducted at a private slaughterhouse in Taichung in central Taiwan. The duration of the study was from December 2010 to May 2013. A total of 2,542 lungs were examined in this study. All pigs in this slaughterhouse were from an auction house in the nearby county. Therefore, the source of the pigs was unknown, but believed to represent a great number of herds.

Lung lesion scoring: The severity of pleuritis in the pigs was evaluated by using the SPES (Dottori, 2007; Fraile et al., 2010; Meraldi et al., 2012). An SPES score was obtained on the basis of the location and extent of the pleural adherence as described before (Table 1) (Dottori, 2007). In addition to use the SPES score directly to point out the prevalence of pleuritis, the frequency of lesions with an SPES score≥2 would be considered to be the result of A. pleuropneumoniae infection, thus the severity of A. pleuropneumoniae infection in the batch could be computed as the A. pleuropneumoniae index (APPI): (the percentage of pigs with SPES≥2) × (the mean SPES score of pigs with SPES≥2).

<table>
<thead>
<tr>
<th>Score</th>
<th>Lesion characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No pleural lesions</td>
</tr>
<tr>
<td>1</td>
<td>Pleural adhesion between cranial and ventral lobes, or ventral and caudal lobes</td>
</tr>
<tr>
<td>2</td>
<td>Single focal pleural adhesion on unilateral diaphragmatic lobe</td>
</tr>
<tr>
<td>3</td>
<td>Focal pleural adhesion on bilateral diaphragmatic lobe or extended unilateral lesion</td>
</tr>
<tr>
<td>4</td>
<td>Bilateral extended pleural adhesion</td>
</tr>
</tbody>
</table>

Serum sample collection and serological analysis: Blood samples were randomly collected from swine pulmonary artery after macroscopic examination and lung scoring. A total of 665 sera were collected from the slaughterhouse and tested for antibodies against A. pleuropneumoniae ApxIV/Tbp2 using the LSIVet APP SUIS (LSIVet) indirect enzyme-linked immunosorbent assay (ELISA) kit. After incubation, the optical density (OD) of each sample was measured at 405 nm. The OD value was used for calculation of the relative index (IRPC): [(OD_{sample} − OD_{negative control}) / (OD_{positive control} − OD_{negative control})] × 100. IRPC>60 was taken as a positive result, and IRPC≤20 was recorded as a negative one.

Lung tissue collection and nested PCR operation: Lung samples were randomly chosen and collected at the slaughterhouse by slicing down part of the pleuritic lesion containing the pleura and the lung substance under the pleural lesion. One gram of lung tissue was placed in a mortar and homogenized with 1 mL of Hank’s balanced salt solution. Tissue debris was removed by a mesh, and the filtrates were used as samples proceeding DNA extraction following the manufacturer’s protocol (Genomic DNA Mini Kit, Geneaid). The PCR amplification process was performed as reported previously based on the A. pleuropneumoniae-specific gene, apxIVA (Schaller et al., 2001), with the primers ApxIVA-1L (5′-TGGGACCTGACCCTGATGACGGT-3′) and ApxIVA-1R (5′-GGCCATCGACTCAACCAT-3′). Nested PCR was performed with the primary PCR product and apxIVA-specific primers APXIVANEST-1L (5′-GGGGACGTAACTCGGTGATT-3′) and APXIVAN EST-1R (5′-GCTCACAACTGTTTGCAT-3′). The positive-PCR product was 377 bp in size.

Statistical analysis: The SPES scores from the slaughterhouse-collected samples were analyzed using Chi-square or Fischer exact tests to evaluate the association between batches and the percentage of dorsocardial pleuritis (SPES score≥2). To study the continuous non-normally distributed APPI and seroprevalence data, the Kruskal-Wallis and Dunn’s multiple comparison tests were used to assess the batch ranking and post hoc analysis. Analysis of Variance (ANOVA) and Chi-square tests were used to find the association between apxIVA-positive rate and lung pathology in each batch of lungs.

RESULTS

The prevalence of pleuritis in central Taiwan: The surveillance period was from December 2010 to May 2013. A total of 2,542 lungs were examined, and 37.6% (955/2,542) showed pleuritis with an SPES score≥1 and 56.5% (540/955) of them were scored more than 2. The overall percentage of SPES≥2 was 21.2% (540/2,542), indicating a severe situation of A. pleuropneumoniae infection in the swine industry. The highest three APPI occurred in August, March, and July, whereas the lowest APPI were in November, May, and April (Table 2). The prevalence of pleuritis with an SPES score≥2 in August was significantly higher than November (P<0.05).
The correlation between antibody level and SPES score: The detection of ApxI/Tbp2 antibody is a dependable indication of A. pleuropneumoniae infection. In this study, 665 serum samples were evaluated using a commercial ELISA kit that presented the antibody titer as IRPC. The median antibody titer (IRPC) of SPES scores 0 to 4 was 35.9, 53.6, 65.0, 69.1, and 42.6, respectively (Fig. 1 and Table 3). The presence of seropositive by SPES scores 0 to 4 was 28.4% (74/261), 37.8% (54/143), 58.8% (70/119), 59.8% (58/97), and 35.6% (14/45), respectively (Table 3). The rank of IRPC of scores 2 and 3 were significantly higher than score 0 (P<0.001). The pigs that were seropositive to A. pleuropneumoniae had 2.65-fold higher risk of showing pleuritis (SPES score≥2) than the seronegative pigs (P<0.0001).

The relationship between SPES score and apxIVA detection: Randomly selected 224 lung tissues were examined for infection of A. pleuropneumoniae by nested PCR, and the overall positive rate was 23.7% (53/224). The detection rates of apxIVA of groups of SPES score 0 to 4 were 5.9% (4/68), 22.2% (14/63), 33.3% (16/48), 35.1% (13/37), and 34.3% (12/35), respectively (Fig. 2A and Table 3). There was a significant association between SPES score and apxIVA detection rate (P<0.0001), indicating lungs with higher SPES scores were usually accompanied by a higher nucleic acid detection rate (Fig. 2B). The risk of pigs suffering from pleuritis (SPES≥2) in the nucleic acid-positive group was 3.98 times higher than the apxIVA-undetectable samples (P<0.0001).

The SPES score was the main factor correlated with the IRPC level: For the samples with SPES scores of 1 and 2, the IRPC values of apxIVA were lower than those for apxIVA- samples (Fig. 3). However, when SPES was more than 2, the IRPC values of apxIVA and apxIVA gradually became discrepant. Discrepancy of variance (ANOVA) was used to evaluate the influence of the SPES score and apxIVA detection on the IRPC value, and the results indicated that the SPES score was strongly correlated with the serum IRPC value regardless of whether apxIVA was detected.

### DISCUSSION

A. pleuropneumoniae is a pathogen of high concern in the swine industry around the world. The present work aimed to use three different scoring systems to evaluate the infection of A. pleuropneumoniae in central Taiwan and figure out the correlation among the evaluation systems. According to our investigation in the slaughterhouse, the prevalence of pleuritis was 37.6% (955/2,542) and half of them were diaphragmatic lesions (540/955), indicating the severity of respiratory disease in central Taiwan. Comparing the prevalence to that in other countries, 24% in Denmark, 19% in New Zealand, 20% in Switzerland, and 26.8% in Spain (Stark et al., 1995; Cleveland-Nielsen et al., 2002; Fraile et al., 2010), our results showed that pleuritis is a relatively severe problem in Taiwan.

A. pleuropneumoniae may occur in cooperation with some factors such as insufficient ventilation and ages (Maes et al., 2001; Gottschalk, 2012; Jager et al., 2012). The season may play a role in the incidence of respiratory illness as reported before (Eze et al., 2015). In Taiwan, the seasons with the highest incidence of temperature alterations are the summer and winter; we found that the highest APPIs were present in August, March, and July (Table 2), indicating the pigs that went through their growing or finishing period in such weather-unstable seasons had a higher risk to develop pleuritis.

The prevalent serovars differ across countries and areas. There are over 15 different serovars of A. pleuropneumoniae were determined, and the highly virulent serovar 1 was the most prevalent serotype isolated in Taiwan (80%) (Chang et al., 2002; Yang et al., 2011; Sarkoz et al., 2015). In this study, we used specific primers to amplify apxIVA gene which is conserved in different serovars instead of apxI, apxII, and apxIII which presented strong interspecific specificity (Seo et al., 2013; Zhang et al., 2016). The seroprevalence and nucleic acid-positive rate increased in lungs with pleuritis, suggesting that pleural lesions in pigs reflect infection by A. pleuropneumoniae prior to slaughter. The IRPC value presented the strongest antibody titer when the lungs were scored for SPES 2 and 3, indicating an evident relationship among A. pleuropneumoniae infection, lesion site, and lesion size. When A. pleuropneumoniae colonizes the lung tissue and secretes specific toxins, it causes pneumonia or pleuropneumonia, and the intense inflammation also alerts the host immune system to fight against the pathogen and limit the influence of the lesion. If A. pleuropneumoniae had been controlled or eliminated by the host immune system, the lesion would be localized instead of being extensive, and the apxIVA detection rate would also not be recognized easily because of the low bacteria load in the tissue. However, for the lungs recorded as SPES 4, the reason for the low IRPC value

### Table 2: Pleuritis percentage with SPES score and APPI from December 2010 to March 2013

<table>
<thead>
<tr>
<th>Month</th>
<th>Number of samples</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>≥2</th>
<th>APPI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jan</td>
<td>213</td>
<td>123 (57.7)</td>
<td>43 (20.2)</td>
<td>28 (13.1)</td>
<td>13 (6.1)</td>
<td>6 (2.8)</td>
<td>47 (22.1)</td>
<td>0.56</td>
</tr>
<tr>
<td>Feb</td>
<td>184</td>
<td>103 (56.0)</td>
<td>36 (19.6)</td>
<td>32 (17.4)</td>
<td>10 (5.4)</td>
<td>3 (1.6)</td>
<td>45 (24.5)</td>
<td>0.58</td>
</tr>
<tr>
<td>Mar</td>
<td>205</td>
<td>122 (59.5)</td>
<td>36 (17.6)</td>
<td>21 (10.2)</td>
<td>22 (10.7)</td>
<td>4 (2.0)</td>
<td>47 (22.9)</td>
<td>0.60</td>
</tr>
<tr>
<td>Apr</td>
<td>252</td>
<td>157 (62.3)</td>
<td>50 (19.8)</td>
<td>23 (9.1)</td>
<td>14 (5.6)</td>
<td>8 (3.2)</td>
<td>45 (17.9)</td>
<td>0.48</td>
</tr>
<tr>
<td>May</td>
<td>256</td>
<td>170 (66.4)</td>
<td>37 (14.5)</td>
<td>32 (12.5)</td>
<td>15 (5.9)</td>
<td>2 (0.8)</td>
<td>49 (19.1)</td>
<td>0.46</td>
</tr>
<tr>
<td>Jun</td>
<td>203</td>
<td>135 (66.5)</td>
<td>22 (10.8)</td>
<td>25 (12.3)</td>
<td>16 (7.9)</td>
<td>5 (2.5)</td>
<td>46 (22.7)</td>
<td>0.58</td>
</tr>
<tr>
<td>Jul</td>
<td>162</td>
<td>102 (63.0)</td>
<td>24 (14.8)</td>
<td>19 (11.7)</td>
<td>10 (6.2)</td>
<td>7 (4.3)</td>
<td>36 (22.2)</td>
<td>0.59</td>
</tr>
<tr>
<td>Aug</td>
<td>103</td>
<td>60 (58.3)</td>
<td>15 (14.6)</td>
<td>16 (15.5)</td>
<td>8 (7.8)</td>
<td>4 (3.9)</td>
<td>28 (27.2)</td>
<td>0.70</td>
</tr>
<tr>
<td>Sep</td>
<td>206</td>
<td>139 (67.5)</td>
<td>24 (11.7)</td>
<td>23 (12.1)</td>
<td>15 (7.3)</td>
<td>3 (1.5)</td>
<td>43 (20.9)</td>
<td>0.52</td>
</tr>
<tr>
<td>Oct</td>
<td>201</td>
<td>124 (61.7)</td>
<td>35 (17.4)</td>
<td>31 (15.4)</td>
<td>6 (3.0)</td>
<td>5 (2.5)</td>
<td>42 (20.9)</td>
<td>0.50</td>
</tr>
<tr>
<td>Nov</td>
<td>292</td>
<td>195 (66.8)</td>
<td>45 (15.4)</td>
<td>31 (10.6)</td>
<td>18 (6.2)</td>
<td>3 (1.0)</td>
<td>52 (17.8)</td>
<td>0.44</td>
</tr>
<tr>
<td>Dec</td>
<td>265</td>
<td>157 (59.2)</td>
<td>48 (18.1)</td>
<td>30 (11.3)</td>
<td>26 (9.8)</td>
<td>4 (1.5)</td>
<td>60 (22.6)</td>
<td>0.58</td>
</tr>
</tbody>
</table>

Total (%) | 2,542 | 1,587 (62.4) | 415 (16.3) | 313 (12.3) | 173 (6.8) | 54 (2.1) | 340 (21.2) | 0.54 |
but a parallel apxIVA detection rate was suggested as the influence of other pathogens. The lungs scored for SPES 4 were usually accompanied by adhesive pericarditis or polyserositis, which might be caused by other organisms such as Streptococcus suis, Mycoplasma hyorhinis, or Haemophilus parasuis (Kang et al., 2012; Palzer et al., 2016). In the sampling process, we preferred to collect the pleuritic lesion under the pleura and checked for the presence of A. pleuropneumoniae, which might be the consequence of a secondary infection but not the principal reason for the severe and massive pleuropneumonia. Moreover, massive pleuritis caused by severe pneumonia might be fatal and not usually be seen in the slaughterhouse. Therefore, the further study should be done to identify the significance of SPES 4 affects APPI.

Conclusions: The average prevalence of A. pleuropneumoniae in central Taiwan measured by SPES, ELISA, and PCR was 21.2%, 40.6%, and 23.7%, respectively. According to the cross-comparison and statistical analysis of our data, the serum IRPC was especially strongly correlated with the SPES. When pigs are infected by A. pleuropneumoniae during the growing or finishing period, the host immune system is activated and produces specific antibodies to get rid of the pathogen, and both antibody and lesion can persist for a period of time even after the pathogen is under control of the host immune system. Our study identified a significant correlation between SPES and A. pleuropneumoniae infection, providing a convenient and useful evaluation pattern for the clinical A. pleuropneumoniae study.

Authors contribution: YCC and THC conceived and supervised this research. SWL and JJJ designed, operated the experiments and drafted the manuscript. FC, WCL, and YCW assisted in tissue sample collection. SLH and CJK helped in data analysis.

REFERENCES


