獸醫學博士 學位論文

Analysis of *apxIVA* gene of *Actinobacillus pleuropneumoniae* Korean isolates and immunogenicity of *Saccharomyces cerevisiae* expressing *apxIA* and *apxIIA* genes by oral administration

국내분리 *Actinobacillus pleuropneumoniae apxIVA*의 유전적 특성과 *apxIA* 및 *apxIIA* 유전자 발현 *Saccharomyces cerevisiae*의 경구투여에 따른 면역원성 분석

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Abstract

Analysis of *apxIVA* gene of *Actinobacillus pleuropneumoniae* Korean isolates and immunogenicity of *Saccharomyces cerevisiae* expressing *apxIA* and *apxIIA* genes by oral administration

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Actinobacillus pleuropneumoniae is the etiologic agent of porcine pleuropneumonia, a highly contagious pulmonary disease in pigs with major economic losses for pig producers worldwide. Fifteen serotypes of *A. pleuropneumoniae* express four different Apx toxins that belong to the pore-forming repeats-in-toxin (RTX) group of toxins, ApxI, ApxII, ApxIII and ApxIV, which are major virulence factors. Although ApxI, ApxII and ApxIII are secreted by different serotypes in various combinations, ApxIV has distinctive features that

are produced by all serotypes of *A. pleuropneumoniae*, and are expressed only *in vivo*. Apx toxins are highly immunogenic, thus inducing a strong antibody response to *A. pleuropneumoniae* infection. In particular, ApxI and ApxII are necessary for full virulence in the development of clinical signs and typical lung lesions. Therefore, Apx toxins appear to be central in any effective approach as antigens in the development of diagnostic method and vaccine against *A. pleuropneumoniae*.

In the present study, we described the complete sequencing and organization of the ApxIVA isolated in Korea (Kor-ApxIVA) and the homology of *apxIVA* to other *A. pleuropneumoniae* serotypes. The structural characteristics of ApxIVA showed RTX proteins, including N-terminal hydrophobic domains, signature sequences for potential acylation sites, and repeated glycine-rich nonapeptides in the C-terminal region of the protein. Thirty glycine-rich nonapeptides with the consensus sequence, L/V-X-G-G-X-G-N/D-D-X, were found in the C-terminus of the Kor-ApxIVA. This genetic analysis of the Kor-ApxIVA might be an important foundation for future biological and functional research on ApxIVA.

The current report describes the development of an ELISA based on the recombinant ApxIA, ApxIIA and ApxIIIA antigens and evaluated the application of the developed assays on the prevalence of anti-Apx toxin antibodies among pigs in Korea. Consequently, the developed ELISAs may be useful for *A. pleuropneumoniae* vaccination strategy as a screening tool for pig herds as well as for detection of specific antibodies to Apx exotoxins.

To develop an effective vaccine for *A. pleuropneumoniae* based on transgenic

antigen expression, the neutralizing epitope of ApxIIA (ApxIIA#5) was selected as a vaccine candidate for *A. pleuropneumoniae*. In the present study, we demonstrated that *S. cerevisiae* expressing surface-displayed ApxIIA#5 has the immunogenic potential as an oral vaccine, helping to improve both systemic and mucosal immune responses. Presentation of ApxIIA on activated DCs to CD4+ T cells from mice orally administered with *S. cerevisiae* expressing surface-displayed ApxIIA#5 elicited specific T cell proliferation. In addition, mice orally administered with *S. cerevisiae* expressing surface-displayed ApxIIA#5 showed higher antigen-specific IgG and IgA antibody responses in serum and spleen, Peyer' s patches, and lamina propria than those with the vector-only *S. cerevisiae* or non-treated. Furthermore, the vaccinated mice induced Th1-type immune responses based on increased levels of IgG2a in serum and predominant increase of IFN-γ producing cells in spleen, and lamina propria.

Ultimately, *S. cerevisiae* expressing ApxIA and surface-displayed ApxIIA#5 were developed to serve as an oral vaccine candidate in pigs. Pigs immunized orally with *S. cerevisiae* expressing ApxIA and surface-displayed ApxIIA#5 showed higher specific IgG and IgA antibody activities than that with the vector-only *S. cerevisiae* and non-treated. Additionally, the induced immune responses are found to protect the pigs infected with *A. pleuropneumoniae* according to the analysis of clinical signs and the gross and microscopic pulmonary lesions. These results suggested that *S. cerevisiae* expressing ApxIA and surface-displayed ApxIIA#5 might be a potential oral vaccine to prevent pigs against porcine pleuropneumonia. Thus the

present study is expected to contribute to the development of a live oral vaccine against porcine pleuropneumonia as alternatives to current conventional vaccines.

Keywords: *Actinobacillus pleuropneumoniae,* Apx toxins, oral immunization, *Saccharomyces cerevisiae*

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List of abbreviations

APCs	Antigen presenting cells
BALF	Broncho-alveolar lavage fluid
BSA	Bovine serum albumin
CDD	Conserved domain database
CFSE.	Carboxyfluorescein succinimidyl ester
CFU	Colony forming unit
CP	Capsular polysaccharide
DCs	Dendritic cells
DMEM	Dulbecco's modified eagle's medium
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
GM-CSF	Granulocyte-macrophage colony- stimulating factor
GRAS	Generally recognized as safe
IEDB	Immune epitope database
IFN- γ	Interferon-γ
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB broth	Luria-bertani broth
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex

NAD	β -Nicotinamide adenine dinucleotide
Ni-NTA	Nickel-Nitrilotriacetic acid
NO	Nitric oxide
OD	Optical density
PBS	Phosphate-buffered saline
PE	Phycoerythrin
PCR	Polymerase chain reaction
ROC	Receiver operating characteristic
RTX	Repeats in toxins
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TNF- α	Tumor necrosis factor-α
TMB	3,3′,5,5′-Tetramethylbenzidine
TSB	Tryptic soy agar
SPSS	Statistical Package for Social Sciences software

General introduction

Actinobacillus pleuropneumoniae is a causative agent of porcine pleuropneumonia, a highly contagious endemic disease of pigs worldwide, inducing significant economic losses worldwide (Haesebrouck *et al.*, 1997; Bosse *et al.*, 2002; Zhou *et al.*, 2008). *A. pleuropneumoniae* can result in various clinical signs from peracute to chronic, with infected pigs typically showing a hemorrhagic, necrotizing pneumonia often associated with fibrinous pleuritis (Haesebrouck *et al.*, 1997). Virulence factors of *A. pleuropneumoniae* include capsular polysaccharides, outer membrane proteins, Apx exotoxins, lipopolysaccharides, permeability factors, and iron-regulated proteins (Frey, 1995; Dubreuil *et al.*, 2000; Baltes *et al.*, 2002; Bosse *et al.*, 2002). Although the respective contributions of the virulence factors to the infection and disease are not fully understood, Apx toxins are believed to be involved in the virulence of *A. pleuropneumoniae* (Tascon *et al.*, 1994; Frey, 1995; Reimer *et al.*, 1995). Apx toxins appear to be central in any effective approach as antigens in the development of diagnostic method and vaccine against *A. pleuropneumoniae*.

For *A. pleuropneumoniae*, it is advisable to develop vaccine strategies inducing both mucosal and systemic immune responses because the mucosal surface is the main site for invasion and development of lesions in the host. Although parenterally administrated vaccines mainly stimulated systemic responses, mucosal vaccines are capable of inducing both mucosal and systemic immune responses,

thus leading to two layers of host protection. In addition, secretory IgA (sIgA) involved in mucosal immunity binds to microorganisms and toxins and neutralizes them by blocking their entry into the host.

Yeast has been used as a tracer for the oral application of vaccines and drugs. Yeast-based expression systems have been developed and successfully used to produce recombinant proteins. Moreover, yeast cell-wall components, such as β-1,3-d-glucan and mannan, have an adjuvant potential. Cell-surface display in *S. cerevisiae*, designed as an antigen delivery system, provide the stable maintenance of surface-expressed epitopes with a high-density displayed protein.

ApxIIA, expressed in either *Saccharomyces cerevisiae* or *Nicotiana tabacum*, has previously been reported to be capable of inducing protective immune responses against *A. pleuropneumoniae* in mice (Shin *et al.*, 2005; Lee *et al.*, 2006; Shin *et al.*, 2007). *Saccharomyces cerevisiae* expressing ApxIA has been also developed and confirmed previously (Shin *et al.*, 2003). The antigenic determinants of ApxIIA from *A. pleuropneumoniae* serotype 2 isolated in Korea was identified and found to induce a strong protective immune response against *A. pleuropneumoniae* (Seo *et al.*, 2011). To develop an effective vaccine for *A. pleuropneumoniae* based on transgenic antigen expression, the neutralizing epitope of ApxIIA (ApxIIA#5) was selected as a vaccine candidate for *A. pleuropneumoniae*. This smaller fragment can be expressed in large quantities due to the difficulty in the expression of large fragments in a heterologous expression system (Kim *et al.*, 2010; Seo *et al.*, 2011).

The purpose of this study was to perform genetic and immunological analysis of

Apx toxins as vaccine and diagnostic antigens, to develop *S. cerevisiae* expressing Apx toxins, and to investigate a successful induction of mucosal immune responses of the transgenic *S. cerevisiae* for developing oral vaccine of porcine pleuropneumonia. This is origanized into four chapters. The genetic analysis of ApxIVA as a new virulence factor was investigated in chapter I. ELISA method against *A. pleuropneumoniae* was developed using the recombinant ApxIA, ApxIIA, and ApxIIIA in chapter II. In chapter III, the mucosal immune responses of the surface-displayed ApxIIA#5 expressing *S. cerevisiae* were evaluated in mice. The potential use of this was carried out with pigs in chapter IV.

Literature Review

Porcine pleuropneumonia

Porcine pleuropneumonia is a severe and contagious respiratory disease, leading to major economic losses in pig farming worldwide (Fenwick and Henry, 1994; Frey, 1995; Haesebrouck *et al.*, 1997). *Actinobacillus pleuropneumoniae* can result in various clinical signs from peracute to chronic, with infected pigs typically showing a hemorrhagic, necrotizing pneumonia often associated with fibrinous pleuritis (Haesebrouck *et al.*, 1997). Many large units may have chronic problems with flare-ups of the acute disease (Zhou *et al.*, 2008). The substantial economic losses in these herds per year can be up to £25,000 for a typical 600 sow breeder-feeder farm in the UK (Zhou *et al.*, 2008). Also, it was the most common identified pathogen in cases of porcine respiratory diseases in the South Korea between 2009 and 2011.

Actinobacillus pleuropnemoniae

The genus *Actinobacillus* are small, gram-negative, pleomorphic, coccobacillary rods that are facultatively anaerobic, indole negative, ß-galactosidase, and urase positive (Marsteller and Fenwick, 1999). The host range of different spieces tends to be limited. Most are associated with farm animals, but *Actinobacillus hominis*

and *Actinobacillus ureae* are opportunistic pathogen of human (MacInnes, 2010).

1. Epidemiology

Actinobacillus pleuropneumoniae has two biotypes based on their requirement for nicotinamide adenine dinucleotide (NAD). Based on NAD requirements, *A. pleuropneumoniae* can be divided into: biotype 1, strains which are NAD-dependent; and biotype 2, strains which are NAD-independent. Up to date, 15 serotypes of *A. pleuropneumoniae* have been described based on their capsular polysaccharides (Blackall *et al.*, 2002). Serotype 1 and 5 are further differentiated into 1a and 1b and 5a and 5b, respectively, according to minor differences in the polysaccharide structure. The detailed structure of cell surface polysaccharides has been a key for the development of serological tests to detect infection, for serotyping and epidemiological studies. Though PCR tests for serotyping based on capsule and Apx toxins have been developed, conventional serological tests such as slide agglutination complement fixation, and indirect hemagglutination are still used (Rayamajhi *et al.*, 2005; Zhou *et al.*, 2008).

A. pleuropneumoniae is rapidly endemic in many countries where pigs are intensively raised. A limited serotype tend to be reported in a specific region, although there have been few recent reports about the prevalence of different seroptyes of *A. pleuropneumoniae*. Generally the most prevalent serotypes were 1, 2, and 5 in Asia and Australia (MacInnes, 2010). In Great British and Europe,

serotypes 2 and 9 were found most often, whereas serotypes 1 and 5 were predominant in Canada, the United States, and Mexico (MacInnes, 2010). In addition, serotypes 2, 5, and 6 were found most often in South Korea, but potential outbreak involving with other serotypes can not be eliminated. Recently, serotype 15 has been reported in Australia and Japan (Blackall *et al.*, 2002).

The virulence of *A. pleuropneumoniae* is multifactorial, and the factors involved in pathogenesis are capsular polysaccharides, lipopolysaccharides, outer membrane proteins, adhesion factors, proteases, and exotoxins (Udeze *et al.*, 1987; Dom *et al.*, 1994; Tascon *et al.*, 1994; Frey, 1995; Haesebrouck *et al.*, 1997; Baltes *et al.*, 2002; Bandara *et al.*, 2003; Negrete-Abascal *et al.*, 2003). Successful control of the infection needs understanding of the virulence factors of the bacterium and the pathogenesis of the disease.

Table Ⅰ. Properties and distribution of *A. pleuropneumoniae* serotypes.

Serotype	*K antigen*	*Capsular group*	*O antigen*[a]	*Biotype*	*Toxins*[b]	*Virulence potential*[c]	*Country, region*[d]
1	K1	I	O1[a]	1	ApxI, ApxII, ApxIV	High	Widespread
2	K2	III	O2	1	ApxII, ApxIII, ApxIV	Low → high	Europe
3	K3	III	O3[b]	1	(ApxII)[e]ApxIII, ApxIV	Low → high	China, Europe
4	K4	1	O4[c]	1	ApxII, ApxIII, ApxIV	Low	Spain
5	K5	II	O5	1	ApxI, ApxII, ApxIV	High	Widespread
6	K6	III	O6	1	ApxII, ApxIII, ApxIV	Low	Widespread
7	K7	III	O7	1	ApxII, ApxIV	High	Canada, Europe
8	K8	III	O3	1	ApxII, ApxIII, ApxIV	Low	Mexico, United Kingdom
9	K9	III	O9	1	ApxI, ApxII, ApxIV	High	Europe
10	K10	II	O10	1	ApxI, ApxIV	Very high?	Europe
11	K11	III	O1	1	ApxI, ApxII, ApxIV	High	Netherlands
12	K12	1	O12	1	ApxII, ApxIV	Low	Europe, Canada
13	K13	3	O7	2	ApxII, ApxIV	Low	Europe

14	"K14"	ND[d]	O14	2	ApxI, ApxIV	Low	Europe
15	K15	1	O3	1	ApxII, ApxIII, ApxIV	High	Australia, Japan

[a] Several O-antigens are structurally and antigenically very similar, for example, O9 and O1; O6 and O3; O7 and O4.

[b] The apxIBD genes are found in all serotypes except serotype 3. As a result, ApxII is not exported.

[c] The virulence can vary markedly from country to country; for example, serotype 3 strains are associated with disease in Asia, but are considered benign in Europe; European serotype 2 strain are more virulent than serotype 2 isolates that are occasionally isolated in North America.

[d] The Structure of the K14 antigen has not been reported.

Adapted from MacInnes *et al*., 2010.

2. Apx toxins

Apx toxins are believed to be involved in the virulence of *A. pleuropneumoniae*, although the respective contributions of the virulence factors to the infection and disease are not fully understood (Tascon *et al.*, 1994; Frey, 1995; Reimer *et al.*, 1995). Apx toxins are highly immunogenic, thus inducing a strong antibodies response to *A. pleuropneumoniae* infection (Frey and Nicolet, 1991; Frey *et al.*, 1992). Fifteen serotypes express four different Apx toxins belonging to the pore-forming repeats-in-toxin (RTX) toxins (Haesebrouck *et al.*, 1997; Blackall *et al.*, 2002). ApxI is strongly hemolytic and cytotoxic, while ApxII is weakly hemolytic and moderately cytotoxic. However, ApxIII is nonhemolytic, but strongly cytotoxic (Frey, 1995; Schaller *et al.*, 1999). Recombinant ApxIV shows weak hemolytic activity and cohemolytic synergy with sphingomyelinase (beta-toxin) of *Staphylococcus aureus*, but the biological and functional characteristics of ApxIV are unclear (Schaller *et al.*, 1999). ApxI, ApxII, and ApxIII are also produced by other *Actinobacillus* species such as *A. rossii*, *A. suis*, and *A. porcitonsillarum* (Schaller *et al.*, 2001; Gottschalk *et al.*, 2003), whereas ApxIV is highly specific to *A. pleuropneumoniae* (Schaller *et al.*, 1999).

The Apx toxin is encoded by the *apx* operon, which contains four genes in the order *apxCABD*: the activator gene, *apxC*, the pretoxin structural gene, *apxA*, and the secretion apparatus-encoding genes, *apxB* and *apxD* (Frey, 1995).

Apx toxins play a role in evasion of the host first line defence. High concentration

of RTX toxins form pores in membranes of phagocytic and other target cells, resulting in osmotic swelling and cell death (Haesebrouck *et al.*, 1997). In addition, they are involved in development of lung lesions and provoked oxidative burst in macrophage and neutrophil (Haesebrouck *et al.*, 1997). The importance of Apx toxins in protective immunity against porcine pleuropneumonia was demonstrated by immunization with Apx toxins in combination with other bacterial compounds. In these experiments the Apx toxins were essential vaccine components to confer protection against challenge (Frey, 1995; Haesebrouck *et al.*, 1997).

Table Ⅱ. Apx toxins of *Actinobacillus pleuropneumoniae*

	Operon			*Activity*		*MW*	*Serotype*
	Activator	Structural	Export	Haemolytic	Cytotoxic	*(kDa)*	
ApxI	*apxIC*	*apxIA*	*apxIBD*[a]	Strong	Strong	105-110	1, 5a, 5b, 9, 10, 11
ApxII	*apxIIC*	*apxIIA*	none[a]	Weak	Moderate	103-105	All but 10
ApxIII	*apxIIIC*	*apxIIIA*	*apxIIIBD*	none	Strong	120	2, 3, 4, 6, 8
ApxIV[b]	ORF1?[c]	*apxIVA*	none[a]	Weak	ND[d]	200[e]	all

This table was compiled using data from Frey et al. and Schaller et al.

[a] The apxIBD genes are found in all serotypes exept serotype 3. Secretion of ApxII occurs via Apx IBD. Nothing is yet knwn regarding secretion of ApxIV.

[b] Unlike the other Apx toxins, ApxIV is only produced in vivo.

[c] Orf1 seems to be requiredfor activity of ApxIV, although it shares no homology with ApxIC, ApxIIC, or ApxIIIC.

[d] ND: not determined.

[e] The molecular mass of ApxIV is predicted from protein sequence.

Adapted from Bosse *et al*., 2002.

3. Infection and Disease

Actinobacillus pleuropneumoniae is present in nasal secretion and lung exudates of acutely infected animals and transmission is by aerosols or direct contact with infected pigs (MacInnes, 2010). Porcine pleuropneumonia occurring in pigs of all ages, increased incidence with conditions such as overcrowding, poor ventilation, and large temperature fluctuations (MacInnes, 2010). *A. pleuropneumoniae* can result in various clinical syndromes, which vary in degrees of severity from peracute to chronic, with infected pigs typically showing a hemorrhagic pneumonia often associated with necrosis and fibrinous pleuritis (Haesebrouck *et al.*, 1997). In peracute or aute disease, pigs may show some of the following clinical signs: high fever, increased respiratory rate, coughing, sneezing, dyspnea, anorexia, ataxia, and severe respiratory distress with cyanosis (Gottschalk *et al.*, 2003). Carrier animals can be the origin of propagation of *A. pleuropneumoniae* (Shin *et al.*, 2011b).

Pulmonary lesions in peracute or acute form are characterized by severe edema, inflammation, hemorrhage, and necrosis. The thoracic cavity is often filled with serosanguinous fluid and fibrin clots, and diffuse fibrinous pleuritis (Bertram, 1988). Polymorphonuclear leukocyte (PMN) infiltration, edema, and fibrinous exudate are apparent in early stage of the disease (Bertram, 1988; Bosse *et al.*, 2002; MacInnes, 2010). Macrophage infiltration and necrotic areas are apparent, leading to hemorrhage by severe necrotizing vasculitis in the lung of the later stage (Bertram, 1988; Bosse *et al.*, 2002; MacInnes, 2010). Chronically infected pigs

may also harbour *A. pleuropneumoniae* in chronically affected lungs as well as in tonsillar crypts (Sidibe *et al.*, 1993; Taylor, 1999).

4. Pathogenesis of *Actinobacillus pleuropneumoniae*

Alveolar macrophages and neutrophils are important in the pathogenesis and/or defense system of the lung against *Actinobacillus pleuropenumoniae*. When *A. pleuropneumoniae* is inhaled and enters the lung alveoli, *A. pleuropneumoniae* is phagocytized by macrophages as innate immune responses. *A. pleuropneumoniae* produces several factors which may contribute to survive within macrophages (Bosse *et al.*, 2002). One of the major factors is Apx toxins, which are involved in damage of phagocytic function, and stimulation of oxidative metabolism (Cruijsen *et al.*, 1992). Activation of alveolar and intravascular macrophages leads to release of toxic oxygen metabolites and various cytokines. In addition, damage to endothelial cells by Apx toxins as well as by LPS initiates the coagulation, and fibrinolysis systems. These systems results in characteristic of acute porcine pleuropneumonia (Bosse *et al.*, 2002). Pathogenesis of acute pleuropenumonia has three basic stages: colonization, evasion of host clearance mechanisms, and damage to host tissues (MacInnes, 2010).

1) Colonization

Colonization, which is the ability of a pathogen to adhere to host cells or surfaces

and to multiply within the host, is often a necessary requirement to the production of disease. Although *A. pleuropneumoniae* can be isolated from the tonsils and nasal cavities of pigs, the bacteria bind preferentially to cells of the lower respiratory tract (Bosse *et al.*, 2002). Fimbriae are present on a lot of pathogens and play their role in adherence. The production of fimbriae by *A. pleuropneumoniae* appears to be unstable; intact fimbriae were not detected on bacteria grown on supplemented BHI plates, whereas fimbriae were present when bacteria were cultured on defined medium under microaerophilic conditions (Zhang *et al.*, 2000). Expression of fimbriae is though to be regulated in *A. pleuropneumoniae* by growth conditions *in vivo*.

After adherence to host cells, infection depends on the ability of the bacteria to acquire all essential nutrients for growth. Within the respiratory tract environment, the variety and quantity of available carbohydrates and certain nutrients is restricted (MacInnes, 2010). The ability to overcome nutritional limitation within the host may be considered a pathogenic mechanism. Mechanisms for overcoming iron–restriction have been worked out in *A. pleuropneumoniae*. *A. pleuropneumoniae* expresses a number of factors, which are involved in the acquisition and uptake of iron. It is capable of using porcine transferrin as an only source of iron, but not other animal transferrin (Bosse *et al.*, 2002). Two distinct transferrin-binding proteins are produced under iron-restricted growth conditions: TbpA, a protein forming a transmembrane channel for transport of iron across the outer membrane; TbpB, a lipoprotein which may be anchored to the outer membrane (Gerlach *et al.*, 1992). The *tbpA* and *tbpB* genes are linked to, and co-

transcribed with, the *exbBD* genes (Tonpitak *et al.*, 2000; Bosse *et al.*, 2002). ExbB and ExbD forming an inner membrane protein complex are associated with TonB, which provides energy to high affinity receptors enabling transport of iron across the outer membrane (Bosse *et al.*, 2002). Expression of ExbB and ExbD is essential for utilisation of transferrin-bound iron by *A. pleuropneumoniae* (Tonpitak *et al.*, 2000). Furthermore, both *exbB* and *tonB* mutants of *A. pleuropneumoniae* are avirulent, indicating the central role of this complex in iron uptake (Baltes *et al.*, 2002).

Nickel is also the limited nutrient within the respiratory tract environment that may affect the ability of *A. pleuropneumoniae* to survive and cause disease, like iron (MacInnes, 2010). It is required for urease activity in *A. pleuropneumoniae* and is only available in extremely low concentrations within mammalian hosts (Bosse *et al.*, 2001). The contribution of nickel to virulence of *A. pleuropneumoniae* is indicated via its role in urease activity (Bosse *et al.*, 2001). Urease activity could also be considered a nutrient acquisition mechanism by producing ammonia, a preferred nitrogen source for many bacteria (Bosse *et al.*, 2002).

2) Avoidance of host clearance mechanisms

Effective host defence against bacterial infections in the lung depends on rapid clearance of bacteria from the respiratory tract. The respiratory immune system can be divided into non-specific (mucociliary function, complement, phagocytes), and

specific lymphocyte-mediated mechanisms (Bosse *et al.*, 2002).

The mucociliary clearance mechanism is very important in protecting lungs from infection. Though the effects of *A. pleuropneumoniae* infection on mucociliary activity have not yet been investigated, suppression of mucus production and ciliary activity by treatment with atropine and xylocaine, increased the severity of disease when pigs were challenged endobronchially with low doses of *A. pleuropneumoniae* (Narita *et al.*, 1995; Bosse *et al.*, 2002).

Bacteria not cleared by normal mucociliary function may still be eliminated by the action of phagocytic cells. *A. pleuropneumoniae* can survive for more than 90 min within macrophages, during which time release of Apx toxins may result in lysis of these phagocytes (Cruijsen *et al.*, 1992). *A. pleuropneumoniae* produces several factors which may contribute to its ability to survive within macrophages (Bosse *et al.*, 2002). The major factors involved in damage of phagocytic function of both macrophages and PMNs are the three RTX-toxins (ApxI, ApxII, and ApxIII) (Frey *et al.*, 1993). These toxins damage macrophage chemotactic and phagocytic function, while macrophage and PMN oxidative metabolism is stimulated (Dom *et al.*, 1992; Bosse *et al.*, 2002). The possible contribution of the recently described ApxIV toxin to macrophage and PMN damage has yet to be determined.

Phagocytosis of *A. pleuropneumoniae* by macrophages and neutrophils seems only to occur in the presence of antibodies against bacterial surface antigens (Haesebrouck *et al.*, 1997). In addition, antibodies to Apx toxins protected the neutrophils from being killed and neutrophils cleared the ingested bacteria efficiently (Cruijsen *et al.*, 1992; Haesebrouck *et al.*, 1997). It has been shown that antibodies directed against LPS, capsule, outer membrane proteins, and Apx toxins

can serve as effective opsonins, enabling phagocytosis of *A. pleuropneumoniae* by neutrophils (Haesebrouck *et al.*, 1997).

3) Damage to host tissues

Most of the pathological consequences of porcine pleuropneumonia can be attributed to the Apx toxins which exert cytotoxic effects on various cell types both directly as well as indirectly by stimulating release of inflammatory mediators from activated phagocytes (Bosse *et al.*, 2002). Activation of alveolar and intravascular macrophages, due to Apx toxins and LPS, leads to release of toxic oxygen metabolites such as superoxide anion, hydrogen peroxide, and hydroxyl radical as well as proteolytic enzymes, and various cytokines (Udeze *et al.*, 1987; Sibille and Reynolds, 1990; Dom *et al.*, 1992; Pabst, 1996). Damage to endothelial cells by Apx toxins, as well as direct activation of factor XII by LPS, initiates the coagulation, fibrinolysis, and kinin systems (Bertram, 1988). Activation of the coagulation pathway results in platelet activation and the formation of microthrombi, localised ischaemia, and subsequent necrosis which are characteristic of acute porcine pleuropneumonia (Bertram, 1988). Furthermore, pigs infected with a mutant of a serotype 1 strain of *A. pleuropneumoniae* lacking ApxI and ApxII, but with normal LPS, do not develop clinical disease or significant lung lesions (Tascon *et al.*, 1994). This indicates that the contribution of LPS to lesion development may be minimal in the absence of Apx toxins. The critical role of Apx toxins in development of clinical disease and tissue damage has been confirmed using recombinant toxins (rApxI, rApxII and rApxIII) (Kamp *et al.*, 1997). Endobronchial inoculation of pigs with either rApxI or rApxIII resulted in severe clinical disease and lesions indistinguishable from those seen in acutely

infected animals (Kamp *et al.*, 1997). These results indicate that there may be other cell-associated toxins or virulence determinants contributing to the severe pulmonary lesions caused by these strains. Recently, a fourth RTX toxin (ApxIV) was reported to be produced by all serotypes of *A. pleuropneumoniae* (Schaller *et al.*, 1999). The contribution of this RTX toxin to pathogenesis of *A. pleuropneumoniae* remains to be explicated.

5. Prevention and Control

Managements such as controlling temperature and hygiene, avoiding overcrowding, and maintaining "all-in all-out" production are essential to control *A. pleuropneumoniae* (MacInnes, 2010). In an infection, treatment of pigs with acute form needs direct injection of highly susceptible antibiotics following antibiotic susceptibility testing of isolates (MacInnes, 2010). However, antibiotics increase their environmental impact, their side effects and residues in food animal products (Shams, 2005). In addition, these have several adverse effects, such as laborious, time-consuming, and expensive procedures, and the stress of the pig, and can be limited to use due to the rapid progression of the disease (MacInnes, 2010).

Infection with one serotype of *A. pleuropneumoniae* provides complete protection against subsequent infection with the homologous serotype, and at least partial protection against heterologous serotype (Haesebrouck *et al.*, 1996). Therefore, vaccination is though to be a potential alternative to antibiotic treatment. Most vaccines for pigs have been developed as parenteral immunization, which has

several adverse effects, such as laborious and time-consuming procedures, the induction of inflammatory reactions at the injection site, and the stress of the pig (Shin *et al.*, 2005). Even though these conventional vaccines have been conductive to the elevation of animal and public health, they are far from the ideal animal and public health. Moreover, although many studies have been tried, a safe vaccine that offers complete protection has not yet developed (Ramjeet *et al.*, 2008).

6. Current vaccine research for *A. pleuropneumoniae*

Many studies have been reported and several vaccines have been commercialized but complete satisfaction has not been obtained in the protection of pigs against pleuropneumonia infection. The whole-cell bacterins were the first commercialized vaccines against *A. pleuropneumoniae* infection and consisted of heat-killed bacteria or formalin-treated whole-cells (Ramjeet *et al.*, 2008). New developments in inactivated whole-cell bacterial vaccines have shown a promising strategy in *A. pleuropneumoniae* vaccination. Genetically-inactivated ghost vaccines have the advantage over bacterins of sharing functional and antigenic determinants with their living counterparts (Witte *et al.*, 1990; Witte *et al.*, 1992; Hensel *et al.*, 2000; Huter *et al.*, 2000; Ramjeet *et al.*, 2008).

Live attenuated *A. pleuropneumoniae* vaccines have a number of drawbacks including the risk of inoculating animals with incompetently attenuated pathogens, the possible reverting of pathogenic state of the attenuated bacteria and the possible

spread of the pathogens to other animals (Ramjeet *et al.*, 2008). In spite of the several disadvantages mentioned above, the use of attenuated live vaccine is a good approach in vaccination against porcine pleuropneumonia is that pigs surviving natural infection were found to be completely protected against homologous infection and partially against heterologous serotypes of *A. pleuropneumoniae* (Haesebrouck *et al.*, 1997). Moreover, live attenuated vaccine can confer cross-protection via *in vivo* induced antigens.

Several virulence factors of *A. pleuropneumoniae* have been explored for their protective potential. Despite all the advances in the field, the subunit vaccines commercialized using virulence factors do not provide complete protection against *A. pleuropneumoniae* infection. Therefore, researches are still progressing in the finding of new virulence factors such as *in vivo*-expressed immunogenic antigens (Schaller *et al.*, 1999; Ramjeet *et al.*, 2008).

Currently, main concern in *A. pleuropneumoniae* vaccination is to find the best vaccination strategies to stimulate an appropriate mucosal immune response. Up to date, three porcine mucosal vaccines are licensed in North America: two using the intranasal route of immunization against transmissible gastroenteritis virus and *Bordetella bronchiseptica*, and one using the oral route against rotavirus (Gerdts *et al.*, 2006; Ramjeet *et al.*, 2008). Many live attenuated *A. pleuropneumoniae* vaccines have been applied in intranasal immunization experiments and shown a protective immune responses (Hensel *et al.*, 1994; Haesebrouck *et al.*, 1997; Maas *et al.*, 2006). Although intranasal vaccination induces efficiently local immune

responses in the respiratory tracts, development of oral vaccine for *A. pleuropneumoniae* is thought to be a practical value on application to pig farming. ApxIIA, expressed in either *Saccharomyces cerevisiae* or *Nicotiana tabacum*, has previously been reported to be capable of inducing protective immune responses against *A. pleuropneumoniae* in mice (Shin *et al.*, 2005; Lee *et al.*, 2006; Shin *et al.*, 2007).

Saccharomyces cerevisiae

Saccharomyces cerevisiae is generally regarded as safe organism (GRAS) for oral use, it can be used in pharmaceutical, livestock feed, and food industry applications (Park *et al.*, 2007). Two promising technologies in the development of oral vaccines are live attenuated bacteria and transgenic organisms. The safe and immunogenic live organisms already were developed as oral vaccines. Several oral delivery systems have been attempted to use a live oral vaccine such as a *Salmonella* typhimurium mutant, *Lactobacillus* spp., and *Saccharomyces cerevisiae* (Scheppler *et al.*, 2002; Shin *et al.*, 2005; Kulkarni *et al.*, 2010). Out of these delivery systems, yeast expression system, *S. cerevisiae*, has several advantages: high expression levels, ease of scale-up, low cost, adjuvant potential of yeast cell-wall components, such as ß-1,3-d-glucan and mannan, and an eukaryotic system executing post-translational modifications (Stubbs *et al.*, 2001; Park *et al.*, 2007). Yeast-based expression systems have been developed and successfully used to

produce recombinant proteins (Schreuder *et al.*, 1996; Stubbs *et al.*, 2001).

Yeast cell surface-display technology

Display of heterologous proteins on the cell surface of microorganisms has been studied as an important objective for many applications in microbiology and molecular biology. The expression of proteins on the surfaces of bacteriophage and bacteria has been actively studied for the past years. These systems are expected to be useful for construction of microbial biocatalysts, whole-cell adsorbents, and live vaccines. Recently, heterologous proteins have been displayed on the surfaces of yeast cells by a genetic engineering technique as yeast cell-surface display (Ueda and Tanaka, 2000). It could be a good candidate for a live oral vaccine carrier, providing the stable maintenance of surface-expressed epitopes with a high-density displayed protein (Ueda and Tanaka, 2000). Several antigens displayed on the surfaces of yeast cells have been developed as new oral vaccine (Schreuder *et al.*, 1996).

The cell surface of the yeast *Saccharomyces cerevisiae* was engeneered by anchoring active glucoamylase protein on the cell wall. The gene encoding *Rhizopus oryzae* glucoamylase with its secretion signal peptide was fused with the gene encoding the C-terminal half (320 amino acid residues from the C terminus) of yeast α-agglutinin, a protein involved in mating and covalently anchored to the cell wall. The constructed plasmid containing this fusion gene was introduced into

S. cerevisiae and expressed under the control of the glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter from *S. cerevisiae* (Murai *et al.*, 1997).

As shown in Figure Ⅰ, protein secretion in *S. cerevisiae* covers transfer through various membrane-enclosed compartments constituting the secretory pathway (Ueda and Tanaka, 2000; Sakuragi *et al.*, 2011). Secreted proteins are firstly translocated into the lumen of the endoplasmic reticulum (ER), and then transported from the ER to the Golgi apparatus and from the Golgi apparatus to the plasma membrane. Fusion of the Golgi-derived secretory vesicles with the plasma membrane releases the secreted proteins to outside of the cell. Cell wall anchorage of α-agglutinin is accomplished by addition of ß1,6-glucan to the GPI anchor residue of α-agglutinin (Ueda and Tanaka, 2000). Surface-displayed ApxIIA expressing *S. cerevisiae* used in the present study was developed according to the scheme mentioned above and further confirmed by immunofluorescence microscopy (Figure Ⅱ).

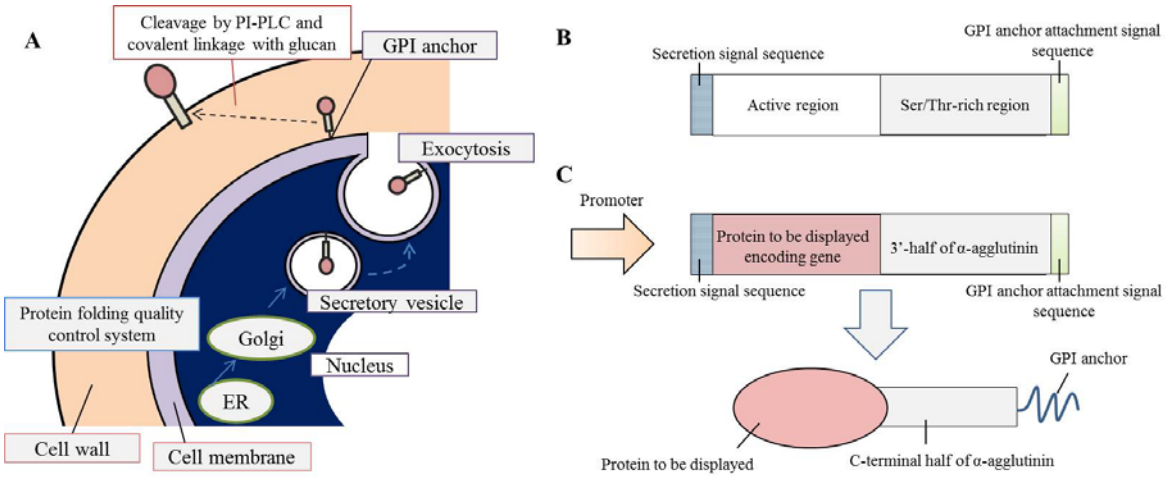


Figure Ⅰ. Mechanism of cell surface display of proteins by cell surface engineering, A; Molecular structure of *α*-agglutinin, B; molecular design of cell surface-displayed enzyme, C. Adapted from Sakuragi *et al.*, 2011(Sakuragi *et al.*, 2011).

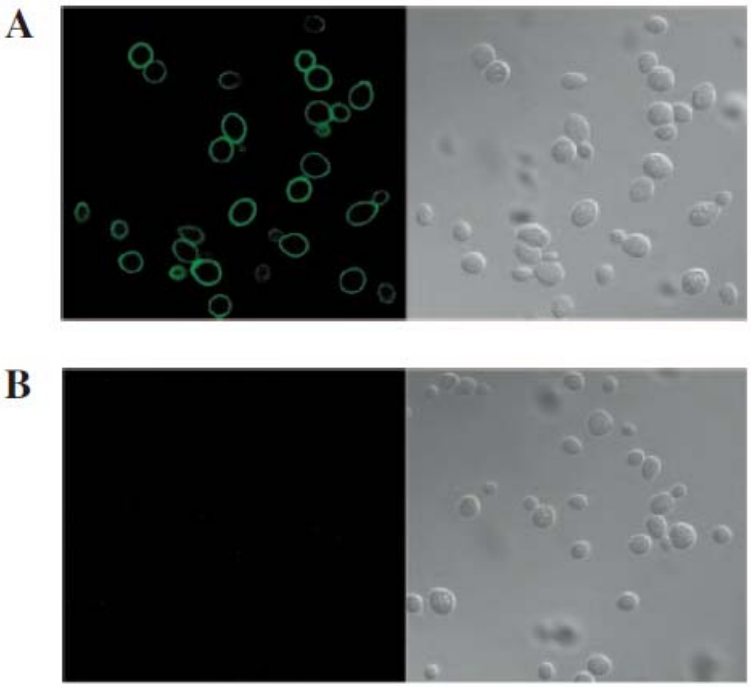


Figure Ⅱ. Immunofluorescence micrograph of recombinant yeast cells. Fluorescence (left) and matching light (right) microscopic images are shown. A, Fluorescence microscopy revealed FITC fluorescence as a ring around the cell surface. B, No fluorescence was observed in the control cells. Adapted from Kim *et al.*, 2010.

Antigen-presenting cells

Antigen-presenting cells (APCs) are cell population that are specialized to capture microbial and other antigens, display them to lymphocytes, and provide signals that stimulate the proliferation and differentiation of the lymphocytes (Abbas *et al.*, 2007).

APCs are required for T cell activation. Purified CD4+ T cells do not respond to a protein antigen by itself but do respond to the antigen in the presence of APCs. The function of the APCs is to display peptides derived from the antigen to T cells. APCs also express costimulators that are important for T cell activation (Abbas *et al.*, 2007).

Dendritic cells are the most effective APCs for activating naïve CD4+ and CD8+ T cells, and therefore for initiating T cell responses. Macrophages present antigens to differentiated (effector) CD4+ T cells in the effector phase of cell-mediated immunity, and B lymphocytes present antigens to helper T cells during humoral immune responses. Dendritic cells, macrophages, and B lymphocytes express MHC class II molecule and costimulators, and are capable of activating CD4+ T lymphocytes. However, APCs are sometimes used to refer only to dendritic cells because these are the only cell type whose principal function is to capture and present antigens, and to initiate T cell responses (Figure Ⅲ).

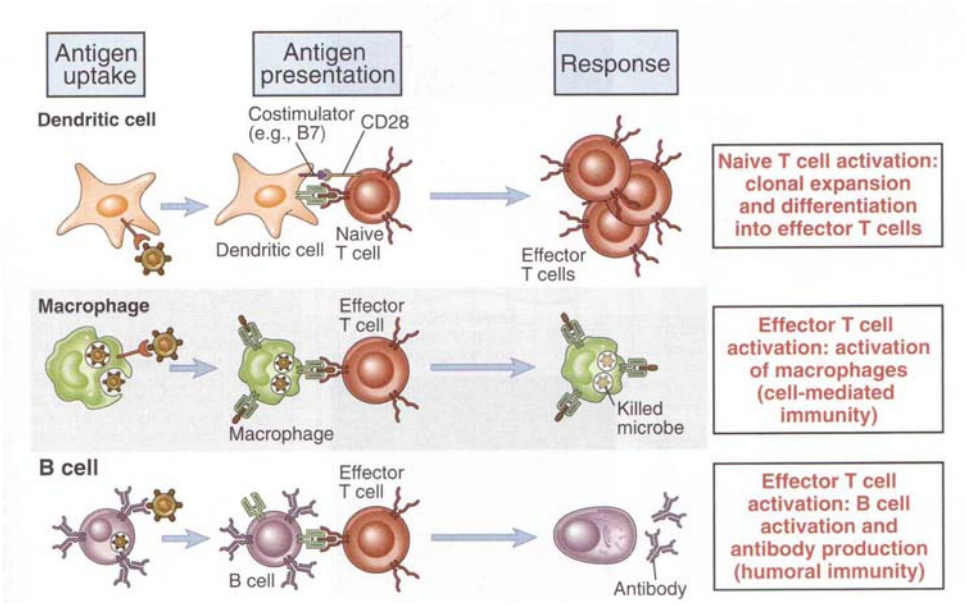


Figure Ⅲ. Function of different antigen-presenting cells. Adapted from Abbas *et al.*, 2007.

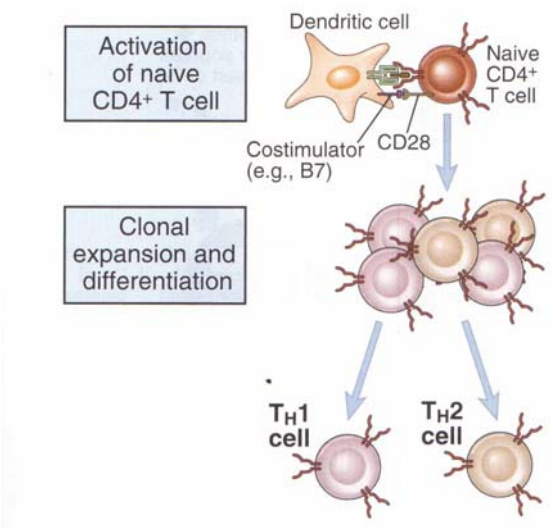
T lymphocyte activation

Dendritic cells that have encountered microbes and internalized their antigens begin to mature and migrate to the T cell zones of secondary lymphoid organs such as the lymph nodes. When they reach these T cell areas, the the dendritic cells present antigens on MHC molecules and also express costimulators that can provide second signals to naïve T cells. Class I and II MHC molecules are associated presentation of cytosolic antigen to cytotoxic T lymphocytes and of extracellular antigen to helper T cells, respectively. Antigen-stimulated T cells that have received both "signal one" through the antigen receptor and "second signal" via costimulatory receptors may be induced to secrete cytokines and to express cytokine receptors. The cytokine interleukin-2 (IL-2) provides autocrine signals to activated T cells, leading to expansion of antigen-specific clones. IL-2 and other cytokines produced by the T cells and by APCs also stimulate the differentiation of the T cells into effector and memory cells. Some of activated T cells leave the lymphoid organ and enter the circulation. Other activated CD4+ T cells remain in the lymphoid organ, where they help B lymphocyte to differentiate into antibody-secreting plasma cells (Abbas *et al.*, 2007).

Th1 and Th2 subsets of CD4+ T cells

CD4+ T cells may differentiate into subsets of effector cells that produce distinct

sets of cytokines and therefore perform effector functions. Specialization of adaptive immunity is the ability of CD4+ T lymphocytes to activate diverse effector mechanisms in response to different types of microbes. CD4+ T cells differentiate Th1 and Th2 subsets, and the pattern of differentiation is determined by stimuli present early during immune responses. The most important differentiation-inducing stimuli are cytokines, with IFN-γ, and IL-12 as the major inducers of Th1 cells and IL-4 of Th2 cells (Figure Ⅳ) (Abbas *et al.*, 2007).



Property	T_H1 subset	T_H2 subset
Cytokines produced		
IFN-γ	+++	–
IL-4, IL-5, IL-13	–	+++
IL-10	+/–	++
IL-3, GM-CSF	++	++
Cytokine receptor expression		
IL-12R β chain	++	–
IL-18R	++	–
Chemokine receptor expression		
CCR4, CCR8, CXCR4	+/–	++
CXCR3, CCR5	++	+/–
Ligands for E- and P-selectin	++	+/–
Antibody isotypes stimulated	IgG2a (mouse)	IgE, IgG1 (mouse)/IgG4 (humans)
Macrophage activation	Classical	Alternative

Figure Ⅳ. Properties of Th1 and Th2 subsets of CD4+ helper T cells. Adapted from Abbas *et al.*, 2007

The principal function of Th1 cells is in phagocyte-mediated defense against infections. IFN-γ produced by Th1 cells stimulates the microcidal activities of phagocytes, thereby promoting the intracellular destruction of phagocytosed microbes. IFN-γ also stimulates the production of opsonizing and complement-fixing IgG antibodies, which promote the phagocytosis of microbes (Abbas *et al.*, 2007).

CD4+ T cells that differentiate into Th2 cells secrete IL-4 and IL-5. IL-4 acts on B cells to stimulate production of antibodies. IL-4 is also an autocrine growth and differentiation cytokine for Th2 cells. IL-5 activets eosinophils, a response that is important for defense against parasites like helminth. In addition, cytokines from Th2 cells also inhibit macrophage activation and Th1-mediated reations (Figure Ⅴ) (Abbas *et al.*, 2007).

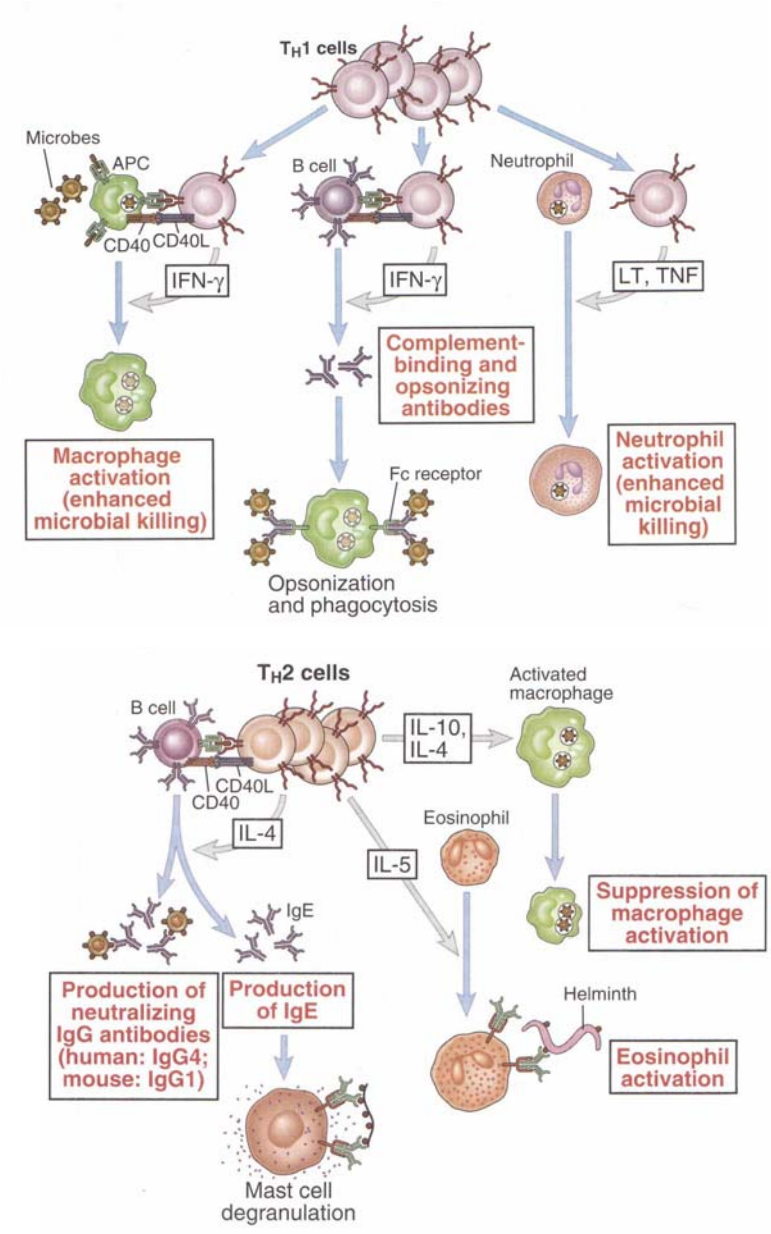


Figure Ⅴ. Effector functions of Th1 and Th2 cells. Adapted from Abbas *et al.*, 2007

Mucosal immunity

Mucosal and skin surfaces are enveloped with special epithelial layers as the first line of defense against pathogenic antigens. The mucosal immune system has main functions, such as (i) to prevent colonization and invasion by harmful microbes, (ii) to limit transmission of pathogens between individuals, and (iii) to prevent harmful immune responses to food and airborne antigens (Cuburu *et al.*, 2007). The mucosa-associated lymphoid tissue (MALT) includes gut-associated lymphoid tissue (GALT), bronchial/tracheal-associated lymphoid tissue (BALT), nose-associated lymphoid tissue (NALT), and vulvovaginal-associated lymphoid tissue (VALT) (Abbas *et al.*, 2007). The MALT is composed of lymphoid cells, or it may include small solitary lymph nodes.

Mucosal vaccines are capable of inducing both mucosal and systemic immune responses, thus leading to two layers of host protection (Kweon, 2011). Administration by injection as a traditional vaccine type has several adverse effects, such as laborious and time-consuming procedures, the induction of inflammatory reactions at the injection site, and the stress (Shin *et al.*, 2005), while mucosal administration of vaccine offers overcoming the problems mentioned above. sIgA related to mucosal immune responses binds to the microorganisms and toxins and neutralize them by blocking their entry into the host (Neutra and Kozlowski, 2006). In addition, mucosal immunization can promote sIgA antibody in mucosal sites distant from the immunization site, according to the so-called “common mucosal

immune system" (Cuburu *et al.*, 2007). Owing to the migration of IgA antibody-secreting cells (ASCs), local mucosal immunization leads to antigen-specific IgA production at distant mucosal sites (Kweon, 2011).

Oral vaccines

Oral administration of vaccines is an attractive alternative to parenteral vaccination, with several advantages, including not only induce locally and systemically protective immune responses against infectious disease but also to be a safe and convenient way. In particular, oral administration elicits a local, mucosal immune response (IgA) in the gut that will act as the first line of defense for diseases that start on mucosal surfaces (Larhed *et al.*, 2004). The adaptive humoral immune responses at mucosal surfaces are involved in secretory IgA (sIgA) antibodies. sIgA prevents direct contact of pathogens with the mucosal surface and block the microbial surface molecules that involved in epithelial attachment (Hutchings *et al.*, 2004).

Oral immunization is recognized to induce strong IgA responses in the gastrointestinal tract and mammary glands but is comparatively less efficient in the respiratory and reproductive tracts (Quiding *et al.*, 1991). Although intranasal immunization induces sIgA antibodies in the respiratory and reproductive tracts, it can cause safety problems such as redirecting vaccine antigens into the central nervous system (van Ginkel *et al.*, 2000). Ninty-five percent and over of the

bacterial cells enter the guts of pigs within 24 h and bacteria in lungs are removed by mucociliary clearance; therefore under natural conditions, not only bronchus-associated lymphoid tissues but also gut-associated lymphoid tissues are required in the induction of immunity (Gerlach *et al.*, 1986).

Chapter I

Predicting genetic traits and epitope analysis of *apxIVA* in *Actinobacillus pleuropneumoniae*

Abstract

Actinobacillus pleuropneumoniae causes a severe hemorrhagic pneumonia in pigs. Fifteen serotypes of *A. pleuropneumoniae* express four different Apx toxins that belong to the pore-forming repeats-in-toxin (RTX) group of toxins. ApxIV, which is conserved and up-regulated *in vivo*, could be an excellent candidate for the development of a protective cross-serotype immunity vaccine, and could aid in the differential diagnosis of diseases caused by *A. pleuropneumoniae*. We identified and sequenced *apxIVA* from *A. pleuropneumoniae* serotype 2 isolated in Korea (Kor-ApxIVA). The Kor-ApxIVA was closely related to Switzerland (AF021919), China (CP000687), and China (GQ332268), showing 98.6%, 98.4% and 97.2% amino acid homology, respectively. The level of amino acid homology, however, was higher than the nucleotide homology. The structural characteristics of ApxIVA showed RTX proteins, including N-terminal hydrophobic domains, signature sequences for potential acylation sites, and repeated glycine-rich nonapeptides in

the C-terminal region of the protein. Thirty glycine-rich nonapeptides with the consensus sequence, L/V-X-G-G-X-G-N/D-D-X, were found in the C-terminus of the Kor-ApxIVA. In addition, the Kor-ApxIVA was predicted for the linear B-cell epitopes and conserved domains with determined peptide sequences. This genetic analysis of the Kor-ApxIVA might be an important foundation for future biological and functional research on ApxIVA.

Key words: *Actinobacillus pleuropneumoniae*, RTX toxins, ApxIV, sequences

Introduction

Actinobacillus pleuropneumoniae causes a highly contagious pleuropneumonia in pigs, leading to major economic losses in pig farming worldwide (Nielsen, 1988; Fenwick and Henry, 1994; Frey, 1995; Haesebrouck *et al.*, 1997). *A. pleuropneumoniae* can result in various clinical degrees of pleuropneumonia from peracute to chronic, with infected pigs typically showing a hemorrhagic, necrotizing pneumonia often associated with fibrinous pleuritis (Haesebrouck *et al.*, 1997). The virulence of *A. pleuropneumoniae* is multifactorial, and the factors involved in pathogenesis are capsular polysaccharides, lipopolysaccharides, outer membrane proteins, adhesion factors, proteases, and exotoxins (Udeze *et al.*, 1987; Dom *et al.*, 1994; Tascon *et al.*, 1994; Frey, 1995; Haesebrouck *et al.*, 1997; Baltes *et al.*, 2002; Bandara *et al.*, 2003; Negrete-Abascal *et al.*, 2003). However, the respective contributions of the virulence factors to the infection and disease are not known. Among these factors, exotoxins have been reported to be strongly related to pathogenesis, and indicate the virulence of the different serotypes (Tascon *et al.*, 1994; Frey, 1995; Haesebrouck *et al.*, 1997; Kamp *et al.*, 1997; Bosse *et al.*, 2002; Boekema *et al.*, 2004). Fifteen serotypes express four different Apx toxins belonging to the pore-forming repeats-in-toxin (RTX) toxins (Haesebrouck *et al.*, 1997; Blackall *et al.*, 2002). ApxI is strongly hemolytic and cytotoxic, while ApxII is weakly hemolytic and moderately cytotoxic. However, ApxIII is nonhemolytic, but strongly cytotoxic (Frey, 1995; Schaller *et al.*, 1999). Recombinant ApxIV

shows weak hemolytic activity and cohemolytic synergy with sphingomyelinase (*beta-toxin*) of *Staphylococcus aureus*, but the biological and functional characteristics of ApxIV are unclear (Schaller *et al.*, 1999). ApxI, ApxII, and ApxIII are also produced by other *Actinobacillus* species such as *A. rossii*, *A. suis*, and *A. porcitonsillarum* (Schaller *et al.*, 2001; Gottschalk *et al.*, 2003), whereas ApxIV is highly specific to *A. pleuropneumoniae* (Schaller *et al.*, 1999).

The Apx toxin is encoded by the *apx* operon, which contains four genes in the order *apxCABD*: the activator gene, *apxC*, the pretoxin structural gene, *apxA*, and the secretion apparatus-encoding genes, *apxB* and *apxD* (Frey, 1995). *ApxIV* has a similar sequence to the *frpA* and *frpC* genes of *Neisseria meningitidis*, and is located immediately downstream of the *A. pleuropneumoniae lacZ* gene and upstream of the ORF1 gene (Anderson and MacInnes, 1997; Schaller *et al.*, 1999). ORF1 seems to be involved in activation and is required for the observed hemolytic and cohemolytic (CAMP) phenotypes (Schaller *et al.*, 1999).

Compared to ApxI, ApxII, and ApxIII, ApxIV has distinctive features that are produced by all serotypes of *A. pleuropneumoniae*. These features are highly specific to *A. pleuropneumoniae* and are expressed only *in vivo* (Schaller *et al.*, 1999; Cho and Chae, 2001; Schaller *et al.*, 2001; Dreyfus *et al.*, 2004; Turni and Blackall, 2007). An ApxIV-based serological test was developed as a differential diagnostic tool to identify infected or carrier pigs (Dreyfus *et al.*, 2004). Furthermore, a subunit vaccine composed of recombinant ApxI, ApxII, ApxIII, the N-terminal half of ApxIV, as well as the recombinant outer membrane protein

(OMP) of *A. pleuropneumoniae* showed strong protection against *A. pleuropneumoniae* infection. Therefore, it may be inferred that ApxIV plays a positive role in immunoprotection (Wang *et al.*, 2009). However, ApxIV itself cannot induce sufficient immune protection against *A. pleuropneumoniae* infection (Wang *et al.*, 2009).

In the present study, we described the complete sequencing and organization of the ApxIVA isolated in Korea (Kor-ApxIVA), and the homology of *apxIVA* to other *A. pleuropneumoniae* serotypes. The Kor-ApxIVA was analyzed and characterized using determined amino acid sequences. We suggest that the genetic analysis of Kor-ApxIVA is an important foundation for future biological and functional research on ApxIVA.

Materials and Methods

Bacterial strains and growth conditions

A. pleuropneumoniae KSID serotype 2 isolated from infected Korean pigs with pleuropneumonia was used to investigate the *apxIVA* gene. *A. pleuropneumoniae* was grown in Luria–Bertani (LB) broth (Difco Laboratories Inc., Detroit, MI, USA) with 0.01% β-nicotinamide adenine dinucleotide (NAD) for 24 h at 37°C.

PCR amplification

Total genomic DNA of *A. pleuropneumoniae* KSID serotype 2 was isolated using a GenElute^{TM} Bacterial Genomic DNA kit (SIGMA, St. Louis, MO, USA) and subsequently used for PCR gene amplification. PCR was conducted with the following components: 1 μL of genomic DNA template, 5 μL of 10X PCR buffer (20 mM Mg^{2+}, Intron Biotechnology Inc., Seoul, Republic of Korea), 5 μL of 10 mM dNTP mixture (2.5 mM of each: dATP, dCTP, dGTP, and dTTP, Intron Biotechnology Inc.), 1 μL of external forward primer (10 pmol μL^{-1}), 1 μL of external reverse primer (10 pmol μL^{-1}), 1 μL of i-*Taq* polymerase (5 units μL^{-1}, Intron Biotechnology Inc.), and 36 μL of triple-distilled H_2O in a total volume of 50 μL. Primer sequences for *apxIVA* (Table 1.1) were designed based on *A. pleuropneumoniae apxIVA* from Switzerland (GenBank accession no. AF021919). Amplification products were analyzed using gel electrophoresis with ethidium bromide-stained gels.

DNA sequencing and homology analysis

Amplified *apxIVA* genes were purified with a QIAquick Gel extraction kit (QIAGEN, Hilden, Germany). The purified fragments were sequenced using automatic dye terminator DNA sequencing (ABI PRISM 377 L, Perkin Elmer, USA). The sequence data were assembled and analyzed using MegAlign 5.0 DNASTAR. Homology and the conserved domain for *apxIVA* were examined

using the BLAST at the National Center for Biotechnology Information. The GenBank nucleotide sequence accession numbers of the *apxIVA* that were analyzed are as follows: AF021919, AF030511, CP001091, CP000569, CP000687, and GQ332268; the sequence accession numbers of *apxIVA* differed substantially in length. The Martinez/Needleman-Wunsch method and Lipman-Pearson method were employed to generate DNA and polypeptide alignments using MegAlign 5.0 DNASTAR. Phylogenetic trees of *apxIVA* were constructed via bootstrap analysis (500 repeats) using MEGA 4.0.

Predicting protective linear B-cell epitopes and the conserved domain

Epitope prediction and analysis were performed using Chou-Fasman Beta-Turn Prediction, Emini Surface Accessibility Prediction, Karplus and Schulz Flexibility Prediction, Kolaskar and Tongaonkar Antigenicity, Parker Hydrophilicity Prediction, and Linear Epitope Prediction. The dataset comprised of protective linear B-cell epitopes was derived from the Immune Epitope Database (IEDB). The determined amino acid sequences were found to contain conserved domains and functional annotations of the Kor-ApxIVA. It was derived from the NCBI's Conserved Domain Database (CDD).

Nucleotide sequence accession number

The nucleotide sequences of the *A. pleuropneumoniae* KSID serotype 2 *apxIVA* gene were given in GenBank under the accession number, HM021153.

Results

Genetic organization of *apxIVA*

A consensus 5,856 bp DNA product was obtained and confirmed by nucleotide sequencing. The Kor-ApxIVA was identified through amino acid sequences with a predicted molecular mass of 218,450.05 Da and an isoelectric point of 4.60. The Kor-ApxIVA was preceded upstream by ORF1 and the *mrp* gene. *LacZ* was present in the C-terminal part of *apxIVA* (Figure 1.1). A 474 bp ORF1 lay within the region between the C-terminal end of the *E. coli* methionine-rich protein, MRP (*mrp* gene product), and *apxIVA*. ORF1 encoded a protein of 157 amino acids with a molecular mass of 18,733.32 Da and an isoelectric point of 6.38. The regions of similarity between the Kor-ApxIVA (HM021153) and the GenBank accession number (AF021919) were represented by a dotplot that displays a wordmatch of two sequences (Figure 1.2). The N-terminus of ApxIVA was highly conserved, while the repeats were found at the C-terminus of ApxIVA.

Homology of ApxIVA

The Kor-ApxIVA showed homology with the *apxIVA* GenBank accession numbers: Switzerland (AF021919), Switzerland (AF030511), Germany (CP001091), Canada (CP00569), China (CP000687), and China (GQ332268) (Table 1.2, Figure 1.3). The nucleotide and amino acid sequences of the Kor-ApxIVA were similar to the GenBank accession numbers. In particular, the Kor-ApxIVA was closely related to Switzerland (AF021919), China (CP000687), and China (GQ332268), showing 98.6%, 98.4% and 97.2% amino acid homology, respectively, which was higher than the nucleotide homology.

Predicting protective linear B-cell epitopes

Beta-turn, surface accessible, antigenic, flexible, hydrophilic, and linear epitope regions were distributed in the general region of the Kor-ApxIVA (Figure 1.4). In addition, at the peptide position, which ranged from 568 to 636, high values were measured in the beta-turn, flexibility prediction analysis, surface accessibility, and hydrophilicity.

Structural features of the Kor-ApxIVA

The three different repeated modules constructed the C-terminal region of ApxIVA in variable sizes (Figure 1.5). The C-terminus of the Kor-ApxIVA contained 30

glycine-rich nonapeptides with the consensus sequence, L/V-X-G-G-X-G-N/D-D-X. Five regions of the consensus sequences for DNA polymerase family 2 signatures, which are considered to be involved in Mg^{2+} binding (Argos, 1988), were distributed in the interval of the glycine-rich nonapeptides. The conserved domain RTX C-terminal domain (pfam08339), peptidase M10 serralysin C terminal domain (pfam08548), and multi-domain RTX toxins and related Ca^{2+}-binding proteins (COG2931) were shown in the C-terminal region of ApxIVA (Figure 1.6). The Kor-ApxIVA (HM021153) and the GenBank accession numbers of ApxIVA had a peptidase M10 serralysin C-terminal domain extending from aa 1,089 to aa 1,135, and the RTX C-terminal domain at the C-terminus of ApxIVA. A peptidase M10 serralysin C-terminal domain was repeated in the Kor-ApxIVA (HM021153) extending from aa 1,500 to aa 1,552.

Discussion

Several current studies have focused on identifying genes that are expressed *in vivo* during a natural infection, because these genes may be important in the infection process (Fuller *et al.*, 1999; Deslandes *et al.*, 2010). These genes are thought to be regulated in the host environments encountered by the pathogen, such as conditions of iron-deficiency or a limited number of anaerobic or branched chain amino acids (BCAA) (Baltes and Gerlach, 2004; Wagner and Mulks, 2006;

Deslandes *et al.*, 2007; Wagner and Mulks, 2007; Lone *et al.*, 2009; Deslandes *et al.*, 2010). For *A. pleuropneumoniae*, the up-regulation of many genes has been reported *in vivo* (Fuller *et al.*, 1999; Deslandes *et al.*, 2010). At first, ApxIV was detected only *in vivo*, but recently its expression was discovered *in vitro* following its addition to bronchoalveolar lavage fluid (BALF) (Lone *et al.*, 2009). Although ApxIV was identified nearly a decade ago, its role in virulence and infection remain unclear (Schaller *et al.*, 1999).

The gene encoding ApxIVA from *A. pleuropneumoniae* serotype 2 KSID was amplified, sequenced, and characterized. The nucleotide and amino acid sequences of the Kor-ApxIVA were similar to the GenBank accession numbers of ApxIVA. The Kor-ApxIVA was homologous with the GenBank accession numbers of ApxIVA, Switzerland (AF021919), Switzerland (AF030511), Germany (CP001091), Canada (CP00569), China (CP000687), and China (GQ332268). In particular, the Kor-ApxIVA showed a higher level of homology with Switzerland (AF021919), China (CP000687), and China (GQ332268) than with the other isolates. The structural characteristics of ApxIVA show RTX proteins, including N-terminal hydrophobic domains, signature sequences for potential acylation sites, and repeated glycine-rich nonapeptides in the C-terminal region of the protein (Welch, 1991; Schaller *et al.*, 1999). The RTX family of cytotoxins are related to the pore-forming protein toxins in many Gram-negative pathogens: the a-hemolysin of *Escherichia coli* (HlyA), the bi-functional adenylate cyclase hemolysin of *Bordetella pertussis* (CyaA), the leucotoxins of *Pasteurella*

hemolytica (LktA) and *Actinobacillus actinomycetemcomitans* (AaltA), as well as two iron-regulated exoproteins (FrpA, FrpC) of *Neisseria meningitidis* (Felmlee *et al.*, 1985; Lo *et al.*, 1987; Glaser *et al.*, 1988; Lally *et al.*, 1989; Schaller *et al.*, 1999). The RTX family has tandem-repeat, glycine-rich nonapeptides with the consensus sequence, Leu/Ile/Phe-Xaa-Gly-Gly-Xaa-Gly-Asn/Asp-Asp-Xaa (Schaller *et al.*, 1999). The glycine-rich nonapeptide repeats, and DNA polymerase 2 signature sequences, which are known to bind Ca^{2+} and Mg^{2+}, respectively, are organized together with nonapeptide repeats as a modular structure in the C-terminal region (Argos, 1988; Schaller *et al.*, 1999). Thirty glycine-rich nonapeptides with the consensus sequence, L/V-X-G-G-X-G-N/D-D-X, were found in the C-terminus of the Kor-ApxIVA. An important characteristic of ApxIVA is its various sizes resulting from the different numbers of repeated modules in each serotype, and this characteristic could be used as a reliable method for typing *A. pleuropneumoniae* (Sthitmatee *et al.*, 2003; da Costa *et al.*, 2004; Rayamajhi *et al.*, 2005).

The conserved domain RTX C-terminal domain (pfam08339), peptidase M10 serralysin C terminal domain (pfam08548), and multi-domain RTX toxins and related Ca^{2+}-binding proteins (COG2931) were found in the C-terminal region of the Kor-ApxIVA. The RTX C-terminal domain (pfam08339) describes the C-terminal region of various bacterial hemolysins and leukotoxins (Czuprynski and Welch, 1995). The peptidase M10 serralysin C terminal region (pfam08548) is considered to be important for secretion of the protein through the bacterial cell

wall, which contains the calcium ion-binding domain, pfam00353. This domain is repeated in the C-terminus of the Kor-ApxIVA, and is thought to affect the secretion of the Kor-ApxIVA. However, further experiments are needed to confirm this effect compared to other strains. In addition, the multi-domain RTX toxins and related Ca^2+-binding proteins conserved domain (COG2931) encode secondary metabolite biosynthesis, transport, and catabolism (Coote, 1992).

Mapping B-cell epitopes is important for vaccine design, immunodiagnostic tests, and antibody production (El-Manzalawy *et al.*, 2008). The resulting dataset comprised of B-cell epitopes is believed to closely approximate a dataset of protective linear B-cell epitopes (Sollner *et al.*, 2008). Since the experimental determination of the B-cell epitopes is exhaustive, the computational methods for the dependable recognition of the B-cell epitopes from peptide sequences are required (El-Manzalawy *et al.*, 2008). Several methods for predicting B cell epitopes based on the physico-chemical properties of the amino acids have been developed (Saha and Raghava, 2006). The present study was performed using Chou-Fasman Beta-Turn Prediction, Emini Surface Accessibility Prediction, Karplus and Schulz Flexibility Prediction, Kolaskar and Tongaonkar Antigenicity, Parker Hydrophilicity Prediction, and Linear Epitope Prediction. Beta-turn, surface accessible, antigenic, flexible, hydrophilic, and linear epitope regions are distributed in the general region of the Kor-ApxIVA. A high value is shown at the peptide position from 568 to 636 in the beta-turn and flexibility prediction analysis, and the surface accessibility and hydrophilicity. Two epitopes of ApxIVA were

found through the generation of monoclonal antibodies between 1 and 866 amino acids (Huang *et al.*, 2006). ApxIV also has three more epitopes—one between 867 and 1022 amino acids and two between 1023 and 1863 amino acids (Huang *et al.*, 2006). ApxIV that contained amino acids from 418 to 645 induces immunity and protection against *A. pleuropneumoniae* (Wang *et al.*, 2009).

In the present study, the Kor-ApxIVA showed a high rate of homology compared with the other accession numbers of ApxIVA, and was predicted for linear B-cell epitopes and conserved domains with determined peptide sequences. This genetic analysis of the Kor-ApxIVA is thought to be an important foundation for future biological and functional research on ApxIVA.

Table 1.1. Nucleotide sequence of primers used in sequencing

Primer name	Primer sequences (5'-3')	Position	PCR parameters[1]
mrp-F	GTGGCGAAGAAATACGGTACTAAAGT	*mrp* 241-	94, 57, 72
orf-R	AATATCATCATCTCCTTTTCCTATTTCAG	*apxIVA* 120	20s, 10s, 60s
apxIVAN-F	CACCATGACAAAATTAACTATGCAAGA	*apxIVA* 1-	94, 55, 72
apxIVAN-R	CTAACTTTTTAACTTTTTAACGGCGG	1500	20s, 10s, 90s
apxIVAN#A	AGAAATAAAAGAGGTTGAAAAGGGG	*apxIVA* 1320-	94, 53, 72
apxIVAN#A-R	GTCGAAACTTCACTTCCGCAT	3517	20s, 10s, 60s
ApxIVADWN-L[2]	CACCGCGAAACAATTCGAAGGG	*apxIVA* 2980-	94, 65, 72 20s, 10s,
ApxIVA-1R[2]	CTAGGCCATCGACTCAACCAT	5766	240s
lacZ-F	CGTTTATCGAATGAGCAAACGT	*apxIVA* 5704-	94, 53, 72
lacZ-R	ATATCGCTTAATGGAAAGCGAA	*lacZ* 70	20s, 10s, 30s

[1] Temperatures are tabulated in the first row in degrees Celsius, and the corresponding times are in the second row

[2] Adapted from Schaller *et al*.

Table 1.2. Nucleotide and amino acid similarity of the Kor-ApxIVA (HM021153) with the six different GenBank accession numbers of ApxIVA according to Martinez/Needleman-Wunsch DNA alignment and Lipman-Pearson protein alignment (MegAlign 5.0 DNASTAR)

	Similarity Index of ApxIVA					
Selected strains	**Switzerland (AF021919)**	**Switzerland (AF030511)**	**Germany (CP001091)**	**Canada (CP00569)**	**China (CP000687)**	**China (GQ332268)**
Nucleotide						
Korea (HM021153)	**78.9**	**75.8**	**82.6**	**84.9**	**76.1**	**74.4**
Amino acid						
Korea (HM021153)	**98.6**	**96.7**	**94.7**	**95.8**	**98.4**	**97.2**

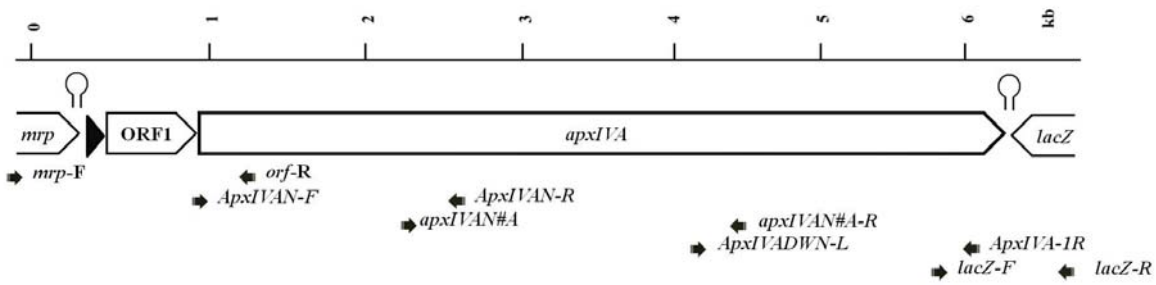


Figure 1.1. Schematic diagram of *apxIVA* and the location of *apxIVA* fragments for sequencing. Boxes with pointed tips indicate *mrp*, ORF1, *apxIVA*, and *lacZ* genes; hairpins indicate putative *rho*-independent transcription termination signals; the black triangle indicates the location of putative promoter sequences; the positions of the primer sequences used for sequencing are indicated by black arrows below the maps.

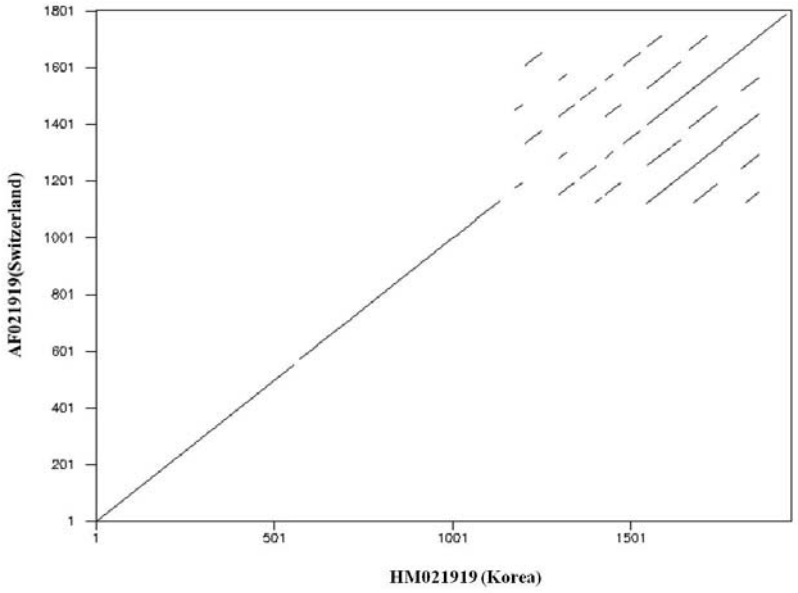


Figure 1.2. A dotplot analysis of the Kor-ApxIVA (HM021153) with the GenBank accession number, AF021919, from Switzerland. A long diagonal line represents the conserved region in the N-terminus of ApxIVA. The C-terminal region of ApxIVA showed repeated regions (parallel diagonal lines); this was derived from EMBOSS server.

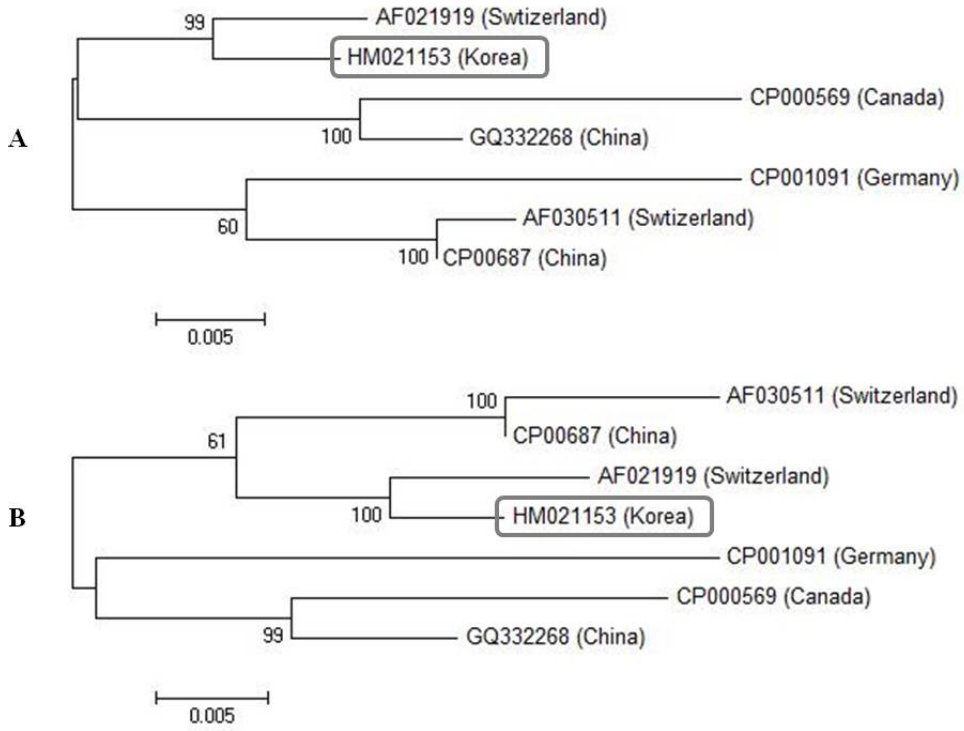


Figure 1.3. Phylogenetic analysis of ApxIVA. The phylogenetic analysis was performed based on nucleotide (A) and amino acid sequences (B) compared with the Kor-ApxIVA (HM021153) and the six different GenBank accession numbers using the MEGA 4.0 program.

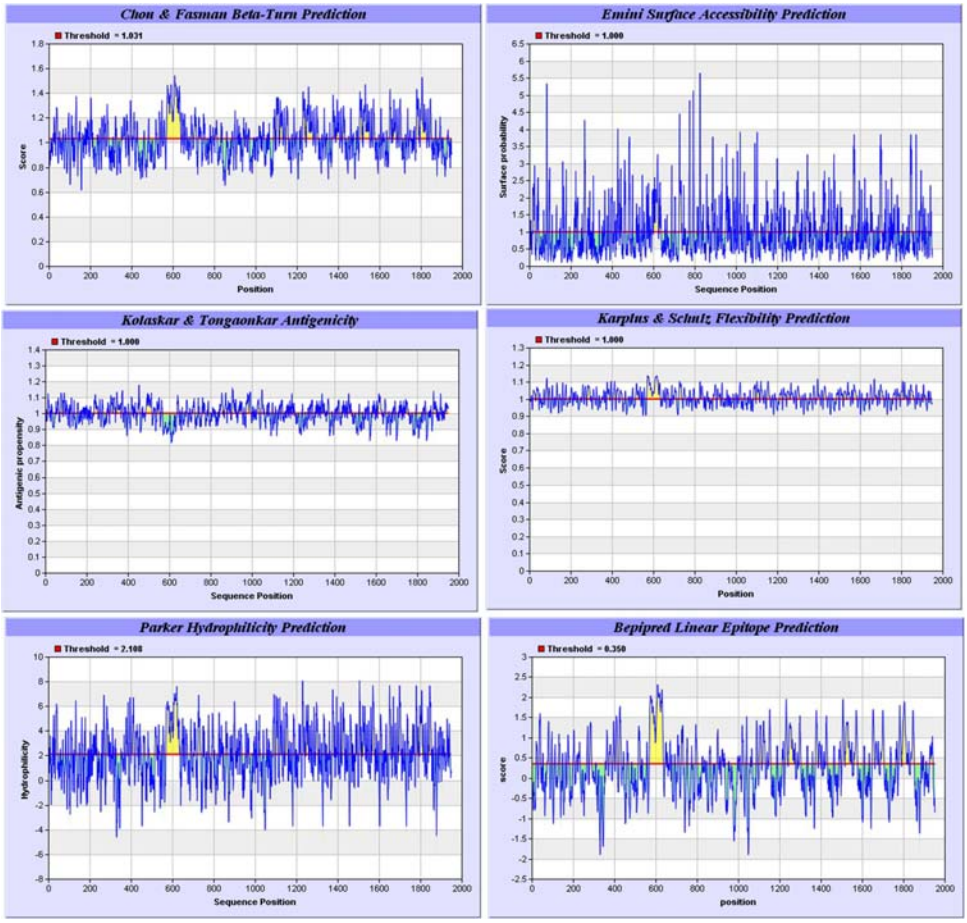


Figure 1.4. Protein sequence analysis of the Kor-ApxIVA (HM021153) including beta-turn, surface accessibility, antigenicity, flexibility, hydrophilicity and linear epitope prediction. Analysis was performed using Chou-Fasman Beta-Turn Prediction, Emini Surface Accessibility Prediction, Karplus and Schulz Flexibility Prediction, Kolaskar and Tongaonkar Antigenicity, Parker Hydrophilicity Prediction, and Linear Epitope Prediction; this analysis was derived from Immune Epitope Database (IEDB).

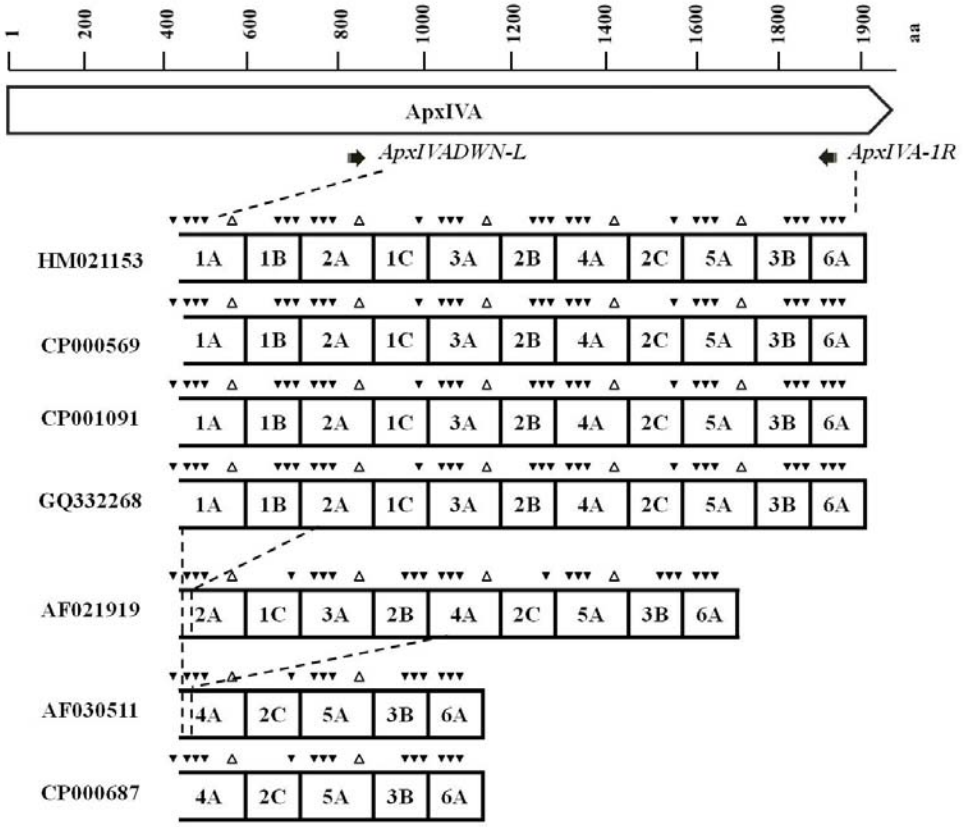


Figure 1.5. Predicted structures of the Kor-ApxIVA (HM021153) and the GenBank accession numbers of ApxIVA. The black arrows with pointed tips in the lower part of the boxes indicate the oligonucleotide primers that amplified the C-terminal region of ApxIVA. The repeated glycine-rich nonapeptides are shown by filled triangles, and the open triangles indicate the positions of the DNA-polymerase family 2 signature sequences. The boxes 1A–6A, 1B–3B and 1C–2C represent the three different repeated modules that construct the C-terminus of ApxIVA. Dotted lines indicate the site of the deletion within the repeated module, as compared to the GenBank accession numbers of ApxIVA.

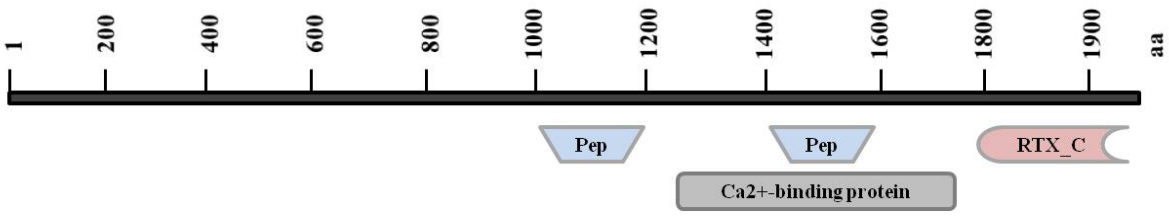


Figure 1.6. Conserved domain of ApxIVA (HM021153). Pep, peptidase M10 serralysin C-terminal domain (pfam08548); RTX_C, RTX C-terminal domain (pfam08339); and Ca^{2+} binding protein, RTX toxins, and related Ca^{2+}-binding proteins.

Chapter II

An immunosorbent assay based on the recombinant ApxIA, ApxIIA and ApxIIIA toxins of *Actinobacillus pleuropneumoniae* and its application to field sera

Abstract

Actinobacillus pleuropneumoniae is the etiologic agent of porcine pleuropneumonia, a highly contagious pulmonary disease in pigs with major economic losses for pig producers worldwide. Whereas *A. pleuropneumoniae* isolates are divided into 15 serotypes, they secret four types of exotoxins, ApxI, ApxII, ApxIII and ApxIV, which are known as major virulence factors. In the current study, the *apxIA*, *apxIIA* and *apxIIIA* genes were amplified and their recombinant proteins expressed in *E. coli* M15 cells. The antigenicity of each recombinant protein was demonstrated by Western blot and enzyme-linked immunosorbent assay (ELISA) using sera from pigs vaccinated with a subunit vaccine. When ELISAs using the recombinant antigens were optimized and then applied to sera from 320 randomized pigs in Korea, an observed increase in seroprevalence was found among sows in comparison with weaned piglets and growing pigs, indicating an age-dependent seroprevalence. The results obtained in

the study suggest that the developed ELISAs may be useful for *A. pleuropneumoniae* vaccination strategy as a screening tool for pig herds as well as for detection of specific antibodies to Apx exotoxins.

Keywords: *Actinobacillus pleuropneumoniae*, Apx toxins, enzyme-linked immunosorbent assay, pigs, prevalence

Introduction

Actinobacillus pleuropneumoniae causes a highly contagious pleuropneumonia in pigs with major economic losses in pig farming worldwide (Fenwick and Henry, 1994; Frey, 1995; Haesebrouck *et al.*, 1997; Chiers *et al.*, 2002). Fifteen serotypes of *A. pleuropneumoniae* have been described based on the differences of capsular polysaccharides (CP) and lipopolysaccharide (LPS) composition.(Beynon *et al.*, 1993; Dubreuil *et al.*, 2000; Blackall *et al.*, 2002) There are several virulence factors such as exotoxins, LPS, CP, membrane proteins, and adhesion factors in *A. pleuropneumoniae* (Tascon *et al.*, 1994; Frey, 1995; Baltes *et al.*, 2002; Negrete-Abascal *et al.*, 2003). Among these virulence factors, the exotoxin, called Apx toxins, is strongly correlated with virulence (Frey, 1995; Reimer *et al.*, 1995; Bosse *et al.*, 2002; Boekema *et al.*, 2004). *A. pleuropneumoniae* produces four different Apx toxins belonging to the pore-forming repeats-in-toxin (RTX) toxins, which include: the strongly hemolytic and cytotoxic ApxI, the weakly hemolytic and moderately cytotoxic ApxII, the nonhemolytic and strongly cytotoxic ApxIII, and the moderately hemolytic recombinant ApxIV (Frey, 1995; Reimer *et al.*, 1995; Schaller *et al.*, 1999). The ApxI, ApxII and ApxIII, which are secreted by different serotypes in various combinations, mainly determine the virulence of the 15 serotypes of *A. pleuropneumoniae* (Frey *et al.*, 1993; Blackall *et al.*, 2002; Bosse *et al.*, 2002). The ApxIV is produced by all serotypes of *A. pleuropneumoniae* (Schaller *et al.*, 1999). The Apx toxin is encoded by the *apx* operon, which contains

four genes: the activator gene *apxC,* the pretoxin structural gene *apxA*, and the secretion-apparatus-encoding genes *apxB* and *apxD,* in the order *apxCABD* (Frey, 1995).

A. pleuropneumoniae can result in various clinical syndromes, which vary in degrees of severity from peracute to chronic, with infected pigs typically showing a hemorrhagic pneumonia often associated with necrosis and fibrinous pleuritis (Haesebrouck *et al.*, 1997). Carrier animals can be the origin of propagation of *A. pleuropneumoniae*. Diagnosis of porcine pleuropneumonia depends on the isolation, identification and serotyping of *A. pleuropneumoniae*, and serodiagnosis. There are several methods used in the serological diagnosis of *A. pleuropneumoniae*: these include radioimmunoassay, tube or microplate agglutination tests, complement fixation test (CFT), hemolysin neutralization (HNT), and the enzyme linked immunosorbent assay (ELISA) (Dubreuil *et al.*, 2000; Montaraz *et al.*, 2006). Although ELISA requires selecting and purifying an antigen, it is more sensitive than other assays (Lo *et al.*, 1998; Montaraz *et al.*, 2006). Commercial ELISA kits were developed for selected serotypes such as serotype 1-9-11, serotype 2, serotype 2-6, serotype 3-6-8 and serotype 4-7 in *A. pleuropneumoniae*. Each ELISA tests for a specific serotype only and it does not offer options to test for the other serotypes. The Apx toxins are major immunogenic factors and stimulate the productions of antibodies to such toxins in infected animals (Frey and Nicolet, 1991; Frey *et al.*, 1992). Moreover, because of its wide distribution, vaccination against *A. pleuropneumoniae* is commonly practiced in Korea. Because it is recognized as a

major virulence factor, an ELISA test that uses a native antigen from the Apx toxin may be a practical approach for the detection of antibodies and control of porcine pleuropneumonia (Dubreuil *et al.*, 2000; Nielsen *et al.*, 2000).

The current report describes the development of an ELISA based on the recombinant ApxIA, ApxIIA and ApxIIIA antigens and evaluated the application of the developed assays on the prevalence of anti-Apx toxin antibodies among pigs in Korea.

Materials and Methods

Bacterial strains and growth conditions

A Korean isolate *A. pleuropneumoniae* serotype 2 was used for the *apx*IIA gene as previously described (Shin, 2003). *A. pleuropneumoniae* serotype 5 and 2 isolated from infected Korean pigs with pleuropneumonia were used for *apx*IA and *apx*IIIA genes, respectively. The organism was grown in Luria–Bertani (LB) broth (Difco Laboratories Inc., Detroit, MI, USA) with 0.01% β-nicotinamide adenine dinucleotide (NAD) (Tokyo Chemical Industry, Tokyo, Japan) for 24h at 37 °C. *E. coli* M15 cells were grown in LB broth with ampicillin^{c} (100 μg/ml) (Duchefa Biochemie, Haarlem, Netherland) and kanamycin (25 μg/ml) (Duchefa Biochemie, Haarlem, Netherland) for 16h at 37 °C.

Polymerase chain reaction amplification and cloning of *apxIA, apxIIA and apxIIIA*

Total genomic DNA was provided for gene amplification from *A. pleuropneumoniae* serotype 2 and 5 using a GenElute^{TM} Bacterial Genomic DNA kit (SIGMA, St. Louis, MO, USA). The sequences of *apx*IA, *apx*IIA and *apx*IIIA and primers were designed on the basis of the GenBank (Table 2.1): for the C-terminal part of *apx*IA, the accession number AF363361 was used; for the full sequences of *apx*IIA, the accession number was AF363362, and for the N-terminal part of *apx*IIIA, the accession number AF363363 was used. The *Apx*IIA gene [2883 base pair; (bp)] was amplified and cloned as previously described (Shin, 2003). Amplified *apx*IA and *apx*IIIA genes were purified with a QIAquick Gel extraction kit (QIAGEN, Hilden, Germany). The PCR products were electrophoresed in a 1.0% agarose gel and observed under UV light after ethidium bromide staining. The *Apx*IA (801 bp) and *apx*IIIA (615 bp) were cloned into the pQE30UA and the pQE31 vector (QIAGEN, Hilden, Germany), respectively. To confirm gene insertion, DNA sequencing was carried out with an ABI 377L automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). The cloned *apx*IA and *apx*IIIA were then transformed into competent *E.coli* M15 cells for expression.

Expression and purification of the recombinant proteins

When the culture reached an optical density of 0.6nm ($OD_{0.6nm}$), isopropyl β-D-1-thiogalactopyranoside (1mM, IPTG) (Duchefa Biochemie, Netherland) was added and then cultured continuously for 4h. The harvested cells were re-suspended in lysis buffer (20mM Tris-hydrogen chloride, 500mM sodium chloride, 8M urea, 40mM imidazole, pH7.0). Nickel–nitrilotriacetic acid (Ni-NTA) chelate affinity chromatography (GE healthcare, Buckinghamshire, United Kingdom) was then performed according to the manufacture's instructions. The bound protein was eluted with elution buffer (20mM Tris-hydrogen chloride, 500mM sodium chloride, 8M urea, 500mM imidazole, pH7.0). The predicted size of the purified recombinant proteins matched the results on the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot, based on amino acids constitution. The purified ApxIA, ApxIIA, and ApxIIIA fusion proteins were analyzed by 12% SDS–PAGE with Coomassie blue staining and transferred to a nitrocellulose membrane; the membrane was immunoblotted with mouse anti-histidine antibodies (IG Therapy Co., Kangwon, Republic of Korea) at a 1:2,000 dilution and an alkaline phosphatase-conjugated goat anti-mouse IgG (H+L) (SIGMA, St. Louis, MO, USA) at a 1:1,000 dilution. Blots were developed with an alkaline phosphatase kit (BIO-RAD, Hercules, CA, USA). The purified recombinant ApxIA, ApxIIA and ApxIIIA were immunoblotted with the serum of a pig vaccinated with a subunit vaccine Porcilis App^{TM} containing ApxI, ApxII and ApxIII toxoids and outer membrane proteins (OMP) at a 1:2,500 dilution and an

alkaline phosphatase conjugated rabbit anti-pig IgG (H+L) (BIO-RAD, Hercules, CA, USA) at a 1:2,000 dilution for the purpose of evaluating diagnostic antigens. Blots were developed with an alkaline phosphatase kit (BIO-RAD, Hercules, CA, USA).

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

The assays were performed using 96-well microplates coated with respective antigen at 4°C overnight. Plates were washed with solution of phosphate buffered saline containing 0.01% Triton X-100 (PBST) after antigen coating and blocked with 10% normal horse serum by incubation at 37°C for 2h. Test reagents (antigen and conjugate) were checkerboard titrated utilizing representative positive and negative sera. Sensitization of recombinant ApxIA, ApxIIA and ApxIIIA antigens was performed by a serial of two-fold dilution of each antigen ranging from 2.4 ng/well to 5,000 ng/well. A serum from a vaccinated pig and a serum from a non-vaccinated pig were used as positive and negative control sera for discrimination, respectively. The plates were incubated at 37 °C for 2h with 100 μl of two-fold serial dilution of each control serum, followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-pig IgG (BETHYL Laboratories Inc., Montgomery, TX, USA) (100 μl, 1:1,000 or 1:2,000 diluted in PBST) at 37 °C for 1h, and washed between incubations with PBST. Color formation was done with

ABTS (SIGMA, St. Louis, MO, USA) as substrate. Optical density was measured at 405 nm using an Emax Precision microplate reader (MDS Inc., Sunnyvale, CA, USA).

To determine optimal cut-off values, 40 sera from pigs vaccinated with Porcilis APP (Intervet International BV, Boxmeer, Netherland) and 25 sera from 10 colostrum-deprived piglets and 15 non-vaccinated piglets were used. The sensitivity, specificity, efficiency and Youden's Index were obtained to evaluate diagnostic accuracy of the ApxIA, ApxIIA and ApxIIIA-ELISAs. The following formulas were calculated:

Sensitivity = Number of true positive / (number of true positive + number of false negative);

Specificity = Number of true negative / (number of true negative + number of false positive);

Efficiency (%) = [(Number of true positive + number of true negative)/(number of true positive + number of false positive + number of true negative + number of false negative)] x 100;

Youden's Index = Sensitivity + Specificity -1.(Crowther, 2000)

Youden's Index is the measure of the probability of correct classifications that is invariant to prevalence (Crowther, 2000). Values for the area under the receiving operator characteristic (ROC) curve (AUC) of the ApxIA, ApxIIA and ApxIIIA-ELISA were evaluated using the statistical program Statistical Package for Social

Sciences software (SPSS) version 17.0 (SPSS Inc., Chicago, IL, USA), at the 95% confidence interval (CI).

Anti-ApxI, ApxII and ApxIII prevalence in domestic pig

A total of 320 field pig sera was chosen randomly according to age, which was divided into suckling piglet (0~4weeks), weaned piglet (4~10 weeks), growing pig (10~20 weeks), and sow classes. Prevalence of antibodies against ApxI, ApxII and ApxIII was investigated with the ELISA in domestic pigs in Korea. Each pig sera were measured three times. The absorbance of samples was measured at 405nm and analyzed.

Results

Production of the recombinant ApxIA, ApxIIA and ApxIIIA

The nucleotide sequences of the cloned genes have confirmed the identity of each toxin (data not shown here). The transformed bacteria were cultured and harvested after IPTG induction. The SDS-PAGE analysis showed that the molecular masses of recombinant ApxIA, ApxIIA and ApxIIIA fusion proteins were approximately 29 kDa, 106 kDa and 23 kDa, respectively, as shown in Figure 2.1, in good

agreement with the software-predicted molecular weights. Western blot analysis also revealed the presence of a clear protein band with an estimated molecular mass of each purified recombinant protein, as presented in Figure 2.2.

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

The results indicated that the optimal concentration of recombinant ApxIA antigen used to coat was 625 ng per well using a 1:100 dilution of serum, and also revealed that the concentrations of recombinant ApxIIA and ApxIIIA antigens were 9.8 and 156.3 ng per well, respectively, using a 1:200 dilution of serum. The largest differences between the absorbance of positive and negative control sera were obtained by the addition of a 1:1,000 dilution of the conjugate for wells coated with the ApxIA antigen under optimized conditions. In case of using wells coated with the ApxIIA antigen or the ApxIIIA antigen, a 1:2,000 dilution of the conjugate made a significantly clear distinction between positive and negative control sera.

Cut-off values

The mean OD and standard deviation (SD) of 25 serum samples (from 10 colostrum-deprived piglets and 15 non-vaccinated piglets) were 0.191 and 0.108 for ApxIA-ELISA, 0.107 and 0.058 for ApxIIA-ELISA and 0.15 and 0.074 for

rApxIIIA-ELISA. A cut-off value was defined as the average value plus SD of the 25 sera, i.e., 0.299 for ApxIA-ELISA, 0.165 for ApxIIA-ELISA and 0.225 for ApxIIIA-ELISA. Such cut-off values were determined to be optimal by ROC analysis of sensitivity and specificity at different cut-off levels, as shown in Figure 2.3, using the 25 control sera noted above and 40 sera from pigs vaccinated with the sub-unit vaccine. The specificity, specificity, efficiency and Youden's index of each ELISA at the corresponding cut-off value are summarized in Table 2.2.

Anti-ApxI, ApxII and ApxIII IgG prevalence in domestic pigs

High seroprevalence was found among domestic pigs in Korea, as shown in Figure 2.4. The rates of antibody positivity for ApxIA, ApxIIA and ApxIIIA antigens were 57.5, 73.13 and 42.19%, respectively. The highest rate was seen for sows when the surveyed pigs were grouped in four herds: suckling or pre-weaning piglets, post-weaning piglets, growing pigs and sows.

Discussion

In the current study, the recombinant ApxIA, ApxIIA and ApxIIIA proteins were used as ELISA antigens instead of employing native proteins. Each recombinant protein was visible as one single band strongly reactive with vaccinated pig sera in

Western blot analysis (Figure 2.2), suggesting that the developed recombinant proteins produced and purified herein have effective antigenicity enough for ELISA tests. Using each of the cut-off values, ApxIA-, ApxIIA-, and ApxIIIA-ELISAs had sensitivities of 1, 0.95, and 0.925 and specificities of 0.96, 0.96, and 0.96, respectively, when 25 serum samples from 10 colostrum-deprived piglets and 15 non-vaccinated piglets and 40 serum samples from vaccinated pigs were used as test serum controls (Table 2.2 and Figure 2.3). The ELISA results clearly indicate that the assay developed can be used for the detection of serum antibodies to three *A. pleuropneumoniae* exotoxins, i.e., ApxI, ApXII and ApxIII. When the present ELISAs were applied to a seroepidemiologic study of anti- *A. pleuropneumoniae* exotoxin antibodies in 320 randomized pigs in Korea, high rates of positive antibody were observed in animals of all groups (i.e., suckling piglets, weaned piglets, growing pigs, and sows), especially in sows, which gave the highest mean OD value in the ApxIIA-ELISA test (Figure 2.4). Such finding may be related to the facts that serotype 2, 5 and 6 are prevalent in Korea, of which all produce ApxIIA, as noted earlier (Frey, 1995; Min and Chae, 1999; Nielsen *et al.*, 2000). Also, the results seemed to show an age-dependent prevalence, except for suckling piglets (Figure 2.4). Considering that the mean OD value of weaned piglets in any one of ApxIA-, ApxIIA- and ApxIIIA-ELISA tests was lower than that of suckling piglets (Figure 2.4), anti-Apx toxin antibody activity in sera of preweaned animals may have been due to maternal antibodies that disappear gradually after weaning. Although there is evidence for geographic boundaries of pig infection by particular

serotypes of *A. pleuropneumoniae*, emergence of an unexpected serotype should always be taken into consideration in naive pigs (Lo *et al.*, 1998). An expanded serologic survey would be needed to understand the present status of *A. pleuropneumoniae* prevalence, which also is important in planning and evaluating *A. pleuropneumoniae* vaccination programs. The exotoxin-specific ELISA developed in the current study may provide a useful screening tool for pleuropneumonia control and prevention in swine farms.

Table 2.1. Primer sequences used for cloning and expression of the *Actinobacillus pleuropneumoniae* ApxIA, ApxIIA and ApxIIIA toxins*

Toxin	Direction	Primer sequences (5'-3')	PCR parameter†
Apx IA (2269-3069) ‡	Forward	GGAGACGACGGTAATGATGTA	94, 57, 72 20s, 10s, 60s
	Reverse	TTAAGCAGATTGTGTTAAATAATTACT	
Apx IIA (1-2883) ‡	Forward	GGATCCATGTCAAAAATCACTTTGTCA	94, 60, 72 20s, 10s, 120s
	Reverse	GGATCCTTAAGCGGCTCTAGCTAATTG	
Apx IIIA (760-1374) ‡	Forward	AGCTTACTTTCAGGAGTAACTGCA	94, 56, 72 20s, 10s, 60s
	Reverse	AGCTTTATGACGAGCATCATAGC	

*PCR = polymerase chain reaction. GenBank accession number in parenthneses.

Table 2.2. Results of *Actinobacillus pleuropneumoniae* toxin ApxIA, ApxIIA and ApxIIIA-enzyme linked immunosorbent assays in control pig sera at each cut-off values*

***Actinobacillus pleuropneumoniae* toxins in control pig sera**						
	ApxIA		**ApxIIA**		**ApxIIIA**	
	Positive	**Negative**	**Positive**	**Negative**	**Positive**	**Negative**
ELISA positive	**40[a]**	**1[c]**	**38[a]**	**1[c]**	**37[a]**	**1[c]**
ELISA negative	**0[b]**	**24[d]**	**2[b]**	**24[d]**	**3[b]**	**24[d]**
Sensitivity†	**1**		**0.95**		**0.925**	
Specificity†	**0.96**		**0.96**		**0.96**	
Percent efficiency†	**98.5**		**95.4**		**93.9**	
Youden's index†	**0.96**		**0.91**		**0.885**	

***** ELISA = enzyme linked immunosorbent assay; Se = Sensitivity; Sp = Specificity

† Formulas: Se = a/(a+b); Sp = d/(c+d); Efficiency = (a+d)/(a+b+c+d) x 100; Youden's index = Se + Sp -1

Table 2.3. Seroplevalence rate of the *Actinobacillus pleuropneumoniae* toxins ApxIA, ApxIIA and ApxIIIA using the developed enzyme linked immunosorbent assay in Korean domestic pigs*

Group	ApxIA	ApxIIA	ApxIIIA
Sucking piglets (n=80)	40	92.5	46.25
Weaned piglets (n=80)	46.25	50	18.75
Growing pigs (n=80)	71.25	51.25	17.5
Sows (n=80)	72.5	98.75	86.25

*Total sample size was 320 pigs.

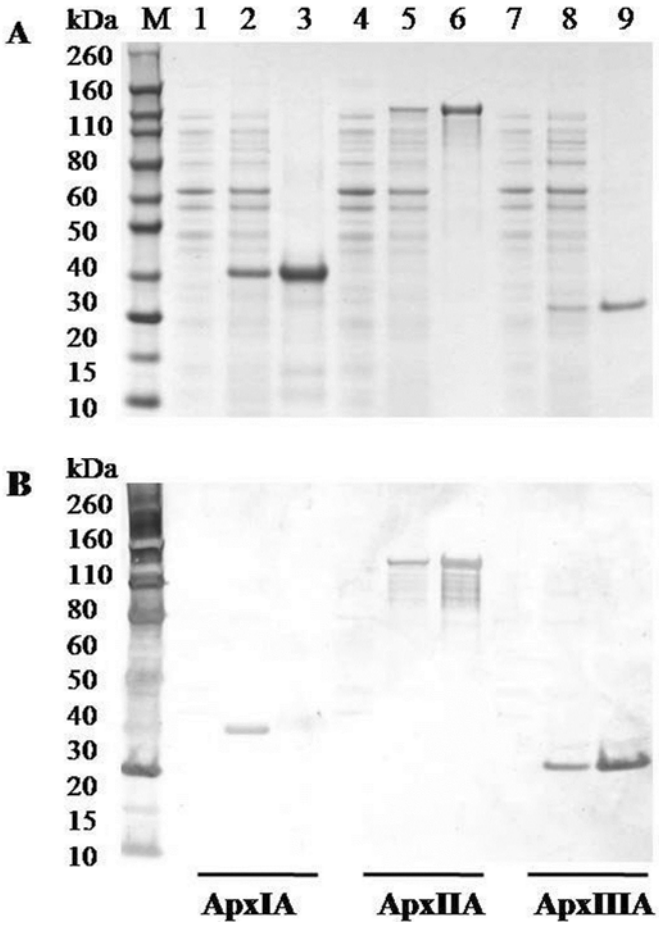


Figure 2.1. Purification of the recombinant *Actinobacillus pleuropneumoniae* toxins ApxIA, ApxIIA and ApxIIIA. The recombinant ApxIA, ApxIIA and ApxIIIA were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (A) and Western blot (B) with an anti-Histidine antibody; lane M, prestained protein molecular mass marker (kDa); lanes 1, 4 and 7, uninduced clone of ApxIA, ApxIIA and ApxIIIA; lane 2, 5 and 8, induced clone ApxIA, ApxIIA and ApxIIIA; lanes 3, 6 and 9, purified ApxIA, ApxIIA and ApxIIIA, respectively.

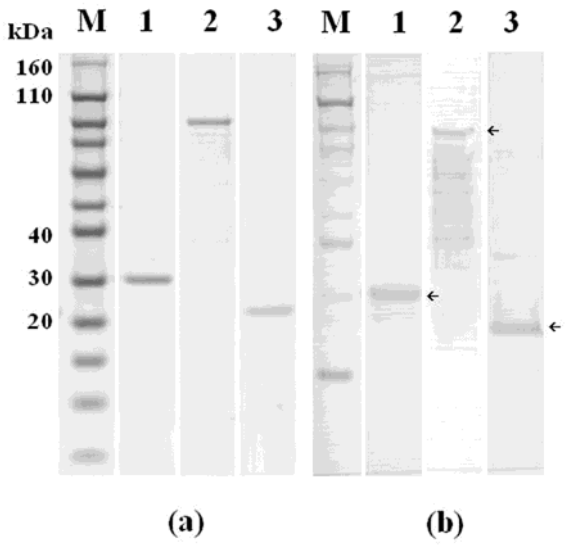


Figure 2.2. Immunoblots of the purified recombinant *Actinobacillus pleuropneumoniae* toxins ApxIA, ApxIIA and ApxIIIA for its antigenicity. The proteins were characterized by SDS-PAGE (a) and Western blot (b) using positive pig serum. Lane M, prestained protein molecular mass marker (kDa); lane 1, recombinant ApxIA; lane 2, recombinant ApxIIA; lane 3, recombinant ApxIIIA.

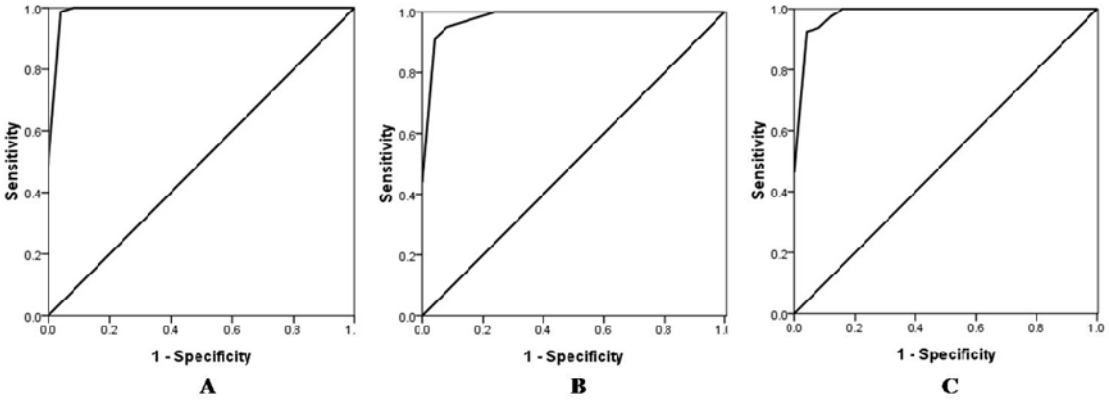


Figure 2.3. Receiver-operating characteristic (ROC) curves of ApxIA(A), ApxIIA(B) and ApxIIIA(C)-ELISA assays for the diagnosis of *A. pleuropneumoniae*. The area under the ROC (AUC) of ApxIA, ApxIIA and ApxIIIA-ELISA assays was 0.999, 0.989, 0.992 (95% confidence interval; P<0.001). The horizontal line belongs to the test curve, and the diagonal line shows a non-informative test curve.

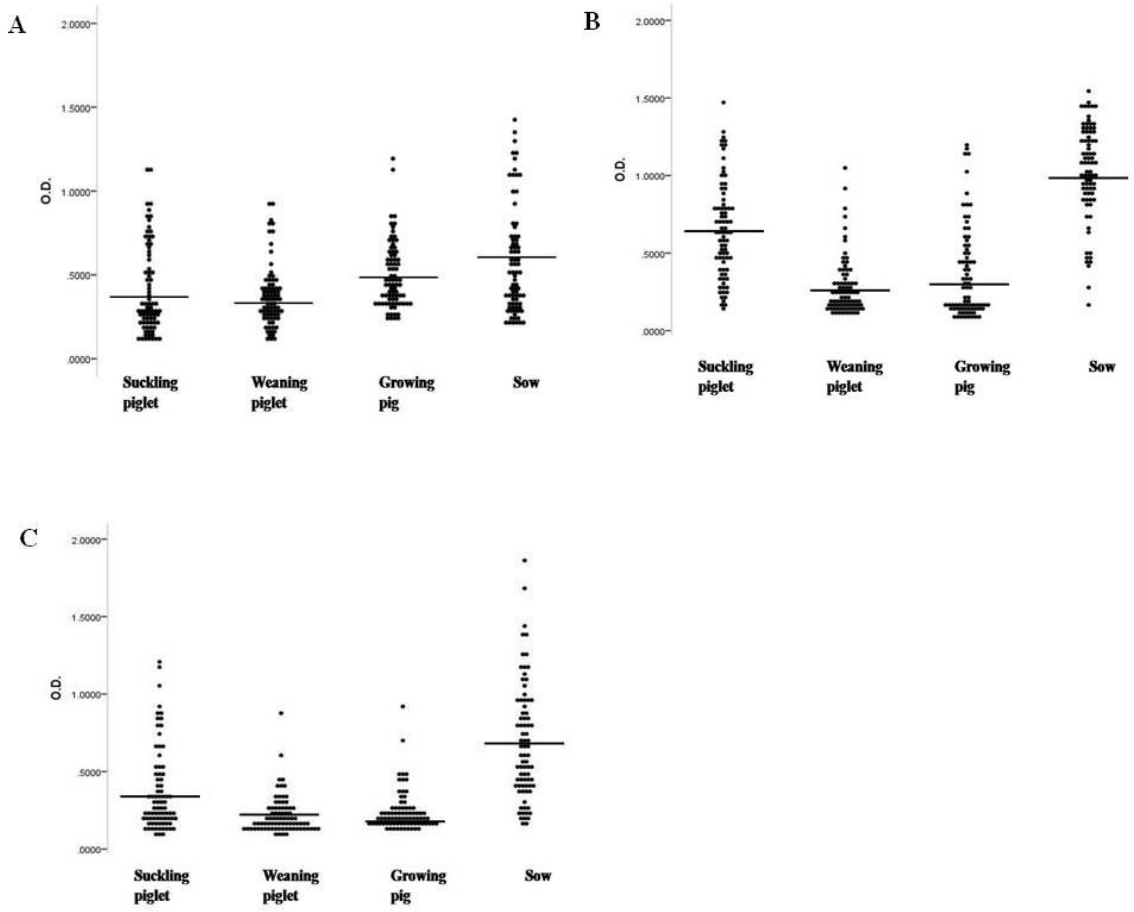


Figure 2.4. Distribution of anti-*Actinobacillus pleuropneumoniae* toxins ApxIA(A), ApxIIA(B) and ApxIIIA(C) antibodies in domestic pigs. Serum samples from 320 pigs were tested by ApxIA, ApxIIA and ApxIIIA- ELISAs. Mean values of optical density $(OD)_{405}$ were noted by a horizontal line in each group, which is divided age-dependently.

Chapter III

Systemic and mucosal immune responses of the surface-displayed neutralizing epitope of ApxIIA exotoxin in *Saccharomyces cerevisiae* by oral vaccination in mice

Abstract

An oral delivery system based on *Saccharomyces cerevisiae* expressing surface-displayed ApxIIA#5 was studied for its potential to induce immune responses. Dendritic cells (DCs) stimulated with *S. cerevisiae* expressing surface-displayed ApxIIA#5 *in vitro* upregulated maturation and activation markers, accompanied by production of TNF-α, IL-1β, IL-12p70, and IL-10. Presentation of ApxIIA on activated DCs to CD4+ T cells from mice orally administered with *S. cerevisiae* expressing surface-displayed ApxIIA#5 elicited specific T cell proliferation. In addition, mice orally administered with *S. cerevisiae* expressing surface-displayed ApxIIA#5 showed higher antigen-specific IgG and IgA antibody responses in serum and higher numbers of IgG and IgA antigen-specific antibody secreting cells in the spleen, Peyer's patches, and lamina propria than those with the vector-only *S.*

cerevisiae or non-treated. Furthermore, the vaccinated mice induced Th1-type immune responses based on increased levels of IgG2a in serum and predominant increase of IFN-γ producing cells in spleen, and lamina propria. These results suggest that *S. cerevisiae* expressing surface-displayed ApxIIA#5 may be a promising candidate for an oral vaccine delivery system to elicit systemic and mucosal immunity.

Keywords: *Saccharomyces cerevisiae*, ApxIIA, oral immunization, mice

Introduction

Saccharomyces cerevisiae has been typically used for the oral application of vaccines and drugs, as a Generally Recognized as Safe (GRAS) organism (Schreuder *et al.*, 1996; Beier and Gebert, 1998). Currently, there is great interest in developing mucosal vaccines, especially by the oral route, because such vaccines could not only induce locally and systemically protective immune responses against infectious disease, but would also be safe and convenient with regard to administration. Several oral delivery systems using a live oral vaccine such as a *Salmonella typhimurium* mutant, *Lactobacillus* spp., or *Saccharomyces cerevisiae* (Scheppler *et al.*, 2002; Shin *et al.*, 2005; Kulkarni *et al.*, 2010) have been attempted. Among these delivery systems, the yeast expression system, *S. cerevisiae*, has several advantages: high expression levels, ease of scale-up, low cost, and the adjuvant potential of yeast cell-wall components such as ß-1,3-d-glucan and mannan (Stubbs *et al.*, 2001). Yeast-based expression systems have been developed and successfully used to produce recombinant proteins (Schreuder *et al.*, 1996; Stubbs *et al.*, 2001). These systems have been used in pharmaceutical, livestock feed, and food industry applications (Park *et al.*, 2007).

Recently, heterogous proteins have been displayed on the surfaces of yeast cells by a genetic engineering technique of yeast cell-surface display (Schreuder *et al.*, 1996; Ueda and Tanaka, 2000; Park *et al.*, 2007; Kim *et al.*, 2010). This system could be a good candidate for a live oral vaccine carrier, providing the stable

maintenance of surface-expressed epitopes with a protein displayed at high-density (Ueda and Tanaka, 2000).

Actinobacillus pleuropneumoniae is a causative agent of porcine pleuropneumonia, a highly contagious endemic disease of pigs, inducing significant economic losses worldwide (Haesebrouck *et al.*, 1997; Bosse *et al.*, 2002). *A. pleuropneumoniae* can result in various clinical signs from peracute to chronic, with infected pigs typically showing a hemorrhagic, necrotizing pneumonia often associated with fibrinous pleuritis (Haesebrouck *et al.*, 1997). ApxII toxin, which is believed to be involved in the virulence of *A. pleuropneumoniae*, has been used as a vaccine protein (Lee *et al.*, 2006). The antigenic determinants of ApxIIA (ApxIIA#5) were found to induce a strong protective immune response against *A. pleuropneumoniae* (Seo *et al.*, 2011). ApxIIA, expressed in either *Saccharomyces cerevisiae* or *Nicotiana tabacum*, has previously been reported to be capable of inducing protective immune responses against *A. pleuropneumoniae* in mice (Shin *et al.*, 2005; Lee *et al.*, 2006; Shin *et al.*, 2007). Moreover, the surface-displayed ApxIIA#5-expressing *S. cerevisiae* was developed, and the induction of antigen-specific immune responses and protection against *A. pleuropneumoniae* in mice were assessed (Kim *et al.*, 2010). In the present study, we demonstrated that surface-displayed ApxIIA#5-expressing *S. cerevisiae* has the immunogenic potential as an oral vaccine, helping to improve both systemic and mucosal immune responses.

Materials and Methods

Preparation of vaccines and oral vaccination of mice

Saccharomyces cerevisiae expressing surface-displayed ApxIIA#5 was prepared as previously described (Kim *et al.*, 2010). Briefly, the yeast was cultured in a selective medium (-URA medium: Casamino acid 5 g, Yeast nitrogen base 6.7 g, Glucose 20 g, Adenine 0.03 g, and Tryptopan 0.03 g in 1L of Distilled Water) for 16 h at 30 °C, and then transferred and cultured in basic medium (YEPD: Yeast extract 10 g, Bactopeptone 20 g, and Glucose 20 g in 1L of DW) for three days at 30 °C. Either yeast harboring control vector or yeast expressing surface-displayed ApxIIA#5 was washed in saline and diluted to a titer of 5 x 10^8 cells/ml in phosphate-buffered saline (PBS).

Female C57BL/6 mice at 5 weeks of age (Central Lab. Animal Inc., Korea) were used throughout this study following policy and regulations for the care and use of laboratory animals (Laboratory animal center, Seoul National University, Korea). All animals were provided with standard mouse chow and water *ad libitum*. The *S. cerevisiae* expressing surface-displayed ApxIIA#5 (vaccinated group) and the vector-only *S. cerevisiae* (vector control group) were orally administered for 2 days with 1.5 x 10^9 cells/day per mouse through an oral gavage at ten days intervals, 3 times. Non-treated mice were also contained in the experimental group as a mock

control. The specimens and serum samples were collected after three days at each immunization.

Preparation of primary cells

Murine dendritic cells (DCs) were isolated from bone marrow progenitors following previously described procedures (Lutz *et al.*, 1999). Bone marrow cells were cultured in RPMI 1640 medium (Gibco Invitrogen, Karlsruhe, Germany) in the presence of 10% heat-inactivated fetal bovine serum (FBS; Gibco Invitrogen, Karlsruhe, Germany), 10 ng/ml recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF) (PeproTech, London, UK) and 5 ng/ml recombinant IL-4 (PeproTech, London, UK). Non-adherent cells were collected and used for further experiments on day 10. The purity of the cells, assessed by flow cytometry using phycoerythrin (PE)-conjugated anti-CD11c mAb (Abcam Inc., Cambridge, UK), was 91.1 ± 0.92 %. Single cell suspensions were obtained from spleen (SP), intestinal lamina propria (LP), and Peyer's patches (PP) for T cell proliferation and ELISPOT assays, as previously described (Jackson *et al.*, 1993; Shin *et al.*, 2011a).

Activation of DC by transgenic *Saccharomyces cerevisiae*

To examine in vitro activation of DCs by transgenic *S. cerevisiae*, immature DCs

(1 x 10^6 cells/ml) were stimulated with *S. cerevisiae* expressing surface-displayed ApxIIA#5 or the vector-only *S. cerevisiae* (1 x 10^6 cells/ml). After 48 h, cells were harvested for flow cytometry, and supernatants were collected and stored at -80°C for analysis of cytokine secretion by quantitative ELISA. The secreted concentrations of TNF-a, IL-1ß, IL-10, and IL-12p70 were measured using the ELISA method (eBioscience Inc., San Diego, CA, USA). The activation and upregulation of costimulatory molecules in DCs were examined using a FACScalibur flow cytometer (BD Biosciences, San Jose, CA, USA). After harvest, DCs were washed and fixed with 4% paraformaldehyde in PBS, followed by labeling with FITC-conjugated antibodies against major histocompatibility complex (MHC) class II, CD40, and CD86 (all from Abcam Inc.). CellQuest software (BD Biosciences) was used for the analysis of flow cytometry data.

CFSE-based T cell proliferation assay

T cells were isolated from spleens of mice immunized with the surface-displayed ApxIIA#5-expressing *S. cerevisiae*, the vector-only *S. cerevisia,* or those that were not immunized, one week after the final administration. The cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) following previously described procedures (Rosa *et al.*, 2011). The labeled cells (5 x 10^6 cells) were cultured for four days with the Apx-activated DCs (1 x 10^6 cells) and stained with anti-mouse CD4 phycoerythrin (PE) monoclonal antibody (Abcam Inc.) for 45 minutes at 4°C.

Cells were then washed twice with Dulbecco's phosphate-buffered saline (DPBS, Gibco Invitrogen) containing 5% FBS and fixed with 4% paraformaldehyde. Cells were acquired on a FACScalibur flow cytometer (BD Biosciences) and then analyzed using FlowJo software (version 7.6.5, Tree Star, San Carlo, CA, USA). The percentage of CFSE-low cells was expressed as the mean ± SEM.

ELISA assays

In order to investigate antigen-specific antibody activities, serum samples were analyzed by an ELISA assay as in a previous study with slight modification (Shin *et al.*, 2011b). The plate was coated with 100 pg of the recombinant ApxIIA suspended in 100 μl of PBS and blocked with PBST containing 1% bovine serum albumin (BSA; Amresco Inc., Solon, OH, USA). The diluted sera (1:20) were added to the plate, and horseradish peroxidase-conjugated goat anti-mouse IgG (H + L) (Bio-Rad Co., Hercules, CA, USA), horseradish peroxidase-conjugated anti-mouse IgA (α-chain specific) (Bethyl Laboratories Inc., Montgomery, TX, USA), or horseradish peroxidase-conjugated anti-mouse IgG1/IgG2a (Serotec Ltd., Oxford, UK) (1:2000 in PBST containing 1% BSA) was used as a secondary antibody. The color development was carried out using 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Sigma Co., St. Louis, MO, USA). The TMB reaction was stopped with 2 M H_2SO_4 and measured at 450 nm using an Emax Precision microplate reader (MDS Inc., Sunnyvale, CA, USA).

Detection of IFN-γ, IL-4, IgG or IgA producing cells by ELISPOT assay

SP, LP, and PP cell suspensions were assayed for frequencies of specific cytokine- and antibody-producing cells by the ELISPOT assay kit for mouse IFN-γ, IL-4, IgG or IgA according to the manufacturer's instructions (Mabtech Inc., Stockholm, Sweden). Spots were counted using an automated reader.

Statistical analysis

Statistical significance (p-values) was calculated using Tukey's test with the statistical program Statistical Package for Social Sciences software (version 17.0, SPSS Inc., Chicago, IL, USA). Differences were considered significant if a value of $p < 0.05$ was obtained. All experiments were repeated at least three times.

Results

Activation of DC by *Saccharomyces cerevisiae* expressing surface-displayed ApxIIA#5

The concentrations of transgenic *S. cerevisiae* for DCs were optimized, followed

by stimulation at different ratios of DCs: transgenic *S. cerevisiae* (4:1, 1:1, or 1:4) and the activity of the DCs were determined by the expression of marker CD86. The highest differences were shown in *S. cerevisiae* expressing surface-displayed ApxIIA#5, compared to the vector-only *S. cerevisiae*, when a ratio of 1:1 was used (data not shown). Treatment of immature DCs with the surface-displayed ApxIIA#5-expressing *S. cerevisiae* or the vector-only *S. cerevisiae* (1:1) induced a significant upregulation of surface MHC class II molecules and the activation markers CD40 and CD86 ($p < 0.05$) (Table 3.1). The DC-stimulatory potential of the transgenic *S. cerevisiae* was also shown by the induction of the cytokines TNF-α, IL-12p70, IL-1β, and IL-10 (Figure 3.1). The surface-displayed ApxIIA#5-expressing *S. cerevisiae* was sufficient to induce strong secretion of the pro-inflammatory cytokines TNF-α, IL-12p70, and IL-1β, and the Th2-inducing cytokine IL-10, compared to the vector-only *S. cerevisiae*.

T cell proliferation after restimulation with ApxIIA-activated DCs

DCs were stimulated with the recombinant ApxIIA to produce ApxIIA-activated DCs and then present to T cells from the experimental mice. T cell proliferation was analyzed by examining CFSE division profiles. CFSE-low cells of the mock control and vector control groups indicated similar levels of 51.4 % and 51.6 %, respectively, but the vaccinated group showed an enhancement of CD4 T cell proliferation, with 81.8% CFSE-low cells. The CD4+ T cell proliferation was four

times greater in the vaccinated group compared with the control groups ($p < 0.001$). Presentation of ApxIIA on activated DCs to T cells from the experimental mice after a third immunization elicited specific proliferation of CD4+ T cells (Figure 3.2).

Enhancement of antigen-specific IgG and IgA antibody responses in mice orally immunized with *Saccharomyces cerevisiae* expressing surface-displayed ApxIIA#5

In order to assess the potential of the surface-displayed antigen-expressing *S. cerevisiae* in an oral delivery system, antigen-specific antibody responses were determined in sera and cell suspensions from SP, LP, PP of mice orally immunized with the vector-only *S. cerevisiae* and the surface-displayed ApxIIA#5-expressing *S. cerevisiae*. As shown in Figure 3.3, high IgG and IgA antibody activities were maintained in sera of the vaccinated group after the final immunization. The group immunized with the surface-displayed antigen-expressing *S. cerevisiae* showed higher specific IgA responses to ApxIIA in sera than those treated with the vector-only *S. cerevisiae* ($p < 0.05$).

The numbers of antigen-specific IgG and IgA antibody-producing B cells increased significantly in the SP, PP, and LP of the vaccinated group ($p < 0.05$) (Figure 3.4). In particular, the numbers of antigen-specific IgA antibody-producing cells in the PP were significantly higher than those in the LP and SP.

Induction of Th1 type responses in mice immunized with *Saccharomyces cerevisiae* expressing surface-displayed ApxIIA#5 by oral route

IgG subclasses were assessed to determine the basis of the Th1 and Th2 type immune responses induced in the serum of the mice immunized with transgenic *S. cerevisiae* by the oral route. The ApxIIA-specific IgG1 (Th2) subclass showed no differences among the experimental groups, while the ApxIIA-specific IgG2a (Th1) subclass increased significantly in the vaccinated group ($p < 0.01$) (Figure 3.3). The numbers of specific and IL-4 producing cells of the vaccinated mice were higher than those of the control mice in SP and CD4+ T cells. In particular, the number of IFN-γ-producing cells of the vaccinated mice predominantly increased in LP, SP, and CD4+ T cells compared with the control groups ($p < 0.05$) (Figure 3.4). Collectively, the levels of Th2-promoting cytokine (IL-4) were less than the levels of Th1-promoting cytokines (IFN-γ).

Discussion

In our previous study, the surface-displayed ApxIIA#5 expressing *S. cerevisiae* and the full ApxIIA-expressing *S. cerevisiae* were developed, and the induction of antigen-specific immune responses and protection against *A. pleuropneumoniae* in

mice by oral immunization was demonstrated (Shin *et al.*, 2005; Kim *et al.*, 2010). However, further study is needed to understand mucosal immune responses induced by transgenic *S. cerevisiae* in order to develop an efficient oral vaccine.

The surface-displayed ApxIIA#5-expressing *S. cerevisiae* was selected as an oral vaccine for porcine pleuropneumoniae, demonstrating the highest specific antibody activities in mice compared to other yeasts including the ApxIIA#5-secreting *S. cerevisiae* and the full-ApxIIA expressing *S. cerevisiae*. DCs induce primary immune responses and have a key role in both innate and adaptive immunity as antigen-presenting cells (APCs) (Lipscomb and Masten, 2002). In an adaptive immune response, the phenotype and function of DCs determine the initiating tolerance, memory, and polarized Th1 and Th2 differentiation (Lipscomb and Masten, 2002). Stimulation of bone marrow-derived DCs with the surface-displayed ApxIIA#5-expressing *S. cerevisiae* in vitro indicated that it could generally induce the secretion of pro-inflammatory cytokines TNF-α, and IL-1β, the Th1-inducing cytokine IL-12p70, and the Th2-inducing cytokine IL-10. Moreover, maturation of APCs was determined by showing upregulation of costimulatory molecules, CD40, and CD86 and surface MHC class II, which are required for efficient stimulation of T cells (Remer *et al.*, 2009).

Mucosal protection requires generation of antigen-specific T cells and antibodies (Fahlen-Yrlid *et al.*, 2009). In addition, $CD11c^{high}$ conventional DCs (cDCs) are reported to be essential for activation of CD4+ T cells and generation of specific antibodies following an ablation of immune responses after oral and nasal

immunization in mice depleted of cDCs in vivo (Fahlen-Yrlid *et al.*, 2009). In the present study, we demonstrated that surface-displayed ApxIIA#5-expressing *S. cerevisiae* helped to improve both systemic and mucosal immune responses by resulting in generation of antigen-specific antibodies and proliferation of CD4+ T cells stimulated with the activated-DCs in mice by oral vaccination.

Presentation of ApxIIA on activated DCs to CD4+ T cells from mice of the vaccinated group elicited specific T cell proliferation. The induction of ApxIIA-specific T cell proliferation demonstrated that ApxIIA was effectively presented on DCs, and the surface-displayed ApxIIA#5-expressing *S. cerevisiae* induced a cellular immune response in mice by the oral route. Ag-specific IgG as well as Ag-specific IgA antibody activities in serum increased in the vaccinated group. Furthermore, the number of both Apx-specific IgG and IgA antibody-producing cells in PP, LP, and SP were significantly higher in the vaccinated group than in the control group. PP and LP serve as an inductive site and as an effector site of intestinal mucosal immune reactions, respectively (Jang *et al.*, 2004). Therefore, the present results support the previous findings that surface-displayed ApxIIA#5-expressing *S. cerevisiae* is helpful in improving the mucosal immune response.

The ApxIIA-specific IgG2a subclass was significantly higher in the serum of the vaccinated group than in that of the control groups. Although specific IL-4 cytokine-producing cells were considerably increased in the SP of the vaccinated group, specific IFN-γ-producing cells were predominantly produced in the LP and SP of the vaccinated group. Consequently, a preponderance of IFN-γ responses and

the ApxIIA-specific IgG2a subclass indicated the induction of a Th1-type immune response. The lymphocyte population in the PP is mainly composed of 80% B cells with T cells, and the LP lymphocyte population is composed of 60% T cells and 30% B cells (Lefrancois and Lycke, 2001). Therefore, we found elevated IgG- and IgA-secreting cells and IFN-γ-producing cells predominantly in the PP and LP, respectively. These results suggest that oral administration of the surface-displayed ApxIIA#5-expressing *S. cerevisiae* induces both systemic and mucosal immune response in mice. Thus, this study is expected to contribute to the application of *S. cerevisiae* engineered by yeast cell-surface display techniques as a live oral vaccine.

Table 3.1. Upregulation of the activation markers CD40, MHC classII and CD86 on the surface of DC after stimulation with the vector-only *S. cerevisiae* and *S. cerevisiae* expressing surface-displayed ApxIIA#5.

	CD40	MHC classII	CD86
Mock control	11.32±2.09	26.4±1.13	18.86±0.64
Vector control	60.3±3.11	65.2±2.40*	62.75±1.41*
S. cerevisiae expressing surface-displayed ApxIIA#5	68.1±0.57	76.05±1.48*	72.075±0.07*

(*, $p < 0.05$)

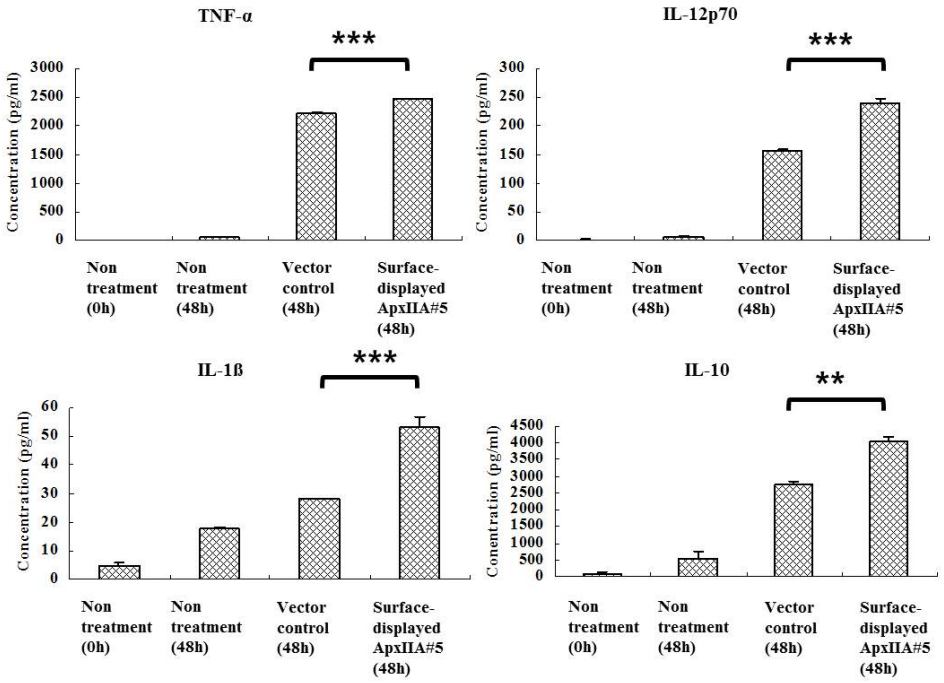


Figure 3.1. Quantitative analysis of cytokines secreted from dendritic cells after stimulation with the vector-only *S. cerevisiae* and the surface-displayed ApxIIA#5-expressing *S. cerevisiae* (**, $p<0.01$; ***, $p<0.001$).

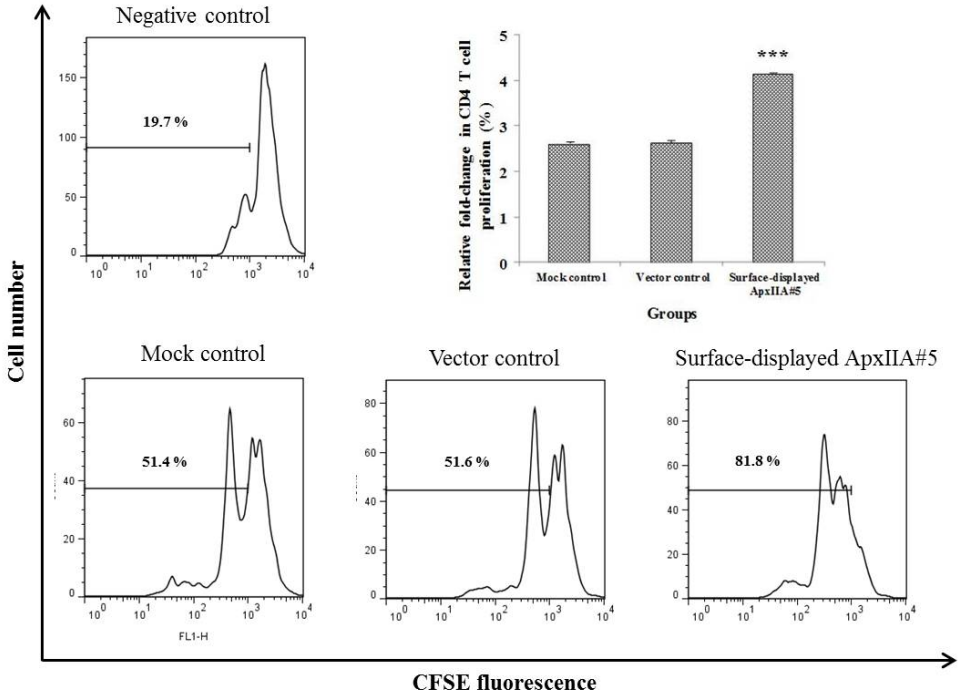


Figure 3.2. Proliferation of CFSE-labeled CD4+ T cells in the host spleen was examined 4 days later by FACS. Percentages within each panel indicate the fraction of cells that underwent proliferation. Negative control, CD4+ T cells without treatment; Mock control, Vector control, and Surface-displayed ApxIIA#5, CD4+T cells from mock control, vector control, and vaccinated group with treatment of ApxIIA-activated DC, respectively. (***, $p < 0.001$)

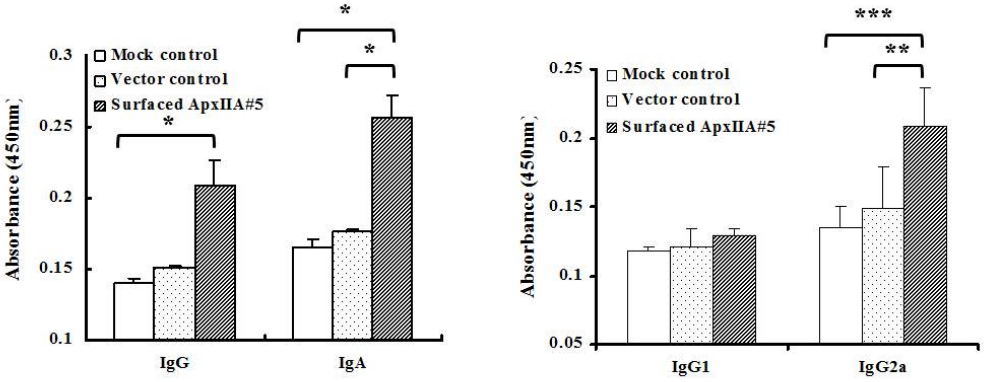


Figure 3.3. The level of ApxIIA-specific antibodies at 1 week after final oral immunization. A, ApxIIA-specific IgG and IgA in sera from mice; B, ApxIIA-specific IgG subclasses in sera from mice (*, $p<0.05$).

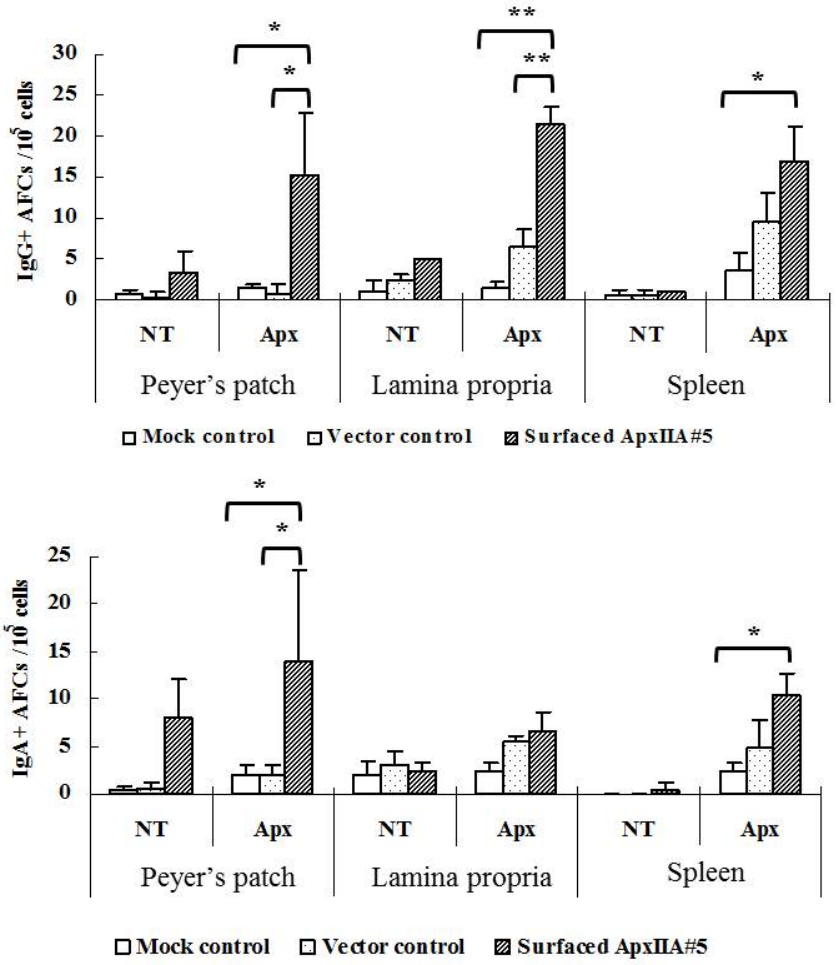


Figure 3.4. Frequency of antigen-specific immune cells in peyer's patch, lamina propria and spleen. Frequency of IgG and IgA-secreting B cells from mice were measured by ELISPOT. NT, non-treatment; Apx, treatment with ApxIIA (*, $p<0.05$; **, $p<0.01$)

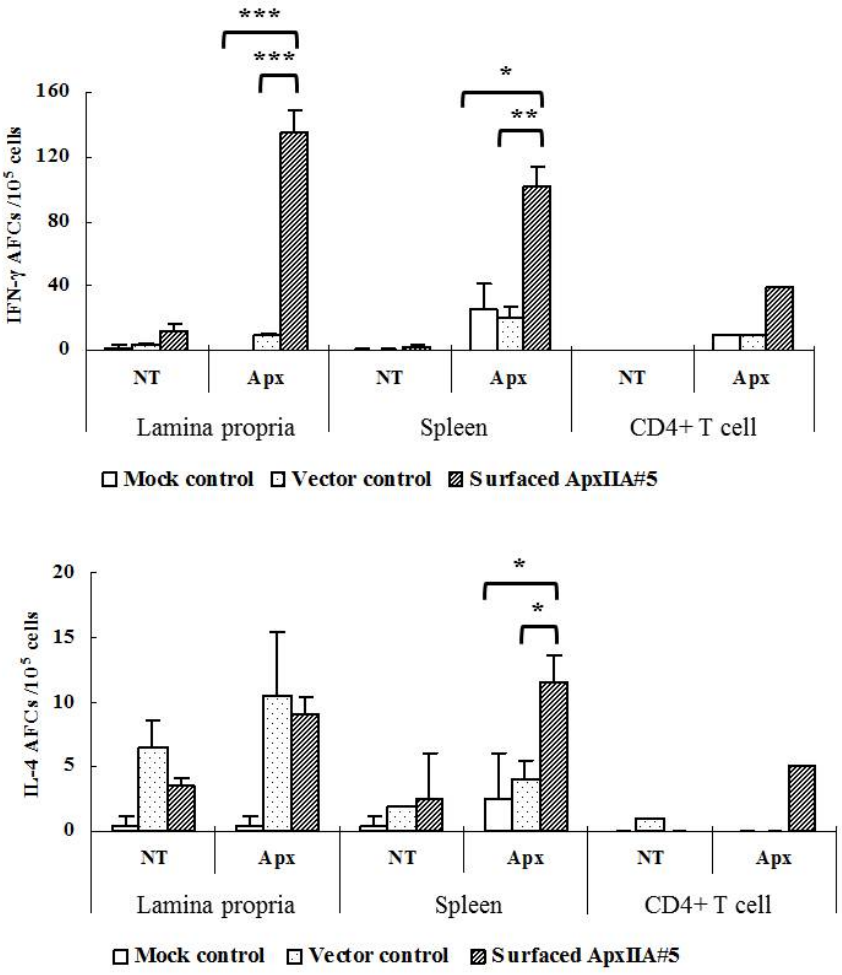


Figure 3.5. Frequency of antigen-specific immune cells in lamina propria, spleen and CD4+ T cell. Frequency of IFN-γ-, IL-4-secreting cells from mice were measured by ELISPOT. NT, non-treatment; Apx, treatment with ApxIIA (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

Chapter IV

Induction of protective immune responses against challenge of *Actinobacillus pleuropneumoniae* by oral administration with *Saccharomyces cerevisiae* expressing Apx toxins in pigs

Abstract

Actinobacillus pleuropneumoniae is a causative agent of porcine pleuropneumonia, a highly contagious endemic disease of pigs worldwide, inducing significant economic losses worldwide. Apx toxins, which are correlated with the virulence of *A. pleuropneumoniae*, were expressed in *Saccharomyces cerevisiae* and its possible use as an oral vaccine has been confirmed in our previous studies using a murine model. The present study was undertaken to test the hypothesis that oral immunization using *S. cerevisiae* expressing either ApxI or ApxII could protect pigs against *A. pleuropneumoniae* as an effective way of inducing both mucosal and systemic immune responses. The surface-displayed ApxIIA#5 expressing *S. cerevisiae* and the ApxIA expressing *S. cerevisiae* were developed to serve as an oral vaccine candidate in pigs. The vaccinated pigs showed higher specific IgG- and IgA-related antibody activities than the non-

treated control and vector control pigs. Additionally, the induced immune responses are found to protect pigs infected with *A. pleuropneumoniae* according to the analysis of clinical signs and the gross and microscopic pulmonary lesions. These results suggested that the surface-displayed ApxIIA#5 and ApxIA in *S. cerevisiae* might be a potential oral vaccine to protect pigs against porcine pleuropneumonia. Thus the present study is expected to contribute to the development of a live oral vaccine against porcine pleuropneumonia as an alternative to current conventional vaccines.

Keywords: *Actinobacillus pleuropneumoniae*, ApxIA, ApxIIA, oral immunization, *Saccharomyces cerevisiae*, pig

Introduction

Actinobacillus pleuropneumoniae is a causative agent of porcine pleuropneumonia, a highly contagious endemic disease of pigs worldwide, inducing significant economic losses worldwide (Haesebrouck *et al.*, 1997; Zhang *et al.*, 2000; Bosse *et al.*, 2002). *A. pleuropneumoniae* can result in various clinical signs from peracute to chronic, with infected pigs typically showing a hemorrhagic, necrotizing pneumonia often associated with fibrinous pleuritis (Haesebrouck *et al.*, 1997). Many large units may have chronic problems with flare-ups of the acute disease (Zhang *et al.*, 2000). The substantial economic losses in these herds per year can be up to £25,000 for a typical 600 sow breeder-feeder farm in the UK (Zhang *et al.*, 2000).

Although the respective contributions of the virulence factors to the infection and disease are not fully understood, Apx toxins are believed to be involved in the virulence of the 15 serotypes of *A. pleuropneumoniae* (Tascon *et al.*, 1994; Frey, 1995; Reimer *et al.*, 1995). Apx toxins are highly immunogenic, thus inducing a strong antibody response to *A. pleuropneumoniae* infection (Frey and Nicolet, 1991; Frey *et al.*, 1992; Bosse *et al.*, 2002). ApxI and ApxII are necessary for full virulence in the development of clinical signs and typical lung lesions (Reimer *et al.*, 1995; Boekema *et al.*, 2004). Therefore, ApxI and ApxII appear to be central in any effective approach as an antigen in the development of a vaccine against *A. pleuropneumoniae*. ApxIIA, expressed in either *Saccharomyces cerevisiae* or

Nicotiana tabacum, has previously been reported to be capable of inducing protective immune responses against *A. pleuropneumoniae* in mice (Shin *et al.*, 2005; Lee *et al.*, 2006; Shin *et al.*, 2007). *Saccharomyces cerevisiae* expressing ApxIA has also been developed and confirmed previously (Shin *et al.*, 2003). The antigenic determinants of ApxIIA from *A. pleuropneumoniae* serotype 2 isolated in Korea was identified and found to induce a strong protective immune response against *A. pleuropneumoniae* (Seo *et al.*, 2011). To develop an effective vaccine for *A. pleuropneumoniae* based on transgenic antigen expression, the neutralizing epitope of ApxIIA (ApxIIA#5) was selected as a vaccine candidate for *A. pleuropneumoniae*. This smaller fragment can be expressed in large quantities due to the difficulty in the expression of large fragments in a heterologous expression system (Kim *et al.*, 2010; Seo *et al.*, 2011).

Baker's yeast (*Saccharomyces cerevisiae*) has been used for the oral application of vaccines and drugs, as a Generally Recognized as Safe (GRAS) organism, because it is relatively stable and nonpathogenic in the gut compared with the other biodegradable vehicles (Schreuder *et al.*, 1996; Beier and Gebert, 1998). There are several advantages of yeast expression systems: high expression levels, ease of scale-up, low cost, and adjuvant potential of yeast cell-wall components, such as β-1,3-d-glucan and mannan (Stubbs *et al.*, 2001). Yeast-based expression systems have been developed and successfully used to produce recombinant proteins (Schreuder *et al.*, 1996; Stubbs *et al.*, 2001). Furthermore cell-surface display in yeast, designed as an antigen delivery system, provide the stable maintenance of

surface-expressed epitopes with a high-density displayed protein (Ueda and Tanaka, 2000).

In our previous study, the surface-displayed ApxIIA#5 expressing *S. cerevisiae*, and the full ApxIIA expressing *S. cerevisiae* were developed and assessed the induction of antigen-specific immune responses and protection against *A. pleuropneumoniae* in murine model (Shin *et al.*, 2005; Kim *et al.*, 2010). Therefore, in the present study, these recombinant yeasts were evaluated by comparison with the antigen-specific immune response in mice for development of a novel oral vaccine against porcine pleuropneumonia. These results showed conclusively that specific immune responses to ApxIIA significantly increased in the mice fed with the surface-displayed ApxIIA#5 expressing yeast. The surface-displayed ApxIIA#5 and the ApxIA expressed in *S. cerevisiae* were ultimately evaluated by analysis of induction of antigen-specific immune response and protectivity against *A. pleuropneumoniae* in pigs.

Materials and Methods

Construction of the vector and transformation of yeast

The full ApxIA (GenBank accession no. AF363361, aa residues 1-1023), the full ApxIIA (GenBank accession no. AF363362, aa residues 1-957) and the

neutralizing epitope of ApxIIA (ApxIIA#5, aa residues 439-801) of Korean *A. pleuropneumoniae* were used as an antigen and *Saccharomyces cerevisiae* 2805 (MATα pep4::HIS3 prb1-δ can1 GAL2 his3 ura3-52) was used as the recipient cell for expression of ApxIA, ApxIIA and ApxIIA#5 (Shin *et al.*, 2003; Shin *et al.*, 2005; Kim *et al.*, 2010; Seo *et al.*, 2011).

The amylase 1A (Ramy1A) signal peptide (ASP) and the ApxIIA#5 gene were fused for yeast expressing secreted ApxIIA#5, and the fusion fragment was combined with the anchor DNA fragment containing the 3′ half of the α-agglutinin gene (AGA1-C320) encoding the C-terminal 320 amino acids in order to construct a surface displaying yeast vector, as described previously (Figure 4.1) (Kim *et al.*, 2010). The fusion fragments were cloned in pYEGPD vector between the glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter and the galactose-1-P uridyl transferase (GAL7) terminator. The full ApxIA and ApxIIA genes were cloned in pYEGPD vector for yeasts expressing ApxIA and ApxIIA, respectively (Shin *et al.*, 2003; Shin *et al.*, 2005). The recombinant vectors were transformed into *S. cerevisiae* 2805 (Shin *et al.*, 2003; Shin *et al.*, 2005; Kim *et al.*, 2010).

Transformed colonies were cultured in a uracil-deficient selective (0.5% casamino acid, 0.67% yeast nitrogen base, 2% glucose, 0.003% adenine, and 0.003% tryptopan) for 16 hours at 30°C, and then transferred and cultured in YEPD medium (1% yeast extract, 2% bactopeptone, and 2% glucose) for three days at 30°C. For the oral vaccine, the recombinant yeasts were lyophilized and ground to make the yeast powder.

Experimental animals, immunization and sample collections

Five-week-old BALB/c female mice (OrientBio Co., Ltd., Kyunggi-do, Korea) were used and provided with standard mouse chow and water *ad libitum*. All oral immunizations were preceded by an overnight fasting of the mice (water was provided *ad libitum*). The surface-displayed ApxIIA#5 expressing *S. cerevisiae*, the ApxIIA#5-secreting *S. cerevisiae*, the full-ApxIIA expressing *S. cerevisiae*, and the vector-only *S. cerevisiae* were lyophilized and ground to make the yeast powder. One hundred-fifty milligrams were dissolved into 1ml of phosphate-buffered saline (PBS) and orally administered at 200 μl (1.5×10^9 CFU/30mg) per mouse through an oral gavage at two weeks intervals, 4 times. Serum samples were collected after each immunization.

Three-week-old cross-bred pigs (Yokshire, Duroc, and Landrace; XPbio, Kyunggi-do, Korea) were used in this study. Three groups were designated as the untreated pigs (n=5, control), the pigs fed with the vector-only *S. cerevisiae* (n=5, vector control), and the experimental pigs fed with the ApxIA expressing *S. cerevisiae* and the surface-displayed ApxIIA#5 expressing *S. cerevisiae* at a time (n=10, vaccinated group), respectively. The yeast vaccines were followed by three-time administrations with the vaccines of 6×10^9 CFU with one week interval. Blood, nasal wash, and fecal samples were collected from the pigs at one week interval during the experimental period. The head was moved gently, and 10 ml of

sterile PBS injected into the nostrils from the pigs were collected to a collection cup for nasal wash. Fecal samples were obtained with a swab and diluted in 1 ml of sterile PBS. The nasal wash, and fecal samples were immediately stored at -20°C until use. All animals were used throughout this study following policy and regulations for the care and use of laboratory animals under the supervision of the Laboratory animal center committee at Seoul National University.

Measurement of Apx-specific antibody immune responses

Apx-specific immune responses were measured in sera of the experimental mice, sera, nasal wash, and fecal samples of the pigs by ELISA. The level of antigen-specific antibodies (IgA or IgG) in the samples was determined using previously described ELISA procedures (Shin *et al.*, 2011b). The diluted mice sera (1:20), pig sera (1:100), pig fecal samples (1:2), and the undiluted pig nasal wash as primary antibodies were then added to the plate, and incubated for 1 h at 37°C. After washing, 100 ml/well (1:1,000) of goat anti-mouse IgG (H + L)–HRP conjugate (Bio-Rad Laboratories Inc., Hercules, CA), goat anti-mouse IgA–HRP conjugate (Zymed Laboratories Inc., South San Francisco, CA), goat anti-pig IgG (H + L)–HRP conjugate (Bio-Rad Laboratories Inc., Hercules, CA), or goat anti-pig IgA–HRP conjugate (Bethyl Laboratories Inc., Montgomery, TX) was added to the plate, and incubated for 1 h at 37°C. Color was developed by adding 100 ml of Horseradish peroxidase substrate solution kit (Bio-Rad Laboratories Inc., Hercules,

CA) to the plate. Optical density was measured at 405 nm using an Emax Precision microplate reader (MDS Inc., Sunnyvale, CA).

Challenge with *A. pleuropneumoniae serotype 5*

After last boosting, three pigs of each control group and six pigs of vaccinated group were challenged intranasally with a dose (1.5×10^9 CFU) of *A. pleuropneumoniae* serotype 5 Korean isolate from a pig with porcine pleuropneumonia.

Clinical and pathological evaluation

The pigs were monitored for ten days to evaluate respiratory signs after the challenge with *A. pleuropneumoniae*. Various clinical signs were graded 0 if absent, and from 1 to 5 if present, including sneezing, nasal discharge, coughing, dyspnea, unable to rise and death, as previously described (Sadilkova *et al.*, 2012). Moreover, rectal temperature was taken on day 2, 5, 7, and 10. The pigs were euthanized and estimated lungs on days 10 after the challenge with *A. pleuropneumoniae*. Gross lesions scoring were used to determine the level of pneumonia in the pig lungs. These methods were performed by calculating the percentage of the lung surface that shows the lesions to give an overall lung score. After examining the lung lesions, a histopathological analysis was performed on 5 representative lesions of

pneumonic lung per a pig, which were fixed in 10% neutral buffered formaldehyde, embedded in paraffin wax, stained with haematoxylin and eosin, and analyzed by light microscopy. The histopathological scores were obtained from 0 to 5 by the severity of the inflammatory response such as haemorrhage, vascular thrombosis, oedema, necrosis and the presence of fibrinous exudate (Marois *et al.*, 2009). To quantify the results, the sum of the 5 lesions score was given for each pig lung, resulting in a maximum score of 25 per pig. To examine for clearance of the challenge bacteria, the cranial lobe of each lung and lung portions exhibiting lesions were plated on chocolate agar and incubated for 48 h at 37℃. Suspect colonies were confirmed as the challenge bacteria by the PCR method (Rayamajhi *et al.*, 2005).

Statistical analysis

Statistical significance was analyzed by Student's t-test with the statistical program Statistical Package for Social Sciences software (SPSS) version 17.0. Differences were considered significant if a value of $p < 0.05$ was obtained.

Results

Comparison of the recombinant yeast vaccines by oral administration in mice

To evaluate the efficacy of oral administration of the recombinant yeast vaccines in inducing immune responses, the levels of ApxIIA-specific antibodies were measured in the sera of the mice orally immunized with the recombinant yeasts: the vector-only *S. cerevisiae*, the ApxIIA#5-secreting *S. cerevisiae*, the surface-displayed ApxIIA#5 expressing *S. cerevisiae*, and the full ApxIIA expressing *S. cerevisiae*. ELISA results showed induction of ApxIIA-specific IgG and IgA in the sera of mice fed with the recombinant yeasts expressing antigen (Figure 4.2). In addition, high IgG antibody activity was maintained in the surface-displayed ApxIIA#5 expressing yeast administered group after the first immunization. In particular, specific IgA responses to ApxIIA in sera of the mice fed with the surface-displayed ApxIIA#5 expressing yeast significantly increased at the final immunization in sera ($p < 0.05$). Although specific IgG and IgA responses to ApxIIA was also increased in both groups fed with the ApxIIA#5-secreting yeast and the full-ApxIIA expressing yeast, the surface-displayed ApxIIA#5 expressing yeast administered group could induce higher immune responses in mice after oral vaccination, compared to the other recombinant yeasts. Therefore, the surface-displayed ApxIIA#5 expressing *S. cerevisiae* was selected as an oral vaccine candidate for application to pigs.

Induction of specific immune responses in pigs

As shown in Figure 4.3, the vaccinated pigs fed with the ApxIA expressing *S. cerevisiae* and the surface-displayed ApxIIA#5 expressing *S. cerevisiae* showed an enhancement of the production of antigen-specific IgG and IgA antibodies, compared to the vector control and control groups. The higher levels of ApxIA and ApxIIA-specific IgG and IgA in sera were observed in the vaccinated group in two weeks after last oral administration ($p < 0.05$). The vaccinated group showed higher secretion of Apx-specific IgA of nasal wash than the control and vector control groups ($p < 0.01$). Specifically, the ApxIIA-specific antibody activities showed significant discrimination in serum and nasal wash between the vaccinated and the control groups, compared with the ApxIA-specific antibody responses. However, there were not meaningful differences in the levels of Apx-specific fecal IgA between the vaccinated group and the control groups (data not shown).

Clinical evaluation after challenge with *A. pleuropneumoniae* serotype 5

The protective potency of the vaccines was examined after challenge with *A. pleuropneumoniae* serotype 5 isolated from Korea. The pigs of non-treated control group suffered from sneezing, coughing, dyspnea, and acute febrile response (39.3-41.3 °C) within ten days after challenge with *A. pleuropneumoniae* (Table 4.1). Although the short rise in rectal temperature was shown in the surface-displayed ApxIIA#5 expressing *S. cerevisiae* administered group at post-challenge day 5, the

non-challenged pigs also showed increase of rectal temperature at the same time. And then they were recovered from the fever at post-challenge day 7; however, rectal temperature of non-treated control and vector control groups showed the increasing current. All pigs in the vaccinated group survived during the experimental period after challenge, while one pig died in non-treated control and two in vector control within 48 h, showing peracute clinical symptoms: dyspnea, anorexia, ataxia, severe respiratory distress, and sudden death (Bosse *et al.*, 2002). In the vaccinated group, one pig showed a mild sneezing and coughing; however, the other pigs remained healthy without any apparent clinical signs. Table 4.1 summarizes the results for clinical scores. The vaccinated group indicated lower clinical scores than non-treated control and vector control groups.

Gross pathological, histopathological and bacteriological examination

The protective effect of oral immunization with the surface-displayed ApxIIA#5 expressing *S. cerevisiae* and the ApxIA expressing *S. cerevisiae* was estimated by gross pathological and histopathological scoring based on the percentage of pneumonic lung lesion and histopathological changes after post-challenge necropsy. In vector control and non-treated control groups, the mean (±SE) total gross lesion scores were 35.4 ± 14.2 % and 49.8 ± 40.1 %, respectively (Table 4.1). The total gross lesion in the vaccinated pigs was 13.2 ± 10.6 %, which was significantly lower than that in vector control ($p < 0.05$) (Table 4.1). Microscopic lung changes

were consisted of alveolar wall congestion and inflammatory infiltration in the non-treated control and vector control groups (Figure 4.4). Alveoli were filled with edema fluid, and neutrophils as acute inflammatory responses. The mean histopathological changes were significant lower in the vaccinated group (7.3 ± 2.6, $p<0.05$), when compared to the non-treated control group (11.2 ± 2.0) (Figure 4.4). Although the thickening of alveolar walls was seen, alveolar lumina were empty in the vaccinated pigs. The challenge strain was reisolated from the lungs of the vaccinated, non-treated control and vector control groups. However, the vaccinated pigs had clearly but not significantly reduced the number of reisolated bacteria (Table 4.1).

Disccussion

Vaccinology in veterinary medicine is implicated in a broad spectrum of chellenges; providing cost effective approaches to control infectious diseases in animals, to consider the animal welfare, and to decrease the cost of production in food animals (Shams, 2005). Moreover, mass vaccination programs lead to a reduction of the consumption in veterinary medication like antibiotics, thus reducing their environmental impact, their side effects and residues in food animal products (Shams, 2005). Most vaccines for pigs have been developed as parenteral immunization, which may induce several adverse effects, such as laborious and

time-consuming procedures, the induction of inflammatory reactions at the injection site, and the stress of the pig (Shin *et al.*, 2005). Even though these conventional vaccines have been conductive to the elevation of animal and public health, they are far from the ideal animal and public health. Therefore, oral vaccination in veterinary medicine is very potential due to their overcoming the problems mentioned above. Moreover, oral vaccination could not only induce locally and systemically protective immune responses against infectious disease but also to be a safe and convenient way (Shin *et al.*, 2005). Although intranasal vaccination induces efficiently local immune responses in the respiratory tracts, it has several limitations including difficulty of administration and safety problems such as redirecting vaccine antigens into the central nervous system (van Ginkel *et al.*, 2000). Owing to the migration of IgA antibody-secreting cells (ASCs), local mucosal immunization leads to antigen-specific IgA production at distant mucosal sites (Kweon, 2011). Therefore, development of oral vaccine for *A. pleuropneumoniae* is thought to be a practical value on application to pig farming.

Our previous studies indicated that the full-length ApxIIA as well as the surface-displayed ApxIIA expressed in *S. cerevisiae* can induce both systemic and mucosal immune responses after oral administration (Shin *et al.*, 2005; Shin *et al.*, 2007; Kim *et al.*, 2010). *S. cerevisiae* used in this study was engineered to anchor the neutralizing epitope (ApxIIA#5) of ApxIIA on the outer layer of the cell for efficient antigen presenting (Kim *et al.*, 2010). The surface-displayed ApxIIA#5 expressing *S. cerevisiae* was chosen as an oral vaccine for porcine

pleuropneumoniae by carrying out specific antibody response assays in mice in order to select a novel oral yeast vaccine out of the recombinant yeast vaccine candidates in the present study. These results demonstrated that the neutralizing epitope of ApxIIA corresponding to aa 439-801 was effective for induction of the immune responses like the full ApxIIA in mice by oral administration (Seo *et al.*, 2011). The ApxIA expressing *S. cerevisiae* was also used as a yeast vaccine in pigs, because induction of immune responses for ApxIA as well as ApxIIA was required to protect against *A. pleuropneumoniae*. Ultimately, the efficacy of the oral administration using the chosen surface-displayed ApxIIA#5 expressing yeast and the ApxIA expressing yeast was investigated in pig as a target animal. The present results indicated that the oral administration of the yeast vaccine was capable to induce immune responses, showing a significant induction of Apx-specific IgG and IgA in sera of pigs fed with the recombinant yeast after oral administration. Especially, significant increase of Apx-specific IgA in nasal wash demonstrated that oral vaccination by transgenic yeasts can induce antigen-specific immune response in nasal mucosa of pigs. On the other hand, it is thought that most mucosal secretions are difficult to assay bring its challenges; rough sample collection, the presence of factors such as viscosity, the presence of bacteria, desquamated cells and bacterial proteases which can quickly break the sIgA (Challacombe, 1995). So there was no significant result in fecal antibodies. Clinical signs, gross lesions, histopathological changes and reisolation of challenge strain have been assessed as a sign of the protective potentials against challenge with *A.*

pleuropneumoniae (Hensel *et al.*, 1995; Maas *et al.*, 2006; Buettner *et al.*, 2008; Sadilkova *et al.*, 2012). In this experiment, clinical signs after the challenge with *A. pleuropneumoniae* were reduced by oral immunization of the transgenic yeasts with a similar pattern of results in the gross lung lesions. In addition, the histophatological observation was also supported the reduced clinical disease of *A. pleuropneumoniae* in the vaccinated group. Though the parenteral vaccine for *A. pleuropneumoniae* may reduce mortality, they did not prevent the expansion of the lung lesions (Hensel *et al.*, 1995). In addition, *A. pleuropneumoniae* bacterins for parenteral administration may also cause toxic side effects and local tissue damage (Hensel *et al.*, 1995). On the other hand, oral immunization with *A. pleuropneumoniae* serotype 9 or microspheres based on formalin-inactivated *A. pleuropneumoniae* serotype 1 induced pulmonary and systemic antibodies and protects against homologous challenge in pigs (Hensel *et al.*, 1995; Liao *et al.*, 2003). Like the proceedings, we obtained very promising results in the pig, indicating that the surface-displayed ApxIIA#5 and the ApxIA in *S. cerevisiae* can induce antigen-specific immune responses and that the induced immune responses can protect the pig infected with *A. pleuropneumoniae*. In addition, oral immunization with the transgenic yeasts expressing Apx protein showed the reduced mortality as well as lung lesions after challenge, according to the results of the present study. There were no clinical side effects observed for oral vaccination with the transgenic yeasts.

Consequently, the surface-displayed ApxIIA#5 in *S. cerevisiae* can be considered

an alternative strategy using cell-surface display technique with a small size of the neutralizing epitope of the ApxIIA. Oral immunization using the *S. cerevisiae* expressing the ApxIA and the surface-displayed ApxIIA#5 may be a good vaccine candidate based on the overall induction of mucosal and systemic immune responses in mice and protective immune responses in pigs. Thus this study is expected to contribute to the development of a live oral vaccine against porcine pleuropneumonia as a new trial in pigs.

Table 4.1. Pathological and clinical analysis for the experimental pigs after challenge with *Actinobacillus pleuropneumoniae* serotype 5

Group	Clinical score[b]	Body temperature (℃)[c]	Lung lesion score (%)[d]	Re-isolation of the challenge strain (CFU/g)
Non-treated[a]	2.67 ± 1.04	39.96 ± 0.25	49.8 ± 40.1	1.1×10^9
Vector control[a]	4.0 ± 0.88	39.93 ± 0.05	35.4 ± 14.2	1.3×10^8
Vaccinated[a]	0.75 ± 0.48[e,f]	39.90 ± 0.08	13.2 ± 10.6[f]	8.6×10^3
Non-challenged	0	39.54 ± 0.04	0.7 ± 2.0	-

[a] Pigs were challenged intranasally with *A. pleuropneumoniae* serotype 5 Korean isolate one week after last immunization.

[b] Pigs were examined for clinical signs of *A. pleuropneumoniae* and scored for ten days using a 0 to 5 scale, following the challenge. Mean value for each group ± standard deviation is given.

[c] Rectal temperature was taken at days 2, 5, 7, and 10 for ten days, following the challenge. Mean value for each group ± standard deviation is given.

[d] The observed lung lesion scores were calculated the percentage of the lung surface that shows the lesions to give an overall lung score.

[e] Statistically significant difference ($p < 0.01$) between the vaccinated group and vector control group.

[f] Statistically significant difference ($p < 0.05$) between the vaccinated group and non-treated control group.

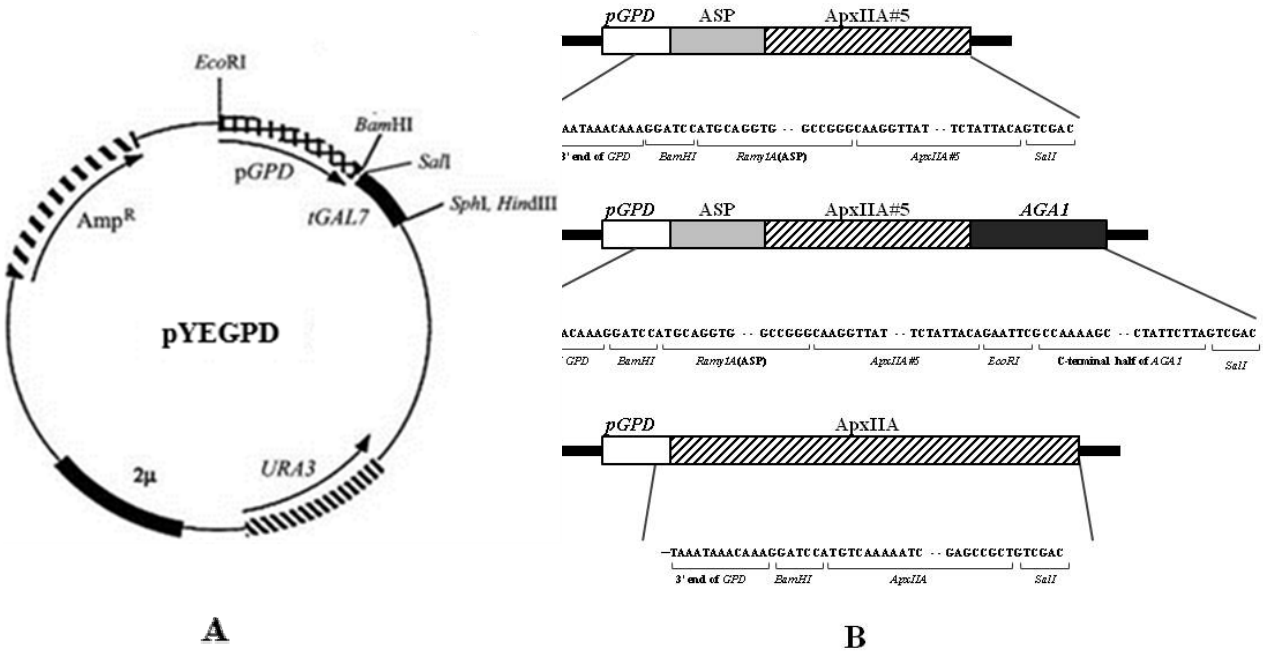


Figure 4.1. Construction of the yeast expression vector for ApxIIA#5 and ApxIIA. A, Schematic diagram of yeast expression vector pYEGPD. The boxes stand for genes or their functional domains. B, Schematic diagram of the fusion construct cloned in the pYEGPD plasmid. pGPD, promoter of glyceraldehyde-3-phosphate dehydrogenase; ASP, rice amylase1A signal peptide; ApxIIA#5 and ApxIIA, neutralizing epitope and full-size of ApxIIA exotoxin from *Actinobacillus pleuropneumoniae*; AGA1, C-terminal half (320 amino acid residues from the C terminus) of yeast α-agglutinin anchoring protein; tGAL7, terminator of galactose-1-P uridyl transferase. Adapted from Kim *et al.*, 2010.

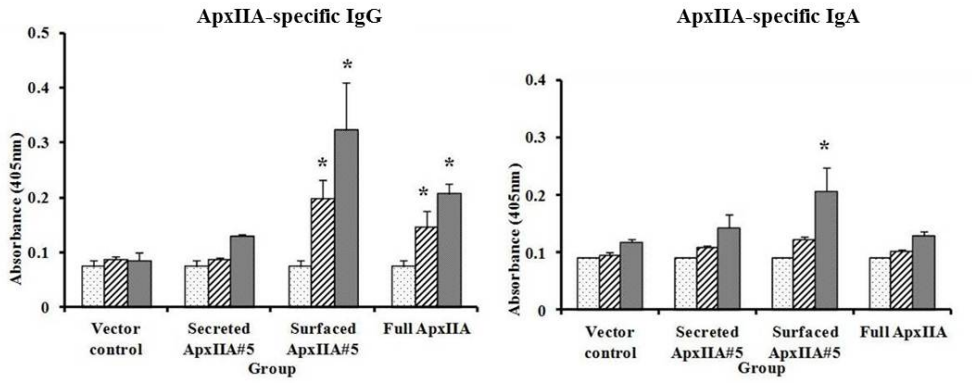


Figure 4.2. Immune responses of specific IgG and IgA against *Actinobacillus pleuropneumoniae* ApxII toxin in sera of mice orally immunized with the transgenic yeasts. (▒, before oral administration; ▨ , after two weeks of first oral administration; ■, after two weeks of fourth oral administration with the vector-only *S. cerevisiae,* the surface-displayed ApxIIA#5 expressing *S. cerevisiae*, the ApxIIA#5-secreting *S. cerevisiae*, and the full-ApxIIA expressing *S. cerevisiae*). Significant differences between the vector control group and the vaccinated group were expressed as *, $P < 0.05$.

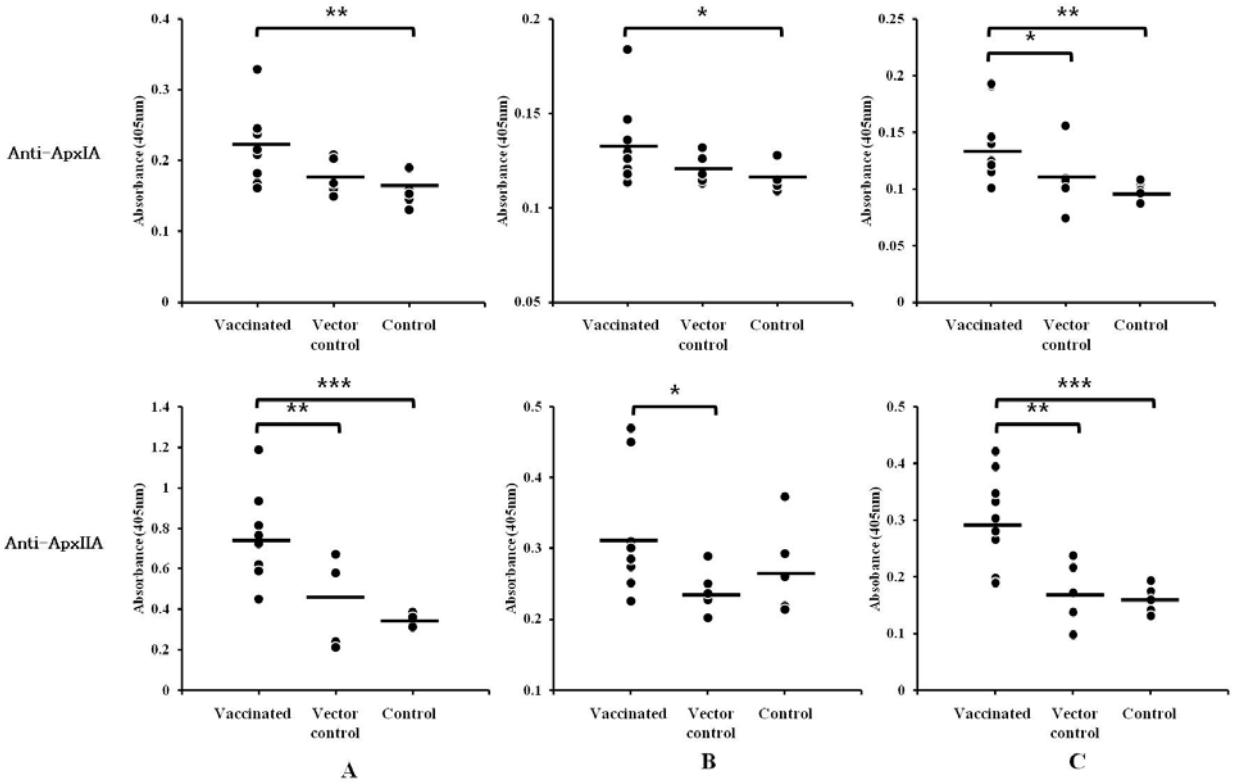


Figure 4.3. Individual levels of antigen-specific antibodies at 1 week after third oral immunization. A, IgG in sera from pigs; B, IgA in sera from pigs; C, IgA in nasal wash from pigs (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

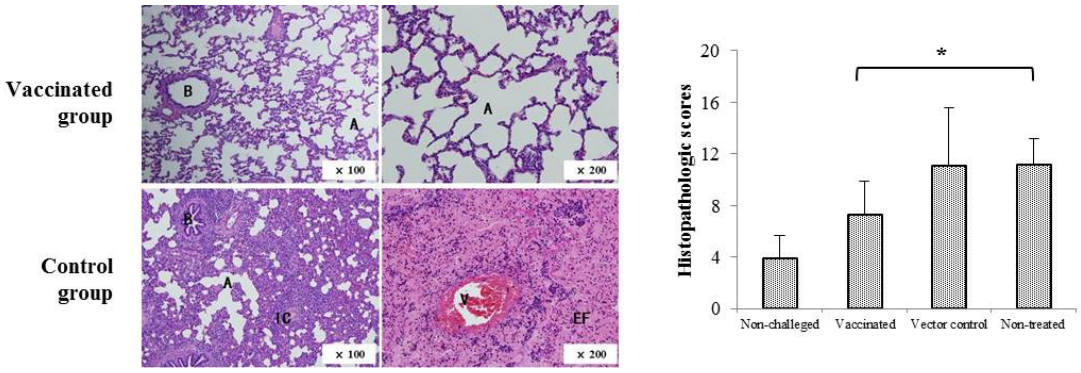


Figure 4.4. Histopathological examination of lung lesions. Lung sections were obtained from pigs on day 10 after challenge with *Actinobacillus pleuropneumoniae* serotype 5. Histopathological scores showed significant difference between the vaccinated and non-treated control groups (*, $p<0.05$). A, alveole; B, bronchus; EF, edema fluid; IC, interstitial consolidation.

General conclusions

Up to date, it is still far from complete understanding pathogenesis and efficient vaccine strategy for *A. pleuropneumoniae* infection. To development effective diagnostic methods and vaccines, it is necessary to understand Apx toxins as major virulence factors, which are involved in virulence of *A. pleuropneumoniae*. The *apx*IVA gene was genetically analyzed as a new virulence factor and the new ELISA methods were developed using recombinant ApxIA, ApxIIA, or ApxIIIA proteins. Moreover, the neutralizing epitope of ApxIIA (ApxIIA#5) expressing *S. cerevisiae* was developed and assessed the induction of immune responses after oral administration in mice and pigs.

1. The nucleotide sequences of the *A. pleuropneumoniae* KSID serotype 2 *apxIVA* gene were given in GenBank under the accession number, HM021153. The Kor-ApxIVA was closely related to other GenBank assession numbers. The structural characteristics of ApxIVA showed RTX proteins, including N-terminal hydrophobic domains, and repeated glycine-rich nonapeptides in the C-terminal region of the protein.

2. A serological diagnosis of *A. pleuropneumoniae* was developed using ELISA with the recombinant ApxIA, ApxIIA and ApxIIIA antigens and evaluated the application of the developed assays on the prevalence of anti-Apx toxin

antibodies among pigs in Korea.

3. The neutralizing epitope of ApxIIA (ApxIIA#5) induced improve both systemic and mucosal immune responses in mice by oral immunization. Furthermore, Th1-type immune responses were predominant in the vaccinated mice.

4. The surface-displayed ApxIIA#5 in *S. cerevisiae* induced protective immune responses in pigs infected with *A. pleuropneumoniae*.

On the biasis of these results, the surface-displayed ApxIIA in *S. cerevisiae* seems to play important role in mucosal and/or cellular immunity by oral route through regulating Th1 immune responses. This study provided a deeper understanding of immune responses to the neutralizing epitope of ApxIIA (ApxIIA#5) expressing *S. cerevisiae* in mice, as well as a practical value of oral vaccination using this transgenic yeast in pigs.

국문초록

국내분리 *Actinobacillus pleuropneumoniae apxIVA*의 유전적 특성과 *apxIA* 및 *apxIIA* 유전자 발현 *Saccharomyces cerevisiae*의 경구투여에 따른 면역원성 분석

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돼지 흉막폐렴은 전세계적으로 양돈산업에 막대한 경제적 손실을 나타내는 전염성 흉막염이며, 원인균은 *Actinobacillus pleuropneumoniae*이다. 지금까지 전 세계적으로 15가지 혈청형이 밝혀져 있고, 흉막폐렴의 병원성은 많은 병원성 인자들중 Repeats in Toxins (RTX)에 속하는 Apx toxins, ApxI, ApxII, ApxIII 그리고 ApxIV 생성에 의한다. ApxI, ApxII 및 ApxIII는 혈청형에 따라서 다양하게 분비되며, 가장 최근에 밝혀진 ApxIV는 병원성 관련 역할은 밝혀지지 않았으나, 모든 혈청형에

서 발현되고 *in vivo*상에서만 발현된다는 특징이 있다. Apx toxins은 강한 면역원성을 나타내기 때문에 *A. pleuropneumoniae* 감염에 강한 항체반응을 유발한다. 특히 많은 연구들이 가장 강한 병원성을 나타내는 것이 ApxI과 ApxII의 독소를 생산하는 혈청형과 밀접한 연관이 있는 것을 지적하고 있다. 그렇기 때문에 Apx toxins은 *A. pleuropneumoniae* 에 대한 진단 및 백신 개발에 항원으로서 효과적인 접근일 수 있다.

본 연구에서는 첫번째로 국내 분리 ApxIV (Kor-ApxIVA)에 대한 전체 염기서열 및 유전적 분석을 실시하고, 다른 혈청형의 *A. pleuropneumoniae* 의 ApxIV와 비교분석하였다. ApxIV의 구조적 특성은 N-말단의 hydrophobic domain과 C-말단의 반복적인 glycine-rich nonapeptides를 보이는 RTX toxin의 특성을 나타내었다. 또한 30번의 glycine-rich nonapeptides (L/V-X-G-G-X-G-N/D-D-X)가 Kor-ApxIVA의 C-말단에서 반복된 것을 확인하였다. 본 연구의 유전적 분석 결과가 ApxIVA의 생물학적 그리고 기능적 특징에 대한 중요한 기초 연구가 될 것으로 생각된다.

둘째로 재조합 ApxIA, ApxIIA 및 ApxIIIA를 이용한 ELISA 진단법을 개발하고, 이를 국내 야외 돼지 혈청에 적용하여 국내 야외 돼지에서 Apx toxins에 대한 항체가를 조사하였다. 결과적으로 개발된 ELISA 기

법은 Apx 항원에 대한 특이 항체를 진단할 뿐만 아니라 백신 항체가 모니터링에 유용할 것으로 생각된다.

셋째로 형질전환 발현 시스템에서 *A. pleuropneumoniae*에 대한 효과적인 백신을 개발하기 위하여, ApxIIA의 neutralizing epitope (ApxIIA#5)를 선택하였다. 본 연구에서 ApxIIA#5 표면 제시 효모를 개발하고, 이를 *A. pleuropneumoniae*에 대한 경구 백신 후보로 선정하였고, 전신면역 및 점막면역 반응을 둘 다 향상 시킨다는 것을 확인할 수 있었다. ApxIIA#5 표면 제시 효모를 경구투여한 마우스에서 분리된 CD4+ T cell에 ApxIIA-activated DC를 제시하였을 때 특이적인 T cell proliferation을 보였다. 또한 ApxIIA#5 표면 제시 효모를 경구투여한 마우스의 혈액과 spleen, peyer's patch 와 lamina propria에서 vector 대조군 및 비처치 대조군과 비교하여 더 높은 항원특이적 IgG 및 IgA 항원 반응을 확인할 수 있었다. 게다가 우세한 IgG2a subclass 및 IFN-γ 의 상승반응에 따라 ApxIIA#5 표면 제시 효모를 경구투여한 마우스에서 Th1-type 면역반응이 우세하게 유도된 것으로 생각된다.

마지막으로 ApxIA 발현 효모와 ApxIIA#5 표면 제시 효모를 돼지에 경구백신 후보로서 투여한 후 효능을 평가하였다. ApxIA 발현 효모와 ApxIIA#5 표면 제시 효모를 경구 투여한 돼지에서 대조군 그룹들에서 보다 혈액 및 비강액에서 더 높은 항원특이 IgG 및 IgA antibody

activities를 보였다. 또한 임상증상 및 육안·조직병리학적 평가를 통해 *A. pleuropneumoniae* 감염된 돼지에서 돼지 흉막폐렴 항원 발현 형질 전환 효모가 방어능을 나타낸 것을 확인하였다. 결론적으로 이러한 결과들은 ApxIA 발현 효모 및 ApxIIA#5 표면제시 효모가 돼지 흉막폐렴을 방어할 수 있는 경구백신의 가능성을 보여주었다. 그러므로 본 연구 결과는 현재 사용되고 있는 주사백신을 대체할 만한 live 경구 백신의 개발에 공헌할 것으로 생각된다.

핵심어: 돼지 흉막폐렴 원인균, Apx 독소, 경구면역, 효모

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감사의 글

끝이 없을 것 같았던 학위과정이 마무리되어 드디어 졸업을 하게 되었습니다. 짧지않았던 대학원 생활을 뒤돌아 보니 즐거웠던 일도 힘들었던 일도 모두 소중한 추억으로 기억됩니다. 그 동안 저에게 힘이 되어주시고, 소중한 추억을 함께 만들었던 모든 분들께 감사한 마음을 전하고 싶습니다. 부족한 저를 끝까지 믿어주시고, 어려운 순간마다 아낌없는 격려와 진실된 충고로 저를 이끌어주신 유한상 교수님께 진심으로 존경과 감사의 마음을 전합니다. 또한 바쁘신 일정에도 불구하고 흔쾌히 논문 심사를 맡아주시고 세심하게 검토하시고 많은 조언을 아끼지않으셨던 김재홍 교수님, 박봉균 교수님, 김대용 교수님, 정병열 박사님께도 진심으로 감사드립니다.

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