Efficacy Evaluation of Combination Vaccine of Recombinant C-Terminal Fragments of ApxIA, ApxIIA and ApxIIIA in Piglets
(Penilaian Kemujaraban Gabungan Vaksin Rekombinan Terminal-C Serpihan ApxIA, ApxIIA dan ApxIIIA pada Anak Babi)

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YEONG HWAN CHOI, MIN SOO Choi & JIN HUR*

ABSTRACT

The efficacy of the combination vaccine of the individual C-terminal fragments of ApxIA, ApxIIA and ApxIIIA of Actinobacillus pleuropneumoniae (APP) was evaluated in piglets. Twenty piglets were divided equally into 2 groups (n=10). All piglets were intramuscularly primed at 4 week-of-age (0 week post prime inoculation (WPPI)) and were intramuscularly boosted at 6 week-of-age (2 WPPI). Group A piglets were inoculated with sterile PBS and group B piglets were inoculated with the combination vaccine. Concentrations of each of the C-terminal fragment-specific IgG as determined by ELISA were significantly higher in group B than in group A from 2 WPPI until the end of this study. Clinical signs were observed from only 10% of group B piglets after the challenge with the mixture of APP serotypes 1, 2 and 5 at 4 WPPI, while 50% of group A piglets were protected against APP infections. Overall, intramuscular inoculation with the vaccine candidate can efficiently protect piglets against APP infection.

Keywords: Actinobacillus pleuropneumoniae; immunization; piglets; porcine pleuropneumonia; protection

INTRODUCTION

Porcine pleuropneumonia (PP) is a severe, contagious, swine pulmonary disease caused by Actinobacillus pleuropneumoniae (APP). This disease affects pigs of all ages and has a major impact on economics, ecology and animal welfare in the pig-rearing industry (Haesebrouck et al. 2004). For control of PP, vaccination is crucial (Fenwick & Henry 1994). However, the many serotypes of APP have made effective vaccination difficult. Various studies have been carried out to identify vaccine candidates for efficient cross-protection, such as killed bacterin vaccines and subunit vaccines (Blackall et al. 2002; Haesebrouck et al. 2004; Zhou et al. 2013). These vaccine candidates do not prevent colonization and are not widely cross-protective (Tumamao et al. 2004). Therefore, further efforts need to be made for development of more efficient vaccines.

Among several virulence factors, Apx [repetitive glycine-rich sequences in repeats-in-toxins exotoxins (RTX toxins)] toxins are recognized as major virulence factors of APP (Haesebrouck et al. 2004; Kamp et al. 1997). The importance of Apx toxins in protective immunity against PP has been demonstrated in many studies (Haesebrouck et al. 2004; Shin et al. 2005). The 15 serotypes of APP produce four different Apx toxins including ApxI, ApxII, ApxIII and ApxIV (Zhou et al. 2013). ApxIA and ApxIIA toxins contribute to hemolytic and cytotoxic functions (Frey & Kuhnert 2002). ApxIIIA has no hemolytic activity but shows strong cytotoxic activity towards alveolar macrophages and neutrophils (Cruysen et al. 1992; Rycroft et al. 1991). In South Korea, serotypes 1, 2 and 5 have been known to dominate since 2000 (Kim et al. 2001; Lee et al. 2015; Yoo et al. 2014). The objective of this study
was to evaluate the efficacy of the combination vaccine of the individual C-terminal recombinant proteins of ApxIA, ApxIIA and ApxIIIA in order to improve the prevention of PP in piglets.

**MATERIALS AND METHODS**

**BACTERIAL STRAINS AND GROWTH CONDITIONS**

Wild type APP, serotype 1, HJL6, serotype 2, HJL67 and serotype 5, HJL263, were used for amplification of the genes encoding each C-terminal fragment of ApxIA, ApxIIA and ApxIIIA. In addition, wild type APP isolates, HJL6, HJL67 and HJL263, were used as the virulent challenge strains. These serotypes were kindly supplied by the National Veterinary Research and Quarantine Service (Anyang, Gyeonggi, South Korea) (Table 1). The strains were grown in chocolate agar at 37°C.

**CONSTRUCTION OF ESCHERICHIA COLI STRAINS EXPRESSING APXIA, APXIIA AND APXIIIA**

Each of the C-terminal fragment gene of ApxIA, ApxIIA and ApxIIIA were amplified from APP genomic DNA using the specific primers (Table 2). The PCR fragments of each gene were digested with restriction enzymes and were subsequently cloned into pET21a. These plasmids were transformed into E. coli BL21 (DE3) pLysS to create HJL401, HJL402 and HJL403. The individual recombinant C-terminal fragments were prepared from an affinity purification process with nickel-nitrilotriacetic acid-agarose (Qiagen, Valencia, CA, USA). The identities of the purified antigens were confirmed via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). All purified antigens were stored at -70°C until use.

**PREPARATION OF THE COMBINATION VACCINE**

In order to prepare combination vaccine that consists of the individual C-terminal fragments, the individual C-terminal fragments (consisting of approximately 30 μg per each C-terminals protein) were resuspended in 10 mg/mL of aluminum hydroxide, which was used here as a parenteral adjuvant. The combination vaccine was stored at 4°C until use.

**IMMUNIZATION AND SAMPLE COLLECTION**

Twenty Large Yorkshire piglets were divided equally into two groups. All piglets were intramuscularly primed at 4 weeks of age (0 week post prime inoculation (WPPI)) and were intramuscularly boosted at 6 weeks of age (2 WPPI). Group A piglets were inoculated with 2 mL of sterile PBS as the control. Group B piglets were inoculated with 2 mL of the combination vaccine. Blood samples were collected at 0, 2 and 4 WPPI for the evaluation of immune response. The animal experiments mentioned in this study were conducted with ethics approval (CBU 2011-0017) from the Chonbuk National University Animal Ethics Committee in accordance with the guidelines of the Korean Council on Animal Care.

**IMMUNE RESPONSE BY ELISA**

IgG concentrations against the individual recombinant C-terminal fragments in serum were evaluated by ELISA using a modified method from a previous study (Hur & Lee 2014). Briefly, sera were diluted 1:100 in PBS. The plates were treated with horseradish peroxidase-conjugated goat anti-swine IgG antibody. Enzymatic reactions were produced through the addition of substrate containing o-phenylenediamine (Sigma-Aldrich, St. Louis, MO, USA) and measured using an automated ELISA spectrophotometer (Thermo Scientific Multiskan GO, Thermo Fisher Scientific Oy, Ratatatie, Vantaa, Finland) at 492 nm. A standard curve was generated to represent the relationship between the concentrations of the standards and their absorbance, and the concentration of antibodies in each sample was determined using this curve. The results of ELISA are expressed as the mean ± standard deviation.

**TABLE 1. Bacterial strains and plasmids used for this study**

<table>
<thead>
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<th>Strain/plasmid</th>
<th>Description</th>
<th>Source of reference</th>
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<td><strong>Strains</strong></td>
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<tr>
<td>E. coli</td>
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<tr>
<td>BL21(DE3)pLysS</td>
<td>F. ompT, hsdS, (r, m, c), dcm, gal, λ(DE3), pLysS, Cm</td>
<td>Lab stock</td>
</tr>
<tr>
<td>HJL401</td>
<td>E. coli BL21 with pET21a-ApxIA C-terminal</td>
<td>This study</td>
</tr>
<tr>
<td>HJL402</td>
<td>E. coli BL21 with pET21a-ApxIIA C-terminal</td>
<td>This study</td>
</tr>
<tr>
<td>HJL403</td>
<td>E. coli BL21 with pET21a-ApxIIIA C-terminal</td>
<td>This study</td>
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<td><strong>A. pleuropneumoniae</strong></td>
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<tr>
<td>HJL6</td>
<td>Actinobacillus pleuropneumoniae serotype 1</td>
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<td>Actinobacillus pleuropneumoniae serotype 2</td>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>pET21a</td>
<td>IPTG-inducible expression vector; Km</td>
<td>Novagen</td>
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CHALLENGE EXPERIMENTS

All piglets were challenged intranasally with the mixture of HJL6, HJL67 and HJL263 at 4 WPPI. A total of 6 × 10^9 CFU in 2 mL of sterile PBS were used as challenge dose for piglets. All challenged piglets were monitored daily for mortality and abnormal behavior during 7 days after the challenge. At 7 days after challenge, all piglets were euthanized and challenge strains were examined from the swabs of lung observed abnormally in gross examination. The swabs were streaked onto chocolate agar and incubated at 37°C for 24 h. Challenge strains were confirmed by PCR (Table 2) using Apx IA, ApxIIA, or ApxIIIA-specific primers (Jessing et al. 2008, 2003). If isolates from the lung swab contained PCR amplicon products from at least one of the serotype-specific primers (Table 2) according to the previously described method (Jessing et al. 2008, 2003), the pneumonic lung sign was classified as induced by the challenge.

STATISTICAL ANALYSIS

Analyses were performed with SPSS version 16.0 software (SPSS, Chicago, IL). A Student’s t test was used to analyze statistical differences in the immune responses between the immunized groups and an unimmunized control group. Statistical significance was determined at p<0.05.

RESULTS

PRODUCTION OF RECOMBINANT PROTEINS

SDS-PAGE analysis of proteins purified after expression in HJL401, HJL402 and HJL403 showed prominent bands at approximately 50, 43.1 and 50 kDa, respectively (Figure 1). Humoral immune response in immunized piglets

PROTECTION OF PIGLETS AGAINST VIRULENT CHALLENGE

All piglets were intranasally challenged with the mixture of the challenge strains at 4 WPPI. Among 10 piglets of group A, 2 were dead within 7 days after challenge and the challenge strains were isolated from lung swab of 5 piglets (containing dead piglets) with pneumonic lung lesions observed upon gross examination. Among 10 piglets of group B, however, the lungs of 9 piglets were observed as normal in gross examination.

### Table 2. PCR primers used in this study and their product sizes

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence(5′→3′)</th>
<th>Size (bp)</th>
<th>Accession number</th>
<th>Reference</th>
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<tr>
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<td>GQ369732</td>
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<td>Apx IAC-R</td>
<td>GTTCGAC AGT ACC ATC GCT GC</td>
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<tr>
<td>Apx IIA-F</td>
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<td>1110</td>
<td>AY736188</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apx IIIA-F</td>
<td>GGGATCC GCA CCA ATT ACT TT</td>
<td>1293</td>
<td>X68815</td>
<td>This study</td>
</tr>
<tr>
<td>Apx IIIA-R</td>
<td>TGTGTCGAC AAG CAC ATT AAA ACC</td>
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<tr>
<td>HPF</td>
<td>AAG GTT GAT ATG TCC GCA CC</td>
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<td>AB007587.1</td>
<td>Jessing et al. 2003</td>
</tr>
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<tr>
<td>Ap1F</td>
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<td>754</td>
<td>AF518558.1</td>
<td>Jessing et al. 2008</td>
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<td>Ap1R</td>
<td>GAA AGA ACC AAG CTC CTG CAA T</td>
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<tr>
<td>Ap2F</td>
<td>ACT ATG GCA ATC AGT CGA TTC AT</td>
<td>500</td>
<td>AY357726.1</td>
<td>Jessing et al. 2003</td>
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<tr>
<td>Ap2R</td>
<td>CCT AAT CGG A A CGC CAT TCT G</td>
<td></td>
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<tr>
<td>Ap5F</td>
<td>TTT ATC ACT ATC ACC GTG CAC ACC T</td>
<td>1,100</td>
<td>AF053723.1</td>
<td>Jessing et al. 2003</td>
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<tr>
<td>Ap5R</td>
<td>CAT TCG GGT CTT GTG GCT ACT AA</td>
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</table>

Underlines indicate the sites of restriction enzymes, such as BamHI and SalI

**Figure 1.** Expression and purification of each C-terminal fragment of ApxIA, ApxIIA or ApxIIIA. Coomassie blue-stained SDS-PAGE gel showing each C-terminal fragments of ApxIA, ApxIIA or ApxIIIA purified from HJL401, HJL402 and HJL403 using His-tag affinity chromatography. Lanes: M, protein marker; IA, ApxIA- C-terminal fragment; IIA, ApxIIA- C-terminal fragment; and IIIA, ApxIIIA- C-terminal fragment.

In Figure 2, antibody responses against the recombinant C-terminal fragments are shown. Serum IgG concentrations against all the individual antigens in group B were significantly increased compared to those of group A from 2 WPPI until the end of the study (p<0.05).

**Figure 2.** Expression and purification of each C-terminal fragment of ApxIA, ApxIIA or ApxIIIA. Coomassie blue-stained SDS-PAGE gel showing each C-terminal fragments of ApxIA, ApxIIA or ApxIIIA purified from HJL401, HJL402 and HJL403 using His-tag affinity chromatography. Lanes: M, protein marker; IA, ApxIA- C-terminal fragment; IIA, ApxIIA- C-terminal fragment; and IIIA, ApxIIIA- C-terminal fragment.

Underlines indicate the sites of restriction enzymes, such as BamHI and SalI.
FIGURE 2. Serum IgG concentrations against the individual C-terminal fragments in piglets immunized intramuscularly with the combination vaccine. Data are presented as the mean of all piglets in each group and error bars show the standard deviation (SD). Asterisks indicate significant differences between the values of the inoculated group B and those of the control group A (*p<0.05)

DISCUSSION

Among available vaccines for APP, the most commonly used are formalin-killed APP bacterin (Liao et al. 2003). These vaccines do not effectively prevent colonization and are not widely cross protective (Tumamao et al. 2004). As other pathogenic bacteria of multiple serotypes, a major point in the prevention of APP infections is to identify conserved antigens or proteins involved in immunogenicity, which may provide cross-protection against distinct APP serotype infections (Chen et al. 2012; Haesebrouck et al. 2004). The Apx toxins, such as ApxI, ApxII and ApxIII are virulence factors that play a predominant role in the pathogenesis of APP. Since each of the Apx toxins confer only partial protection against PP and the distribution of Apx toxins vary among the different serotypes (Lu et al. 2011; Ramjeet et al. 2008), a combination of three Apx toxins in the vaccine constructs is necessary to protect against the broad range of APP infections.

In the present study, the individual B-subunit proteins of Apx IA, Apx IIA and Apx IIIA were constructed and the efficacy of the combination vaccine against APP infections was examined in piglet models. Secretory IgA transported and secreted across the mucosal epithelium into the lumen can inhibit attachment of microorganisms and/or neutralize exotoxins (Holmgren et al. 2005; MacDonald 2003). This defense mechanism against pulmonary bacterial infection depends on the clearance of the pathogens from the respiratory tract and mucosal immunity by systemic immune response is necessary for effective vaccination against PP (Haesebrouck et al. 2004). We confirmed that the intramuscular inoculation procedure effectively stimulated all the antigen-specific IgG immune response. Following intranasal inoculation with the mixture of wild type virulent APP strains, the survival ratio in each group of piglets was examined and 90% of the immunized piglets were protected against virulent APP infection after challenge, while only 50% of non-immunized group of piglets were protected. This finding showed that intramuscular immunization with our combination vaccine can effectively protect the challenge strain.

In conclusion, the systemic immune response was markedly induced by intramuscular immunization with the combination vaccine of individual C-terminal fragments of three Apx toxins. Clinical signs were observed in a total of only 10% from intramuscular-primed and intramuscular-boosted piglets after challenge with virulent APP strains. However, 50% clinical signs were observed in the control. Therefore, intramuscular immunization of piglets with our combination vaccine candidate may effectively protect piglets from PP caused by APP.

ACKNOWLEDGMENTS

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antigens entrapped in
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Adhesion protein AfpA of Actinobacillus pleuropneumoniae
is required for pathogenesis and is a potential target for
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