Original Article

Protective Immunity of the Pore-Forming Domains of *Actinobacillus pleuropneumoniae* Apx Toxins in a Mouse Model

Kamonpun Chuekwon[1,2] Chun-Yen Chu[2,3] Li-Ting Cheng[2,3*]

Abstract

The hemolytic/leukolytic toxins ApxI, II and III play a central role in the pathogenicity of *Actinobacillus pleuropneumoniae* and may serve as effective vaccine antigens. However, since these toxins are large in size, we aimed to determine whether only the pore-forming domain of the toxins may be sufficient for protective immunity. Mice were vaccinated with the pore-forming domains for immune response analysis and challenge test. Significant antibody response was observed for the ApxI and II vaccine groups. For cellular immune response, $CD4^+$ and $CD8^+$ T cell expansion was observed for ApxI. Pro-inflammatory cytokine (IL-1β, IL-6) and T_H2-type cytokine (IL-4, IL-10) gene expression was detected for the ApxI and II groups. Finally, in a challenge test with a *A. pleuropneumoniae* serotype 1 strain, the pore-forming domains of ApxI, II, III and three of pore-forming domains conferred 100%, 40%, 0% and 100% protection, respectively. The pore-forming domain of the Apx toxins shows promise as a vaccine antigen against *A. pleuropneumoniae*.

Keywords: *Actinobacillus pleuropneumoniae*, Apx toxin, vaccine, pore-forming domain

[1]*Department of Tropical Agriculture and International Cooperation, National Pingtung University of Science and Technology, 1, Shuefu Road, Neipu, Pingtung, 91201 Taiwan*

[2]*Graduate Institute of Animal Vaccine Technology, College of Veterinary Medicine, National Pingtung University of Science and Technology, 1, Shuefu Road, Neipu, Pingtung, 91201 Taiwan*

[3]*International Program in Animal Vaccine Technology, International College, National Pingtung University of Science and Technology, Pingtung, Taiwan*

****Correspondence****: chenglt@mail.npust.edu.tw* (L.T. Cheng)

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Introduction

Actinobacillus pleuropneumoniae is a Gram-negative bacterium that infects the respiratory tract of pigs and causes hemorrhagic pneumonia, leading to significant economic loss (Yang *et al*., 2011). Among the many virulent factors of *A. pleuropneumoniae* (Chiers *et al*., 2010), the pore-forming RTX (repeats-in-toxin) exotoxins ApxI, ApxII and ApxIII are considered the most important for pathogenesis (Jansen *et al*., 1994; Jarma and Regassa, 2004; Jung *et al*., 2019; Sassu *et al*., 2018) and may serve as effective vaccine antigens (Loera-Muro *et al*., 2018). However, since the RTX toxins are large proteins (>100 kDa) (Frey, 2019), heterologous expression of recombinant toxins may result in low yield. Therefore, this study sought to determine if the pore-forming domains alone may provide protective immunity.

RTX toxins are typically encoded on a polycistronic operon, pCABD. Gene "A" codes for the structural toxin, "C" for the activating acyltransferase (Stanley *et al*., 1998) and "B" and "D" for membrane proteins that together form the type 1 secretion system (T1SS) (Frey *et al*., 1994; Yoo *et al*., 2020). After synthesis in the cytoplasm, the inactive pro-toxin A is activated through acylation and then exported across the cytoplasmic and outer membranes simultaneously through the T1SS. RTX toxins effect on their target cells are hemolytic and leukolytic. The presence of RTX toxins can be easily detected *in vitro* by the characteristic hemolytic halo around colonies grown on blood agar (Marsteller and Fenwick, 1999). However, it is now known that RTX toxins exert their pathogenic effect by targeting leukocytes of the innate immune system, i.e., macrophages and neutrophils (Chen *et al*., 2011). By binding to β_2-integrins on leukocytes, RTX toxins induces apoptosis, cell death and tissue lesions that lead to disease (Atapattu and Czuprynski, 2007; Aulik *et al*., 2012; Frey, 2019; Hiyoshi *et al*., 2019; Lally *et al*., 1999; Los *et al*., 2013). Different Apx toxins exhibit varying levels of hemolytic and cytotoxic activity: ApxIA shows the strongest hemolytic and cytotoxic activity, ApxIIA toxin is weakly hemolytic and moderately cytotoxic and ApxIIIA is cytotoxic but non-hemolytic (Frey *et al*., 1993).

The RTX toxin contains several structural features. Starting from the N-terminus is an amphipathic segment followed by a hydrophobic region that together are responsible for pore-forming activity (Basler *et al*., 2007; Bellalou *et al*., 1990). The hydrophobic domains can form α-helical structures that insert into target membrane to create ion cannel (Wiles and Mulvey, 2013). Following the pore-forming region are two conserved lysine residues that must be acylated for the activation of the toxin. Then the defining region of RTX toxins can be found containing 6~50 copies of glycine and aspartate-rich nonapeptide repeats (hence repeats-in-toxin) (Guo *et al*., 2019). These repeats form β-roll motifs that bind to Ca^{2+} ions for pore-forming activity. Finally, found at the C-terminus of the RTX toxin is a non-cleavable secretion signal that interacts with the T1SS for export (Delepelaire, 2004).

Of the various domains of the RTX toxin, evidence points to the pore-forming domain as a promising vaccine candidate. Seah *et al*. (2002), and Mei *et al*. (2006), found that the pore-forming domain (residues 40-380) of ApxI provided protection against lethal challenge of *A. pleuropneumoniae* in mice. Another study using a portion of the pore-forming domain (residues 1-200) of ApxII found partial protection (40%) against a challenge test in mice (Seo *et al*., 2011).

In this study, we analyzed the protective potential of the pore-forming domains of all three Apx toxins. Mice were immunized with the pore-forming domains of ApxI, II and III. Analysis of humoral and cellular immunity was performed and a challenge test was carried out with *A. pleuropneumoniae* serovar 1, the dominant serovar in Taiwan (Yang *et al*. 2011).

Materials and Methods

Bacteria Strain: *A. pleuropneumoniae* serotype 1 strain 4074 (ATCC® 27088™) was obtained from the American Type Culture Collection (ATCC) and grown in Brain-Heart Infusion Broth supplemented with 15 mg/mL nicotinamide adenine dinucleotide (NAD) at 37 °C.

Recombinant Protein Expression of Apx toxin pore-forming domains and vaccine formulation: Expression plasmids for the Apx toxin pore-forming domains were constructed. Genomic DNA from *A. pleuropneumoniae* strain 4074 was used for the cloning of ApxI and II pore-forming domains. For ApxIII, genomic DNA (a gift from Dr. Chun-Yen Chu) from a *A. pleuropneumoniae* serotype 2 strain ATCC® 27089™ was used. Primers shown in Table 1 were used to amplify the pore-forming regions through Polymerase Chain Reaction. For primer design, protein sequences of ApxI, II and III were aligned and the pore-forming region was defined as the first 380 residues of ApxI (Seah *et al*., 2002). After restriction enzyme digestion, the amplicons were ligated into the expression vector pET32a (Novagen, Darmstadt, Germany). Escherichia coli DH5α (Yeastern Biotech, Taipei, Taiwan) was used for plasmid propagation and the gene clones were sent for sequence confirmation (Genomics, Taipei, Taiwan).

For recombinant protein expression of the Apx toxin pore-forming domains, plasmid constructs were used to transform chemically competent *E. coli* BL21(DE3) (Yeastern Biotech, Taipei, Taiwan) according to the manufacturer's instructions. Protein expression was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma, Darmstadt, Germany) for 4 hours at 37°C. Expressed protein was purified using Bio-scale Mini Profinity IMAC cartridges (1 mL) (Bio-Rad, Hercules, CA, USA). The quality and quantity of the recombinant protein were analyzed using 12% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Western blotting was also performed to confirm the identity of the expressed proteins. As the primary antibody, 6X-His Tag Antibody (Gentex, Hsinchu, Taiwan) at 1:5,000 dilution was used and peroxidase-conjugated goat anti-mouse antibody (Gentex, Hsinchu, Taiwan) at 1:5,000 dilution was used as the secondary antibody. Western Lighting PLUS (PerkinElmer, Waltham, MA, USA) was used for color development. Using the ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit (GenScript, Piscataway, NJ,

USA), the endotoxin level of the protein preparations was shown to be less than 0.125 EU/mL.

Five vaccine formulations were prepared from the recombinant proteins: (1) ApxI-PF, (2) ApxII-PF, (3) ApxIII-PF (4) ApxI-PF+ApxII-PF+ApxIII-PF and (5) PBS as the negative control group. Purified recombinant proteins (at 25 µg/dose final concentration) were mixed with the water-in-oil-in-water adjuvant Montanide ISA 206 (Seppic, Paris, France) at a 1:1 ratio for a final injection volume of 200 µL per mouse.

Table 1 Primers for gene cloning and recombinant protein construction.

Target gene	Oligonucleotide Sequence (5′ to 3′)	Tm (°C)	Gene	Target gene
ApxI-PF	F: GC*GGATCC* ATGGCTAACTCTCAGCTCG	57	1140	ATCC® 27088™
	R: A***CTCGAG***AGCCCCAACACCTGCGGAAC			
ApxII-PF	F: GC*GGATCC*ATGTCAAAAATCACTTTGTCATCAT	57	1140	ATCC® 27088™
	R: CG**CTCGAG**AGCTCCAACTCCACCGGAGAT			
ApxIII-PF	F: CA*GGATCC*ATGAGTACTTGGTCAAGCAT	58	1173	L12145.1
	R: CG**CTCGAG**GGCGGCCGTTCCAGCTGCG			

Italics and Bold in the primers represent restriction enzyme sites, GGATCC: BamHI Enzyme; CTCGAG: XhoI Enzyme.

Immunization: For immunization, 35 five-week-old female ICR mice from BioLASCO Taiwan Co., Ltd, (Taipei, Taiwan) were randomly assigned into four groups of seven mice for the five vaccine formulations. Immunization was performed twice intraperitoneally on days 0 and 14. For analysis of immune response, blood samples were collected on days 0, 7, 14, 21 and 28 from three mice per group. All animal experimental protocols (NPUST-109-064) were approved by the Animal Care and Use Committee, National Pingtung University of Science and Technology (NPUST).

Antibody Response Analysis: Antibody response elicited by the vaccines was analyzed by indirect enzyme-linked immunosorbent assay (ELISA). Collected whole blood was permitted to coagulate and then centrifuged at 500×*g* for 7 mins to obtain serum. High-binding 96-well ELISA plates were coated with 10 µg/well of corresponding purified recombinant protein (i.e. ApxI for anti-ApxI serum analysis) overnight at 4°C. The plates were subsequently washed and blocked with skim milk at 37 °C for 1h. Afterwards, serum samples at 1×10^6 dilution were added as the primary antibody. Horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Sigma, Carlsbad, CA, USA) at 1:5,000 dilution was used as a secondary antibody. Finally, the Peroxidase Kit (KPL, Gaithersburg, MD, USA) was used for color development and the plates were read at 450 nm on the Multiskan™ FC Photometer (Thermo Fisher Scientific, Vantaa, Finland).

T-cell response analysis: On day 28, two mice from each vaccine group were sacrificed and splenocytes were isolated for T-cell response analysis. Harvested spleens were mashed through cell strainers and spleen cells were collected in RPMI 1640 Media (Gibco Invitrogen, Carlsbad, CA, USA). Erythrocytes were lysed by treatment with ACK lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2-EDTA, pH 7.4) and the remaining splenocytes were washed three times with Phosphate Buffer Saline (PBS) before resuspension (at 1x10^6 cells/mL) in RPMI-1640 Media supplemented with 5% fetal bovine serum (Gibco Invitrogen, Carlsbad, CA, USA).

For analysis of the percentages of $CD4^+$ and $CD8^+$ T cells in the splenocytes on day 14 and 28, cells were stained with anti-CD4-APC or anti-CD8-FITC antibodies (Sino Biological Inc, Wayne, PA, USA) in PBS at 4°C for 30 mins. The splenocyte cells with light scattering properties were then assessed by immunofluorescence on a FACScan cytometer using the Cellquest software (BD Biosciences, San Diego, CA, USA). A total of 10,000 gated events were acquired for each sample.

Cytokine response analysis: To determine the cytokine response of the splenocytes from vaccinated mice, cells (1x10^6 cells/mL) were stimulated with 10 µg/mL of the recombinant proteins for 3 hours. Total RNA was then extracted with the Total RNA Extraction Miniprep system (Viogene, Taipei, Taiwan) and complementary DNA (cDNA) was synthesized using the Reverse Transcription Kit (Applied Biosystems, Foster, CA, USA). Real-time PCR was then carried out in the SmartCycler I (Cepheid, Sunnyvale, CA, USA) with primers for the cytokines listed in Table 2. Expression levels of the cytokine genes were normalized to that of thehousekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and expressed as N-fold increase or decrease relative to the PBS control group.

Table 2 Primers for cytokine genes

Gene	Oligonucleotide Sequence (5′ to 3′)	Tm (°C)	Gene length (bp)	NCBI Reference Sequence
IL-1β	F:AGTTGACGGACCCCAAAAGAT R: CATGGAGAATATCACTTGTT	57	412	M15131.1
IL-4	F:CGAAGAACACCACAGAGAGTGAGCT R:GACTCATTCATGGTGCAGCTTATCG	50	175	M25892.1
IL-6	F:CTTCCATCCAGTTGCCTTCTTG R:AATTAAGCCTCCGACTTGTGA	57	141	M24221.1
IL-8	F: CAAGGGCCAAGAGAATATCC R: TTACTATAACATCTTTATAA	55	445	BC013615.1
IL-10	F: AAGGCAGTGGAGCAGGTGAA R: CCAGCAGACTCAATACACAC	55	155	NM_010548.2
IL-12p40	F: CAGAAGCTAACCATCTCCTGGTTTG R: CCGGAGTAATTTGGTGCTCCACAC	55	396	BC103610.1
IFN-γ	F:AGCGGCTGACTGAACTCAGATTGTAG R:GTCACAGTTTCAGCTGTATAGGG	55	243	NM_008337.4
TNF-α	F:GGCAGGTCTACTTTGGAGTCATTGC R:ACATTCGAGGCTCCAGTGAATTCGG	55	300	NM_001278601.1
GADPH	F: CGGCACAGTCAAGGCCGAGAAT R:AGCCTTCTCCATGGTGGTGAA	57	154	M32599.1

Note: IL: interleukin; IFN-γ: interferon gamma; TNF-α: Tumor Necrosis Factor alpha; GAPDH: glyceraldehyde 3 phosphate dehydrogenase.

Challenge test: Vaccinated mice (n=5 per group) were challenged intraperitoneally with $5x10^8$ CFU (20 LD_{50}) *A. pleuropneumoniae* ATCC 27088 on day 28. Mice were monitored for seven days and moribund mice satisfying criteria for humane endpoints (as defined by the Animal Use Protocol of NPUST) were sacrificed. All mice were sacrificed at the end of the seven day period.

Statistical analysis: One-way analysis of variance (ANOVA) and Student's *F*-test were used to compare the differences between groupsfor the analysis ofantibody response, cytokine mRNA levels, and percentages of $CD4^+$ and $CD8^+$ T cells. Data was expressed as mean ± standard error of mean (SEM), and P value less than 0.05 was considered statistically significant. All statistical analyses were performed using SAS version 9.0.

Results

Pore-forming domains of the Apx toxins were expressed and formulated as vaccines: The pore-forming domains of ApxI, II and III (Figure 1A and 1B) were cloned and expressed in *E. coli*. SDS-PAGE analysis showed the target proteins at approximately 63 kDa (pET32a inserts a 20-kDa Trx-His-S-enterokinase tag) for the three toxins (Figure 1C). Western blotting using anti-His antibody confirmed the identity of the recombinant proteins (Figure 1D). Protein quantitation showed that the expression quantity of the pore-forming domains can reach as high as 400 mg/mL (ApxIII). The recombinant proteins were formulated with a water-in-oil-in water adjuvant into five vaccines: (1) ApxI-PF, (2) ApxII-PF (3) ApxIII-PF (4) ApxI-PF+ApxII-PF+ApxIII-PF and (5) PBS as the negative control. Mice were vaccinated twice and immune response analysis performed.

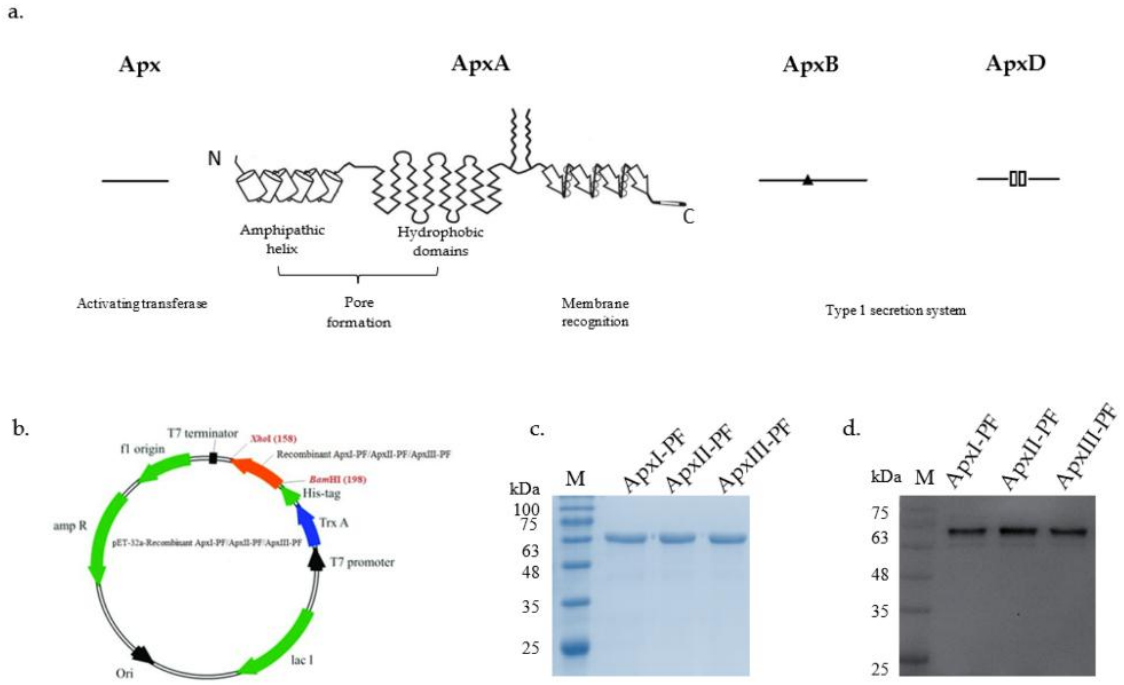

Figure 1 Recombinant protein production of the pore-forming domain of ApxI, II and III. (A) Schematic diagram of a typical RTX toxin gene arrangement, pCABD, is shown. ApxC encodes the activating acyltransferase, ApxA the structural toxin and ApxB and ApxD the type 1 secretion system. (B) Map of constructed pET32a expression vector. (C) SDS-PAGE and (D) Western blot analyses of the purified recombinant proteins.

ApxI pore-forming domain elicited the highest level of antibody response: Analysis of antibody response using indirect ELISA showed that the pore-forming domain of ApxI elicited the highest levels of antibody response among the three toxins on Day 28 (Figure 2). Antibody levels elicited by the pore-forming domains of ApxIII appeared to be only marginally higher (not statistically significant; $P > 0.05$) than that of the PBS group. For the vaccine group that included the pore-forming domains of all three toxins, the antibody level was comparable to that of the ApxI group.

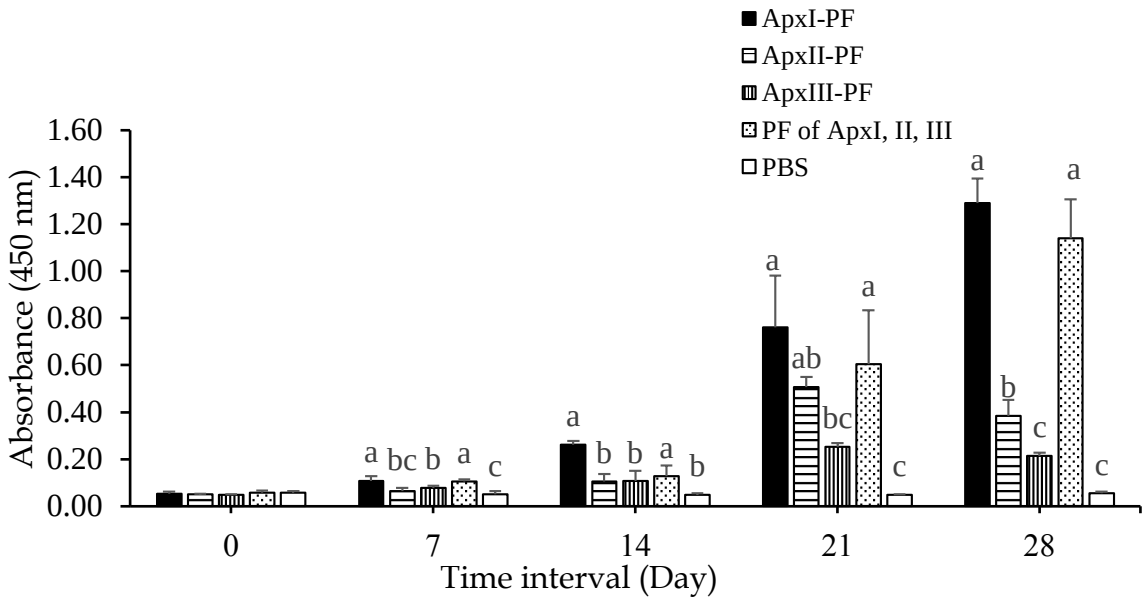

Figure 2 Antigen-specific antibody response of immunized mice. Mice were immunized twice with ApxI-PF, ApxII-PF, ApxIII-PF, PF of Apx I-III and PBS. Serum antibody levels were analyzed by indirect ELISA. Data was presented as mean±SEM. Different superscript letters indicate significant differences ($P<0.05$) between treatment groups.

ApxI pore-forming domain elicited the highest level of CD4⁺ and CD8⁺ T Cell Expansion: Cellular immune response elicited by the vaccines was examined by measuring the extent of $CD4^+$ and $CD8^+$ T cell expansion of the splenocytes from the vaccinated mice. For the ApxI group, the percentages of $CD4^+$ and $CD8^+$ T cells in the splenocytes reached around 19% and 16%, respectively, on both Day 14 and 28 (Figure 3). This is significantly higher than those of the ApxII and III groups, indicating strong stimulation of cellular immune response by the pore-forming domain of ApxI. For the vaccine group that included the pore-forming domains of all three toxins, the percentages of CD_{4+} and CD_{8+} T cells in the splenocytes were comparable to those of the ApxI group.

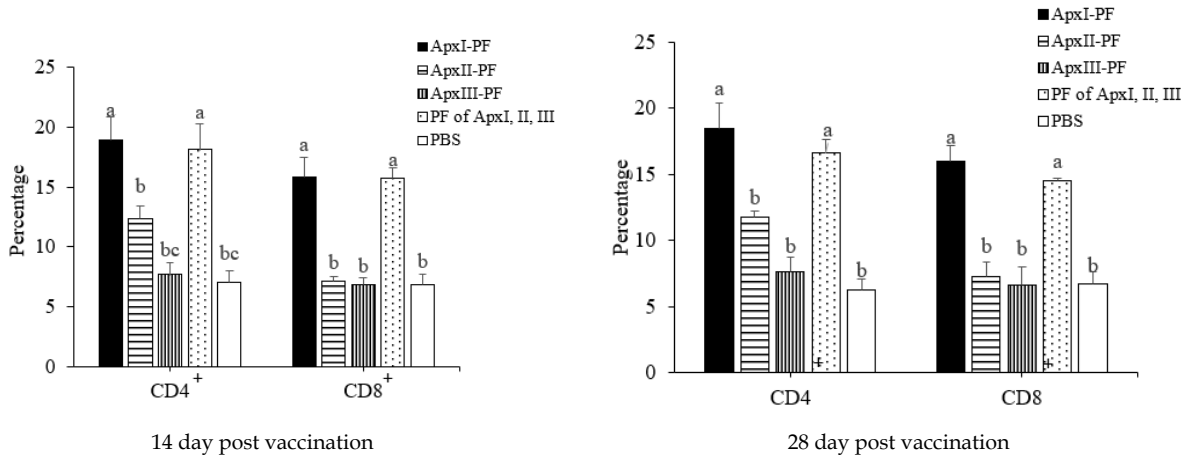

Figure 3 $CD4^+$ and $CD8^+$ T cell expansion analysis of immunized mice. Mice were immunized twice with ApxI-PF, ApxII-PF, ApxIII-PF, PF of Apx I-III and PBS. On Day 28, two mice from each treatment group were sacrificed and the percentage of $CD4^+$ and $CD8^+$ cells in the splenocytes was determined. Data was presented as mean±SEM. Different superscript letters indicate significant differences ($P<0.05$) between treatment groups.

ApxI and II pore-forming domains elicited higher levels of cytokine production than the ApxIII pore-forming domain: The cytokine gene expression profile of splenocytes from vaccinated mice (Day 28) was examined. In terms of proinflammatory cytokines, prominent gene expression of IL-1β and IL-6 could be observed for the ApxI and II groups. However, for the ApxIII group, very low levels were observed for the four cytokines (IL-1β, IL-6, IL-8, and TNF- α) analyzed (Figure 4A). This indicates that ApxI and II are more immunostimulatory than ApxIII.

Gene expression of T_H1-type (IL-12 and IFN-γ) and T_H2-type (IL-4 and IL-10) cytokines was also evaluated. For the ApxI and II groups, both T_H1-type (IFN-γ) and T_H2-type (IL-4 and IL-10) cytokine gene expressions were observed. In contrast, for the ApxIII group, only a low level of IL-10 expression was detected (Figure 4B). Overall, ApxI and II pore-forming domains elicited higher levels of both T_H1 and T_H2-type cytokine production than the ApxIII pore-forming domain, indicating a more balanced immune response. Furthermore, for the vaccine group that included the pore-forming domains of all three toxins, the cytokine expression profile resembled that of the ApxI vaccine group.

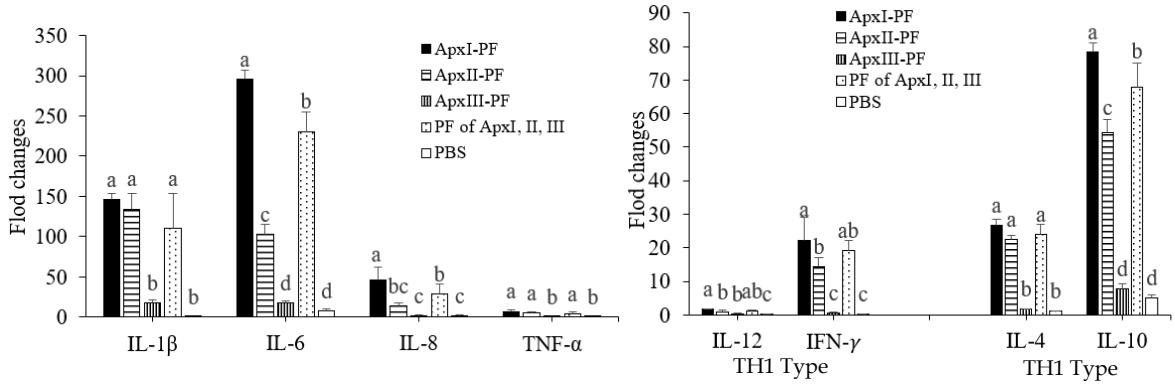

Figure 4 Cytokine gene expression of splenocytes from immunized mice. Mice were immunized twice with ApxI-PF, ApxII-PF, ApxIII-PF, PF of Apx I-III and PBS. On Day 28, two mice from each treatment group were sacrificed and isolated splenocytes were stimulated with purified antigens. Relative mRNA expression levels of various cytokines were determined. Data were presented as mean±SEM. Different superscript letters indicate significant differences ($P<0.05$) between treatment groups.

ApxI pore-forming domain provided 100% protection against lethal challenge: Vaccinated mice were challenged using the *A. pleuropneumoniae* serotype 1 strain. Results showed that the pore-forming domain of ApxI provided 100% protection (Figure 5). Partial protection of 40% was observed for the ApxII vaccine group. The ApxIII group, just as the PBS control group, afforded no protection. For the vaccine group that included the pore-forming domains of all three toxins, 100% protection was observed. The vaccine including ApxI-PF groups provided a significant boost to protective efficacy. The lung gross lesions of sacrificed mice are shown in Fig 6., the vaccine including with ApxI-PF groups showed significantly less bleeding than PBS groups.

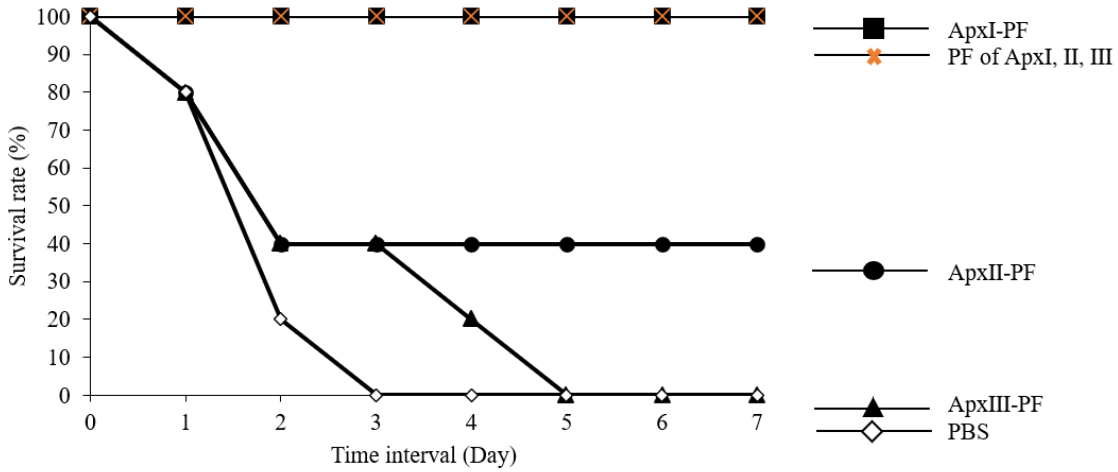

Figure 5 Survival rate of immunized mice when challenged with *Actinobacillus pleuropneumoniae* strain 4707. Mice (n=5) were immunized twice with ApxI-PF, ApxII-PF, ApxIII-PF and PBS and challenged with 5x10^8 CFU/dose *A. pleuropneumoniae*.

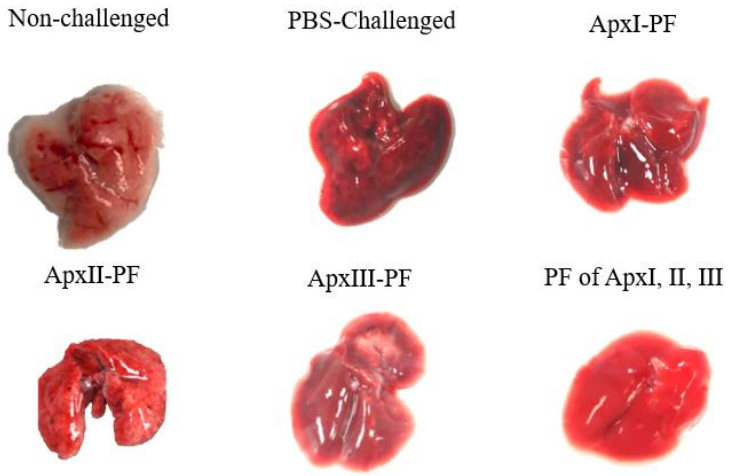

Figure 6 Gross lesions of the lungs of mice after challenge with *Actinobacillus pleuropneumoniae* strain 4707. Mice (n=5) were immunized twice the five vaccine formulations and challenged with 10 LD_{50} with 5x10^8 CFU/dose *A. pleuropneumoniae*.

Discussion

In this study, we found significant differences in the protective efficacy of the pore-forming domains of the three different Apx toxins against *A. pleuropneumoniae* serovar 1: ApxI (100%), ApxII (40%), and ApxIII (0%). Also, for the vaccine group that included the pore-forming domains of all three toxins, 100% protection was observed, the same as the ApxI group. There are currently 19 recognized serotypes of *A. pleuropneumoniae* (Stringer *et al*., 2021) and different serotypes express various combinations of the Apx toxins (Jung *et al*., 2019). For example, serovar 1 expresses ApxI and II while serovar 2 expresses ApxII and III (Boekema *et al*., 2004). Since ApxIII is not expressed by the serotype 1 strain used for the challenge test, it is not too surprising that the ApxIII pore-forming domain afforded no protection. However, as a counter point, amino acid sequence comparison of ApxI and ApxIII pore-forming domains showed 51.3% homology, suggesting the possibility of cross-protection. It is still possible that cross-protective epitopes remain in the ApxIII pore-forming domain and that further epitope isolation or adjuvant enhancement could make the ApxIII pore-forming domain a protective antigen even against *A. pleuropneumoniae* serovar 1. Nevertheless, based on the protective rates observed in our study, a logical vaccine design could be to combine the pore-forming domains of all three toxins for protective efficacy evaluation against the numerous serotypes of *A. pleuropneumoniae*.

In trying to pinpoint the protective domains within the Apx toxins, one important factor to consider is the role the pore-forming domain plays in the hemolytic vs. leukolytic activity of the toxin (Ostolaza *et al*., 2019). Since it is now known that leukolytic activity is the main contributor of pathogenesis, it is essential to know whether leukolytic activity resides, at least in part, within the pore-forming domain (Linhartová *et al*., 2010). For example, one study mapped the leukolytic and pro-apoptotic epitopes to the N-terminal (residues 1-160), activation and C-terminal (residues 825-1000) domains of ApxIII (Seah and Kwang, 2004). *In vitro* evaluation using porcine macrophages can help pinpoint epitopes involved in leukolytic activity.

While the initial evaluation of protective efficacy was performed in mice in our study, final confirmation of protection in pigs is important since the leukolytic activity of RTX toxins is known to be species-dependent. One study showed that ApxIII can induce cell death of peripheral blood mononuclear cells from pigs and wild boars but not those from llama, humans, dogs, rats, mouse, cattle or goats (Bergh *et al*., 2008). At the molecular level, the target cell receptor for ApxIII has been demonstrated to be the porcine integrin β_2 subunit (CD18), as a non-susceptible human cell line can be made susceptible to ApxIII leukolytic activity by transient expression of an integrin containing porcine CD18 (Bergh *et al.*, 2009; Ristow and Welch, 2019). Given the host-specificity of the RTX toxin leukolytic activity, data obtained from mice experiments will need to be reconfirmed in the target animal.

In conclusion, we performed an initial analysis of the protective efficacy of the Apx toxin pore-forming domains in mice. Results showed a range of protection rates for the three Apx toxins and indicated that the pore-forming domains can be further evaluated for leukolytic activity in porcine macrophages and protective efficacy in pigs.

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