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# Induction of Systemic and Mucosal Immune Responses in Mice Orally Administered with Recombinant Attenuated *Salmonella* Expressing Subunits of P Fimbriae of Avian Pathogenic *Escherichia coli*

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**Abstract :** Avian pathogenic *Escherichia coli* (APEC) causes a number of extraintestinal diseases in poultry. A virulence factor, P-fimbriae is firmly associated with the diseases. In this study, to develop an effective vaccine for the prevention of APEC, recombinant attenuatted *Salmonella* Typhimurium vaccines expressing PapA and PapG of P-fimbriae were evaluated whether these induced protective immune responses in murine models. Female BALB/c mice were primed and boosted orally at 7 and 10 weeks of age. In all immunized mice, the antigen-specific serum IgG levels were remained higher than those in the control mice from the fourth week post inoculation till the end of this study. In addition, antigen-specific serum IgG levels in the prime-booster immunized mice were enhanced as compared to the single immunized mice among each immunized group. The antigen-specific mucosal IgA levels in the mice immunized with each strain also induced higher than those in control mice. In addition, serum IgG and fecal IgA levels in mice administered with the combination of both strains were highly induced compared to those in mice immunized with each strain alone. These results indicated that PapA and PapG worked together for inducing high immune responses. To partly discern the nature of immunity induced by the strains, we quantified serum IgG subtypes IgG1 and IgG2a specific to antigens. The PapA and PapG strains biased the immunity to the Th1-type, as determined by the IgG2a/IgG1 ratio. On the other hand, the immunization with the both strains in combination produced mixed Th1- and Th2-type immune responses. These indicated that immunization with the combination of PapA and PapG could elicit both humoral and cell-mediated immunities.

***Key words*** **:** APEC vaccine, P-fimbriae, mucosal and systemic immune responses.

## Introduction

Avian pathogenic *Escherichia coli* (APEC) is a causative agent for a number of extraintestinal diseases in poultry, including airsacculitis, colisepticemia, and cellulitis. APEC initially infects the respiratory tract of poultry, followed by an invasive phase to the air sac system and vital organs such as liver, heart and spleen (5,6,20,24). APEC is capable of surviving inside macrophages (1), and resistance to phagocytosis may be an important mechanism in the development of collibacillosis (20). These diseases account for significant losses to the poultry industry and mortality can reach 20%, especially under poor environmental conditions and in the presence of concurrent infections such as viral or mycoplasma infections (27).

The pathogenicity of APEC is based on the presence and expression of various virulence factors. One of the factors, P-fimbriae encoded by *pap, prs*, or related gene clusters, is firmly associated with the pathogenecity (5,15). P-fimbriae is thought to act in later stages of the infection and considered to play an important role in the colonization of systemic organs (5).

The prevention of infection by environmental control measures is not sufficient to eradicate APEC. Treatment with antibiotics is expensive and not always effective due to rapid development of multi-resistant pathogenic strains of APEC (2,3,9). A valuable alternative for this *E. coli* infection is prevention by vaccination. There have been many attempts to develop vaccines and have been tested experimentally (8,12,22). P-fimbriae have been considered a suitable vaccine candidate for colibacillosis of chickens (11).

Genetically modified attenuated *Salmonella* have been used as vaccine delivery vehicles for heterologous pathogenic antigens and a balanced lethal host-vector system based on the aspartate β-semialdehyde dehydrogenase (*asd*) gene has been used to specify recombinant antigens from $Asd^+$ plasmids (14,26). Stable $Asd^+$ vectors, pYA3493 and pYA3560 have been constructed to enable stable expression of recombinant antigens in the *Salmonella* systems (10).

In this study, we have constructed recombinant attenuated *S.* Typhimurium vaccine strains expressing PapA and PapG of P-fimbriae. These constructs were then evaluated whether these induced antigen specific immune responses using murine models and examined for the potential as vaccines against APEC.

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## Materials and Methods

### Bacterial strains, plasmid and media

The bacterial strains and plasmids used in this study are listed in Table 1. The *Δcrp-28 ΔasdA16* attenuated *Salmonella enterica* serovar Typhimurium strain, χ8501, and the plasmids pYA3493 and pYA3560 were kindly provided by Ho Young Kang, Department of Microbiology, College of Natural Sciences, Pusan National University, Korea. All strains were cultured by using Luria-Bertani media (LB) (Difco, USA) with or without antibiotics. Diaminopimelic acid (DAP) (Sigma, USA) was added for the growth of $Asd^-$ strains (14). Phosphate-buffered saline containing 0.01% gelatin (BSG) was used for the dilution of the vaccine strains.

### Construction of strains for protein expression and purification of PapA and PapG proteins

The genes for expressing subunit proteins such as PapA and PapG of P-fimbriae were obtained by polymerase chain reaction (PCR) using primers for PapA (F, 5'-CCGCGAATTCGCTCCAACTATTCCACAG3'; R, 5'CCCGCGTCGACTTACTGGTAACTTAAATT-3') and PapG (F, 5'-CCGCGAATTCATGAAAAAATGGTTCCCAGCTTTG3'; R, 5'-CCCGCAAGCTTTTATGGCAATATCATGAGCAGCG-3'). These primers were designed using DNASTAR Lasergene 5.07/5.52 program (DNASTAR Inc., USA). Template DNA was purified from a $P\text{-fimbriae}^+$ *E. coli* isolate JOL443. The amplified PCR products were cloned into the pET28a (Novagen, Germany) or pQE9 vector (Qiazen, Germany). These PapA and PapG expression plasmids were introduced into an *E. coli* Top10 (Invitrogen, USA) or M15 (Qiazen, Germany), respectively, resulted in the strains for protein expression, JOL711 and JOL714. In-frame cloning was confirmed by nucleotide sequencing. The proteins were purified by an affinity purification process with $Ni^{2+}$-nitrilotriacetic acid-agarose support (Qiazen, Germany). The purified proteins were verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the total amount of purified proteins was determined by using a protein assay kit (Bio Rad, USA).

### Production of rabbit antisera against PapA and PapG

Purified protein antigens were administered subcutaneously in Freud complete adjuvant (Sigma, USA) to NewZealand White rabbits in dose of 250 μg of purified protein antigen and then followed booster injection with 250 μg of purified protein antigen in Freud incomplete adjuvant (Sigma, USA) 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 *papA* and *papG* genes of P-fimbriae were cloned into the $Asd^+$ pYA3493 and pYA3560, respectivley. These cloned plasmids were introduced into $Asd^-$ χ6212, to obtain the balanced lethal *E. coli* constructs, and resulted in the strains, JOL754 and JOL748, respectively. The purified plasmids ob-

**Table 1.** Bacterial strains and plasmids used in this study

| Strain or plasmid | Characteristic | Reference |
|---|---|---|
| *E.coli* | | |
| TOP10 | *E. coli* host strain | Invitrogen |
| M15 | *E. coli* host strain, kanamycine resistance | Qiazen |
| χ6212 | $F^-$ $\lambda^-$ ϕ80*Δ(lacZYA-argF) endA1 recA1 hsdR17 deoR thi-1 glnV44 gyrA96 relA1 ΔasdA4* | 14 |
| JOL443 | Wild type $P\text{-fimbriae}^+$ *E. coli* isolate from chicken | This study |
| JOL711 | Top10 (containing pET28a expressing PapA) | This study |
| JOL714 | M15 (containing pQE9 expressing PapG) | This study |
| JOL754 | χ6212 (containing pYA3493 expressing PapA) | This study |
| JOL748 | χ6212 (containing pYA3560 expressing PapG) | This study |
| *S.* Typhmurium | | |
| χ8501 | *hisGΔcrp-28ΔasdA16* | 7 |
| JOL713 | χ8501 (containing pYA3493 expressing PapA) | This study |
| JOL755 | χ8501 (containing pYA3560 expressing PapG) | This study |
| JOL707 | χ8501 (containing pYA3493 only) | This study |
| Plasmids | | |
| pET28a | Protein expression vector, kanamycine resistance | Novagen |
| pQE9 | Protein expression vector, ampicillin resistance | Qiazen |
| pYA3493 | $Asd^+$; pBR *ori*, β-lactamase signal sequence-based periplasmic secretion plamid | 10 |
| pYA3560 | $Asd^+$; p15A *ori* | This study |

tained from JOL754 and JOL748 were then electroporated into the attenuated *S.* Typhimurium strain χ8501, and resulted in the strains, strain JOL713 and JOL755, respectively. Selection for transformants was achieved by growth on LB agar plates without DAP supplementation. Only clones containing the recombinant plasmids were able to grow under these conditions. The expression of the antigen was assessed by an immunoblot analysis.

**SDS-PAGE and immunoblot analyses**

SDS-PAGE was performed by using standard procedures. Antigen proteins of vaccine strains were prepared as follow. The strains grown in LB broth to an optical density at 600 nm ($OD_{600}$) of 0.8 were centrifuged at 7,000 × g for 10 min, and the supernatant were collected for analysis of secreted antigens. The supernatant was filtered by using 0.22 μm-pore size filters, and secreted proteins were precipitated with 10% trichloroacetic acid for 1 h at 4°C. An equal volume of each fraction sample was separated by SDS-PAGE. For immunoblot analysis, proteins were transferred from the SDS-PAGE (12% [wt/vol] polyacrylamide) to 0.2 μm microporous polyvinylidene fluoride membranes (Millipore, USA). The membranes were probed first with the rabbit polyclonal antiserum against PapA and PapG, and then with a goat anti-rabbit IgG conjugated to horseradish peroxidase (Pierce, USA). Detection of PapA and PapG was achieved upon development with a immunoblot detection system kit (Intron, Korea).

**Immunization and sample collection**

Immunization groups in this study were shown in Table 2. Four groups of five inbred 7 weeks old female BALB/c mice were used for the experiment. The group A of mice was immunized orally with $2 \times 10^9$ colony-forming units (CFU) of the PapA strain, JOL713, whereas the group B was immunized with PapG strain, JOL755. The mice in the group C were immunized with both strains. Group D for the vector control received the attenuated *S.* Typhimurium containing the vector pYA3493 only. Food and water were withdrawn 4 hours prior to immunization and resupplied 30 min after immunization. An identical booster dose was administered 3 weeks later. Blood samples were collected via retro-orbital puncture using a Pasteur pipette at two week intervals from week (week 0) before immunization till week 20 post prime immunization (PPI) for the evaluation of serum IgG. Sera were obtained from the whole blood by centrifugation at 4,000 × g for 5 min and stored at −20°C until used. Vaginal secretion specimens were collected in a 100 μl PBS wash and stored at −20°C until used (10).

**Table 2.** Experimental groups of mice in this study

| Group | Strain administered |
|---|---|
| A | JOL713 (PapA vaccine) |
| B | JOL755 (PapG vaccine) |
| C | JOL713 + JOL755 |
| D | JOL707 (containing pYA3493 only) |

**Enzyme-linked immunosorbent assay (ELISA)**

Polystyrene 96-well flat-bottom microtiter plates were coated with 1 μg of purified PapA or PapG per well. Antigens suspended in sodium carbonate coating buffer (pH 9.6) were applied with 100 μl volumes in each well. The coated plates were incubated at 4°C overnight and were blocked with PBS containing 3% skim milk. Vaginal secretions and sera were diluted 1:4 and 1:100, respectively. A 100 μl 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-conjugated goat anti-mouse IgG, IgG1, or IgG2a (Southern Biotechnology Inc., USA) for sera and IgA for vaginal secretions. The reactions were developed with *o*-phenylenediamine (Sigma, USA) in 0.05M phosphate citrate buffer (pH 5.0). The absorbance was measured at 492 nm in an automated ELISA spectrophotometer (TECAN, Austria). 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 and PapG antigens**

As *papG* gene was cloned into the pYA3493, no transformant was isolate, suggesting that PapG could be lethal to *S.* Typhimurium. Therefore, *papG* gene was cloned into the pYA3560, which is middle copy plasmid, while *papA* gene was cloned into the pYA3493. These cloned plasmids were electroporated into the strain χ8501. To observe the PapA and PapG productions in the strain, the culture supernatant of the strains were analyzed by the immunoblot assay. The strains produced the PapA and PapG at approximate molecular weights of 20.3 kDa and 37.5 kDa, respectively, and a large amount of the PapA and PapG proteins were detected in the culture (Fig 1).

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

The strains were inoculated to immunize mice by individual or mixtures. The groups of mice were immunized orally with either JOL713 or JOL755, or a mixture of both strains. The vaginal secretions and sera were collected at biweekly inter-

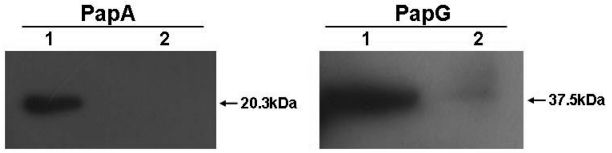


**Fig 1.** Immunoblot analysis of PapA vaccine (left) and PapG vaccine (right). Lane 1, vaccine strain supernatant; Lane 2, vector control supernatant. PapA (20.3 kDa), PapG (37.5 kDa) proteins are indicated by arrows.

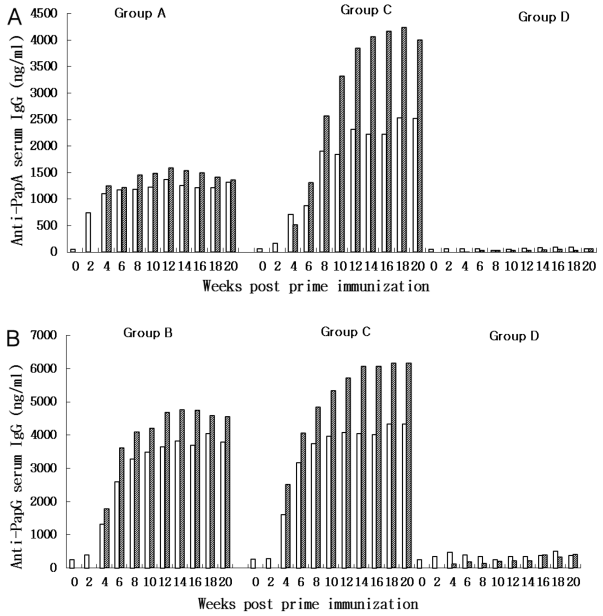


**Fig 2.** Serum IgG responses to PapA and PapG. Antibody titers are shown as geometric mean for each group of 1st immunization (open bars) and 2nd immunization (striped bars). Panel A, serum IgG responses to PapA; Panel B, serum IgG responses to PapG. Immunization groups were GroupA, JOL713 (PapA); GroupB, JOL755 (PapG); GroupC, JOL713 + JOL755; GroupD (vector control), *S.* Typhimurim containing pYA3493 only.

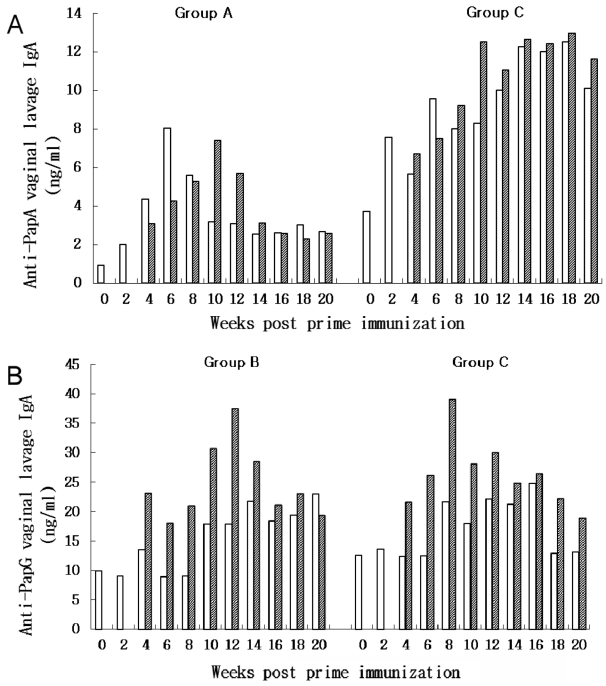


**Fig 3.** Secretory IgA responses to PapA and PapG. Antibody titers are shown as geometric mean for each group of 1st immunization (open bars) and 2nd immunization (striped bars). Panel A, secretory IgA responses to PapA; Panel B, secretory IgA responses to PapG. Immunization groups were GroupA, JOL713 (PapA); GroupB, JOL755 (PapG); GroupC, JOL713 + JOL755.

vals to assess mucosal and serum antibody responses. The antibody levels to PapA or PapG in the sera and the vaginal secretions of the immunized mice are presented in Fig 2 and Fig 3. The PapA vaccine in group A and PapG vaccine in group B induced about 13 times higher levels of antigen-specific IgG antibodies compared with the vector control in group D. Surprisingly, the combination of both strains, group C induced significantly higher levels of IgG than the individual vaccine in group A or group B. Serum IgG levels to the antigens without booster immunization were remained from 4th week PPI till the end of this study. The antigen-specific serum IgG levels were increased by a booster vaccination at 3rd week post booster (6th week PPI). The elicited antigen-specific mucosal IgA levels were also observed in the Group A and group B but not in the group D. Group C showed higher or similar levels of secretory IgA than that of group A or group B. Interestingly, the antigen-specific immune responses in both serum and vaginal lavages induced by group B were approximately 3-fold higher than those induced by group A.

### IgG isotype analyses

The nature of immunity to antigens was further examined by measuring the levels of IgG isotype subclasses IgG2a and IgG1 at 12th week post-immunization. The IgG2a dominant immune responses were observed in group A and group B. In anti-PapA IgG isotype analyses, Group C produced a higher level of IgG2a and IgG1 isotypes than group A and produced a lower

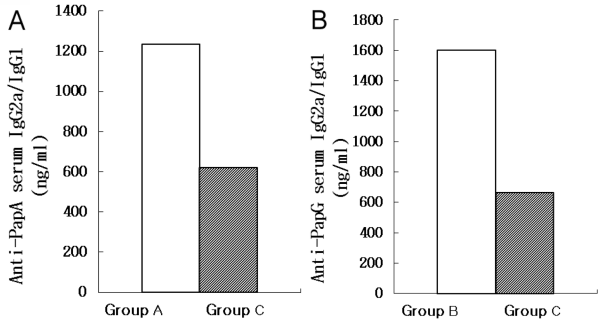


**Fig 4.** Serum IgG2a and IgG1 responses to PapA and PapG. Antibody titers are shown as geometric mean for each group IgG2a (open bars) and IgG1 (striped bars). Panel A, serum IgG subclass responses to PapA; Panel B, serum IgG subclass responses to PapG. Immunization groups were GroupA, JOL713 (PapA); GroupB, JOL755 (PapG); GroupC, JOL713 + JOL755.

IgG2a/IgG1 ratio (Fig 4). In anti-PapG IgG isotype analyses, the group B and group C produced a similar level of IgG2a and IgG1 isotypes and induced a similar ratio of isotypes.

## Discussion

The advantage of mucosal vaccination over parenteral routes of vaccination is that this mode can induce not only systemic

immune response but also mucosal responses, which results in two layers of host protection against many pathogens whose infection routes are mucosal tissues (17,28). Additional advantage of mucosal vaccination is the convenience of administration (16). Among the mucosal routes of vaccinations, the oral administration is possibly the most favored route while orally administered antigen must be resistant to the acidic environment and the activity of proteases of the gut (28). For these convenience and obstacles of the oral vaccination, attenuated *Salmonella* Typhimurium has been studied for mucosal delivery of various antigens (4,26). In this study, the recombinant attenuated *Salmonella* vaccines for P-fimbriae virulence factor were constructed using the strain *S.* Typhimurium χ8501 and the plasmids pYA3493 and pYA3560 (10) as a live vehicle for production and oral delivery of PapA and PapG of P-fimbriae. Production and secretion of PapA and PapG from the constructs were confirmed by the immunoblot assay, and induction of strong antigen-specific mucosal and systemic antibody responses was proved in murine model after oral immunization, which was an indication of the feasibility as vaccine candidates. In addition, boost immunization induced higher immune responses than those induced by single dose of immunization as suggested by other previous study (21). Interestingly, the antigen-specific mucosal and systemic immune responses were significantly higher in the group of mice immunized with the *Salmonella*-PapG vaccine than those in the group of mice immunized with the PapA vaccine. Kariywasam *et al.* (11) also reported that PapG, adhesin tip of P-fimbriae induced strong antibody responses in chickens experimentally infected with APEC. Several previous studies indicated that PapG protein has the high degree of antigenic conservation, which can induce highly effective immune responses and provide good protection against APEC (11,18).

Administration of mixture of both PapA and PapG vaccine strains generally increased the antigen-specific immune responses as compared to those immune responses by administration of each individual dose. The PapA-specific immune responses were more enhanced than the PapG responses in the mixture administration. The usage of adjuvant such as cholera toxin for vaccine antigens induces the antigen-specific Th2- type immune responses (13). Similarly, the Th2-type immune responses for PapA were observed in this study, which suggested that PapG functioned as adjuvant or carrier in the mixture administration and enhanced the immune responses for PapA.

In T-cell-dependent immunity, Th1-type immune responses are associated with the development of cell mediated immune response and promote class switching to IgG2a, while Th2-type immune responses provide B-cell antibody production and promote class switching to IgG1 (23). In this study, antigen-specific Th1-type responses were observed in the group immunized with the individual PapA or PapG vaccines, as suggested by several reports that *Salmonella* delivery system induced predominance of Th1-type responses against passenger and *Salmonella* antigens (25). On the other hand, immunization with the mixture of the PapA and PapG vaccine strains elicited both Th1- and Th2-type immune responses. Thus, immunization with the mixture of both vaccine strains can be better effective mode of vaccination against APEC than that with the individual strain, considering good protections against intracellular infections at mucosal surfaces requires both Th1 and Th2-type immune responses (19).

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# 마우스에서 조류 병원성 대장균의 P Fimbriae subunits을 발현하는 약독화 살모넬라균 경구 접종 후 면역 반응 유도 실험

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**요 약** : 조류병원성 대장균 (APEC)은 가금에서 많은 장관외 질병을 야기한다. 병원성 인자 중 하나인 P-fimbriae 또한 질병과 밀접하게 관련되어 있다. 이번 연구에서 마우스에 P-fimbriae의 subunit 들인 PapA와 PapG를 발현하는 재조합 약독화 *Salmonella* Typhimurium 백신균주 접종 후 APEC 방어에 필요한 면역반응 유도 여부를 알아보기 위한 실험을 수행하였다. 각 백신 균주로 경구 접종 후 각 항원에 대한 serum IgG 항체 역가는 접종 후 4주째부터 대조군에 비해 항체 역가가 높게 유도되기 시작하여 실험이 끝날 때까지 높은 항체 역가가 계속 유지 되었다. 한번 접종 된 경우보다 추가 접종 된 군에서 보다 높은 항체 역가가 관찰되었다. 더불어 각 항원에 대한 mucosal IgA 역가 또한 높게 유도되었다. 두 균주를 혼합하여 접종 하였을 경우에는 각 백신 균주를 접종하였을 경우보다 높은 serum IgG와 mucosal IgA의 역가가 관찰되었다. 이 결과는 이들 백신 균주가 면역원성이 있음을 확인하는 결과였다. 또한 helper T cells type을 알아보기 위해 IgG1과 IgG2a의 항체 역가를 측정하여 본 결과 각 백신 균주를 접종하였을 경우에는 IgG2a의 항체 역가가 IgG1의 항체 역가 보다 월등히 높은 이들 백신 균주를 접종 하였을 경우에는 Th1-type의 면역반응이 유도되지만 두 백신 균주를 혼합 접종하였을 경우에는 Th1-과 Th2-type 모두를 유도하는 것으로 관찰되었다. 이 결과를 통해서 이들 두 백신 균주를 혼합 접종 하였을 경우에는 세포성 면역뿐만 아니라 체액성 면역 모두를 유도할 수 있음을 확인할 수 있었다.

**주요어** : 조류 병원성 대장균 백신, P-fimbriae, 점막 및 전신 면역 반응