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Immune Response to the Dual Antigen Vaccine of *Actinobacillus pleuropneumoniae* in a Mouse Model

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Abstract

Actinobacillus pleuropneuminiae is the major cause of invasive respiratory disease in swine. To develop an efficient subunit vaccine against pathogens, we designed a dual antigen in a single vaccine that increased the potential for an immune response. We present a dual antigen vaccine (pore-forming domain and nutritional immunity) that induces humoral and cell-mediated T-cell responses, antibodies and T_H1 and T_H2 cells. Mice were immunized with the dual antigen and individual antigen and then subjected to immune response analysis. A significant antibody response was observed for the dual antigen group. For the cellular immune response, $CD4^+$ and $CD8^+$ T-cell expansion, proinflammatory cytokine (IL-1β, IL-6) and T_H2-type cytokine (IL-4, IL-10) gene expression was observed for the dual antigen. Finally, in a challenge test with *A. pleuropneumoniae* serotype1, the dual antigen, individual antigen and PBS vaccine conferred 100, 40 and 0 % protection. In conclusion, the dual antigen presents critical antigens and increases the efficacy of the vaccine.

Keywords: *Actinobacillus* pleuropneumoniae, Apx toxin, TonB2 system, Subunit vaccine

Introduction

A. pleuropneumoniae is a Gram-negative bacterium that is an important respiratory pathogen in swine. It has caused great commercial losses in the porcine industry [1]. A vaccine is the best way to decrease the mortality, clinical sign, production losses of *A. pleuropneumoniae* infection, reduce the use of antibiotic drugs and support consumer food safety [2]. An inactivated whole bacteria vaccine has been developed, but the vaccine is not sufficient to protect against disease [3]. Because the bacteria secrete exotoxins into the medium, some proteins were damaged during the process of developing the whole-bacteria vaccine. A subunit vaccine was then developed, it is mainly based on the conserved virulent toxins and outer membrane proteins (OMPs). But it exhibit partial cross-protection [4,5], effective *A. pleuropneumoniae* vaccine protection can be provided by multi-component protein single target vaccines will increase protective immunity in subunit vaccine [6] . To date, a pathogen comprising 19 serotypes of *A. pleuropneumoniae* have been identified worldwide [7].

TonB is essential for virulence protein and transferrin uptake in gram-negative bacteria. The TonB2 system consists of three transmembrane exbB2-exbD2-tonB2 complexes involved in iron uptake (**Figure 1**). The host secretes proteins, including hemoglobin, myoglobin, transferrin and ferrin, which then bind to free iron and transport it into the cell. That is important defense mechanism which restricts the growth of the invading pathogens completely, a strategy called "nutritional immunity" [8]. The TonB2 system plays an essential role in *A. pleuropneumoniae* gaining access to iron for growth, colonization, and survival during host infection. The pathogen mechanism involves the secretion of siderophores to bind and chelate free iron and transports into the periplasmic space via TonB2 system to avoid nutritional immunity. The TonB2 system is a surface-exposed epitope that triggers protective immunogenicity [9], which is a characteristic that can be exploited to develop vaccines against *A. pleuropneumoniae* [10].

Apx recombinant protein as a vaccine antigen has shown potential efficacy in terms of safety and protection. Apx toxins are highly immunogenic, which induces strong production of antibodies. They were selected as the main virulence factor in the first subunit vaccine formula [11]. There are 4 different Apx

toxins that typically encode the structural gene "A", the activator gene "C" and the T1SS genes "B" and "D". The structural features of gene "A" contains with amphipathic helix, pore-forming region, the glycine-rich repeats and export signal (**Figure 1**). However, the structure of RTX proteins are the large size of the protein and the toxin protein expression may result in low yield. The recombinant protein of sub-unit vaccine is not the best way for vaccine development [12]. Therefore, pervious study identified the pore-forming region is as virulence of cytotoxic bacteria, the pore-forming region (residues 40-380) of ApxI provided protection [13]. ApxII toxin is weakly hemolytic and moderately cytotoxic. We identified the pore-forming region of ApxII (termed ApxIIPF) to be test as an antigen because of its lower molecular weight and importance in the hemolytic activity of the toxin.

It is difficult to generate strong immunogenicity from subunit vaccines with a single antigen. Because a single protein may lack epitopes suitable for universal immune recognition, it cannot be used against all serotypes of *A. pleuropneumoniae*. Multiple antigens induce stronger immune responses than those triggered by a single component [14]. This is our strategy immunization with subunit vaccines combined with individual potential structural and functional proteins from the same target would be predicted to trigger the development of a strong host protective immune response.

Materials and methods

Bacteria strain

A. pleuropneumoniae serotype 1 strain 4074 (serotype1, ATCC® 27088™) was bought from the American Type Culture Collection (ATCC) and cultured in Brain-Heart Infusion Broth with 15 mg/mL nicotinamide adenine dinucleotide (NAD) at 37 °C in incubator.

Recombinant protein expression of pore-forming domains, nutritional immunity recombinant protein and vaccine formulation

ApxII-PF and TonB2 from *A. pleuropneumoniae* serotype 1 were cloned into an expression plasmid. The primers shown in **Table 1** were used to amplify the ApxII-PF and TonB2 genes via polymerase chain reactions. Then, restriction enzyme digestion was performed, and the PCR products were ligated into the expression vector pET32a (Novagen, Darmstadt, Germany). The expression vector carrying the ApxII-PF and TonB2 genes was transformed into *Escherichia coli* DH5α (Yeastern Biotech, Taipei, Taiwan), and the plasmid was sent for sequencing and compared with sequence data in the National Center for Biotechnology Information database to confirm the gene sequences were correct.

Table 1 Primers for gene cloning and recombinant protein construction.

Target gene	Oligonucleotide Sequence (5′ to 3′)	Tm (°C)	Gene length (bp)	NCBI Reference Sequence
TonB	F: CC***GAATTC***ATGAAGAAAAAACATTCTGCG	60	816	ATCC® 27088™
	R: CC***AAGCTT***TTATTCAATCGAGAATTTCAC			
ApxII-PF	F: GC***GGATCC***ATGTCAAAAATCACTTTGTCATCAT	57	1140	ATCC® 27088™
	R: CGCTCGAGAGCTCCAACTCCACCGGAGAT			

Italics and Bold in the primers represent restriction enzyme sites, GAATTC: *Eco*RI Enzyme, AAGCTT: HindIII Enzyme, GGATCC: *Bam*HI Enzyme; CTCGAG: *Xho*I Enzyme

The successful recombinant proteins were used to transform into *E. coli* BL21 (DE3) competent cells according to the manufacturer's instructions. Protein expression was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma, Darmstadt, Germany) incubator at 37 °C for 4 h. The expression proteins were harvested and purified using Bio-scale Mini Profinity IMAC cartridges (1mL) (Bio-Rad, Hercules, CA, USA) according to the manufacture's instruction. The quantification and quantitation of the purified soluble ApxII-PF and TonB2 proteins were verified by 12 % sodium dodecylsulfate polyacrylamise gel electrophoresis (SDS-PAGE) and western blotting with an anti-6X-His Tag Antibody (Gentex, Hsinchu, Taiwan) in 1:5,000 dilution and a secondary peroxidase-conjugated goat anti-mouse antibody (Gentex, Hsinchu, Taiwan) at 1:5,000 dilution. Visualization of the western Lighting PLUS (PerkinElmer, Waltham, MA, USA) was used for color development by the ToxinSensor™ Chromogenic LAL Endotoxin AssayKit (GenScript, Piscataway, NJ, USA), endotoxin levels of the purified proteins were confirmed to be less than 0.125 EU/mL.

Immunization and sample preparation in mice

Four vaccine formulations were prepared from the recombinant proteins and mixed with the commercial water-in-oil-in-water emulsion adjuvant: Montanide ISA206 adjuvant (Seppic, Courbevoie, France) at a ratio of 1:1 for a final injection volume of 200 μL per mouse: (1) the ApxII-PF group, (2) the TonB2 group, (3) the dual-antigen group with both ApxII-PF and TonB2 protein, and (4) PBS as the negative control group. The 28 female ICR mice at 5 weeks of age were purchased from BioLASCO Taiwan Co., Ltd. (Taipei, Taiwan). The mice were randomly divided into 4 groups of 7 mice to receive the 4 vaccine formulations. All studies were performed in accordance with the guidelines of the National Pingtung University of Science and Technology Institutional Animal Care and Use Committee. All mouse groups were immunized twice intraperitoneally with 25 μg of recombinant proteins alone, a combination of 2 antigens 25 μg of each recombinant proteins [15] on days 0 and 14 with the indicated different vaccine formulations.

Measurement of antibody response by ELISA

Antibody responses to vaccine formulations were analyzed by the indirect enzymes linked immunosorbent assay on days 0, 7, 14, 21 and 28. The mouse serum samples against antigen were measured using ELISA. The wells of a 96 wells plate were coated with 50 ng of antigen in 0.5 % skim milk volume 100 μL overnight at 4 °C. The wells were washed three times with PBS contain 0.05 % Tween 20, blocked with 5 % skim milk for 1.5 h at 37 °C, washed with PBST and then incubated with the diluted serum samples ($1:10^6$ dilution) as the primary antibody for 2 h at 37 °C. The wells were washed and incubated with Horseradish peroxidase (HRP)-conjugated anti-mouse IgG at 1:5,000 dilution (GeneTex, Irvine, CA, USA) was used as secondary antibody. Finally, the Peroxidase Kit (KPL, Gaitherburg, MD, USA) was used for color development at room temperature for 30 min and the plates were read at 450 nm on the Multiskan^{TM} FC Photometer (Thermo Fisher Scientific, Vantaa, Finland).

Splenocytes and cytokine analysis

The spleens of mice immunized with vaccines (n = 2 per group) were aseptically removed and mashed through cell strainers at day 28. Splenocytes were lysed and resuspended in ACK lysis buffer (150 mM NH_4CL, 10 mM $KHCO_3$, 0.1 mM Na_2-EDTA, pH7.4), washed twice with RPMI 1640 media, and cultured in RPMI 1640 media (Gibco Invitrogen, Carlsbad, CA, USA). supplemented with 10 % fetal bovine serum (Gibco Invitrogen, Carlsbad, CA, USA), 1 mM sodium pyruvate, 100 U/mL penicillin 100 μg/mL streptomycin and 50 μM β-mercaptoethanol at 37 °C under 5 % CO_2 in a humidified atmosphere. The splenocytes were determined cytokine production and $CD4^+$ and $CD8^+$ T cell response. The splenocytes were stimulated with antigen for 72 h at 0.5 μg/mL of antigen dissolved in RPMI 1640 media. The end of experiment, the cell culture was harvested and determined for cytokine response. Total RNA was extracted from splenocytes using Miniprep system and complementary DNA was synthesized using the Reverse Transcription Kit. Real-time PCR was performed with the SmartCycler I targeting pro-inflammatory, T_H1 and T_H2. The data presented were normalized to GAPDH mRNA. To determine the relative mRNA expression levels, we used the ΔΔCt method. Primer sequences are shown in **Table2**.

Table 2 Primers for cytokine genes.

Gene	Oligonucleotide Sequence (5′ to 3′)	Tm (°C)	Gene length (bp)	NCBI Reference Sequence
IL-1β	F: AGTTGACGGACCCCAAAAGAT	57	412	M15131.1
	R: CATGGAGAATATCACTTGTT			
IL-4	F: CGAAGAACACCACAGAGAGTGAGCT	50	175	M25892.1
	R: GACTCATTCATGGTGCAGCTTATCG			
IL-6	F: CTTCCATCCAGTTGCCTTCTTG	57	141	M24221.1
	R: AATTAAGCCTCCGACTTGTGA			
IL-8	F: CAAGGGCCAAGAGAATATCC	55	445	BC013615.1
	R: TTACTATAACATCTTTATAA			

Gene	Oligonucleotide Sequence (5′ to 3′)	Tm (°C)	Gene length (bp)	NCBI Reference Sequence
IL-10	F: AAGGCAGTGGAGCAGGTGAA	55	155	NM_010548.2
	R: CCAGCAGACTCAATACACAC			
IL-12p40	F: CAGAAGCTAACCATCTCCTGGTTTG	55	396	BC103610.1
	R: CCGGAGTAATTTGGTGCTCCACAC			
IFN-γ	F: AGCGGCTGACTGAACTCAGATTGTAG	55	243	NM_008337.4
	R: GTCACAGTTTCAGCTGTATAGGG			
TNF-α	F: GGCAGGTCTACTTTGGAGTCATTGC	55	300	NM_001278601.1
	R: ACATTCGAGGCTCCAGTGAATTCGG			
GADPH	F: CGGCACAGTCAAGGCCGAGAAT	57	154	M32599.1
	R: AGCCTTCTCCATGGTGGTGAA			

Note: IL: Interleukin; IFN-γ: Interferon gamma; TNF-α: Tumor necrosis factor alpha; GAPDH: Glyceraldehyde 3 phosphate dehydrogenase

T-cell response analysis

On day 28, the splenocytes were stained with anti-$CD4^+$-APC and anti-$CD8^+$-FITC antibodies (Sino Biological Inc, Wayne, PA, USA) in PBS at 4 ºC for 30 min and washed with PBS 2 times. The cells were analyzed by the BD Accuri™ C6 flow cytometer. All animal experimental protocols (NPUST-109-064) were approved by the Institutional Animal Care and Use Committee of the National Pingtung University of Science and Technology, Taiwan.

Challenge test

Vaccinated mice (n = 5 per group) were challenged intraperitoneally with 5×10^8 CFU (20 LD_{50}) *A. pleuropneumoniae* ATCC 27088. Mice were observed for 7 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 7-day period.

Statistical analysis

All data were analyzed using IBM Statistical Package for the Social Sciences ver. 22.0. To examine of antibody response, cytokine mRNA levels, percentages of $CD4^+$ and $CD8^+$ T cells whether there were any statistically significant differences. Statistical significance was set as $p < 0.05$.

Results and discussion

Production of recombinant proteins

SDS-PAGE analysis of proteins purified after expression in *E. coli* showed the target proteins at approximately 61.8 and 49.92 kDa for the ApxII and TonB2 recombinant proteins. Western blotting using an anti-His antibody confirmed the identity of the recombinant proteins (**Figure 1**). These findings demonstrate that both recombinant proteins were expressed as expected.

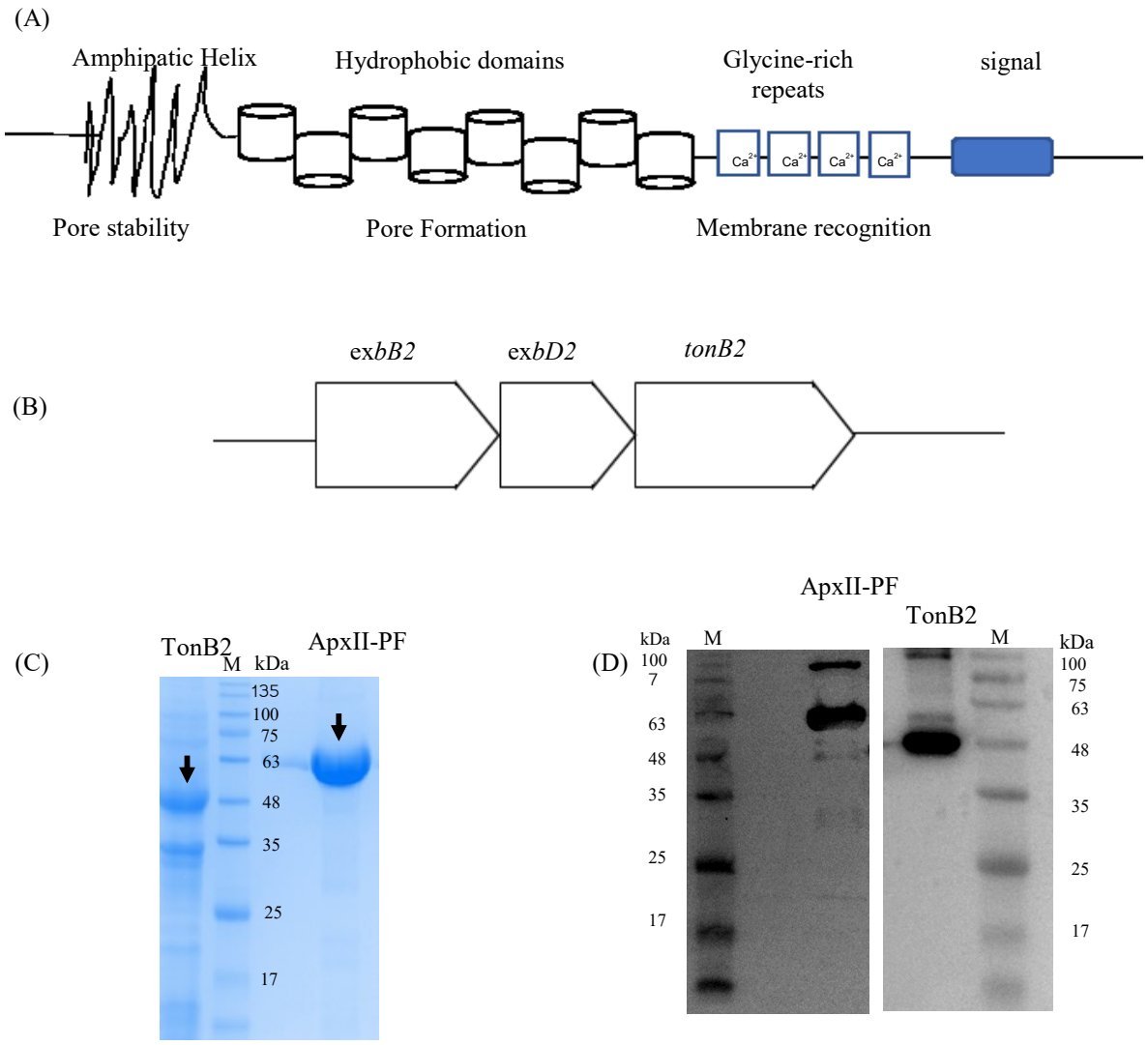

Figure 1 Characterization, expression of Apx toxin and TonB2. (A) Schematic organization of Apx toxins. The different domains of the proteins are indicated as follows: Amphipatic Helix, Hydrophobic domains, Glycine-rich repeats and signal. (B) Schematic of TonB2 dependent iron uptake. (C) SDS-PAGE analysis for the recombinant protein production of the pore-forming domain of ApxII-PF and TonB2. Lanes M, molecular weight marker. (D) Western blot analysis for the recombinant protein production of the pore-forming domain of ApxII-PF and TonB2.

The dual antigen *A. pleuropneumoniae* vaccine elicited a humoral immune response

The mice were immunized with 2 boosters, and serum samples were collected on days 0, 7, 14, 21 and 28 in each group for measurement of IgG levels by indirect ELISA. The serum IgG levels of all groups were increased by relatively high margins compared with the baseline levels the day before immunization. The IgG levels of the dual protein group were significantly elevated relative to the single antigen groups after priming and boost vaccination. The single antigen elicited a higher IgG level than the PBS control (**Figure 2**). Our vaccine is expected to produce a focused immune response. When we determined the fold change in the antigen-specific IgG antibodies, we observed that this difference could predominantly be detected as a significant increase in cellular immunity and antibody response after day 7 vaccination and the dual-antigen immunized mice compared to single antigen-immunized mice, dual antigen was good sensitivity antigen [16,17].

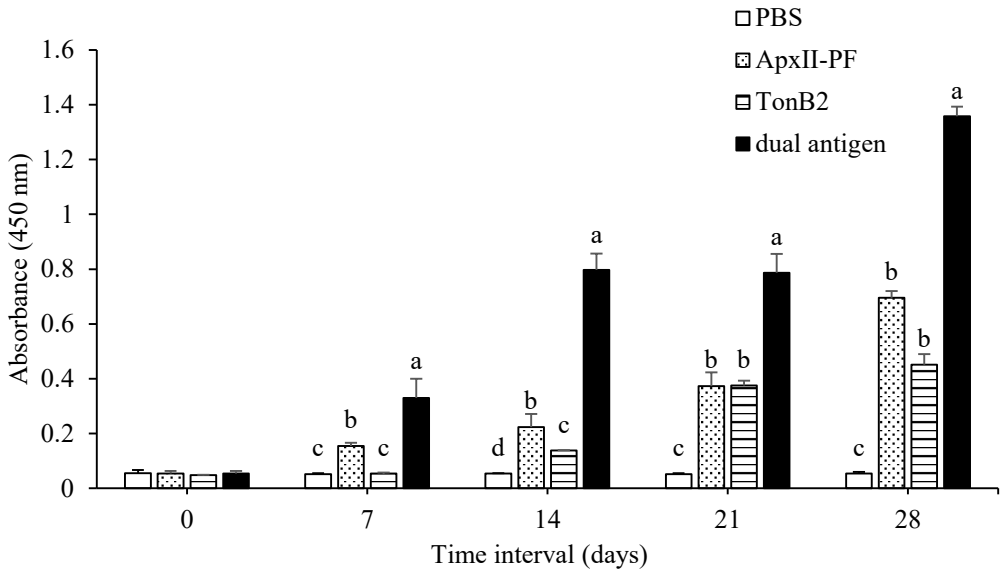

Figure 2 Antigen-specific antibody response of immunized mice. Mice were immunized twice with ApxII-PF, TonB2, dual antigen and PBS. Serum antibody levels were analyzed by indirect ELISA. Data were presented as mean±SEM. Different superscript letters indicate significant differences ($p < 0.05$) between treatment groups.

Dual antigen *A. pleuropneumoniae* vaccine elicited the highest level of $CD4^+$ and $CD8^+$ expansion

The distribution of $CD4^+$ and $CD8^+$ T cells was evaluated by FACS in the splenocytes from the immunized and control mice. At day 28, the percentages of $CD4^+$ and $CD8^+$ T cells were higher in immunized mice than in the control group. The $CD4^+$ (20.05 %) and $CD8^+$ (16.32 %) cells were the highest in the dual antigen group as shown in **Figure 3**, significantly higher than in the control group ($p < 0.05$). Currently, the T cells CD4+ and $CD8^+$ as Th17 cells are resulted with immune responses against *A. pleuropneumoniae* in pig [18].

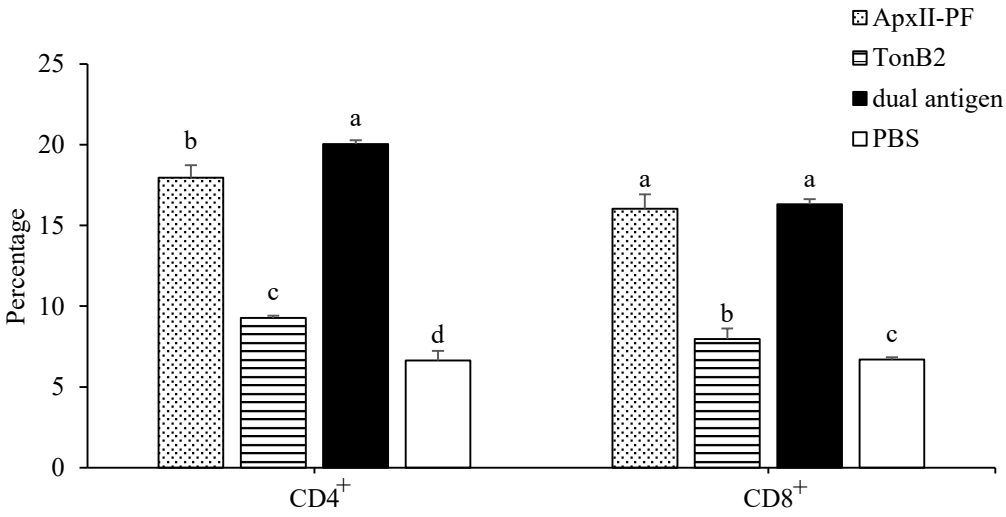

Figure 3 $CD4^+$ and $CD8^+$ T cell expansion analysis of immunized mice. Mice were immunized twice with ApxII-PF, TonB2, dual antigen and PBS. On day 28, 2 mice from each treatment group were sacrificed and the percentage of $CD4^+$ and $CD8^+$ cells in the splenocytes was determined. Data were presented as mean±SEM. Different superscript letters indicate significant differences ($p < 0.05$) between treatment groups.

The dual antigen *A. pleuropneumoniae* vaccine induced cytokine expression

Splenocytes from mice immunized with the single antigen, dual antigen vaccine and PBS control were restimulated to produce IL-1β, IL-6, IL-8, and TNF-α. The result showed IL-1β, IL-6 production in the spleen increased post vaccination 28 days. The prevention of IL-1β, IL-6 levels was correlated with severity of disease. mRNA expression levels of T_H1-type (IL-12 and IFN-γ), pathway to fight bacteria and T_H2-type (IL-4 and IL-10) cytokines, which up regulate antibody production were evaluated, and the dual antigen elicited higher levels of cytokine production than the single antigen vaccine and the PBS control as shown in **Figure 4**.

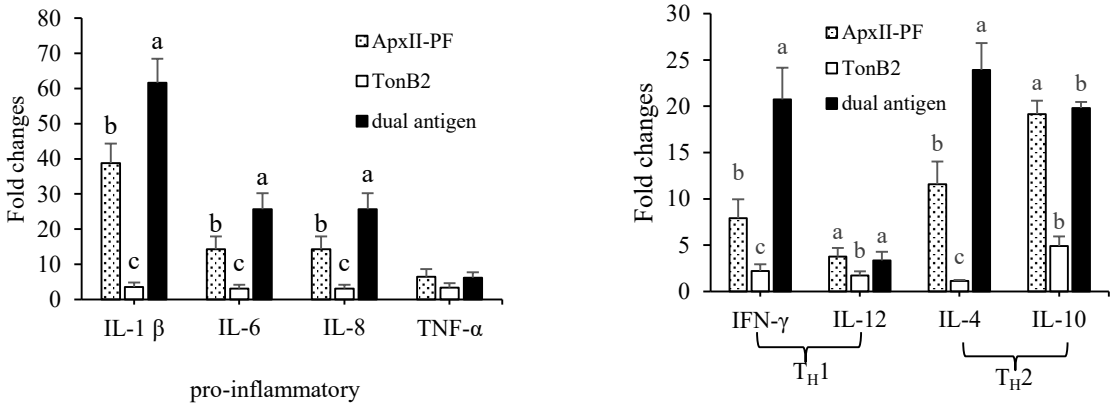

Figure 4 Cytokine gene expression of splenocytes from immunized mice. Mice were immunized twice with ApxII-PF, TonB2, dual antigen and PBS. On day 28, 2 mice from each treatment group were sacrificed and isolated splenocytes were stimulated with purified antigens. Relative mRNA expression levels of various cytokines were determined. Data were presented as mean±SEM. Different superscript letters indicate significant differences ($p < 0.05$) between treatment groups.

The dual antigen provided 100 % protection against lethal challenge

Vaccinated mice were challenged using the *A. pleuropneumoniae* serotype 1 strain. Results showed that the highest bacterial clearance and 100 % protection were observed in the group vaccinated with the dual antigen (**Figure 5**). The individual antigen protection of 40 % was observed for ApxII-PF and TonB2 antigen. The negative group was no protection. This study showed that the protection rate from the antibody against each component is additive, as this is a basic assumption about the mechanism of the individual responses combined to confer more efficacy of the vaccine in an individual fighting an infection [19,20]. Finally, a limitation of this study is the usage of all female mice to test the vaccine, considering that the goal is to protect pigs against *A. pleuropneumoniae* infection. This study provides a basis for the more rational construction of multicomponent vaccine formulations by combining the pore-forming domain of the ApxII toxin and the nutritional immunity antigen of *A. pleuropneumoniae* into a single vaccine.

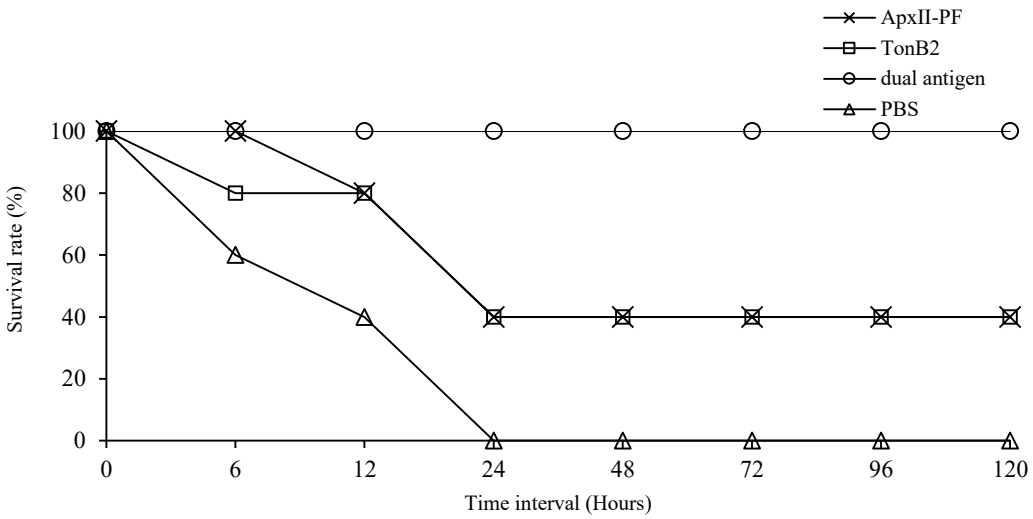

Figure 5. Survival rate of immunized mice when challenged with *Actinobacillus pleuropneumoniae* strain 4707. Mice (n = 5) were immunized twice with ApxII-PF, TonB2, dual antigen and PBS and challenged with 5×10^8 CFU/dose *A. pleuropneumoniae*.

Conclusions

We developed a dual antigen vaccine (TonB2 protein and the ApxII pore-forming domain of exotoxin protein) with the goal of improving its efficacy. The dual antigen could provide a useful improvement by approximately 2.5-fold compared to each of the individual antigen vaccines. After administration of the priming and boosting vaccines, mice immunized with the dual antigen demonstrated a significantly higher antigen-specific IgG antibody response than mice immunized with either individual antigen. Therefore, this dual antigen vaccine targeting both exotoxin and nutritional immunity may be more effective in triggering antigen-elicited humoral and cellular immune responses against pathogens. The dual antigen provides more protection to the animals against *A. pleuropneumoniae* infection. Overall, the results of this study demonstrate that the developed anti-gene vaccines as an answer to the ineffectiveness of whole bacterial vaccines that have been developed and applied in protecting against disease.

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