



## RESEARCH ARTICLE

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

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#### ARTICLE HISTORY (16-359)

Received: December 29, 2016  
Revised: April 14, 2017  
Accepted: April 18, 2017  
Published online: May 31, 2017

#### Key words:

*Actinobacillus pleuropneumoniae*  
*apxIVA*  
Pleuritis  
Serum antibody  
Slaughterhouse pleurisy evaluation system

#### 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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**To Cite This Article:** Liao SW, Lee JJ, Chen F, Lee WC, Wu YC, Hsuan SL, Kuo CJ, Chang YC and Chen TH, 2017. Evaluation of lung scoring system and serological analysis of *Actinobacillus pleuropneumoniae* infection in pigs. Pak Vet J, 37(3): 340-344.

#### 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; Sarkozi *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

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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 (Merialdi *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; Merialdi *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  $\geq 2$  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  $\geq 2$ )  $\times$  (the mean SPES score of pigs with SPES  $\geq 2$ ).

**Table 1:** Scoring table of SPES

Score	Lesion characteristics
0	No pleural lesions
1	Pleural adhesion between cranial and ventral lobes, or ventral and caudal lobes
2	Single focal pleural adhesion on unilateral diaphragmatic lobe
3	Focal pleural adhesion on bilateral diaphragmatic lobe or extended unilateral lesion
4	Bilateral extended pleural adhesion

### 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* ApxI/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):  $[(OD_{\text{sample}} - OD_{\text{negative control}}) / (OD_{\text{positive control}} - OD_{\text{negative control}})] \times 100$ . IRPC  $> 60$  was taken as a positive result, and IRPC  $\leq 20$  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'-TGGCACTGACGGTGATGACGGT-3') and *ApxIVA*-1R (5'-GGCCATCGACTCAACCAT-3'). Nested PCR was performed with the primary PCR product and *apxIVA*-specific primers APXIVANEST-1L (5'-GGGGACGTAACCTCGGTGATT-3') and APXIVANEST-1R (5'-GCTCACCAACGTTTGCTCAT-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 dorsocaudal pleuritis (SPES score  $\geq 2$ ). 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  $\geq 1$  and 56.5% (540/955) of them were scored more than 2. The overall percentage of SPES  $\geq 2$  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  $\geq 2$  in August was significantly higher than November ( $P < 0.05$ ).

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

Month	Number of samples	No. of samples (%) in each SPES score						APPI
		0	1	2	3	4	≥2	
Jan	213	123 (57.7)	43 (20.2)	28 (13.1)	13 (6.1)	6 (2.8)	47 (22.1)	0.56
Feb	184	103 (56.0)	36 (19.6)	32 (17.4)	10 (5.4)	3 (1.6)	45 (24.5)	0.58
Mar	205	122 (59.5)	36 (17.6)	21 (10.2)	22 (10.7)	4 (2.0)	47 (22.9)	0.60
Apr	252	157 (62.3)	50 (19.8)	23 (9.1)	14 (5.6)	8 (3.2)	45 (17.9)	0.48
May	256	170 (66.4)	37 (14.5)	32 (12.5)	15 (5.9)	2 (0.8)	49 (19.1)	0.46
Jun	203	135 (66.5)	22 (10.8)	25 (12.3)	16 (7.9)	5 (2.5)	46 (22.7)	0.58
Jul	162	102 (63.0)	24 (14.8)	19 (11.7)	10 (6.2)	7 (4.3)	36 (22.2)	0.59
Aug	103	60 (58.3)	15 (14.6)	16 (15.5)	8 (7.8)	4 (3.9)	28 (27.2)	0.70
Sep	206	139 (67.5)	24 (11.7)	25 (12.1)	15 (7.3)	3 (1.5)	43 (20.9)	0.52
Oct	201	124 (61.7)	35 (17.4)	31 (15.4)	6 (3.0)	5 (2.5)	42 (20.9)	0.50
Nov	292	195 (66.8)	45 (15.4)	31 (10.6)	18 (6.2)	3 (1.0)	52 (17.8)	0.44
Dec	265	157 (59.2)	48 (18.1)	30 (11.3)	26 (9.8)	4 (1.5)	60 (22.6)	0.58
Total (%)	2,542	1,587 (62.4)	415 (16.3)	313 (12.3)	173 (6.8)	54 (2.1)	540 (21.2)	0.54

**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  $\geq 2$ ) 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% (53/244). 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  $\geq 2$ ) 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*<sup>+</sup> were lower than those for *apxIVA*<sup>-</sup> samples (Fig. 3). However, when SPES was more than 2, the IRPC values of *apxIVA*<sup>+</sup> and *apxIVA*<sup>-</sup> gradually became discrepant. Analysis 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.

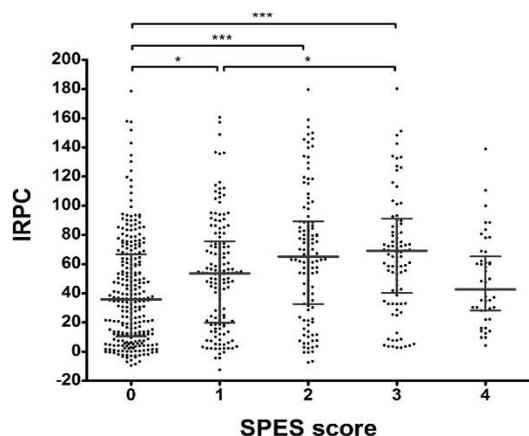
## DISCUSSION

*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

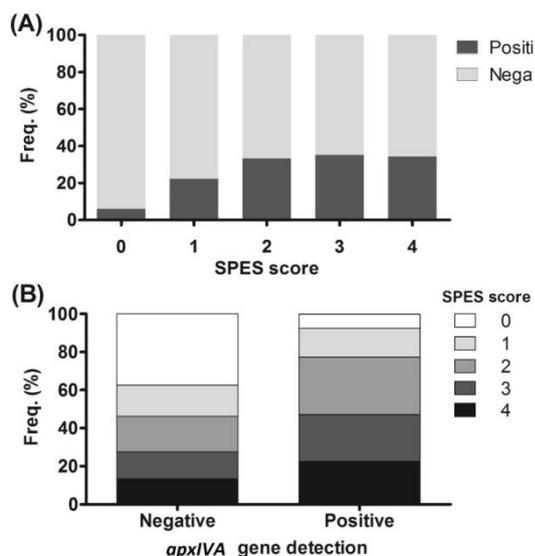
and figure out the correlation among the evaluation systems. According to our investigation in the slaughterhouse, the prevalence of pleuritis was 37.6% (955/2,542) and half of them were diaphragmatic lesions (540/955), indicating the severity of respiratory disease in central Taiwan. Comparing the prevalence to that in other countries, 19% in New Zealand, 20% in Switzerland, and 26.8% in Spain (Stark *et al.*, 1998; 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.

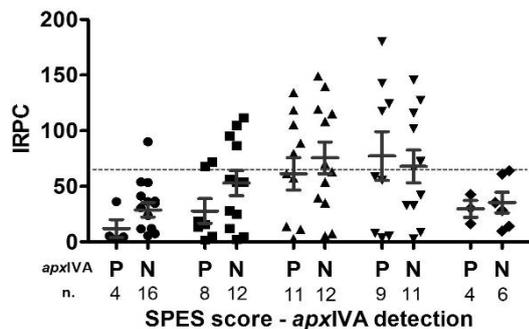
The prevalent serovars differ across countries and areas. There are over 15 different serovars of *A. pleuropneumoniae* were determinate, and the highly virulent serovar 1 was the most prevalent serotype isolated in Taiwan (80%) (Chang *et al.*, 2002; Yang *et al.*, 2011; Sarkozi *et al.*, 2015). In 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



**Fig. 1:** Serum antibodies to ApxI/Tbp2 by corresponding SPES score. Bars indicate the median with the interquartile range (IQ1–IQ3). SPES 0: 35.9 (10.5–66.8), SPES 1: 53.6 (19.8–75.5), SPES 2: 65.0 (32.7–89.2), SPES 3: 69.1 (40.3–91.2), SPES 4: 42.6 (28.2–65.2). Sample number in each SPES group from 0 to 4 is 45, 97, 119, 143, and 261, respectively. \*  $P < 0.05$ ; \*\*\*  $P < 0.001$ .



**Fig. 2:** *A. pleuropneumoniae* nucleic acid detection. (A) The positive rate of *apxIVA* detection in lung tissue corresponded to the SPES score. The positive rate of each SPES score from 0 to 4 is 5.9%, 22.2%, 33.3%, 35.1%, and 34.3%, respectively. The sample number in each group is 68, 63, 48, 37, and 35, respectively. (B) The composition of SPES scores in *apxIVA*-positive and *apxIVA*-negative groups. The risk of pigs suffering from pleuritis (SPES $\geq$ 2) in the *apxIVA*-positive group ( $n=59$ ) was 3.98 times higher than the *apxIVA*-undetectable samples ( $n=192$ ) ( $P < 0.0001$ ).



**Fig. 3:** The cross-comparison of IRPC value, SPES score, and *apxIVA* positivity. The serum IRPC corresponds with the *apxIVA* detection rate and SPES score. However, the main factor that correlated with the IRPC of the samples in each group was the SPES score according to the statistical analysis (ANOVA,  $P = 0.002$ ); SPES score:  $\bullet$ (0),  $\blacksquare$ (1),  $\blacktriangle$ (2),  $\blacktriangledown$ (3),  $\blacklozenge$ (4); n.: number of samples; P/ N: positive/negative for *apxIVA* detection.

**Table 3:** The results of serological and molecular examination

	SPES score				
	0	1	2	3	4
ELISA-test	(n=261)	(n=143)	(n=119)	(n=97)	(n=45)
Seropositive (%)	28.4	37.8	58.8	59.8	35.6
Median IRPC	35.9	53.6	65.0	69.1	42.6
PCR-test	(n=68)	(n=63)	(n=48)	(n=37)	(n=35)
<i>apxIVA</i> -positive (%)	5.9	22.2	33.3	35.1	34.3

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 JLL designed, operated the experiments and drafted the manuscript. FC, WCL, and YCW assisted in tissue sample collection. SLH and CJK helped in data analysis.

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