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# A predictive model for the level of sIgA based on IgG levels following the oral administration of antigens expressed in *Sacchromyces cerevisiae*

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**Oral vaccination may be the most efficient way of inducing an immune response at the remote mucosal site through the common mucosal immune network. Antigen-specific secretory IgA (sIgA) is the major immunoglobulin type generally detected in the secretions of experimental animals following an effective oral immunization. *Actinobacillus pleuropneumoniae* causing disease in the lung of pig initially interacts, colonizes, and infects the host tissues at the mucosal surface of the respiratory tract. Also, importantly for *A. pleuropneumoniae* protection, the quantity of sIgA in the lung had merits associated with the mucosal immunity. However, there is no simple method to monitor the level of sIgA as an indicator for the induction of local immune responses by an oral vaccination in the target tissue. Therefore, the relationship between sIgA and IgG was analyzed to evaluate the induction of local immune responses by an oral immunization with *Saccharomyces cerevisiae* expressing the *apx*IA and *apx*IIA genes of *A. pleuropneumoniae* in this study. The correlation coefficient of determination ($r^2 \times 100$) for paired samples in both vaccinated and control groups showed a significant positive-relationship between IgG in sera and sIgA in the lung or intestine. These results indicated that IgG antibody titers in sera could be useful to indirectly predict local immune response, and sIgA, in the lung or intestine to evaluate the efficacy of an oral vaccination.**

**Key words:** correlation coefficient, IgG, oral vaccine, sIgA

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## Introduction

*Actinobacillus pleuropneumoniae* is the etiological agent of porcine pleuropneumonia, which is characterized by haemorrhagic pneumonia and fibrinous pleuritis, and had a high incidence of mortality worldwide [2,5,13]. Virulence factors that have been described for *A. pleuropneumoniae* include capsular polysaccharides, outer membrane proteins, Apx toxins, lipopolysaccharides, permeability factors, and iron-regulated proteins [5,19]. Of the virulent factors, Apx toxins have been proven to be of particular importance for the induction of protective immunity as previously demonstrated with several different mutants such as spontaneous, chemically induced, and transposon mutagenesis [1,7,8,16,17,21]. Although the virulence of *A. pleuropneumoniae* is multifactorial, these studies indicate that the virulence of *A. pleuropneumoniae* is strongly correlated with the production of Apx exotoxins, in particular, with serovars producing Apx I and Apx II being the most virulent [7,16,17,22]. Similar to other respiratory pathogens, *A. pleuropneumoniae* gain access to their host through the mucosal surfaces [10,11]. It is therefore desirable to develop vaccination strategies that lead to mucosal immune responses [4,14,21]. Oral vaccines are convenient for mass administration and allow the risk of intramuscular injection of toxins to be avoided. In addition, oral formulations are safer than injections because of the specialized protective and detoxifying properties of the digestive system. They also stimulate the gut-associated lymohoid tissue, with a subsequent development of immunoglobulin A (IgA)-secreting plasma cells in the other mucous membrane [15,21].

A natural infection with *A. pleuropneumoniae* results in a protective immunity against a challenge infection, and specific immunoglobulin A (IgA) is elicited after a natural and experimental infection [11,15]. It is well documented that secretory IgA (sIgA) antibodies found in secretions are produced locally by plasma cells in the respiratory mucosa, and such antibodies may protect the host from both bacterial

colonization and disease [11,21]. sIgA functions to prevent the adsorption of pathogens or their toxic products at the mucosal epithelium [6,15]. A further feature of sIgA is the participation in the disposal of antigens by mechanisms mostly devoid of inflammatory consequences, which is essential if sensitive organs such as the lungs are involved [6,11,14,15].

However, there are no simple methods to measure the level of antigen-specific sIgA induced by an oral immunization. In addition, the complex procedures such as sacrificing animals and sample preparations are needed to directly measure sIgA in the tissues. Therefore, in this study, an attempt was carried out to indirectly predict sIgA inducton following an oral immunization with *Saccharomyces cerevisiae* (*S. cerevisiae*) expressing *apx*IA and *apx*IIA genes by analyzing the relationship between mucosal sIgA and systemic IgG.

## Materials and Methods

### Vaccine preparations

*A. pleuropneumoniae* serotypes 2 and 5 isolated from the lungs of Korean pigs with pleuropneumonia were used for the cloning of the *apx*I A and *apx* II A genes. *apx*IA and *apx*IIA genes were cloned and sequenced [20]. The cloned genes were subcloned in *Sacchromyces cerevisiae* 2805 with YEpGPD expression vector using LiAc method and expressed as previously described [18,19]. *S. cerevisiae* expressing *apx*IA or *apx*IIA genes were prepared as previously described and used as an oral vaccine in this study [18,19].

### Experimental animals, immunization and sample collections

Five-week-old BALB/c female mice (Laboratory Animal Center, Seoul National University, Korea) were used throughout this study following policy and regulations for the care and use of laboratory animals (Seoul National University, Korea). All animals were provided with standard mouse chow and water *ad libitum*. Each experimental group consisting of 20 mice was allocated to one of 4 oral immunization regimens; non-treated groups, vector control group, oral-vaccinated group with 10 mg of *S. cerevisiae* expressing *apx*IA and oral-vaccinated group with 10 mg of *S. cerevisiae* expressing *apx*IIA.

All oral immunizations were preceded by an overnight fasting of the mice (water was provided *ad libitum*). Either control vector- or YEpGPD-TER-*apx* genes-harboring yeast were lyophilized and ground to make the yeast powder [19]. Forty milligrams of the yeast powder were dissolved into 1 ml of 0.9% saline and administered at 250 µl per mouse (10 mg per mouse) through an esophageal cannula four times at 10 day intervals. Three to four mice from each group were sacrificed before one day at each time of immunization as described below. The lung and small intestine were collected from individual mice as described below. Before sacrificing the mice, the mice were deeply anesthetized with a mixture of xylazine hydrochloride (Rompun; Bayer, Korea) and ketamin hydrochloride (Ketamin; Yuhan, Korea), blood was then collected, and sera were collected by centrifugation at 2,500 *g* for 20 min at 4°C after clotting. The sera were stored at –20°C until use. For preparations of the lung and intestine homogenates, the mice were perfused intracardially with 0.9% saline at a rate of 70 ml/min with a perfusion pump (Masterflex, USA) to remove whole blood. Lung and intestine homogenates were obtained from parts of lung and small intestine by a 10,000 RPM homogenization (Polytron PT3000; Kinematica, USA). The samples were stored at 4°C overnight, followed by a centrifugation at 12,000 *g* for 10 min at 4°C. Supernatants were collected and stored at –20°C for subsequent analysis. Total protein concentrations of each sample were measured using a BCA protein assay kit (Pierce, USA) and normalized to 5 mg just before performing the assay.

### Measurement of ApxIA or ApIIA antigen-specific antibody immune responses

The level of antigen-specific antibodies (IgA or IgG) in small intestine, lung and serum samples was determined using enzyme-linked immunosorbent assay (ELISA). Ten µg of rApxIA or rApIIA suspended in 100 µl of coating buffer (14.2 mM $Na_2CO_3$, 34.9 mM $NaHCO_3$, 3.1 mM $NaN_3$, pH 9.6) was added to a microplate for ELISA (Greiner, Australia) and incubated overnight at 4°C. The plate was washed three times with PBST (0.05% Tween 20 in PBS) and blocked with PBST containing 1% bovine serum albumin (BSA) for 1 hr at 37°C. As the first antibody, mice sera collected from immunized mice and 5 mg of total protein from each homogenized sample described above were used for IgG and IgA analysis, respectively. One to ten or 1 to 100 diluted primary antibodies were then added to the plate, and incubated for 1 hr at 37°C. After washing with PBST, 100 µl of goat anti-mouse IgG (H+L)- HRP conjugate (Bio-Rad, USA) or anti-mouse IgA (α-chain specific)-HRP conjugate (Sigma Aldrich, USA) was added to the plate, and incubated for 1 hr at 37°C. Color was developed by adding 100 µl of ABTS substrate solution (Bio-Rad, USA) to the plate. After 20 min of incubation at room temperature, the O.D. value was measured at 405 nm using an ELISA reader (Molecular Device, USA).

### Statistical analysis

Correlation coefficient r and coefficient of determination $r^2 \times 100$ for paired samples were examined for statistical significance by analysis of variance for linear regression using Excel 2002 program (version 10.2614.2625; Microsoft, USA) and GraphPad Prism software package version 4.03 (GraphPad Software, USA). The relationship between IgG and IgA was exhibited by linear regression equation.

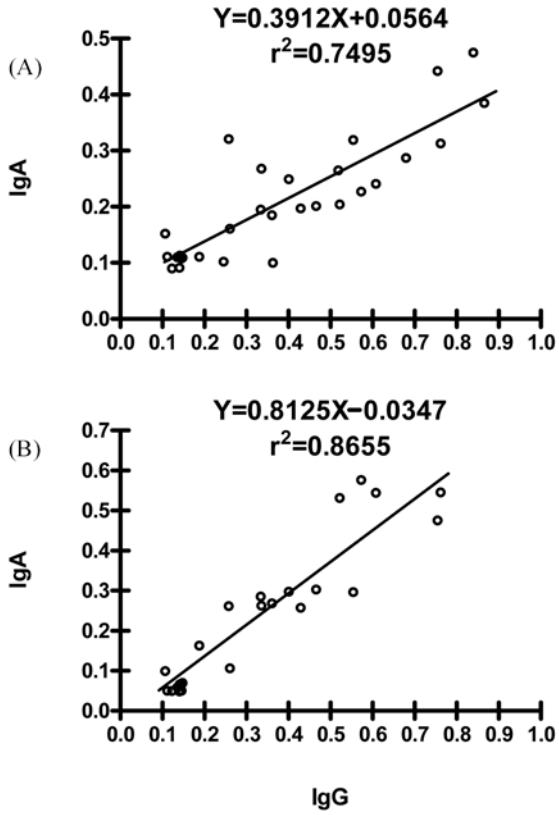


**Fig. 1.** Correlation between serum IgG and mucosal sIgA titers to Apx IA using a linear regression. A: The relationship between pulmonary sIgA and serum IgG. B: The relationship between intestinal sIgA and serum IgG. $r^2$ = coefficient of determination, Y = simple linear regression equation. The number in X- and Y-axis indicates the optical density at 405 nm.

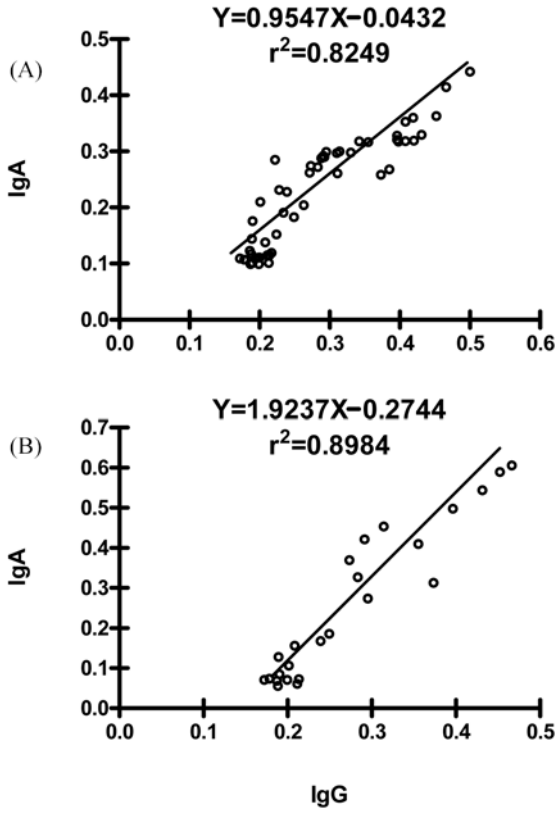


**Fig. 2.** Correlation between serum IgG and mucosal sIgA titers to Apx IIA using a linear regression. A: The relationship between pulmonary sIgA and serum IgG. B: The relationship between intestinal sIgA and serum IgG. $r^2$ = Coefficient of determination, Y = simple linear regression equation. The number in X- and Y-axis indicates the optical density at 405 nm.

## Results

To determine the induction of mucosal immunity in both the lung and small intestine after an oral immunization of mice with *S. cerevisiae* expressing *apx*IA and *apx*IIA genes, the relationship between mucosal sIgA in the lung or small intestine and systemic IgG was examined. Y = 0.3912X + 0.0564 in the lung and Y = 0.8125X – 0.0347 in the small intestine were exhibited in the relationship of ApxIA-specific antibodies, and the correlation-coefficient r was significantly high in both the lung (r = 0.87) and small intestine (r = 0.93) (Fig. 1).

In the relationship of ApxIIA-specific antibodies, Y = 0.9547X – 0.0432 in the lung and Y = 1.9327X – 0.2744 in small intestine were represented. All correlation-coefficient r between ApxIIA-specific sIgA in the lung or in small intestine and ApxIIA-specific IgG in sera were also significantly high at 0.85 and 0.95, respectively (Fig. 2).

## Discussion

Recently, mucosal immunity through oral vaccination has been focused on because the diseases of the mucosal surfaces such as the intestine and lung are the most common causes of mortality and morbidity in all species, particularly affecting young animals. Moreover, the vast majority of infections take place at, or originate from, mucosal surfaces. Topical application of the vaccine may be the most efficient way of inducing an immune response at the mucosal level [9,10,12,21].

The mucosal and systemic immune systems could be regarded as independent but closely interrelated entities, each which its own compartmentalization and specialization [3,4,14,15,21]. The mucosal immunity, although essentially similar to the systemic system in terms of afferent, efferent and regulatory components, has developed certain peculiar characteristics and adaptive mechanisms for responding to the foreign antigens to which it is constantly exposed [14, 15]. Both of them acting independently are essential for protecting the host from infections on one hand, and from undesirable immunological reaction to innocuous environmental antigens on the other hand. They are constantly exchanging immunological messages and tend to complement each other in their respective responses [3,14,15]. While the

mucosal system responds mainly to locally presented antigens, it is also capable of responding to systemic antigens that might be transported to through the blood circulation [14,15]. The systemic immune system might also mount immune responses to mucosally presented antigens, which escape the local responses and find their way into circulation [15,21].

Antigen-specific sIgA response in target organs is characteristic for oral vaccine administration while the systemic immunity produces predominantly IgG [6,11,15]. The sIgA antibodies defend the mucosal surfaces in the upper respiratory tract or the intestinal mucosa against micro-organisms by reducing colonization rates as well as by preventing adherence to epithelial surfaces [6,14,15]. Thus, micro-organisms entrapped in the mucous layer can be cleared from the airway or from the intestinal tract [6,14,15,21].

It is important to determine the titer of antigen-specific sIgA in the target site to understand whether the local immune responses are successfully induced by an oral immunization. However, the available methods to directly measure antigen-specific sIgA in the targeted organ or inductive site of specific local immune responses are quite limited due to the requirement for labor-intensive works such as sacrificing animals, sample collections and preparations.

In this study, we attempted to determine local immune responses effectively induced by an oral vaccination with *S. cerevisiae* expressing *A. pleuropneumoniae apx*IA and *apx*IIA genes through analyzing the relationship between antigen-specific IgA in the lung or intestine and IgG in the serum.

The correlation coefficient of determination ($r^2 \times 100$) for paired samples between ApxIA or ApxIIA-specific sIgA in the small intestine and serum IgG showed a higher relationship than those between pulmonary sIgA and serum IgG because the primary site of antigen contact was the intestine in an oral vaccination [3,6,21]. In addition, specialized M cells which cover the Peyer's patches in the small intestine pass antigenic material to lymphocytes below the epithelium, where the processed antigens are presented to IgA precursor B cells or T cells [3,6,15]. A portion of the lymphocytes primed by antigens at the intestine migrate via the lymphatic system to secondary mucosal sites such as the lung and then give rise to IgA-secreting plasma cells [3,15, 21]. However, the correlation coefficient of determination ($r^2 \times 100$) between each antigen-specific sIgA in the lung and serum IgG of paired samples showed a statistically significant relationship by representing 75% for ApxIA and 83% for ApxIIA, respectively ($p < 0.05$). Also, the different relationship according to the type of antigens could be dependent on their antigenic properties because the mucosal system presents different ranges of antigens and produces different subtypes of antibodies [3,14,15].

However, the increase in titers of sIgA in serum was not statistically paralleled by changes in serum IgG levels even though high levels of serum sIgA were observed in vaccinated groups ($p > 0.05$) (data not shown).

These results suggested that the antigen-specific immune responses induced by an oral vaccination in both the primary site and targeting site could be indirectly predicted by analyzing the levels of IgG in serum.

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