

Original Article



Development of *Actinobacillus pleuropneumoniae* ApxI, ApxII, and ApxIII-specific ELISA methods for evaluation of vaccine efficiency

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ABSTRACT



Among various vaccines against *Actinobacillus pleuropneumoniae*, subunit vaccines using recombinant proteins of ApxI, ApxII, and ApxIII as vaccine antigens have shown good efficacy in terms of safety and protection. Therefore, subunit vaccines are being applied worldwide and the development of new subunit vaccines is actively being conducted. To evaluate the efficacy of the subunit vaccines, it is important to measure immune responses to each Apx toxin separately. However, the cross-reactivity of antibodies makes it difficult to measure specific immune reactivity to each toxin. In the present study, specific antigen regions among the toxins were identified and cloned to solve this problem. The antigenicity of each recombinant protein was demonstrated by Western blot. Using the recombinant proteins, we developed enzyme-linked immunosorbent assay (ELISA) methods that can detect specific immune responses to each Apx toxin in laboratory guinea pigs. We suggest that the ELISA method developed in this study can be an important tool in the evaluation of vaccine efficiency and vaccine development.

Keywords: *Actinobacillus pleuropneumoniae*; Apx toxins; vaccines, subunit; ELISA

INTRODUCTION

Porcine pleuropneumonia is a highly contagious respiratory disease that causes massive economic losses in the global pork industry [1-3]. *Actinobacillus pleuropneumoniae* is known to be the major causative pathogen of this disease [1,2]. It has been reported that there are 15 serotypes of *A. pleuropneumoniae*, showing susceptibility to pigs of all ages [4-6]. Pigs recovered from acute infection and chronically infected pigs can be carrier animals that shed the pathogen continuously without clinical symptoms. Thus, it is difficult to control and eradicate this disease in infected herds [4,7].

Apx toxins, which belong to the pore-forming repeat-in-toxin (RTX) family of *A. pleuropneumoniae*, are known to play an important role in the pathogenesis of *A.*

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Conflict of Interest

The APQA and BK21 PLUS and Research Institute for Veterinary Science had no role in study design, data collection and interpretation, or the decision to submit the work for publication. Choong Ang Vaccine Laboratories Company developed full-length Apx toxins for vaccination and conducted animal experiments. No third-party funding or support was received in connection with this study or the writing or publication of the manuscript. The authors declare that there were no conflicts of interests.

Author Contributions

Conceptualization: Jung M; Data curation: Jung M; Formal analysis: Jung M, Won H, Shin MK; Investigation: Oh MW, Shim S, Yoon I; Supervision: Yoo HS; Writing - original draft: Jung M, Won H; Writing - review & editing: Yoo HS.

pleuropneumoniae as major virulence factors [4,8]. The importance of Apx toxins in protective immunity against *A. pleuropneumoniae* infection has also been reported in many studies [4,9,10-12]. There are four different Apx toxins, ApxI, ApxII, ApxIII, and ApxIV, each of which are encoded by the *apx* operon. The *apx* operon includes four genes, the activator gene of *apxC*, the pretoxin structural gene of *apxA*, and the secretion-apparatus-encoding genes of *apxB* and *apxD* [4,11,12]. Among these toxins, ApxI is strongly hemolytic and cytotoxic, while ApxII is weakly hemolytic and moderately cytotoxic, and ApxIII is nonhemolytic and strongly cytotoxic. The toxins of ApxI, ApxII, and ApxIII, which are secreted by different serotypes in various combinations, mainly determine the virulence of the 15 serotypes of *A. pleuropneumoniae* [4,10,11]. The ApxIV toxin is produced by all serotypes of *A. pleuropneumoniae* in infected pigs, but not under *in vitro* culture conditions; therefore, antibody response to ApxIV toxin has been used as a marker for infected pigs [4,10,11,13,14].

In an effort to control the disease, vaccination has been actively conducted in the swine industry around the world. Currently available vaccines can be classified based on the antigens such as bacterins (killed whole bacteria), purified toxoid vaccines, and mixed vaccines (killed whole bacteria and purified toxins) [10,14,15]. Most of these *A. pleuropneumoniae* vaccines are primarily based on immunization against Apx toxins, which could be achieved effectively using subunit vaccines with recombinant proteins of ApxI, ApxII, and ApxIII [10,15]. It has also been reported that subunit vaccines using recombinant Apx toxins showed safety and cross-protection against different serotypes of *A. pleuropneumoniae* [16]. Therefore, subunit vaccines are being applied worldwide and the development of new subunit vaccines is actively being conducted [16-20].

It is important to measure immune responses to each Apx toxin separately when evaluating vaccine efficiency during the development of subunit vaccines containing the recombinant toxin antigens of ApxI, ApxII, and ApxIII [4,7,21]. Immune responses to *A. pleuropneumoniae* could be detected using various diagnostic methods such as radioimmunoassay, hemolysin neutralization, the microplate agglutination test, and complement fixation [5,22,23]. However, these approaches are not suitable for evaluation of the immune responses to each Apx toxin. Enzyme-linked immunosorbent assay (ELISA) using purified recombinant protein antigens is more sensitive than other methods and is able to determine antibody titers against each antigen [5,24-26]. Thus, ELISA methods have an advantage in evaluating vaccine efficiency for the development of subunit vaccines of *A. pleuropneumoniae* [3,19,20,26]. However, currently available commercial ELISA methods are difficult to apply to the evaluation of vaccine efficiency because they have been developed only to diagnose serotypes of *A. pleuropneumoniae*, while they cannot measure antibody titers against ApxI, ApxII, and ApxIII, respectively [11,24,25]. Moreover, antibodies against Apx toxins have been reported to have cross-reactivity to each other, making the determination of levels of immune responses to each toxin more difficult [21,27-29]. Therefore, the development of ELISA methods that can detect specific antibodies against each toxin should take precedence for the development of *A. pleuropneumoniae* subunit vaccines.

In this study, we developed ELISA methods that can evaluate specific antibodies against each toxin of ApxI, ApxII, and ApxIII. Specific peptide regions in each toxin were identified and candidate partial Apx toxins for ELISA were purified. The ELISA conditions using the recombinant partial toxins were then established. Using the sera of guinea pigs vaccinated with each toxin of ApxI, ApxII, and ApxIII of the commercial vaccine, the identified ELISA conditions were assessed to determine if they could be used in vaccine development as a tool for vaccine efficiency evaluation based on the quantification of ApxI, ApxII, and ApxIII-specific antibody responses.

MATERIALS AND METHODS

Bacterial strains and culture conditions

A. pleuropneumoniae serotype 2, isolated in Korea, was used for the genes of *apxIIA* and *apxIIIA* [12]. The *apxIA* gene was obtained from *A. pleuropneumoniae* serotype 5 isolated from a Korean pig with pleuropneumonia. The bacteria were cultured in Luria-Bertani (LB) broth (Difco, USA) with 0.01% β -nicotinamide adenine dinucleotide for 24 h at 37°C.

Homology analysis of *apxIA*, *apxIIA*, and *apxIIIA*

Protein sequence information regarding *apxIA* (AF363361), *apxIIA* (AF363362), and *apxIIIA* (AF363363) was retrieved from the National Center for Biotechnology Information database. Homology and conserved domains were investigated using multiple sequence alignment of the ClustalW2 program (<http://www.ebi.ac.uk/Tools/clustalw>) to identify specific gene regions among toxins.

Identification and cloning of specific genes

The gene sequence of each toxin was divided into four regions, the N-terminal (Nt), middle part 1 (M1), middle part 2 (M2), and C-terminal (Ct), for investigation of specific gene regions. Specific gene regions showing low levels of homology were determined based on the results of alignment of the four gene regions of each toxin. The specific gene regions identified for each toxin (partial Apx toxins) were amplified by polymerase chain reaction (PCR) using the primers described in **Table 1**. Total genomic DNA extracted from *A. pleuropneumoniae* serotype 2 and 5 was used for PCR templates (GenElute; Sigma-Aldrich, USA). The PCR products were electrophoresed in 1.0% agarose gel and then purified with a gel extraction kit (QIAquick; Qiagen, Germany). Amplified gene regions for each toxin were cloned with the Chaperon pET100 vector (Invitrogen, USA) into Top10 chemical-component *Escherichia coli* cells (Invitrogen) according to the manufacturer's protocols. To confirm gene insertion, DNA sequencing was conducted using an automated DNA sequencer (ABI377L; Applied Biosystems, USA). Following confirmation of gene insertion by DNA sequencing, the cloned partial Apx toxins were transformed into component *E. coli* M15 cells for expression.

Table 1. Primers for recombinant partial toxins of ApxIA, ApxIIA, and ApxIIIA

Region		Sequence	Size	Location
ApxIA				
Nt	F	CACCAatggctaaactctcagctcgatagag	525	1–525
	R	cgaaccactgagctcctcacc		
Ct	R	CACCTttgagtgtaacaacaactgattattagg	660	2,407–3,066
	R	agcagattgtgtaataaactactgaa		
ApxIIA				
Nt	F	CACCAatgtcaaaaatcactttgtcatcat	522	1–525
	R	ttctaattgattaggatctttattttgaa		
M2	F	CACCGtacagctagataacaaaaactgattat	582	1,543–2,124
	R	cgtcacagtataaccagtatgaaaac		
Ct	F	CACCGatatttatgtccataaaacagggcg	507	2,359–2,865
	R	agcggctctagctaattgaatatt		
ApxIIIA				
Nt	F	CACCAatgagtacttggtcaagcatgtag	549	1–549
	R	gagctctgaaccgccaataa		
M2	F	CACCGacttttagcaaagtagttttcgtatcc	600	1,576–2,175
	R	attacctgattgcgtaattcataatc		
Ct	F	CACCTtaggcaatggttttaattgtgct	705	2,452–3,156
	R	ttaagctgcttagctaggttacctag		

Nt, N-terminal; M1, middle part 1; M2, middle part 2; Ct, C-terminal.

Expression and purification of the recombinant proteins

E. coli M15 cells were grown in LB broth with ampicillin (100 µg/mL) at 37°C. When the culture reached an optical density (OD) of 0.6 nm, isopropyl β-D-1-thiogalactopyranoside (1 mM; Duchefa Biochemie, the Netherlands) was added and then cultured continuously for 4 h. The harvested cells were subsequently re-suspended in lysis buffer (20 mM Tris-hydrogen chloride, 500 mM sodium chloride, 8 M urea, 40 mM imidazole, pH 7.0). Nickel-nitrilotriacetic acid chelate affinity chromatography (GE Healthcare, UK) was conducted based on the manufacturer's protocols, after which the bound proteins were eluted with elution buffer (20 mM Tris-hydrogen chloride, 500 mM sodium chloride, 8 M urea, 500 mM imidazole, pH 7.0). The concentration of the purified recombinant proteins was then determined using a BCA Protein Assay kit (Pierce, USA)

Sodium dodecylsulfate-polyacrylamide gel electrophoresis and Western blot analysis

The purified partial-Apx toxins were separated by 12% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie blue R-250. For Western blot analysis, the separated proteins from the SDS-PAGE were electrotransferred onto an iBlot Gel Transfer Stacks nitrocellulose membrane (Invitrogen), which was then immunoblotted with sera (1:1,000) of guinea pigs vaccinated with subunit vaccines containing ApxI, ApxII, and ApxIII toxoids, respectively. An alkaline phosphatase-conjugated rabbit anti-pig IgG (H+L) at 1:2,000 dilution was used as the secondary antibody and the specific reaction was detected using an AP-conjugated substrate kit (BioRad, USA). The predicted size of the purified partial-Apx toxins matched the results of the SDS-PAGE and Western blot analyses based on amino acids constitution.

Guinea pig vaccination with ApxI, ApxII, and ApxIII toxoids

The full-length ApxIA, ApxIIA, and ApxIIIA are recombinant proteins and major components of the *A. pleuropneumoniae* vaccine newly developed by Choong Ang Vaccine Laboratories Company (Daejeon, Korea). Guinea pig vaccination was carried out three times with these full-length Apx toxoids by intramuscular injection of 200 µg of each toxin at two-week intervals, respectively. Sampling of sera was performed two days after the last administration. All experiments using guinea pigs were conducted in accordance with the procedures permitted by the Institutional Animal Care and Use Committees of Choong Ang Vaccine Laboratories Company (No. 160129-01).

Development enzyme-linked immunosorbent assays for ApxIa, ApxIIa, and ApxIIIA-specific detection

The assays were developed through checkerboard titration of 96-well microplates utilizing representative positive and negative sera. Sensitization of partial recombinant ApxIA, ApxIIA, and ApxIIIA antigens was performed using a two-fold serial dilution of each antigen at levels ranging from 3.125 ng/well to 200 ng/well. Antigens of partial-recombinant Apx toxins were coated onto 96-well microplates with 50 mM coating buffer (15 mM sodium carbonate and 35 mM sodium bicarbonate, pH 9.6), then incubated at 4°C overnight. Next, microplates were washed with a solution of phosphate-buffered saline containing 0.05% Tween 20 (PBST), after which they were blocked at room temperature for 2 h using 3% bovine serum albumin fraction V (BSA; Fisher Scientific, USA) in PBST. Sera from vaccinated guinea pigs with each full-length Apx toxin and serum from a nonvaccinated guinea pig were used as positive and negative controls, respectively. The microplates were incubated at room temperature for 2 h with 100 µL of two-fold serial diluted sera, after which they

were incubated with 100 μ L of 1:10,000 diluted horseradish peroxidase-conjugated goat anti-guinea pig IgG (H+L) (BioRad) at room temperature for 1 h. Sera and antibody used in ELISA were diluted with 1% BSA in PBST. Color development was performed with 3,3',5,5'-tetramethylbenzidine liquid substrate (Sigma-Aldrich) at room temperature for 10 min. The color development was stopped with 1 N sulfuric acid, after which the microplates were read at 450 nm. Based on the optimal ELISA conditions confirmed by checkerboard titration, ApxI, ApxII, and ApxIII-specific ELISA conditions were established under the same conditions of the checkerboard titration; however, 5% (for blocking) and 3% (for dilution of sera and antibody) skim milk in PBST were used.

Statistics

Data are presented as the means \pm standard deviation. Statistical significance was determined by Student's *t*-tests using SPSS version 23.0 (SPSS, USA). A *p* < 0.05 was considered to indicate statistical significance.

RESULTS

Homology among apxIA, apxIIA, and apxIIIA

All of the Apx toxins showed > 40% homology with each other (**Table 2**). The results of homology analysis between regions within each toxin (Nt, M1, M2, and Ct) are presented in **Table 3**. The M2 region was considered to be inadequate for the specific gene region based on high levels of homology. Based on the low percent identity observed upon homology analysis, specific gene regions were determined for ELISA antigen candidate peptides as

Table 2. Homology among ApxIA, ApxIIA, and ApxIIIA

Proteins and accession No.	Percent identity (%)		
	ApxIA	ApxIIA	ApxIIIA
ApxIA (1022aa)			
AF363361	-	44.0	50.9
ApxIIA (956aa)			
AF363362	44.0	-	44.6
ApxIIIA (1052aa)			
AF363363	50.9	44.6	-

Table 3. Homology among the Nt, M1, M2, and Ct regions within Apx toxins

Region	Percent identity (%)		
	ApxIA	ApxIIA	ApxIIIA
ApxIA			
Nt (1-250)	-	47.4	40.0
M1 (251-515)	-	59.1	62.6
M2 (516-774)	-	41.0	59.7
Ct (775-1022)	-	23.5	39.5
ApxIIA			
Nt (1-250)	47.4	-	44.6
M1 (251-514)	59.1	-	60.6
M2 (515-772)	41.0	-	44.1
Ct (773-956)	23.5	-	25.7
ApxIIIA			
Nt (1-261)	40.0	44.6	-
M1 (262-526)	62.6	60.6	-
M2 (527-789)	59.7	44.1	-
Ct (790-1052)	39.5	25.7	-

Nt, N-terminal; M1, middle part 1; M2, middle part 2; Ct, C-terminal.

follows: Nt and Ct regions in ApxIA; Nt, M2, and Ct regions in ApxIIA; Nt, M2, and Ct regions in ApxIIIA. The determined sequence regions are shown in yellow in Fig. 1.

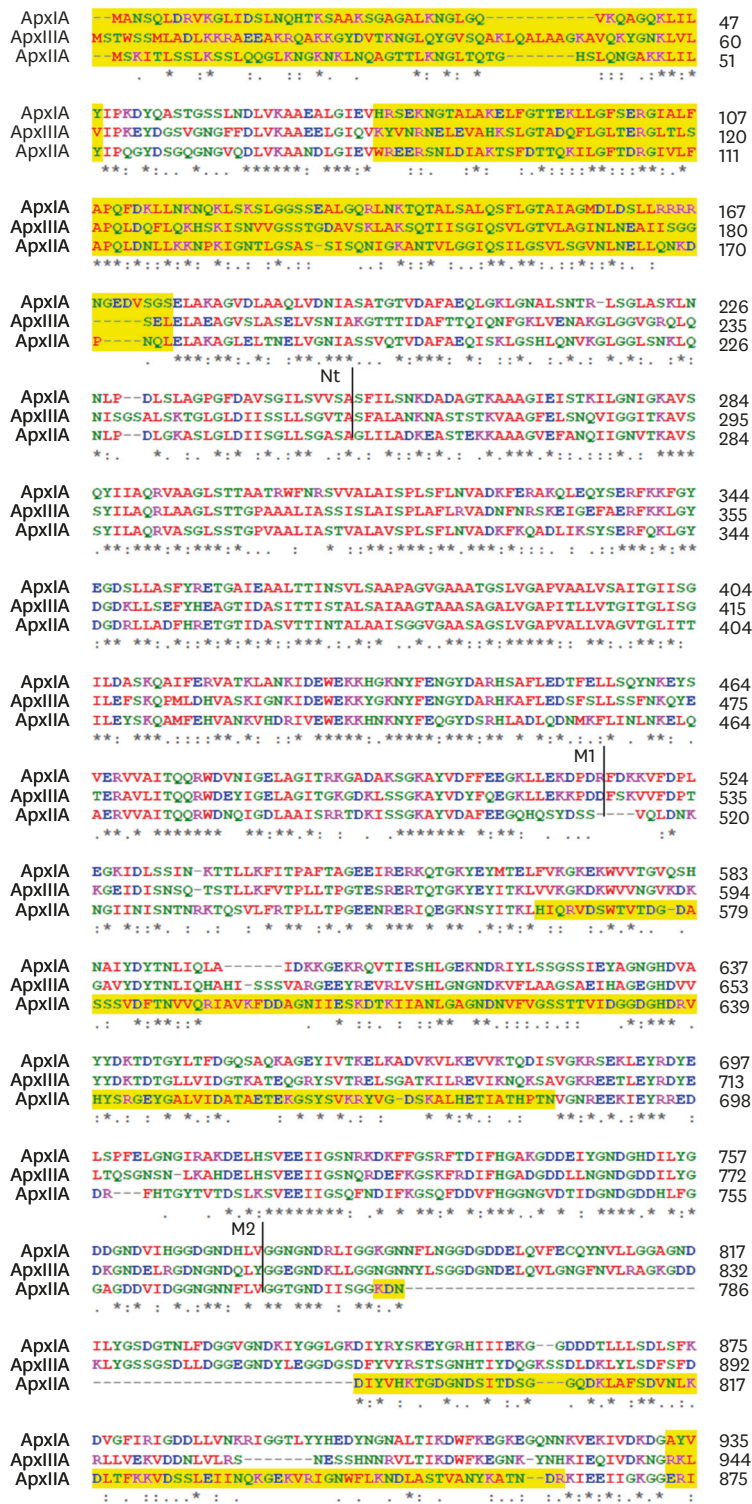


Fig. 1. Multiple sequence alignment of the Apx proteins of *Actinobacillus pleuropneumoniae*. Multiple alignment was performed using ClustalW2. The selected sequences, which showed a lesser degree of similarity, are shaded yellow. (continued to the next page)

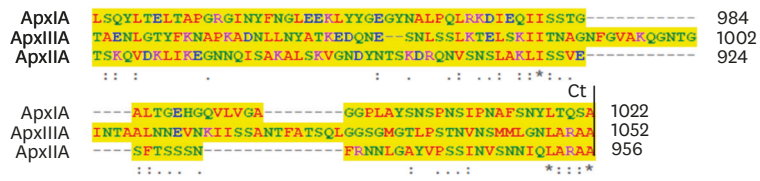


Fig. 1. (Continued) Multiple sequence alignment of the Apx proteins of *Actinobacillus pleuropneumoniae*. Multiple alignment was performed using ClustalW2. The selected sequences, which showed a lesser degree of similarity, are shaded yellow.

Production of the recombinant partial toxins of ApxIA, ApxIIA, and ApxIIIA

Based on the results of homology analysis, all selected specific gene regions except ApxIIA Ct were amplified using the primers shown in **Table 1** and then cloned for production of recombinant proteins. As shown in **Fig. 2**, the molecular masses of the recombinant proteins were in concordance with the predicted molecular weights (ApxIA Nt, 19.3 kDa; ApxIA Ct, 24.2 kDa; ApxIIA Nt, 19.1 kDa; ApxIIA M2, 21.3 kDa; ApxIIIA Nt, 20.1 kDa; ApxIIIA M2, 22 kDa; ApxIIIA Ct, 25.9 kDa). Upon Western blot analysis, the recombinant proteins were found to be responsive to the positive control of guinea pig sera, with cross reactions observed between the purified partial toxins upon homology analysis as expected.

Development of ApxIA, ApxIIA, and ApxIIIA-specific enzyme-linked immunosorbent assays

Checkerboard titration was performed to establish the optimal ELISA conditions making a clear distinction between positive and negative sera. The optimal concentrations of recombinant partial toxins used to coat the microplates were as follows: 100 ng for ApxIA Nt, 200 ng for ApxIA Ct, 200 ng for ApxIIA Nt, 50 ng for ApxIIA M2, 200 ng for ApxIIIA Nt, 200 ng for ApxIIIA M2, and 100 ng for ApxIIIA Ct (**Supplementary Figs. 1-3**). To establish ELISA conditions for measuring ApxIA, ApxII, and ApxIIIA-specific antibody levels using the recombinant partial toxins, purified proteins of ApxIA Ct, ApxIIA Nt, and ApxIIIA Nt

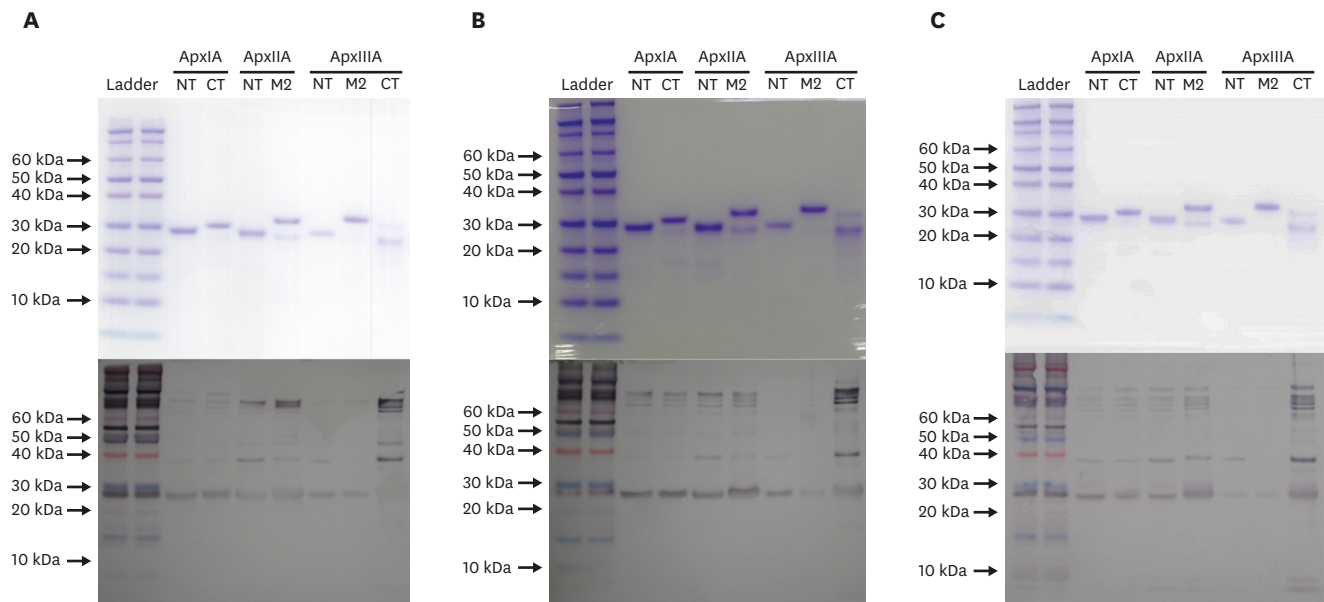


Fig. 2. Confirmation of purification and antigenicity or recombinant partial Apx toxins by SDS-PAGE and Western blot. Upper and lower panels represent SDS-PAGE and Western blot, respectively. Western blot was conducted using positive guinea pig serum of ApxIA (A), ApxIIA (B), and ApxIIIA (C), respectively. SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis.

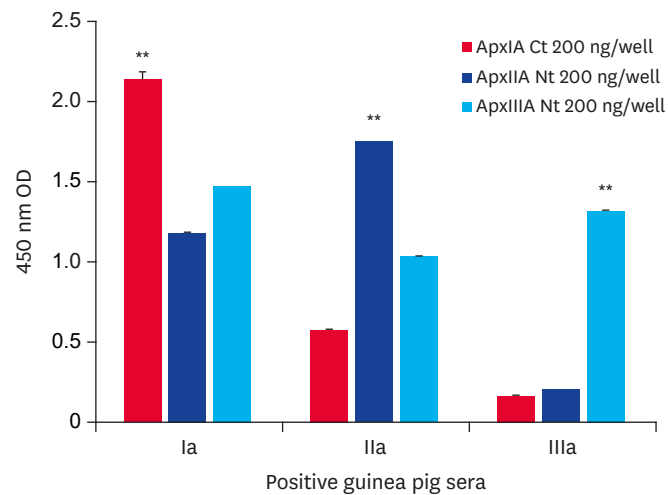


Fig. 3. The ELISA results for each Apx toxin (ApxIA, ApxIIA, and ApxIIIA)-positive guinea pig sera under the optimal conditions. The determined antigen concentrations (ng/well) and serum dilution rate (1:320) are described in the graph. Each Apx toxin-positive guinea pig serum could be identified by ELISA using ApxIA Ct, ApxIIA Nt, and ApxIIIA Nt. ELISA, enzyme-linked immunosorbent assay; Nt, N-terminal; Ct, C-terminal; **, $p < 0.01$.

were selected as ELISA antigens considering the cross-reactivity (**Fig. 3**). Based on the checkerboard results and selected antigens, a 1:320 dilution ratio of guinea pig sera was found to show the most precise results. As shown in **Fig. 3**, ApxIA Ct (200 ng/well) had higher reactivity with ApxIA positive guinea pig sera than ApxIIA Nt (200 ng/well) and ApxIIIA Nt (200 ng/well). ApxIIA Nt and ApxIIIA Nt also showed greater responses to each positive serum than the other two antigens.

DISCUSSION

Vaccination appears to be the most effective way to control *A. pleuropneumoniae* infection and has therefore been adapted in many countries around the world. It has also been reported that subunit vaccines against *A. pleuropneumoniae* using recombinant Apx toxins showed safety and cross-protection against different serotypes [16]. For this reason, many currently available vaccines use the recombinant Apx toxins as their key antigens [10,15] and new subunit vaccines are actively being developed [16-20]. Therefore, the measurement of immune responses to purified Apx toxin antigens has been an important milestone in vaccine development as a tool for vaccine efficiency evaluation [4,7,21].

ELISAs are widely used to investigate immunization levels induced by *A. pleuropneumoniae* vaccines. However, most commercial ELISAs have been developed for serotype determination of *A. pleuropneumoniae* and cannot be easily applied to measure Apx-specific immune responses when evaluating the efficiency of Apx toxins-based subunit vaccines [11,24,25]. In addition, experimental ELISAs reported in previous studies are also still difficult to differentiate levels of immunization against respective Apx toxin antigens [3,26,29]. This problem is believed to be due to cross-reactivity among ApxI, ApxII, and ApxIII as a result of their high homology [21,27-29], which was also observed in homology analysis conducted in the present study (**Table 2**). By excluding antigen regions showing high homology among Apx toxins, therefore, we attempted to identify specific antigen regions to purify ELISA antigens for differential serological detection. To accomplish this, Apx toxin amino sequences were divided into

regions of Nt, M1, M2, and Ct, and specific regions of Apx toxins were then cloned to obtain purified recombinant Apx toxin proteins. Nevertheless, positive guinea pig sera to each toxin of ApxI, ApxII, and ApxIII were reacted with all of the partial Apx toxins upon Western blot analysis. These nonspecific reactivities were thought to be attributed to the high levels of homology among partial proteins, as was observed during homology analysis (**Table 3**). Although specific antigen-antibody reactions were not observed upon Western blot analysis, the antigenicity of purified partial Apx toxins was confirmed.

To establish the optimal ELISA conditions for each recombinant partial toxin, checkerboard titration was conducted using guinea pig sera positive to each toxin of ApxI, ApxII, and ApxIII. Considering the need for ELISA to serve as a simple and convenient diagnostic tool, we attempted to set serum dilution conditions to the same ratio. Antigen coating conditions were determined to be the concentration at which the changes in the OD value of 450 nm were most prominent according to the serum dilution. Optimal ELISA conditions were attempted to be set to give an OD value of about 1.5 for each positive serum at the determined serum dilution and antigen concentration.

The results of Western blot and ELISA checkerboard titration suggested that the recombinant partial Apx toxins of ApxIA Nt, ApxIA Ct, ApxIIA Nt, ApxIIA M2, ApxIIIA Nt, and ApxIIIA Ct had a high possibility of being useful as ELISA antigens. The ApxIIIA M2 partial toxin was ruled out based on its low antigenicity to ApxIIIA positive guinea pig serum in the ELISA checkerboard titration. Based on the optimal ELISA conditions, an antigen combination of ApxIA Ct, ApxIIA Nt, and ApxIIIA Nt was determined by additional ELISA experiments (**Fig. 3**). In the additional ELISA experiments, all procedures were conducted using the optimal ELISA conditions; however, skim milk solution for blocking (5% in PBST) and antibody diluent (3% in PBST) were used. The skim milk solution was adapted to further reduce nonspecific reactions. As shown in **Fig. 3**, each of the antigens (ApxIA Ct, ApxIIA Nt, and ApxIIIA Nt) showed a higher OD value in response to each positive serum than the other two antigens. As reported in previous studies, cross-reactivity was also observed in the ELISA using ApxIA Ct and ApxIIA Nt antigens, but they could be distinguished based on the difference in OD value. In addition, ELISA using ApxIII Nt antigen showed excellent reactivity to its positive guinea pig serum when compared to the other two positive control guinea pig sera (**Fig. 3**).

The results of this study confirmed the high homology among toxins of ApxIA, ApxIIA, and ApxIIIA, which has been considered to be an important factor in the cross-reactivity of antibodies to each toxin. Due to this cross-reactivity, it has been difficult to measure and differentiate antibody levels to each of the three toxins. In this study, the recombinant partial Apx toxins were purified to reduce the interference of homology observed among the three toxins, and the produced partial Apx toxins were used to develop an ELISA method capable of measuring the level of each toxin-specific antibody. This Apx toxin-specific ELISA will be useful as an important tool for evaluation of vaccine efficiency by measuring specific antibody responses to each toxin during the development of subunit vaccines against *A. pleuropneumoniae*.

SUPPLEMENTARY MATERIALS

Supplementary Fig. 1

Determination of the optimal conditions for concentrations of ApxIA antigens (100 ng/well for ApxIA Nt and 200 ng/well for ApxIA Ct) and serum dilution rate (1/320) for ELISA using

guinea pig serum already known to be positive for ApxIA. The arrows indicate the optimal and most reliable ELISA conditions. (A, B) Checkerboard ELISA results of positive guinea pig serum. (C, D) Checkerboard ELISA results of positive and negative guinea pig sera at the optimal concentrations of coating antigens.

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Supplementary Fig. 2

Determination of the optimal conditions for concentrations of ApxIIA antigens (200 ng/well for ApxIIA Nt and 50 ng/well for ApxIIA M2) and serum dilution rate (1/320) for ELISA using guinea pig serum already known to be positive for ApxIA. The arrows indicate the optimal and most reliable ELISA conditions. (A, B) Checkerboard ELISA results of positive guinea pig serum. (C, D) Checkerboard ELISA results of positive and negative guinea pig sera at the optimal concentrations of coating antigens.

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Supplementary Fig. 3

Determination of the optimal conditions for concentrations of ApxIIIA antigens (200 ng/well for ApxIIIA Nt, 200 ng/well for ApxIIIA M2, and 100 ng/well for ApxIIIA Ct) and serum dilution rate (1/320) for ELISA using guinea pig serum already known to be positive for ApxIA. The arrows indicate the optimal and most reliable ELISA conditions. (A-C) Checkerboard ELISA results of positive guinea pig serum. (D-F) Checkerboard ELISA results of positive and negative guinea pig sera at the optimal concentrations of coating antigens.

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REFERENCES

1. Dayao DA, Gibson JS, Blackall PJ, Turni C. Antimicrobial resistance in bacteria associated with porcine respiratory disease in Australia. *Vet Microbiol* 2014;171:232-235.
[PUBMED](#) | [CROSSREF](#)
2. Opriessnig T, Giménez-Lirola LG, Halbur PG. Polymicrobial respiratory disease in pigs. *Anim Health Res Rev* 2011;12:133-148.
[PUBMED](#) | [CROSSREF](#)
3. Shin MK, Kang ML, Cha SB, Lee WJ, Sung JH, Yoo HS. An immunosorbent assay based on the recombinant ApxIa, ApxIIa, and ApxIIIA toxins of *Actinobacillus pleuropneumoniae* and its application to field sera. *J Vet Diagn Invest* 2011;23:736-742.
[PUBMED](#) | [CROSSREF](#)
4. Bossé JT, Janson H, Sheehan BJ, Beddek AJ, Rycroft AN, Kroll JS, Langford PR. *Actinobacillus pleuropneumoniae*: pathobiology and pathogenesis of infection. *Microbes Infect* 2002;4:225-235.
[PUBMED](#) | [CROSSREF](#)
5. Dubreuil JD, Jacques M, Mittal KR, Gottschalk M. *Actinobacillus pleuropneumoniae* surface polysaccharides: their role in diagnosis and immunogenicity. *Anim Health Res Rev* 2000;1:73-93.
[PUBMED](#) | [CROSSREF](#)
6. Xu Z, Chen X, Li L, Li T, Wang S, Chen H, Zhou R. Comparative genomic characterization of *Actinobacillus pleuropneumoniae*. *J Bacteriol* 2010;192:5625-5636.
[PUBMED](#) | [CROSSREF](#)
7. Dreyfus A, Schaller A, Nivollet S, Segers RP, Kobisch M, Mieli L, Soerensen V, Hüseyin D, Miserez R, Zimmermann W, Inderbitzin F, Frey J. Use of recombinant ApxIV in serodiagnosis of *Actinobacillus pleuropneumoniae* infections, development and prevalidation of the ApxIV ELISA. *Vet Microbiol* 2004;99:227-238.
[PUBMED](#) | [CROSSREF](#)

8. Boekema BK, Kamp EM, Smits MA, Smith HE, Stockhofe-Zurwieden N. Both ApxI and ApxII of *Actinobacillus pleuropneumoniae* serotype 1 are necessary for full virulence. *Vet Microbiol* 2004;100:17-23.
[PUBMED](#) | [CROSSREF](#)
9. Inzana TJ, Todd J, Ma JN, Veit H. Characterization of a non-hemolytic mutant of *Actinobacillus pleuropneumoniae* serotype 5: role of the 110 kilodalton hemolysin in virulence and immunoprotection. *Microb Pathog* 1991;10:281-296.
[PUBMED](#) | [CROSSREF](#)
10. Ramjeet M, Deslandes V, Gouré J, Jacques M. *Actinobacillus pleuropneumoniae* vaccines: from bacterins to new insights into vaccination strategies. *Anim Health Res Rev* 2008;9:25-45.
[PUBMED](#) | [CROSSREF](#)
11. Schaller A, Kuhn R, Kuhnert P, Nicolet J, Anderson TJ, MacInnes JI, Segers RP, Frey J. Characterization of apxIVA, a new RTX determinant of *Actinobacillus pleuropneumoniae*. *Microbiology* 1999;145:2105-2116.
[PUBMED](#) | [CROSSREF](#)
12. Shin SJ, Cho YW, Yoo HS. Cloning, sequencing and expression of apxIA, IIA, IIIA of *Actinobacillus pleuropneumoniae* isolated in Korea. *Korean J Vet Res* 2003;43:247-253.
13. Shin MK, Cha SB, Lee WJ, Yoo HS. Predicting genetic traits and epitope analysis of apxIVA in *Actinobacillus pleuropneumoniae*. *J Microbiol* 2011;49:462-468.
[PUBMED](#) | [CROSSREF](#)
14. Oishi E, Kitajima T, Nakamura H, Matsuda C, Amimoto K, Yasuhara H. A field trial of oil adjuvanted trivalent *Actinobacillus pleuropneumoniae* vaccine. *J Vet Med Sci* 1997;59:421-423.
[PUBMED](#) | [CROSSREF](#)
15. Sjölund M, Wallgren P. Field experience with two different vaccination strategies aiming to control infections with *Actinobacillus pleuropneumoniae* in a fattening pig herd. *Acta Vet Scand* 2010;52:23.
[PUBMED](#) | [CROSSREF](#)
16. Xu K, Zhao Q, Wen X, Wu R, Wen Y, Huang X, Huang Y, Yan Q, Han X, Ma X, Chang YF, Cao S. A trivalent Apx-fusion protein delivered by *E. coli* outer membrane vesicles induce protection against *Actinobacillus pleuropneumoniae* of serotype 1 and 7 challenge in a murine model. *PLoS One* 2018;13:e0191286.
[PUBMED](#) | [CROSSREF](#)
17. Lu YC, Li MC, Chen YM, Chu CY, Lin SF, Yang WJ. DNA vaccine encoding type IV pilin of *Actinobacillus pleuropneumoniae* induces strong immune response but confers limited protective efficacy against serotype 2 challenge. *Vaccine* 2011;29:7740-7746.
[PUBMED](#) | [CROSSREF](#)
18. Shin MK, Jung MH, Lee WJ, Choi PS, Jang YS, Yoo HS. Generation of transgenic corn-derived *Actinobacillus pleuropneumoniae* ApxIIA fused with the cholera toxin B subunit as a vaccine candidate. *J Vet Sci* 2011;12:401-403.
[PUBMED](#) | [CROSSREF](#)
19. Shin MK, Kang ML, Jung MH, Cha SB, Lee WJ, Kim JM, Kim DH, Yoo HS. Induction of protective immune responses against challenge of *Actinobacillus pleuropneumoniae* by oral administration with *Saccharomyces cerevisiae* expressing Apx toxins in pigs. *Vet Immunol Immunopathol* 2013;151:132-139.
[PUBMED](#) | [CROSSREF](#)
20. Shin MK, Lee WJ, Jung MH, Cha SB, Shin SW, Yoo A, Kim DH, Yoo HS. Oral immunization of mice with *Saccharomyces cerevisiae* expressing a neutralizing epitope of ApxIA exotoxin from *Actinobacillus pleuropneumoniae* induces systemic and mucosal immune responses. *Microbiol Immunol* 2013;57:417-425.
[PUBMED](#) | [CROSSREF](#)
21. Nielsen R, van den Bosch JF, Plambeek T, Sørensen V, Nielsen JP. Evaluation of an indirect enzyme-linked immunosorbent assay (ELISA) for detection of antibodies to the Apx toxins of *Actinobacillus pleuropneumoniae*. *Vet Microbiol* 2000;71:81-87.
[PUBMED](#) | [CROSSREF](#)
22. Gottschalk M, Lacouture S. *Actinobacillus pleuropneumoniae* serotypes 3, 6, 8 and 15 isolated from diseased pigs in North America. *Vet Rec* 2014;174:452.
[PUBMED](#) | [CROSSREF](#)
23. Montaraz JA, Fenwick B, Hill H, Rider M. Evaluating antibody isotype-specific ELISA, complement fixation, and Apx 1 hemolysin neutralization tests to detect serum antibodies in pigs infected with *Actinobacillus pleuropneumoniae* serotype 1. *Swine Health and Production* 1996;4:79-83.
24. Costa G, Oliveira S, Torrison J, Dee S. Evaluation of *Actinobacillus pleuropneumoniae* diagnostic tests using samples derived from experimentally infected pigs. *Vet Microbiol* 2011;148:246-251.
[PUBMED](#) | [CROSSREF](#)
25. Opriessnig T, Hemann M, Johnson JK, Heinen S, Giménez-Lirola LG, O'Neill KC, Hoang H, Yoon KJ, Gottschalk M, Halbur PG. Evaluation of diagnostic assays for the serological detection of *Actinobacillus pleuropneumoniae* on samples of known or unknown exposure. *J Vet Diagn Invest* 2013;25:61-71.
[PUBMED](#) | [CROSSREF](#)

26. Teshima K, Lee J, To H, Kamada T, Tazumi A, Hirano H, Maruyama M, Ogawa T, Nagai S, Turni C, Tsutsumi N. Application of an enzyme-linked immunosorbent assay for detection of antibodies to *Actinobacillus pleuropneumoniae* serovar 15 in pig sera. *J Vet Med Sci* 2017;79:1968-1972.
[PUBMED](#) | [CROSSREF](#)
27. Devenish J, Rosendal S, Johnson R, Hubler S. Immunoserological comparison of 104-kilodalton proteins associated with hemolysis and cytolysis in *Actinobacillus pleuropneumoniae*, *Actinobacillus suis*, *Pasteurella haemolytica*, and *Escherichia coli*. *Infect Immun* 1989;57:3210-3213.
[PUBMED](#)
28. Giménez-Lirola LG, Jiang YH, Sun D, Hoang H, Yoon KJ, Halbur PG, Opriessnig T. Simultaneous detection of antibodies against Apx toxins ApxI, ApxII, ApxIII, and ApxIV in pigs with known and unknown *Actinobacillus pleuropneumoniae* exposure using a multiplexing liquid array platform. *Clin Vaccine Immunol* 2014;21:85-95.
[PUBMED](#) | [CROSSREF](#)
29. Gottschalk M. The challenge of detecting herds sub-clinically infected with *Actinobacillus pleuropneumoniae*. *Vet J* 2015;206:30-38.
[PUBMED](#) | [CROSSREF](#)