Evaluation of systemic and mucosal immune responses in mice administered with novel recombinant *Salmonella* vaccines for avian pathogenic *Escherichia coli*

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Abstract : Avian pathogenic *Escherichia coli* (APEC) is a causative agent for a number of extra intestinal diseases and account for significant losses to the poultry industry. Since protective immunity against APEC is largely directed to virulence antigens, we have individually expressed four different virulence antigens, *papA*, *papG*, *IutA*, and *CS31A*, using an attenuated *Salmonella* Typhimurium and a plasmid pBB244. Following oral immunization of mice with combination of two or four of these strains, serum IgG and mucosal IgA responses were elicited against each antigen represented in the mixture. The antigen-specific mucosal IgA responses were significantly higher in the group of mice immunized with the heat-labile *Escherichia coli* enterotoxin B subunit (LTB) strain than those in the group of mice immunized without the LTB strain. While, there was no significant difference between these two groups in antigen-specific serum IgG responses. The results showed that LTB could act as mucosal immune adjuvant. To assess the nature of immunity, the distribution of antigen-specific IgG isotypes was analyzed. All groups promoted Th1-type immunity as determined by the IgG2a/IgG1 ratio. Thus, our findings provided evidence that immunization with a combination of several vaccine strains is one of the strategies of developing effective vaccines against APEC.

Keywords : Avian pathogenic *Escherichia coli*, heat-labile *Escherichia coli* enterotoxin B subunit, immune response, pBB244, *Salmonella* vaccine delivery

Introduction

Avian pathogenic *Escherichia coli* (APEC) is the most common secondary bacterial infection of commercial poultry flocks and may also be a primary pathogen. Disease syndromes caused by the *Escherichia (E.) coli* include both disseminated systemic conditions like colisepticemia and focal infections such as cellulitis [4, 5, 19, 23]. Many putative virulence factors of APEC have been known [15, 22] and among the factors, the CS31A surface antigen, encoded by the *clp* operon; P-fimbriae, encoded by the *pap* gene cluster (*papA* and *papG*); the aerobactin receptor, encoded by the gene *IutA* are the most common virulence factors found on APEC isolates in Korea.

Although antibiotic therapy is available to treat this infection, the development of an effective prophylactic or therapeutic vaccine will be of enormous benefit. The use of oral routes for immunization against infective diseases is desirable because oral vaccines are easier to administer and mucosal surfaces, on which immunity can be acquired by the routes, are the portals of entry for many pathogenic micro-

bial agents [17, 25]. However, oral vaccine need more and repeated antigen doses to achieve the protective immune response level, therefore, it is necessary to developing strategies to identify mucosa-active immunostimulating agents, adjuvants [10].

Attenuated *Salmonella* strains have been developed as potential live oral vaccines against salmonellosis and as carriers for the delivery of heterologous antigens to the mammalian immune systems. The *Salmonella* strains secreting the antigens induced a more pronounced immune response than did the strain expressing the antigen in the cytosol [2, 7]. A foreign antigen expression vector, plasmid pBP244 which was incorporated with the key components of the type-II Sec-dependent secretion system, has been constructed to improve the secretion of antigen proteins in the *Salmonella* vaccine system [13].

In this study, we have constructed an attenuated *Salmonella (S.)* Typhimurium vaccine system with pBB244 that can express the virulence factors of APEC and heat-labile *Escherichia coli* enterotoxin B subunit (LTB). We examined the possibility that an immunization with combination of

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Table 1. Bacterial strains and plasmids used in this study

Strain / Plasmid	Characteristic	References
<i>E. coli</i>		
M15	<i>E.coli</i> host strain, kanamycine resistance	Qiagen
JM109	<i>E.coli</i> host strain	promega
JOL718	Wild type IutA ⁺ CS31A ⁺ <i>E.coli</i> isolate from chicken	this study
JOL708	Top10 (containing pGEM T-easy expressing LTB)	this study
JOL711	Top10 (containing pET28a expressing <i>papA</i>)	this study
JOL714	M15 (containing pQE9 expressing <i>papG</i>)	this study
JOL742	M15 (containing pQE10 expressing IutA)	this study
JOL483	JM109 (containing pQE9 expressing CS31A)	this study
<i>S. Typhimurium</i>		
×8501	<i>hisGAcrp-28ΔasdA16</i>	Guig and Curtiss [9]
JOL401	Wild type <i>S.Typhimurium</i> isolate from chicken	this study
JOL912	<i>S. Typhimurium</i> JOL 401 <i>Alon ΔcpxR ΔasdA16</i>	this study
JOL924	×8501 (containing pBP244 and expressing <i>papA</i>)	this study
JOL928	JOL912 (containing pBP244 and expressing <i>papG</i>)	this study
JOL930	JOL912 (containing pBP244 and expressing IutA)	this study
JOL954	×8501 (containing pBP244 and expressing CS31A)	this study
JOL758	×8501 (containing pYA3560 and expressing LTB)	this study
JOL921	×8501 (containing pBP244 only)	this study
Plasmids		
pQE9	Protein expression vector, ampicillin resistance	Qiagen
pQE10	Protein expression vector, ampicillin resistance	Qiagen
pYA3560	Asd ⁺ ; p15A ori	this study
pBP244	Asd ⁺ ; containing <i>SecA</i> (ATPase) <i>SecB</i> (chaperone), <i>LepB</i> (signal peptidase) genes	Kim <i>et al.</i> [13]

E. coli: *Escherichia coli*, *S. Typhimurium*: *Salmonella Typhimurium*, LTB; heat-labile *Escherichia coli* enterotoxin B subunit.

these vaccines might elicit antigen-specific immune responses to every APEC antigen represented in the mixture. We also investigated the adjuvant effects of LTB with respect to mucosal and systemic immune responses of the vaccines.

Materials and Methods

Bacterial strains, plasmids, media, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. The *Alon ΔcpxR ΔasdA16* attenuated *S. Typhimurium*, JOL912, were used as hosts. JOL912 were a derivative of JOL401, *S. Typhimurium* isolated from chicken. Plasmid pBP244 was used for extracellular secretion of antigen protein. This vector was developed with the application of Sec-dependent protein secretion components, including *LepB*, *SecA*, and *SecB* proteins [13]. All strains were grown at 37°C in Luria-Bertani media (LB) (Difco, USA) with or without antibiotics. Diaminopimelic acid (DAP) (Sigma-Aldrich, USA) was added for the growth of Asd⁻ strains [16]. Phosphate-buffered saline containing 0.01% gelatin (BSG) was used for the resuspension of the vaccine strains.

Construction of strains for protein expression and purification of proteins

Genes required for antigen proteins were obtained by polymerase chain reaction (PCR) using a pair of primers. Sequences of oligonucleotide primers used for PCR amplification and cloning are listed in Table 2. The amplified PCR products were cloned into the pET28a (Novagen, Germany), pQE9 or pQE10 (Qiagen, Germany). These expression plasmids were introduced into an *E. coli* Top10 (Invitrogen, USA) or M15 (Qiagen) or JM109 (Promega, USA). In-frame cloning was confirmed by nucleotide sequencing. The proteins were purified by an affinity purification process with Ni²⁺-nitrilotriacetic acid-agarose support (Qiagen). The purified proteins were verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and protein concentrations were estimated by using Bradford kit (Bio Rad, USA).

Production of rabbit antisera against antigen proteins

Hyperimmune rabbit sera against *papA*, *papG*, IutA and CS31A were generated by subcutaneous immunization of NewZealand White rabbits with each purified protein. Each

Table 2. Sequences of oligonucleotide primers used for polymerase chain reaction amplification and cloning

Gene	Primer	Sequence (5'-3')
<i>papA</i>	F	CCGCGAATTCGCTCCAACTATTCCACAG
	R	CCC GCGTCGACTTACTGGTAACTTAAATT
<i>papG</i>	F	CCGCGAATTCATGAAAAAATGGTCCCAGCTTTG
	R	CCC GCAAGCTTTTATGGCAATATCATGAGCAGCG
IutA	F	CGTGGATCCGCAACAAACCGATGATGAAACG
	R	CGGAAGCTTTCAGAACAGCACAGAGTAGITCAGACC
<i>clpG</i>	F	CCGGAATTCATGTGGACCACTGGTGATTTTAAT
	R	CCGAAGCTTTTAGTTATAAGTTACTGCCA
<i>eltB</i>	F	CCGCGAATTCGCTCCCCAGTCTATTACAG
	R	CCGCAAGCTTCTAGTTTCCATACTGATTG

Table 3. Experimental groups used in this study

Group	Vaccine strain
group A	JOL 924 (<i>papA</i>) + JOL 928 (<i>papG</i>) + JOL 758 (LTB)
group B	JOL 924 + JOL 928 + JOL 930 (IutA) + JOL954 (CS31A) + JOL 758
group C	JOL 924 + JOL 928 + JOL 930 + JOL954
group D	PBS

rabbit was primed with 250 µg of purified protein antigen in Freud complete adjuvant (Sigma-Aldrich) and then followed booster injection with the same concentration of each protein in Freud incomplete adjuvant (Sigma-Aldrich) at 2 weeks later. The rabbits were bled through the marginal ear vein at 2nd week after the last injection, then sera were obtained and stored at -20°C until used.

Construction of vaccine strains

The genes for P-fimbriae (*papA* and *papG*), Aerobactin receptor (IutA), CS31A surface antigen (*clpG*) were cloned into the Asd⁺ pBP244, respectively. The LTB were cloned into the Asd⁺ pYA3560. These cloned plasmids were introduced into the Asd⁻ χ 6212, to obtain the balanced lethal *E.coli* construct. The purified plasmids obtained from the *E. coli* constructs were then electroporated into an attenuated *S. Typhimurium* strain JOL912, and resulted in the strains, JOL924 (*papA*), JOL928 (*papG*), JOL930 (IutA), JOL954 (CS31A) and JOL758 (LTB), respectively. Selection for transformants was achieved by growth on LB agar plates without diaminopimelic acid supplementation. Only clones containing the recombinant plasmids were able to grow under these conditions. The expression of the antigen was assessed by immunoblot analysis.

SDS-PAGE and immunoblot analyses

SDS-PAGE and immunoblot analyses were performed by the same procedures as described in our previous study [18]. The secreted antigen proteins of vaccine strains were obtained from the cell-free culture supernatants of the TCA-precipitation process.

Immunization and sample collection

Overnight cultures of the vaccine strains were harvested from LB broth and resuspended in BSG to total concentration of 3×10^9 CFU. Four groups of five inbred 7 weeks old female BALB/C mice were immunized orally with 20 µL of the bacterial suspension. Immunization groups in this study were shown in Table 3. The group A of mice was immunized with a mixture of the vaccine strains including *papA*, *papG* and LTB strains. The mice in the group B were immunized with a mixture of the vaccine strains including *papA*, *papG*, IutA, CS31A and LTB strains, whereas the group C were immunized with a mixture of the vaccine strains including *papA*, *papG*, IutA and CS31A strains. Group D for the control received PBS only. Food and water were withdrawn 4 h prior to immunization and resupplied 30 min after immunization. Blood samples were obtained by retro-orbital puncture with heparinized capillary tubes at biweekly intervals. Sera were obtained from the whole blood by centrifugation at $4,000 \times g$ for 5 min and stored at -20°C until used. Vaginal secretions were collected by washing the vaginal tracts with 100 µL of sterile PBS and stored at -20°C until used [12]. The animal experiment described in this study was conducted with approval (CBU 2011-0017) from the Chonbuk National University Animal Ethics Committee in accordance with the guidelines of the Korean Council on Animal Care.

Enzyme-linked immunosorbent assay (ELISA)

Polystyrene 96-well flat-bottom microtiter plates were coated with 1 µg of purified antigens per well and blocked with PBS containing 3% skim milk at 4°C overnight. Vaginal secretions and sera were diluted 1 : 4 and 1 : 100, respec-

tively. A 100 μ L volume of diluted sample was added to individual wells in duplicate and incubated for 1.5–2 h at 37°C. Plates were treated with horseradish peroxidase-conju-

gated goat anti-mouse IgG, IgG1, or IgG2a (Southern Biotechnology, USA) for sera and IgA for vaginal secretions. The reactions were visualized with *o*-phenylenediamine (Sigma-Aldrich) and stopped by H₂SO₄. The absorbance in each well was measured by an automated ELISA spectrophotometer (TECAN, Austria) at a wave length of 492 nm. The concentrations of antibodies were determined by comparing the result with two concentrations of the standard immunoglobulin protein.

Results

Recombinant attenuated *S. Typhimurium* vaccines expressing *papA*, *papG*, *IutA*, CS31A and LTB.

Plasmid pBP244 was designed to effectively translocate antigens into extracellular space. The expression of proteins derived from *Salmonella* vaccines was confirmed by immunoblot analyses. The strains produced the *papA*, CS31A and LTB at approximate molecular weights of 21 kDa, 29 kDa and 11 kDa respectively, and a large amount of these proteins were detected in the culture supernatant (Fig. 1). The *papG* and *IutA* proteins were barely detected in the supernatant and were mostly detected in the cell pellets.

Fig. 1. (A) Immunoblot analysis of *papA* vaccine, (B) CS31A vaccine, (C) LTB strain. Lane 1: vector control supernatant and lane 2: vaccine strain supernatant. *papA* (21 kDa), CS31A (29 kDa), LTB (11 kDa) proteins are indicated by arrows.

Fig. 2. Antigen-specific serum IgG responses. Antibody titers are shown as geometric mean titers for each group single oral immunization. Panel *papA*: serum IgG responses to *papA*, panel *papG*: serum IgG responses to *papG*, panel *IutA*: serum IgG responses to *IutA*, and panel CS31A: serum IgG responses to CS31A.

Fig. 3. Antigen-specific mucosal IgA responses. Antibody titers are shown as geometric mean titers for each group single oral immunization. Panel A: mucosal IgA responses to *papA*, panel B: mucosal IgA responses to *papG*, panel C: mucosal IgA responses to *IutA*, and panel D: mucosal IgA responses to CS31A.

Systemic and mucosal immune responses induced by recombinant *S. Typhimurium* vaccines

To evaluate the protective immune responses elicited by immunization with vaccine strains in combinations, BALB/c mice were immunized with a mixture of two or four vaccine strains. After immunization, the vaginal samples and sera were obtained at biweekly intervals to measure antigen-specific antibody responses. Adjuvant effects of recombinant LTB were also investigated. The antigen-specific serum IgG and mucosal IgA titers generated by immunization with vaccine strains were significantly higher than those by administration with PBS only. Serum anti-*papA*, -*IutA*, and CS31A IgG responses peaked at six weeks postimmunization, and the responses gradually declined. Serum *papG*-specific IgG responses increased slowly, and maximal levels detected at 10 weeks postimmunization in group B and group C, immunization groups with four vaccine strains. Group A, immunization with two vaccine strains in the presence of the LTB, induced higher serum anti-*papG* IgG responses as com-

pared to group B and group C. There was no significant difference between group B and group C in antigen-specific serum IgG responses (Fig. 2). In vaginal samples, however, antigen-specific IgA titers rose faster and higher in group B in the presence of LTB than in group C in the absence of it. Maximal anti-*papG* and -*IutA* levels were detected at 8 weeks postimmunization, while maximal anti-*papA* and -CS31A levels were detected at 12 weeks postimmunization, and these gradually declined afterward (Fig. 3).

IgG isotype analyses

To further evaluate the nature of immunity following immunization, we determined the levels of IgG isotype subclasses IgG2a and IgG1 at 6th week postimmunization. The IgG1 immune response was detected in lower than 100 ng/mL titer, and data not shown. The IgG2a dominant immune responses were observed in all groups and the group B and group C produced more antigen-specific IgG2a isotype than the group A did (Fig. 4).

Fig. 4. Antigen-specific serum IgG2a responses. Serum IgG2a antibody titers are shown as geometric mean titers at 6 weeks.

Discussion

There are a variety of virulence-associated proteins of APEC playing important roles in the pathogenesis of colibacillosis. It has been proved that several virulence factors could be vaccine candidates [8, 14, 20]. However multiple virulence factors are produced by APEC strains and immune responses against one factor are not protective against APEC bearing different virulence factors. One of the strategies to overcome these is to combine different virulence factors in order to provide better protection. In this study, the recombinant attenuated *Salmonella* vaccines for *papA*, *papG*, *IutA* and CS31A virulence factors were constructed using the *S. Typhimurium* χ 8501, JOL912 and the plasmid pBP244 [13] as a live vehicle for production and oral delivery of these virulence factors.

The antigen placed in the periplasm and extracellular fluid induced significantly higher levels of IgG than that mostly retained in the cytoplasm [11]. Immunoblot analyses showed that *papA* and CS31A proteins mostly detected in the culture supernatant, whereas *papG* and *IutA* protein barely detected in the supernatant and mostly detected in the cell pellet (data not shown). However, the ELISA results showed that the *papG*- and *IutA*-specific immune responses were of similar titer to the *papA*- and CS31A-specific immune responses regardless of the immunoblot data, which suggested that considerable amount of *papG* and *IutA* proteins were located in the periplasm and induced the immune responses.

In this study, all immunized animals achieved high antibody titers against each of the guest APEC antigens, which demonstrated that immunization with a combination of vaccines elicits immune responses to every antigen represented in the mix. The other studies [3] also reported that immunization with a combination of several virulence-associated proteins or their polyclonal antibodies might elicit better protection against pneumococcal infections.

Mucosal vaccination necessitates a suitable adjuvant, and LTB have been shown to enhance the immune response to co-administered antigens [6]. Our data showed that the antigen-specific mucosal IgA responses were significantly higher

in the group of mice immunized with LTB than those in the group of mice immunized without LTB. Thus, this suggested that LTB could also be used as a mucosal immune adjuvant for these vaccines consisting of the APEC virulence factors.

In mice, T-helper type 1 (Th1) and Th2 cells stimulate antigen-specific B cells to secrete IgG2a and IgG1, respectively [21]. The ratio of IgG2a and IgG1 is therefore an indirect indication of the bias of the immune responses. In this study, immunization with the mixture of four strains expressing P-fimbriae, *IutA* and CS31A produced more IgG2a isotype than immunization with the mixture of two strains expressing P-fimbriae only, which resulted in driving immunity biased to the Th1 pattern, which is related to cell mediated effector responses. Thus, the observation of Th1 type immune response may suggest that immunization with a combination of several virulence-associated vaccines is one of the strategies of developing effective vaccines for APEC, which survives inside macrophages and cell mediated immunity is more required to protect this pathogen [1, 24].

In conclusion, the construction of recombinant attenuated *Salmonella* vaccines expressing several virulence factors of APEC demonstrated that the immunization with the mixture of the vaccine strains is capable of eliciting both mucosal and systemic immune responses to every antigen represented in the mixture. LTB had effects on enhancing immunity in the mixture and can be used a mucosal immune adjuvant for the vaccines expressing APEC virulence factors.

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