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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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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*

ABSTRAK

Keberkesanan kombinasi vaksin oleh serpihan terminal-C individu ApxIA, ApxIIA dan ApxIIIA daripada Actinobacillus pleuropneumoniae *(APP) dinilai pada anak babi. Dua puluh anak babi dibahagikan sama rata kepada 2 kumpulan (*n=*10). Semua anak babi telah mencapai intraotot primer pada umur 4 minggu (0 minggu selepas inokulasi utama (WPPI)) dan telah dirangsang intraototnya pada umur 6 minggu (2 WPPI). Anak babi kumpulan A telah diinokulasi dengan PBS steril dan anak babi kumpulan B telah diinokulasi dengan vaksin kombinasi. Kepekatan setiap IgG fragmen-tertentu terminal-C seperti yang ditetapkan oleh ELISA adalah jauh lebih tinggi dalam kumpulan B daripada kumpulan A daripada 2 WPPI sehingga ke penghujung kajian ini. Tanda klinikal diperhatikan pada 10% daripada anak babi kumpulan B selepas cabaran dengan campuran serotip APP 1, 2 dan 5 pada 4 WPPI, manakala 50% anak babi kumpulan A dilindungi daripada jangkitan APP. Secara keseluruhannya, inokulasi intraotot dengan calon vaksin boleh melindungi anak babi terhadap jangkitan APP dengan cekap.*

Kata kunci: Actinobacillus pleuropneumoniae*; anak babi; imunisasi; perlindungan; porcine pleuropneumonia*

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 (Cruijsen 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, Ratastie, 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

Strain/plasmid	Description	Source of reference
Strains		
E. coli		
BL21(DE3)pLysS	F^-, *ompT*, *hsd*$S_B(r_B^-,m_B^-)$, *dcm*, *gal*, λ(DE3), pLysS, Cm^r	Lab stock
HJL401	*E. coli* BL21 with pET21a-ApxIA C-terminal	This study
HJL402	*E. coli* BL21 with pET21a-ApxIIA C-terminal	This study
HJL403	*E. coli* BL21 with pET21a-ApxIIIA C-terminal	This study
A. pleuropneumoniae		
HJL6	*Actinobacillus pleuropneumoniae* serotype 1	Lab stock
HJL67	*Actinobacillus pleuropneumoniae* serotype 2	Lab stock
HJL263	*Actinobacillus pleuropneumoniae* serotype 5	Lab stock
Plasmids		
pET21a	IPTG-inducible expression vector; Km^r	Novagen

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

Name	Sequence(5′→3′)	Size (bp)	Accession number	Reference
Apx IAC-F	CGGGATCC GGT ATT ATT TCA	1293	GQ369732	This study
Apx IAC-R	GTCGAC AGT ACC ATC GCT GC			
Apx IIAC-F	ATTCGGATCC CAA GGT TAT GA	1110	AY736188	This study
Apx IIAC-R	GCTTGTCGAC TGT AAT AGA ATC ATT T			
Apx IIIAC-F	GGGATCC GCA CCA ATT ACT TT	1293	X68815	This study
Apx IIIAC-R	TGTCGAC AAG CAC ATT AAA ACC			
HPF	AAG GTT GAT ATG TCC GCA CC	956	AB007587.1	Jessing et al. 2003
HPR	CAC CGA TTA CGC CTT GCC A			
Ap1F	GGG CAA GCC TCT GCT CGT AA	754	AF518558.1	Jessing et al. 2008
Ap1R	GAA AGA ACC AAG CTC CTG CAA T			
Ap2F	ACT ATG GCA ATC AGT CGA TTC AT	500	AY357726.1	Jessing et al. 2003
Ap2R	CCT AAT CGG AAA CGC CAT TCT G			
Ap5F	TTT ATC ACT ATC ACC GTC CAC ACC T	1,100	AF053723.1	Jessing et al. 2003
Ap5R	CAT TCG GGT CTT GTG GCT ACT AA			

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

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 APP- , type 1-, type 2- and type 5-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

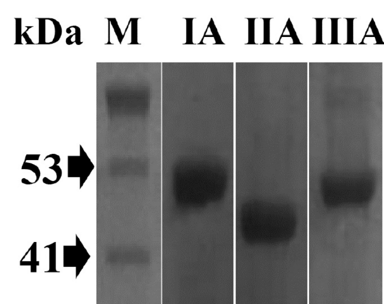

FIGURE 1. Expression and purification of each C-terminal fragment of ApxIA, ApxIIA or ApxIIIA. Coomassie blue-stained SDS-PAGE gel showing each C-terminal fragments of ApxIA, ApxIIA or ApxIIIA purified from HJL401, HJL402 and HJL403 using His-tag affinity chromatography. Lanes: M, protein marker; IA, 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 ($p<0.05$).

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.

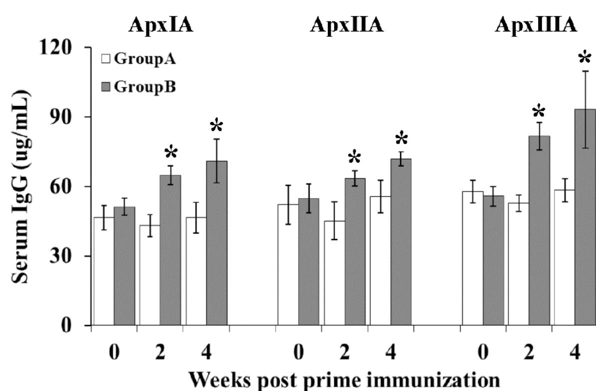

FIGURE 2. Serum IgG concentrations against the individual C-terminal fragments in piglets immunized intramuscularly with the combination vaccine. Data are presented as the mean of all piglets in each group and error bars show the standard deviation (SD). Asterisks indicate significant differences between 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.

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