Serology and DNA-based detection and serotyping of *Actinobacillus pleuropneumoniae* Pohl from backyard pigs in selected provinces of Luzon, Philippines

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Actinobacillus pleuropneumoniae (APP) is the etiologic agent of porcine pleuropneumonia (PP) and one of the pathogens causing the porcine respiratory disease complex (PRDC). PP and PRDC cause significant losses in the swine industry worldwide, hence, proper and accurate diagnoses are important in implementing appropriate measures to manage and control APP diseases. In this study, serology and DNA-based methods were employed to detect the presence of APP, and multiplex PCR based on *Apx* genes was used to determine the prevalent APP serotypes, in 397 backyard pigs from eight provincess in Luzon, Philippines. Enzyme Linked Immunosorbent Assay (ELISA) showed that 88 out of 397 serum samples were positive for ApxIV toxin antibodies while 49 out of the 397 oro-nasal swabs DNA extracts were positive for the *omlA* gene. The ApxIV ELISA results were found to be significantly associated with the number of clinical signs and age of the pigs but not so for the *omlA* gene PCR results. Significant association (P-value < 0.0001) between a positive ApxIV ELISA result and a positive omlA-PCR result was established. Serotype 5 was determined to be the most prevalent serotype followed by serotypes 1 and 12 or 13. This study confirmed the presence of APP infection in backyard pigs in Luzon. The data generated may provide support in the proper management and control of APP infection through the early detection of subclinical carriers to prevent transmission of the pathogen, as well as in the possible incorporation of the prevalent serotypes in the development of new vaccines.

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INTRODUCTION

Actinobacillus pleuropneumoniae (APP) is a Gram-negative, non-motile, nonspore-forming, capsulated rod bacterium belonging to Family Pasteurellaceae (Marsteller and Fenwick 1999; Nicolet 1992). There are sixteen major serotypes of APP which are differentiated based on their antigenic differences particularly in their cell wall lipopolysaccharides (LPS) and capsular polysaccharide (Bossé et al. 2017). The different

serotypes vary considerably in their virulence and can be grouped into two based on their requirement for nicotinamide adenine dinucleotide (NAD). Serotypes 1-12 and 15-16 are clustered in NAD-dependent biovar 1, while serotypes 13 and 14 are grouped in NAD-independent biovar 2 (Sassu et al. 2017).

APP is the etiologic agent of porcine pleuropneumonia, a severe and contagious respiratory disease of swine (Savoye et al. 2000; Sidibe et al. 1993). Porcine pleuropneumonia occurs in all pig-rearing countries. It causes significant economic impact due to high mortality rates caused by pneumonia or reduced production in terms of average daily weight gain and feed conversion rate, and increased medication or vaccination costs in chronic or subclinical cases of the disease (Gottschalk and Lacouture 2014; Paradis et al. 2004). APP is also one of the primary pathogens involved in Porcine Respiratory Disease Complex (PRDC). PRDC is described as pneumonia of multiple etiology involving both viral and bacterial agents causing clinical diseases and failure in weight gain in finishing pigs (Brockeimeier et al. 2002).

APP is an economically-important respiratory pathogen in pigs and had been reported to be present in swine raised in Luzon, Philippines, causing problems to the local swine industry (Castro 1995; Interior 1989; Irorita 2011; Torres et al. 2006). Proper and accurate diagnoses are important in implementing appropriate measures to manage and control APP diseases and avoid significant production losses. Serological surveillance is necessary to determine the current herd immunological status, whereas early detection of subclinically-infected pigs or asymptomatic carriers of APP is important in preventing the spread of the pathogen. Serotyping is also necessary in making informed diagnosis, epidemiological investigation of outbreaks, and detecting newly-emerged serotypes that are rare or previously not found in a geographical area (Bosse et al. 2014). The virulence and distribution of prevalent serotypes also varies in different continents (Gottschalk 2012; Gottschalk and Lacouture 2014).

Serology remains as the most cost effective and preferred method for the diagnosis of APP infection (Gottschalk 2012). Other methods such as bacterial isolation and polymerase chain reaction (PCR) tests are also performed in the detection of APP (Broes et al. 2007). However, isolating APP from contaminated samples is hindered by low sensitivity (Gram et al. 1996). Rapid testing with increased sensitivity can be achieved through the use of several PCR assays developed specifically for APP detection even from mixed bacterial cultures (Gram and Ahrens 1998; Savoye et al. 2001; Schaller et al. 2001). Serotyping by PCR had also been designed to address the cross-reaction problems usually encountered in conventional serotyping as well as the need for preparing a full-set of antisera (Rayamajhi et al. 2005; Angen et al. 2008; Ito et al. 2013).

In this study, serological detection of APP antibodies against ApxIV toxin was conducted with a wide coverage in Philippines particularly in Luzon. In addition, this study could also be the first in the Philippines to use PCR for the detection and serotyping of APP on samples from live animals. Baseline information on the current status of APP infection and its serotypes in backyard pigs from Luzon, Philippines was generated.

MATERIALS AND METHODS

Bacterial Strain

Actinobacillus pleuropneumoniae ATCC 27088 serotype 1 was used as the positive control in the Polymerase Chain Reaction (PCR) using *omlA* specific primers and serotyping by multiplex PCR. The bacterial culture was grown in brain heart infusion broth supplemented with nicotinamide adenine dinucleotide (10 µg/µl), for 24 hours at 37°C under 5% carbon dioxide.

Sample Collection and Examination for Clinical Signs

The sampling locations were selected based on the provinces with the highest production (in terms of swine population) among backyard farms in the Philippines (Philippine Statistics Authority, Bureau of Agricultural Statistics, 2016). A backyard piggery is defined by the Department of Agriculture Administrative Order (AO) 41 series of 2000 as having less than 20 sows and depends on commercial feedmills for feeds.

The sampling size was determined by Winepi software for sampling in detection of disease with a confidence level of 95%, an expected minimum prevalence of 30% and sampling fraction of 0.0001%.The composition of the samples by sources were as follows: Camarines Sur (n=83), Cagayan (n=62), Pangasinan (n=61), Batangas (n=56), Palawan (n=50), Marinduque (n=30), Bulacan (n=28) and Isabela (n=27). The pigs were examined for the presence or absence of clinical signs during sampling.

Blood samples, oral and nasal swabs were collected from 397 pigs of different ages. Pigs that showed clinical signs and/or with previous exposure to respiratory diseases were given priority for sampling. Samples were also taken from healthy pigs when the selected farms reported no history or incidence of the disease. The sampling period was from February to August 2017 and the sampling team included veterinarians and trained personnel, and representatives from the Provincial Veterinary Office, farms, or other cooperating institutions and agencies. A permit from the Institutional Animal Care and Use Committee (IACUC) was secured to ensure that the protocols followed were in compliance with established rules involving proper care and use of vertebrate animals.

The blood samples, oral and nasal swabs were placed in an ice chest and transported to the laboratory within an eight hour period after collection. Oral and nasal swabs were stored at -80°C until further analysis while the EDTA-anticoagulated blood samples were refrigerated at 4°C until serum preparation.

The ApxIV and *omlA* tests are specific for the detection of *Actinobacillus pleuropneumoniae* (Oliveira 2008; Fittipaldi et al. 2003). In this study, the ApxIV test was used for the serological test to check for the presence of the antibodies against ApxIV antigen in serum samples. On the other hand, the omlA test was used to detect for the presence of the *omlA* gene from the oro-nasal swabs of pigs.

Serological Detection of *A. pleuropneumoniae* through ApxIV ELISA

The presence of antibodies against ApxIV toxin of *A. pleuropneumoniae* in all the 397 collected sera was determined using a commercially available *Actinobacillus pleuropneumoniae* (APP) ELISA Test Kit (IDEXX, Westbrook, Maine) following the manufacturer's instructions. Results based on sample-to-positive ratio (S/P %) were interpreted as follows : S/P % ratio < 40% was considered as negative; 40% ≤ S/P % < 50% for suspect; and S/P ratio % ≥ 50% as positive.

Diagnostic *omlA* PCR Assay for *A. pleuropneumoniae* from Oro-Nasal Swabs

The oral and nasal swabs from each pig were pooled by mixing equal volumes (100 µl) of each swab solution into a 1.5 ml sterile microcentrifuge tube. The DNA from each pooled sample was extracted and purified using commercially-available DNA Mini

Table 1: Primers used for serotyping of *Actinobacillus pleuropneumoniae* by multiplex Polymerase Chain Reaction based on *Apx* toxin genes (Rayamajhi et al. 2005).

NAME	SEQUENCE (5' -> 3')	GeneBank ACCESSION NO.	POSITION
ApxIAF	ATC GAA GTA CAT CGC TCG GA	X52899	275-295
ApxIAR	CGC TAA TGC TAC GAC CGA AC	X52899	968-998
ApxIBF	TTA TCG CAC TAC CGG CAC TT	X68595	4102-4121
ApxIBR	TGC AGT CAC CGA TTC CAC TA	X68595	4893-4913
ApxIIF	GAA GTA TGG CGA GAA GAA CG	AY736188	973-993
ApxIIR	CGT AAC ACC AGC AAC GAT TA	AY736188	1918-1938
ApxIIIF	GCA ATC AGT CCA TTG GCG TT	X80055	9558-9578
ApxIIIR	GAC GAG CAT CAT AGC CAT TC	X80055	9934-9954
ApxIVDWN-L*	GCG AAA CAA TTC GAA GGG	AF021919	6456-6442
ApxIV-R*	GGC CAT CGA CTC AAC CAT	AF021919	4111-4128

Table 2: The *Apx* profile of the fifteen serotypes of *Actinobacillus pleuropneumoniae* after multiplex Polymerase Chain Reaction based on *Apx* toxin genes (Rayamahji et al. 2005).

SEROTYPE	*ApxIA* 723bp	*ApxIB* 811bp	*ApxIIA* 965bp	*ApxIII* 396bp	*ApxIV* 1600bp	*ApxIV* 2000bp	*ApxIV* 2800bp
1	+	+	+				
2		+	+	+			+
3			+	+			
4		+	+	+	+		
5a	+	+	+				+
5b	+	+	+				+
6		+	+	+		+	
7		+	+				+
8		+	+	+			+
9	+	+	+		+		
10	+	+					+
11	+	+	+		+		
12		+	+				
13		+	+				
14	+	+					
15		+	+	+			+

+ = presence of amplicon

Kit (QIAamp®, Qiagen, Hilden) following manufacturer's instructions.

The extracted DNA served as the template in the PCR assay. The *omlA* specific-primers used were as follows: Forward- 5'-AAGGTTGATATGTCCGCACC-3' and Reverse 5'-GCCGTAGCACCGATTACG-3' (Savoye et al. 2000). About 5 µl of each DNA template was added to the 25 µl reaction mix containing 12.5 µl of GoTaq® Colorless PCR Master Mix (Promega, United States), 3.5 µl of nuclease-free water and 2.0 µl (10 µM) of each primer. Amplification was carried out in a DNA thermal cycler (ProFlex™ Thermo Fischer Scientific, Singapore) following an initial denaturation at 95°C for 4 min, 40 cycles of denaturation at 94°C for 30 sec, annealing of primers at 57°C for 30 sec and extension at 72°C for 1 min and final extension at 72°C for 10 min. The PCR products (10 ul) were checked on 2% agarose gel in Tris acetate EDTA (TAE) buffer (40 mM Tris-HCl, 20mM acetic acid, 1 mM EDTA, pH 8.2) by gel electrophoresis (Mupid®, Japan) set at 100V. The gel was stained with 3X gel red (15 µl in 50 ml TAE) for viewing and was photographed under a UV transilluminator. For each gel electrophoresis run, molecular size standard VC 100bp Plus (Vivantis, Malaysia) was included.

Serotyping of *A. pleuropneumoniae* based on *Apx* Toxin Genes

Multiplex PCR based on *Apx* toxin genes was employed in serotyping of *A. pleuropneumoniae* (Rayamajhi et al. 2005). Five sets of primers were used to amplify *ApxI*, *ApxII*, *ApxIII*, and *ApxIV* genes in a combination that matched the Apx genotype and phenotype profile of APP for a particular serotype (Table 1) (Rayamajhi et al. 2005). The total volume of the PCR mix used was 25 µL containing 12.5 µl of Qiagen® Multiplex PCR Master Mix, 5 µl of template DNA, 2.5 µl of primer mix (0.2 µM per primer) and 5 µl of RNA free-water. The PCR was run on ProFlex™ thermal cycler (Thermo Fischer Scientific, Singapore) with the following cycling conditions: initial denaturation at 95°C for 15 min; 40 cycles of denaturation at 94°C for 30 sec, annealing of primers at 57°C for 90 sec, and primer extension at 72°C for 3 min; and final extension at 72°C for 10 min. The PCR products (10 µl) were analyzed by gel electrophoresis (Scie-Plas, United Kingdom) using 1.3% agarose at 100V. Bands were visualized by staining with GelRed (3x, 15 µl in 50 ml TAE). Molecular size standard VC 100bp Plus (Vivantis, Malaysia) was included in each gel run. The gel was observed under a UV transilluminator, and the resulting banding patterns and sizes were compared with the established Apx profile of the 15 serotypes of APP (Table 2) (Rayamajhi et al. 2005).

Nucleotide sequencing of the PCR-amplified *omlA* gene

To confirm the identity of the PCR amplicon, the amplified *omlA* gene of a sample with considerably high concentration (based on the band on gel) and obtained from a pig positive for clinical signs was sent for DNA sequencing to Apical Scientific Sdn. Bhd. (Malaysia). The sequence obtained was analyzed using Basic Local Alignment Search Tool (National Center for Biotechnology Information, NCBI).

Data Analyses

Descriptive statistics was used to summarize the data collected in this study. Statistical analyses were performed using STATA version 13.0. Frequency and percentage were used for categorical variables, and mean and SD for interval/ratio

Table 3: Summary of the clinical signs observed in backyard pigs from selected provinces in Luzon, Philippines

Province	Number of Samples	CLINICAL SIGNS*					
		Anorexia	Respiratory	Diarrhea	Runting	Fever	Lethargy
Camarines Sur	83	30 (36.14)	3 (3.61)	6 (7.22)	9 (7.22)	0 (0.00)	2 (2.41)
Cagayan	62	11 (17.74)	7 (11.29)	9 (14.52)	2 (3.23)	2 (3.23)	3 (4.83)
Pangasinan	61	7 (11.48)	13 (21.31)	5 (8.20)	2 (3.28)	4 (6.56)	4 (6.56)
Batangas	56	1 (1.79)	2 (3.58)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Palawan	50	21 (42.00)	12 (24.00)	2 (4.00)	4 (8.00)	3 (6.00)	2 (4.00)
Marinduque	30	6 (20.00)	1 (3.33)	7 (23.33)	3 (10.00)	0 (0.00)	0 (0.00)
Bulacan	28	0 (0.00)	10 (35.71)	16 (57.14)	0 (0.00)	0 (0.00)	0 (0.00)
Isabela	27	7 (25.93)	7 (25.93)	1 (3.70)	0 (0.00)	2 (7.41)	0 (0.00)
Total	**397 (100)**	**83 (20.90)**	**55 (13.85)**	46 (11.59)	20 (5.03)	11 (2.77)	11 (2.77)

Legend: * Frequency (percentage) of each clinical sign per province.

Table 4: Prevalence of antibodies against ApxIV in serum of backyard pigs from selected provinces in Luzon, Philippines.

Provinces	ELISA Results			Total	Prevalence (%)
	Positive	Negative	Suspect		
Bulacan	10	17	1	28	**35.71**
Isabela	9	16	2	27	33.33
Palawan	15	32	3	50	30.00
Batangas	15	40	1	56	26.79
Cagayan	14	48	0	62	22.58
Pangasinan	11	50	0	61	18.03
Camarines Sur	12	69	2	83	14.46
Marinduque	2	28	0	30	**6.67**
Total	**88**	300	9	**397**	**22.16**

Remarks: Negative: S/P % ratio < 40% ; Suspect: 40% ≤ S/P % < 50%; Positive: S/P ratio % ≥ 50%.

variables. Association between the variables were determined using the chi-square test, Fischer's exact test and simple logistic regression. Significant differences were determined at $\alpha = 0.05$.

RESULTS AND DISCUSSION

Examination of pigs for clinical signs

APP affects mainly the respiratory tract of pigs (Gottschalk and Lacouture 2014). The clinical signs observed in the animals during sampling were grouped into six categories namely (1) respiratory signs; (2) anorexia; (3) runting; (4) fever; (5) lethargy / depression; and (6) diarrhea. Coughing, nasal discharge, thumping, panting, dyspnea and cyanosis as observed, were all considered under the respiratory signs. It should be noted however, that APP may not be the sole pathogen that can cause these clinical signs, but other respiratory pathogens such as viruses or other bacteria may also be considered as causative agents of the disease. Examination for clinical signs, for the samples collected, revealed that Bulacan (92.85%) followed by Palawan (88.00%) showed the highest percentages of pigs showing clinical symptoms. Anorexia (20.90%) was the most frequent clinical sign seen among the pigs sampled (Table 3). The highest percentages of anorexic pigs per province were found in Palawan (42.00%) and Camarines Sur (36.14%). The presence of respiratory signs (13.85%) was the second most common clinical sign observed. Bulacan (35.71%), Isabela (25.93%) and Palawan (24.00%) were the provinces with the highest percentages of pigs with respiratory signs.

Serological Assay for ApxIV Antibodies

Serological test is an important and widely used diagnostic tool for APP surveillance (Broes et al. 2007). In this study, an ELISA kit (IDEXX, Westbrook, Maine) was used to detect for the antibodies against ApxIV toxin. The ApxIV toxin is unique to APP and is produced by all APP serotypes (Gonzales et al. 2017; Schaller et al. 1999). The expression of ApxIV occurs *in vivo* and is induced through the infection of pigs with APP (Schaller et al. 1999). The ApxIV toxin is highly immunogenic and it induces strong production of antibodies in infected animals (Dreyfus et al. 2004). It has been used as an important marker for the detection of APP infection in pigs (Gimenez-Lirola et al. 2014).

Based on ELISA, a total of 88 out of the 397 serum samples (22.16%) were positive for antibodies against ApxIV antigen (Table 4). The highest prevalence of ApxIV serum antibodies was found in pigs from the province of Bulacan (35.71%) while the lowest prevalence was seen in pigs from Marinduque (6.67%). The prevalence of APP infection in sera of pigs may be attributed to the geographical location of the provinces. Bulacan is one of the major pig-producing provinces in Central Luzon (Alawneh et al. 2018). In the study by Alawneh et. al (2018), a high prevalence of gross pathological lesions in the lungs of pigs scored at slaughter (47% had lung scores above 6 and 22% had pleurisy) has been reported from Bulacan and Pampanga. The results suggested that a large proportion in Central Luzon (Region 3) are likely exposed to a variety of major respiratory pathogens throughout the production cycle (Alawneh et al. 2018). On the other hand, the island of Marinduque is relatively distant and isolated which may limit transport of pigs to and from the province.

The prevalence of antibodies against APP ApxIV antigens reported in this study was lower (22.16%) compared to the overall pig seroprevalence of 32% reported in a previous study (Irorita 2011). This may be attributed to the differences in the provinces from where the samples were obtained. The scope of this study was also wider covering eight provinces, with a total of 397 pigs compared to the four provinces (Batangas, Cavite, Rizal and Quezon) and 250 pigs sampled in the previous study (Irorita 2011). However, it was cited in the study of Irorita (2011) that low and uneven number of herds were analyzed per province. Quezon province had the lowest number of herds (2 herds) and pigs tested (20 out of 250), but both herds (100%) and 75% of the pigs tested were seropositive. In addition, Cavite had zero herd prevalence in 3 herds and only a 3% pig prevalence. The largely varied results from the previous study

Table 5: Association between the number of clinical signs and the presence of ApxIV antibodies in sera of backyard pigs from selected provinces in Luzon, Philippines.

Number of Clinical Signs	ELISA Result (n = 397)			Total	P-Value
	Positive	Negative	Suspect		
0	44 (49.44)	184 (61.54)	3 (33.33)	231 (58.19)	0.049
1	34 (38.20)	77 (25.75)	5 (55.56)	116 (29.22)	
2	8 (8.99)	35 (11.71)	1 (11.11)	44 (11.18)	
3	3 (3.37)	3 (1.00)	0 (0.0)	6 (1.51)	

Legend: * Frequency (percentage) of each clinical sign per province.

Table 6: Presence of ApxIV antibodies against APP among pigs at different stages of growth.

Age / Stage	ELISA Result (n=397)			TOTAL
	Positive	Negative	Suspect	
Weaner	12	61	1	74
Grower	16	85	3	104
Fattener	20	121	4	145
Sow	27	14	0	41
Others	13	19	1	33
Total	88	300	9	397

have greatly contributed to the discrepancy with the findings of this study.

Nine samples (2.27%) were considered as suspects, with sample-to-positive ratio (S/P %) within 40-50%, and the same result was obtained after second ELISA was performed (Table 4). Suspects are pigs which may have been infected, but have not yet produced significant antibody titer to be considered as positive. In such cases, re-sampling on the same animal is recommended, however, it was not possible for this study.

Fischer's exact test revealed that each clinical sign was not significantly associated with the ELISA results, indicating that the appearance of any of the clinical signs may not necessarily result in the detection of antibodies against ApxIV toxin. However, the data may provide preliminary information to suggest that the observation of anorexia, respiratory symptoms or fever in pigs may likely give a positive ELISA result based on the higher percentages of positive ELISA results over negative ELISA results. In the study of Montaraz et al. (1996), clinical signs of APP disease were characterized by respiratory distress with coughing, lethargy, anorexia and elevated rectal temperatures higher than 105°F.

The pigs tested presented no clinical signs to having one to three clinical signs (Table 5). Chi-square test showed that the number of clinical signs is significantly associated with the results of ELISA (P-value = 0.049) indicating that obtaining a positive ELISA result is more likely in pigs presenting one to three clinical signs than in asymptomatic or apparently healthy pigs. The reliability of serum tests is often questioned in the absence of clinical signs (Montaraz et al. 1996; Dron et al. 2012). The results of this study, therefore, confirmed APP infection in clinically positive pigs which were serologically positive or produced antibodies against ApxIV toxin. It could also indicate that the Apx-IV ELISA is specific and sensitive to detect serum antibodies against ApxIV toxin (Marsteller and Fenwick 1999).

The presence of APP antibodies was examined in pigs of different ages. Based on age/ growth stage, pigs were categorized as follows: (1) weaner - between weaning until ten weeks of age; (2) grower – in the early stage of rearing; (3) fattener – in the latter stage of rearing; (4) sow – breeding female that has farrowed; (5) others – included gilts, boar, native and pigs with no information given (Compassion in World Farming 2013). Results showed that percent prevalences of ApxIV antibodies were increasing based on age group from weaner (13.64%), grower (18.18%), fattener (22.73%) to sows (30.68%) (Table 6). Fischer's exact test indicated that the ages of the pigs were found to be markedly associated with the ELISA results indicating that ApxIV antibody production increased with the age of the pigs (P-value=<0.0001). In the study of Gimenez-Lirola et al. (2014), it was determined that the seroprevalence of anti-ApxIV IgG in field samples was between 18% in suckling pigs to 100% in adults. It is assumed that most sows in an infected herd carry the pathogen and vertically transmit it to the offsprings by close contact as the bacteria are shed in nasal secretions (Gottschalk 2012; Sassu et al. 2017). Infected sow herds are typically seropositive and pass high concentrations of antibodies to their piglets through colostrum (Marsteller and Fenwick 1999). As the passively acquired immunity wanes between 5 to 12 weeks of age, clinical signs mainly appear in 12 to 16 week-old pigs (Opriessnig et al. 2013). Overall, the findings of this study provide evidence that humoral responses to ApxIV and APP prevalence increase with age (Gimenez-Lirola et al. 2014).

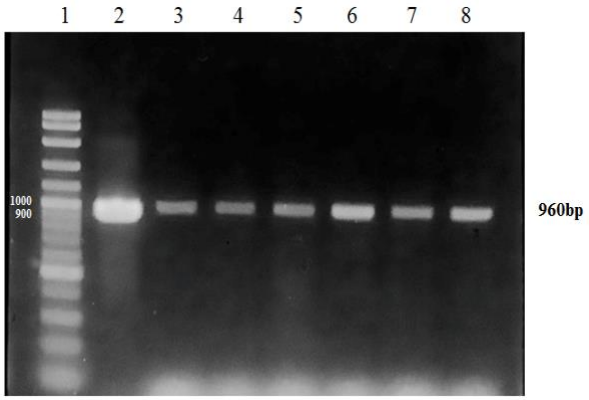

Figure 1: Agarose gel electrophoresis of PCR amplified *omlA* gene from oro-nasal swabs of pigs from selected provinces in Luzon, Philippines. Lane 1 - DNA ladder; Lane 2 - APP culture; Lanes 3- 8 - oro-nasal swab samples positive for *omlA* (Samples no. 56 (Batangas), Sample nos. 100 and 150 (Bulacan), Sample 331 (Pangasinan), Sample nos. 406 and 410 (Palawan), respectively). Expected amplicon size was approximately ~960bp.

Diagnostic *omlA* PCR Assay for Actinobacillus pleuropneumoniae

The extracted DNA from the 397 oro-nasal swab samples were used for the detection of APP by *omlA* PCR. The *omlA* gene encodes for the outer membrane protein, a bacterial lipoprotein widely distributed in β and γ proteobacteria (Vanini et al. 2008). The omlA protein is a virulence factor of APP and it facilitates iron acquisition from the host by binding to transferrin (Baltes

Table 7: Summary results for PCR amplification of *omlA* gene from oro-nasal swabs of pigs from selected provinces in Luzon, Philippines.

Province	PCR Results		Total	% Positive
	Positive	Negative		
Bulacan	7	21	28	25.00
Palawan	12	38	50	24.00
Batangas	13	43	56	23.21
Pangasinan	5	56	61	8.20
Isabela	2	25	27	7.41
Camarines Sur	6	77	83	7.22
Cagayan	4	58	62	6.45
Marinduque	0	30	30	0.00
Total	49	348	397	**12.34**

Table 8: Association between Apx IV enzyme-linked immunosorbent assay and PCR results.

ELISA	PCR*		Odds Ratio	P-value
	Negative	Positive		
Negative	274 (78.74)	25 (51.02)	2.50	<0.0001§
Positive	66 (18.97)	23 (46.94)		
Suspect	8 (2.30)	1 (2.04)		
OD	0.373 ± 0.523	0.794 ± 0.678	2.72	<0.0001§
S/P%	22.43 ± 49.31	61.12 ± 58.24	1.01	<0.0001§

**Frequency (%)/ Mean ±SD; §Simple Logistic Regression*

Table 9: Summary of the frequencies of ApxIV-ELISA positive, omlA-PCR positive, and Apx-ELISA and omlA-PCR positive pigs with or without clinical signs per province.

CLINICAL SIGN	ELISA (+) ONLY	PCR (+) ONLY	ELISA AND PCR (+)
Absent	31 (35.00%*)	12 (24.49%**)	13 (14.77%*;26.53%**)
Present	34 (38.63%*)	14 (28.57%**)	10 (11.36%*; 20.40%**)

Legend: * Based on total number of ELISA positive; **Based on total number of PCR positive

et al. 2002). Metabolism of iron is highly important in the survival and multiplication of APP in the host (Sassu et al. 2017).

The PCR amplification using the *omlA* gene-specific primers yielded a 960 bp amplicon. (Figure 1). The amplicon was sequenced, and sequence analysis results revealed 98 to 99% sequence identity (E-value = 0.0) with *Actinobacillus pleuropneumoniae* outer membrane lipoprotein A (*omlA*). Based on the presence of the 960 bp amplicon, 49 out of the 397 (12.34%) pigs were considered *omlA* positive (Table 7). These results confirmed the APP infection, through the detection of the pathogen in the upper respiratory tract of the pigs tested. APP invades the tonsils and the upper respiratory tract and it can be isolated from nasal cavities, tonsils, middle ear cavity and lungs of infected pigs (Bosse et al. 2002; Sidibe et al. 1993).

The highest number of *omlA*-positive pigs was observed in Bulacan (25%), followed by Palawan (24%) and Batangas (23.21%). These results concurred with the ELISA results as the high prevalence of ApxIV antibodies was also detected from samples obtained from these three provinces. Previous studies conducted in the Philippines (Castro 1995; Interior 1989; Torres et al. 2006) involved the isolation of APP from lung samples using conventional microbiological methods. Based on their results, the isolation rate of APP from pneumonic lungs was quite low ranging from 1.85% to 13.61%. Isolating the pathogen from affected lung tissue is the most adequate in diagnosing APP infection. However, it is difficult to culture APP due to its fastidious nature and complex nutritional requirements (Branka et al. 2004; Vaduva et al. 2010). The rate of isolating APP can be greatly improved by immuno-magnetic separation (IMS) which involves the use of microscopic magnetic beads coated with serotype-specific APP antibodies. However, IMS is time-consuming and expensive (Angen et al. 2001; Gagne et al. 1998).

The major differences of the current study from the previous studies are sampling from live animals (oro-nasal swabs) and detection of APP through PCR assay which is more sensitive than conventional cultivation of APP. Several PCR assays that were developed to diagnose and identify APP from live pigs such as the amplification of *omlA* gene from tracheobronchial lavage fluids and tonsillar biopsies (Savoye et al. 2000), and *dsbE*-like gene from nasal and tonsil swabs (Chiers et al. 2001) are highly sensitive with a detection threshold of 10^2 CFU/50 µl (2.0 x 10^4 CFU/ml sample). A nested PCR using primers for ApxIVA gene was also designed for the identification of APP from nasal swabs of pigs with a sensitivity of 10 fg (Schaller et al. 2001). In this study, *omlA* gene was detected from DNA samples from oro-nasal swabs at concentrations as low as 2.7 ng/µl, indicating the high sensitivity of PCR assay in detecting *omlA* from oro-nasal swabs of pigs. It was observed previously that 2.5 times more positive results were obtained from culture-independent PCR assay than conventional bacterial isolation (Savoye et al. 2000). Moreover, in the study of Chiers et al. (2001), 32 out of 40 animals tested were positive in *dsbE*-PCR, while APP was isolated only in 2 pigs by conventional bacterial cultivation. This was attributed to the difficulty in visually identifying APP colonies due to the heavy contamination of microflora from nose and tonsils (Chiers et al. 2001).

Chi-square test revealed that clinical signs were not significantly associated with the PCR results (P-value = 0.191), indicating that a clinically positive pig may not be *omlA* positive. The apparently healthy animals may have been subclinically infected with APP. The age of the pig was also found not to be

significantly associated with the PCR result (P-value = 0.630) as all ages of pigs may be infected by APP (Marsteller and Fenwick 1999).

Association of ELISA and PCR test results

Table 8 shows the comparison of APP detection using ApxIV-ELISA and *omlA* PCR. Twenty three samples (47.92%) tested positive for both ApxIV antibodies and *omlA* gene, while 25 samples (52.08%) were *omlA*-positive but ApxIV-negative. The *omlA*-negative samples were 340 pigs divided into 66 (19.41%) and 274 (80.59%) for ApxIV-positive and negative samples, respectively. Using simple logistic regression, it was found that the ELISA test results were significantly associated with the results of the PCR test (P-value <0.0001). Furthermore, the optical density reading and S/P% values obtained from the ELISA test were both significantly associated with the PCR results based on simple logistic regression (P-value <0.0001). These results may indicate that positive serological result is associated with a positive PCR result and it is most probable to detect *A. pleuropneumoniae* from the upper respiratory tract in serologically positive pigs. In peracute and acute pulmonary infections, APP can be found in large numbers in nasal discharges along with its presence in pneumonic lung (Gottschalk 2012). Pigs can also become carriers of the pathogen in the nasal cavities, tonsils and/or chronic lung lesions after clinical and subclinical infections (Chiers et al. 2001).

Although the Apx-IV ELISA and PCR results are associated, these two tests provide different information. PCR test results indicate the presence of the pathogen, while serology reveals the host's exposure to the bacterium and its subsequent immune response through antibody production (Chiers et al. 2001). Serological analysis through ELISA has been a classical method of diagnosing, monitoring and controlling APP infections in naïve and positive swine herds (Oliveira 2008; Gottschalk 2012). However, serological tests may sometimes provide inconclusive results (Fittipaldi et al. 2003). Antibody titers that are seen in ELISA may also be interpreted as vaccine- induced antibodies. With this, the vaccination history of animals tested in this study was reviewed, and out of 397 swine tested using ELISA, only 3 animals were known to have been vaccinated against APP, which may suggest that only 1.14% of the positive reactions in this study were due to vaccination.

In this study, APP antibodies against ApxIV toxins were detected from apparently healthy pigs or pigs not showing clinical signs (Table 9). These results may be considered as false positives because the infection was not confirmed through the demonstration of the presence of the pathogen, although prior infection could also be a possible explanation, considering that antibodies for APP can persists for many months (Haesebrouck et al. 1997). The activity of the antibodies in clearing the pathogen might have been the reason for observation of clinically and serologically positive but PCR-negative pigs as antibodies produced by infected pigs could neutralize Apx toxins and reduce the severity of clinical signs as a result of enhanced phagocytosis by opsonization (Cruijsen et al. 1995). The bacterial cells may have also been residing in the deep tonsillar crypts which rendered them inaccessible through oral and nasal swabbing (Fittipaldi et al. 2003).

Clinically positive pigs with APP infection, as confirmed by *omlA* PCR, but without significant antibody production were also identified (Table 9). The result may be explained by the considerable lag between the time of infection and significant increase in antibody titer (Tobias 2014). These animals may still be in the early stage of infection wherein the increase in antibody titer to a maximum may occur after 3 to 4 weeks (Haesebrouck et al. 1997). The results also showed that *omlA*-positive pigs without clinical signs and serologically negative for antibodies against ApxIV toxin were detected (Table 9). These pigs may have been subclinically-infected showing no clinical signs and pathological lesions typical of APP because the animals harbor the pathogen in the upper respiratory tract particularly in tonsils and nasal cavities and does not enter the lungs (Chiers et al. 2002; Fittipaldi et al. 2003). High antibody titers against ApxIV toxin may not be produced during subclinical infections or antibody production against the toxin may not be induced making detection by serology difficult (Chiers et al. 2002; Chiers et al. 2010). Despite the high specificity of ApxIV ELISA, there are few data which show the sensitivity of ApxIV-ELISA in detecting subclinically infected pigs (Broes et al. 2007). It is through PCR assay, that screening for APP from samples obtained from live animals, can be used for early detection of subclinically infected or asymptomatic pigs (Savoye et al. 2000). Subclinically-infected pigs can become carriers of the pathogen and serve as sources of infection for other pigs that may result in recurring disease outbreaks (Gimenez-Lirola et al. 2014). Therefore, animals should be checked for absence of APP before they are mixed with APP-free herd to prevent the transmission of APP and subsequent economic losses (Sassu et al. 2017). The presence of APP antibodies and the *omlA* gene in pigs without clinical signs was also detected (Table 9). The pathogenesis of APP disease is complex and multifactorial (Haesebrouck et al. 1997). The amount and severity of the disease may be attributed to the differences in management, environmental conditions, pathogen load, host's immune status and concurrent diseases (Gottschalk and Broes 2013; Sjölund et al. 2011). The infection may have also been caused by one or more than one low or mildly virulent serotypes causing high levels of antibodies (Gottschalk and Broes 2013). Virulent serotypes (such as serotypes 1, 5 or 7) may also infect some herds without demonstrating any clinical signs or lesions, but may result in occasional clinical outbreaks as a result of co-infections or unfavorable environmental conditions (Gottschalk and Broes, 2013).

Overall, the detection rate of ApxIV-ELISA positive samples was higher (22.16%) in comparison to that of *omlA*-positive samples (12.34%) . Nasal swabs, together with tonsils, are frequently used for the detection of APP by direct PCR (Gottschalk 2015). However, direct PCR using nasal swabs may have been inadequate for complete diagnosis of APP infection or maybe PCR inhibitors might have been present in the sample although it was not checked in this study. Parallel PCR testing of nasal, tonsillar and oropharyngeal swabs may be performed to increase the sensitivity of sampling live pigs (Sassu et al. 2017). Fablet et al. (2010) was successful in detecting more than 30% of healthy carrier sows in positive herds using this technique. Definitive diagnosis of APP infection was confirmed through the combined observation of clinical signs, detection of serum antibodies against ApxIV toxin and demonstration of the presence of pathogen through *omlA*-PCR. Therefore, a single test would be insufficient to declare adequate diagnosis and implement appropriate management and control. Serological and PCR tests can serve as complementary tools in determining immune status and diagnosing APP infection of individual animal or herds. These can be done together with clinical inspection. In cases of ambiguous diagnosis, bacterial isolation or other diagnostic procedures must be done to clarify results. Several diagnostic approaches in dealing with unexpected *Actinobacillus pleuropneumoniae* serological results prescribed by Broes et al. (2007) could also be considered in deciding on proper course of action.

Serotyping based on Apx Toxin Genes of omlA Positive Samples

Figure 2 shows the Apx gene profiles of the APP serotypes detected from oro-nasal swabs of pigs from selected Luzon provinces. The serotype of APP from *omlA*-positive sample was

Table 10: The clinical signs in pigs infected with different serotypes of *Actinobacillus pleuropneumoniae*.

CLINICAL SIGN	FREQUENCY			
	Serotype 5	Serotype 1	Serotype 12 or 13	>1 serotype
Respiratory	2	1		1
Anorexia		3	2	
Respiratory + Anorexia				2
Respiratory + Lethargy	1			
Anorexia + Runting			1	1
Diarrhea + Anorexia				1
Diarrhea + Fever		1		
Anorexia + Diarrhea + Fever	1			
Lethargy + Respiratory + Fever				1
Without CS	7	3	4	5
Total	11	8	7	11

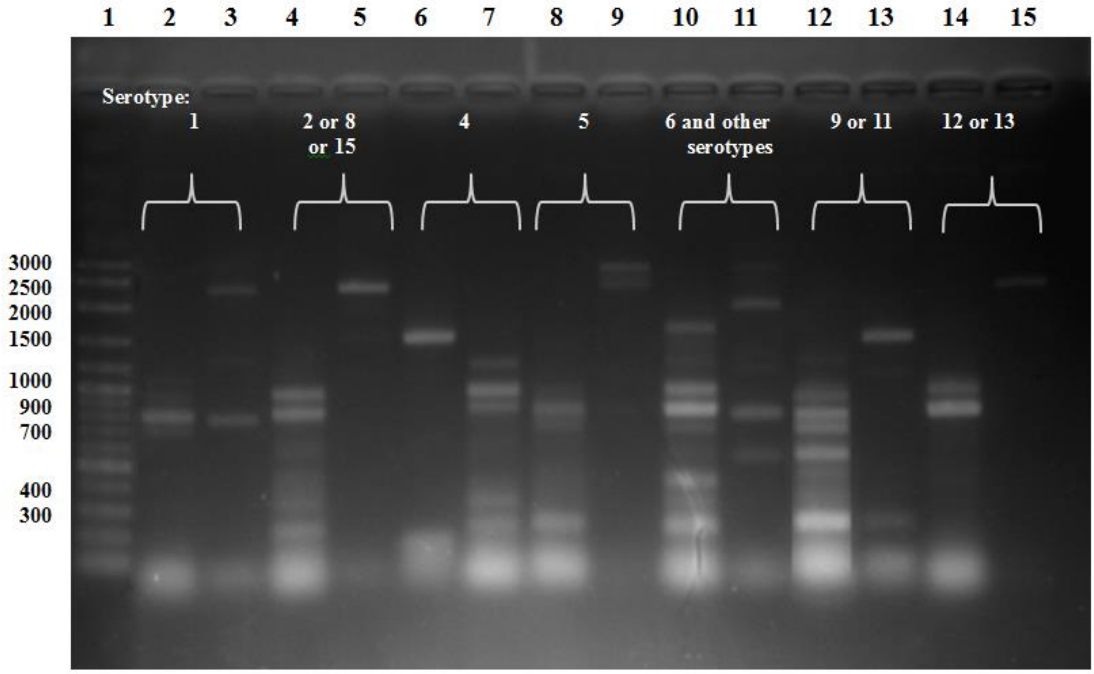

Figure 2: *Apx* profiles of the different serotypes of *Actinobacillus pleuropneumoniae* detected from oro-nasal swabs of backyard pigs raised in selected provinces of Luzon, Philippines.

identified based on the profile of four *Apx* genes (Rayamajhi et al. 2005). Results showed that serotype 5 was the most prevalent (22.45%) followed by serotype 1 (16.33%) and serotypes 12 or 13 (14.29%). Other serotypes observed were 6 (10.20%), 9 or 11 (8.16%), 2, 8 or 15 (6.12%), 4 (4.08%), 10 (2.04%) and an untypable strain (2.04%). It was also noted that more than one serotypes were present in some samples (22.45%). However, the specific serotypes could not be determined because of the presence of more bands than expected and random assignment to a particular toxin profile/serotype could not be made; except for serotype 6, that even in combination with other serotype/s, could be easily distinguished based on the presence of a 2000 bp *ApxIV* gene. The possibilities of detecting mixed serotypes and other *Actinobacillus* species producing *ApxI, ApxII* and *ApxIII* such as *A. suis*, *A. rossii* and *A. porcitonsillarum* which may interfere in serotype identification were higher because no culture method was conducted and the samples contain contaminating flora present in the oral and nasal areas of the pigs (Dreyfus et al. 2004). Sensitivity and specificity of the assay may be increased by performing bacterial isolation prior to serotyping. In most routine laboratories, isolation of APP strain is a requirement for PCR determination of serotype (Gottschalk 2015).

Torres et al. (2006) conducted coagglutination to determine the serotype distribution of APP isolated in the Philippines. In their study, serotype 5 which is a strongly virulent serotype was found to be the most prevalent. An earlier study (Interior 1989) reported serotype 1 as the most common serotype at 99% prevalence. Therefore, the findings of this study support the previous results that these serotypes are predominant in local swine herds. Serotypes 1 and 5 are the serotypes that predominate in North America causing outbreaks and in Asian countries such as Taiwan, Thailand, Korea and Japan among others (Assavacheep et al. 2003; Chang et al. 2002; Gottschalk 2012; Ito et al. 2013; Lee et al. 2015). It was also noticed that serotype 11, a highly virulent strain, has reduced frequency, while serotype 12 (or 13) was not detected prior to this study, but has now increased in number of occurrence. In Canada, a shift from the traditionally predominant and virulent serotypes 1 and 5 to less virulent strains such as serotype 12 was recorded. Strains of low virulence has led to decrease in outbreaks, though these resulted in herds having high seroprevalence (Gottschalk 2012).

The climate of the Philippines is tropical and maritime, which is characterized by relatively high temperature, high humidity and abundant rainfall (Philippine Atmospheric, Geophysical and Astronomical Services Administration, PAG-ASA). These adverse climatic conditions as well as rapid changes in temperature and insufficient ventilation promotes the progression and transmission of the disease, thus may also lead

to morbidity and mortality caused by APP in pigs (Gottschalk 2012). Backyard farms are more exposed to such conditions because of the lack of adequate facilities for environmental control, therefore, the prevalence of APP disease is higher. The serotype distribution among the different provinces was variable. Serotype 5 was the prevalent serotype in Isabela and Pangasinan and was observed in all provinces except Cagayan, while serotype 1 was distributed in four provinces. Various serotypes were observed to be present in Batangas and Palawan with a high incidence of serotypes 12 or 13, whereas serotype 9 or 11 was present in Bulacan and Camarines Sur. Multiple serotypes occurred in higher number of individual pigs in Bulacan and Palawan. The clinical signs observed in pigs infected with different serotypes are listed in Table 10. Pigs infected with serotype 5 of APP manifested respiratory signs alone or together with other clinical signs such as anorexia and diarrhea. Respiratory signs, anorexia, diarrhea and fever were observed in pigs infected with Serotype 1. Milder symptoms such as anorexia and runting were seen in animals with serotype 12 or 13. The infection of more than one serotype in pigs resulted in higher number of pigs which presented with one or combinations of clinical signs including respiratory, anorexia, diarrhea, fever and lethargy. Fischer's exact test result showed that the two most prevalent serotypes (serotypes 1 and 5) and the most frequent clinical signs (anorexia and respiratory) are not correlated. However, it is known that the degree of virulence and severity of clinical signs depend on the serotype present in the population (Marsteller and Fenwick 1999). The various combinations of the three major RTX toxins (*ApxI*, *ApxII* and *ApxIII*) determine the virulence of the different serotypes (Dreyfus et al. 2004). The ability to induce clinical signs vary among APP serotypes, where clinical signs may manifest as fast as 4-12 hour post-infection with some of the more virulent serovars (Opriessnig et al. 2013). Serotypes 1, 5, 9 and 11 are strongly pathogenic and epizootologically most relevant, and serotypes 2, 4, 6, 8 are less pathogenic, but epizootologically relevant (Frey et al. 1993). Serotypes 3, 6, 7 and 12 are least virulent (Marsteller and Fenwick 1999). On the other hand, this study found that the number of serotypes was significantly associated with the number of clinical signs observed in individual pig (P-value = 0.024). The detection of multiple serotypes in the provinces of Bulacan and Palawan might explain the high prevalence of APP infection in those provinces. The results of this study suggest that the manifestation of clinical signs from APP infection may be favored by co-infection with other APP serotypes or maybe other respiratory pathogens (Gottschalk and Taylor 2006).

Because of the increasing emergence of antibiotic resistance and demands concerning food safety, the use of vaccines is becoming more relevant in preventing bacterial diseases like diseases caused by APP (Ramjeet et al. 2008). Registered vaccines in the Philippines include killed APP or bacterins for certain serotypes (serotypes 1-5, 7, 9 and 11), subunit vaccines containing toxoids (ApxI, ApxII and ApxIII) with or without outer membrane proteins (OMP); and combination of toxoid-bacterin vaccines (Department of Agriculture - Bureau of Animal Industry). However, there is lack of vaccines inducing reliable cross-serotype protection (Maas et al. 2006). The prevalent serotypes identified from this study can be incorporated into vaccines, or are essential for choice of bacterins that could provide protection to the strains currently present in the field (Gottschalk 2012; Torres et al. 2006). Furthermore, the determination of the serotypes would allow for assessment of herd and area epidemiology (Gottschalk 2012).

CONCLUSION

In conclusion, this study provides relevant information to confirm that APP infection is prevalent in selected areas of Luzon. Findings from this study can be used in implementing proper management and control of APP infection such as early detection of subclinically-infected pigs showing no clinical symptoms of APP infection, but serve as carriers of the pathogen that can be transmitted to susceptible animals. Furthermore, the knowledge of prevalent serotype may help in making informed decisions in selecting strategies for addressing current status in the field. For future studies, it is recommended to perform bacterial isolation for ambiguous results and to determine the circulating serotypes in the local swine herds. The bacterial cultures could also be used in the development of bacterins that will provide protection against serotypes present in the field. Multiplex PCR for serotyping could be optimized for easier identification of serotypes or other targets, such as capsular genes may be considered, and commercially-available serotype-specific ELISA could be used for validation.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

CONTRIBUTION OF INDIVIDUAL AUTHORS

All authors contributed to the form and content of this paper through data gathering, data analysis, and writing.

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