<Original Article>

# Hens immunized with live attenuated *Salmonella* strains expressing virulence-associated genes in avian pathogenic *Escherichia coli* passively transfer maternal antibodies to chicks

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**Abstract:** We investigated whether maternal antibodies (mAbs) elicited by dams immunized with recombinant vaccine candidates against avian pathogenic *Escherichia coli* (APEC) can passively confer protective immunity to chicks. In the present study, pBP244 plasmids carrying selected antigens of APEC were transformed into *Salmonella* Typhimurium JOL912, which was used as a vaccine candidate against APEC. The hens were immunized with the vaccine candidates using prime or booster doses. The levels of IgG and sIgA specific to the selected antigens increased significantly following prime immunization. To evaluate the persistence of passively transferred mAbs, the levels of IgY and IgA were determined in egg yolks and whites, respectively. The eggs from the immunized group showed consistently increased levels of IgY and IgA until week 16 post-laying (PL) and week 8 PL, respectively, relative to the control group. The presence of mAbs was observed in chicks that hatched from the hens, and titers of plasma IgY were consistently raised in those from the immunized hens by day 14 post-hatching. Further, chicks from the immunized hens were protected from challenge with a virulent APEC strain, whereas those from non-immunized hens showed acute mortality.

**Keywords:** avian pathogenic *Escherichia coli*, immunity, passive antibody transfer, protection, vaccine

## Introduction

Maternal antibody transfer can be defined as the transfer of antibodies by a female to her offspring either through the placenta, colostrum, milk or egg [8]. Birds transmit maternal antibodies to their offspring by depositing the antibodies in the eggs [2]. There are three classes of antibodies in chickens, namely IgY (IgG), IgA, and IgM. In eggs, IgY is predominantly present in the egg yolk, whereas IgA and IgM are present in the egg white as a result of mucosal secretion in the oviduct [3]. The young chickens are susceptible to many pathogens during the first few weeks of age because their immune system is not fully developed. The time at which the newly hatched chicks start to synthesize antibodies endogenously depends on the type of antibody. The endogenous synthesis of the antibodies in chick's plasma has been reported on 3 to 12 days post hatch [15]. Therefore, the maternal antibodies that are passively transferred to the chicks are the primary means of antigen-specific protection against the early age infection such as avian pathogenic *Escherichia coli* (APEC).

The APEC infection causes colibacillosis, a disease of serious economic significance to poultry industry that leads to substantial economic losses worldwide [5]. The infection is mostly characterized by initial colonization of the respiratory tract followed by dissemination to internal organs which leads to pericarditis, perihepatitis, peritonitis and septicemia [4]. The most prevalent genes associated with virulence such as genes for P-fimbriae (*pap* gene cluster), CS31A adhesin (*clpG*) and iron regulated aerobactin receptor (*iutA*) have been discovered in APEC [5]. Due to rapid development of genetically transferred resistance to antibiotics, effective prophylactic or therapeutic vaccination against APEC infection might be useful in either reducing the disease incidence or eradicating the disease. A number of experimental vaccines have already been evaluated for the prevention of colibacillosis [9]. However, none of these vaccines have been shown to offer the protection against the multi-virulent factor bearing APEC strain. The value of passively transferred maternal antibodies in protection against APEC infection has been reported [11, 12]. Since anti-*Escherichia* (*E.*) *coli* antibodies are transferred from immunized hens to their progeny through the yolk [9], control of the APEC infection through vaccination of parent flocks is an alternative approach to protect chicks during the first few weeks post-hatch through maternal antibody transfer. The passively transferred antibodies have been shown to

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offer protection against the heterologous APEC challenge in broiler chicken [11], although these were primarily results based on the protection observed in the chicks after the inoculation of egg yolk antibodies extracted from the hens immunized with APEC antigens. There are also several reports regarding the transfer of other pathogen-specific antibodies from hens to their chicks via the egg and their role in the protection of newly hatched chicks from the pathogens [9, 20], while the mechanism of protection against the virulent bacterial infection in young chicks via passively transferred egg yolk and egg white antibodies have not been studied in detail.

The current study was designed to examine the resistance to APEC infection in chicks by passively transferred APEC antigen specific maternal antibodies via egg yolks and egg whites from hens immunized with a live recombinant *E. coli* polyvalent vaccine set using a *Salmonella* delivery system. In addition, using a time-course approach, we also aimed to explore the persistence of maternal antibodies in the egg yolks and egg whites. The levels of these antibodies were quantified in the hen's plasma, egg yolks, egg whites and the chicks' plasma.

## Materials and Methods

### Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. A wild type *Salmonella* (*S.*) Typhimurium isolate, JOL401, from a chicken was used as a donor strain for the construction of vaccine strains. The attenuated *S.* Typhimurium strain JOL912 was constructed by deletion of the *lon*, *cpxR* and *asd* genes of JOL401 as previously described [10]. The cloned plasmids of APEC antigens such as P-fimbriae (*papA, papG*), Aerobactin receptor (*iutA*) and CS31A surface antigen (*clpG*) were transformed into JOL912 and were selected on LB agar plates. JOL 718, a wild type APEC

isolate derived from the liver of chicken that died of colisepticemia, was used as a challenge strain. Bacterial strains were grown at 37ºC in Luria-Bertani media (LB; Difco, USA). Phosphate-buffered saline (PBS; pH 7.0) was used for the resuspension of the vaccine and challenge strains.

### Immunization of hens

The animal experiments in this study were conducted with the approval of the Chonbuk National University Animal Ethics Committee in accordance with the guidelines of the Korean Council on Animal Care. Forty female 15-week-old Brown Nick chickens were divided into two groups (n = 20), vaccinated and non-vaccinated control group. The animals were provided with water and antibiotic-free food *ad libitum*. The groups were designated as group A, non-vaccinated control, group B, prime and boost immunization by oral route. For group B, prime oral immunization was performed at 15th week of age with 200 μL of bacterial suspension containing $1 \times 10^8$ colony-forming units (CFU) of each APEC vaccine strain and the booster dose was administered orally with the APEC vaccine at the 18th-week of age.

### Collection of plasma and intestinal wash samples

To examine the immune responses, five hens per each group were used for the sample collection at every week after prime and booster vaccination until the laying started. The plasma and intestinal wash samples were collected for six weeks post-prime immunization (PPI). The plasma samples were separated by centrifugation of the peripheral blood. The intestinal wash samples were collected as described previously with some modifications [18]. Briefly, the birds were orally inoculated with the lavage solution (6 mM PEG-3350; 40 mM Na₂SO₄, 20 mM NaHCO₃, 10 mM KCl, and 25 mM NaCl, pH 7.6; Sigma-Aldrich, USA) at 12 mL/kg of body weight. The birds were placed individually into clean empty buckets and mon-

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

| Strains/plasmid | Description | References |
|---|---|---|
| *Escherichia* (*E.*) *coli* | | |
| JOL718 | Wild type ColV+ Tsh+ Iss+ IuC+ IutA+ CS31A+ *E. coli* isolate from chicken | This study |
| *Salmonella* (*S.*) Typhimurium | | |
| JOL401 | Wild type *S.* Typhimurium isolate from chicken | This study |
| JOL912 | *S.* Typhimurium JOL 401 Δ *lon* Δ *cpxR* Δ *asdA16* | This study |
| JOL924 | JOL912 (with pBP245) | This study |
| JOL928 | JOL912 (with pBP246) | This study |
| JOL930 | JOL912 (with pBP247) | This study |
| JOL954 | JOL912 (with pBP248) | This study |
| Plasmids | | |
| pBP244 | Asd+; containing *SecA* (ATPase), *SecB* (chaperone), *LepB* (signal peptidase) genes | [13] |
| pBP245 | pBP244 carrying *papA* gene | This study |
| pBP246 | pBP244 carrying *papG* gene | This study |
| pBP247 | pBP244 carrying *iutA* gene | This study |
| pBP248 | pBP244 carrying *clpG* gene | This study |

itored for 30 min until they began to excrete the feces. At this time, a sterile solution of 5% pilocarpine (Sigma, USA) in water was injected intramuscularly at 50 mg/kg body weight. Administration of pilocarpine was followed by immediate and intermittent excretion of intestinal fluid for 30 min. After collection, the intestinal fluid was vortexed and centrifuged at 700 × g for 10 min. One milliliter of the supernatant was saved and added with a 10 µL of 100 mM phenylmethane-sulfonyl fluoride (PMSF; Sigma) solution, a 10 µL of 1% (w/v) sodium azide, and 5% (w/v) of bovine serum albumin. The samples were stored at −20ºC until use.

### Collection and bacteriological examination of eggs

Almost all hens started laying at 21 weeks of age. To evaluate the antibody pattern in egg yolk and egg white, the first 4 to 5 eggs per group, considered as eggs laid at the first week, were collected for antibody extraction and analysis. Subsequently, the eggs per group were collected for next consecutive four weeks and from the 4th-week onwards the eggs were collected at two weeks interval until 18th week of laying for antibody extraction. Hens were artificially inseminated at the 3rd week of laying to obtain the fertile eggs. The fertile eggs were collected from 3 to 7 days after artificial insemination (AI) and stored at 20ºC until hatching. All the stored eggs were hatched at the same time using an automatic hatching incubator by maintaining the temperature at 37.5ºC and humidity at 65 to 75%. To investigate the vertical transmission of vaccine strains, eggs were evaluated for the bacteriological examination. Eggs of five chickens per group were pooled in one batch at the weekly basis. Upon collection, feces on the surface of the eggs were removed and the eggs were decontaminated by dipping in ethanol (95%) for 1 min. The eggs were broken aseptically, and the total content of the eggs was pooled and homogenized. The homogenate was inoculated on brilliant green agar (BGA) for enumeration and incubated overnight at 37ºC. A volume of forty ml of buffered peptone water (Becton, Dickinson and company, France) was added to the pooled egg content and incubated overnight at 37ºC, followed by culture in Rappaport-Vassiliadis R10 broth (Becton, Dickinson and Company) for 48 h at 42ºC. A loop of the enrichment broth was streaked onto BGA and the grown colonies were examined for typical *Salmonella* species after incubation at 37ºC for 24 h. PCR assay was performed using *S.* Typhimurium specific primers for further confirmation [1].

### Extraction of antibodies from egg white and egg yolk

The antibodies were extracted from the eggs stored at 4ºC. To minimize any differences that may arise due to early and late extraction of antibodies from the egg yolk and egg white, eggs were processed in the order they were laid by completing extraction of the first week eggs before proceeding to the second week eggs. To extract immunoglobulin (Ig) from the egg white, a polyethylene glycol (PEG)-based Ig isolation method described previously [17] was modified for egg whites.

Briefly, the eggshell from the narrowed end was broken, and the egg white was allowed to run into the measuring cylinder by gently inverting the egg to facilitate the flow of egg white and the volume of the egg white was noted. Twice the volume of Dulbecco's PBS (pH 7.2) was added into the cylinder containing egg white, and the contents were mixed thoroughly by shaking. Pulverized PEG-3350 (Sigma-Aldrich, USA) was added to make a final concentration of 3.5% (wt/vol) and mixed thoroughly using a magnetic stirrer until the PEG was completely dissolved. The above mixture was then centrifuged at 14,000 × g for 10 min at room temperature. After centrifugation, the clear supernatant fluid containing the Ig was collected, leaving behind a semisolid pliable layer and stored at −20ºC until analysis. To extract Ig from the egg yolk, a chloroform-based method described earlier [23] was used. The egg yolk was taken out of the eggshell and placed in a clean petri dish. The egg yolk membrane was washed with distilled water and then broken with the help of forceps. The yolk was allowed to run into a measuring cylinder, and its volume was noted after it settled down. Twice the volume of Dulbecco's PBS was added, and the contents were mixed thoroughly by shaking. Chloroform (Duksan, Korea) equal to the volume of egg yolk and PBS was then added, and the contents were mixed vigorously, which resulted in the production of a thick emulsion. The emulsion was then centrifuged at 1,000 × g for 30 min at room temperature. After centrifugation, the mixture was separated into 3 distinct layers in the centrifuge tube: an orange-colored solution of lecithin at the bottom, a semisolid emulsion of yolk in chloroform in the middle, and a watery phase of chicken serum protein on top. The watery phase on the top containing the Ig was removed and stored at −20ºC until analysis.

### Immune response and protection assay in hatched chicks

Approximately, forty chicks per group were received after hatching. The chicks received from the non-immunized hens were designated as group I chicks whereas group II represented the chicks from the immunized hens. The hatched chicks were provided with water and antibiotic-free food *ad libitum*. The chicks were neither vaccinated nor medicated throughout the study. Five chicks per group were bled at 3, 7 and 14 days of age to examine the plasma IgY and IgA responses. The chicks in all groups were introduced to virulent APEC infection via spray at 3rd day of age. For the APEC spray infection, the chicks (n = 30) were placed into clean empty buckets and were sprayed three times with 1 × $10^8$ CFU of bacterial suspension in PBS. After each spray, the buckets were closed and the birds were exposed to the infection for 10 min. After challenge with the virulent APEC strain, the mortality rate was assessed daily for ten days. After the apparent recovery of all remaining animals, all birds were sacrificed on the 10th days post challenge. The gross lesions were assigned to the air sac, heart and liver. A score of 0 indicated no lesion and the higher scores were

associated with more severe lesions. The lesion score for the air sac was considered to be moderate or severe depending on the cloudiness and thickness with the presence of serous or fibrinopurulent exudate. The presence of cloudy and fibrinopurulent exudate in the pericardial cavity was examined for the heart. The presence of whitish perihepatic membrane on liver was considered as the characteristic APEC lesion for the liver. A mean lesion score per group was determined and the differences among the groups were compared statistically.

### Enzyme-linked immunosorbent assay (ELISA)

The presence of plasma IgG, intestinal secretory, egg white and chick's plasma IgA specific to APEC antigens such as PapA, PapG, CS31A and Iut-A was determined by using Chicken IgG and IgA ELISA Quantitation kits (Bethyl laboratories, USA) according to the instructions. The egg yolk and chick's plasma IgY antibodies were measured by using the rabbit anti-chicken IgY antibodies (Pierce Biotechnology, USA). Wells of Microlon ELISA plate (Greiner Bio-One, Germany) were coated with 100 μL of purified *E. coli* antigens at a concentration of 5 μg/mL. The values for plasma IgG and intestinal IgA were determined as the mean plus standard errors for 5 samples assayed at a dilution of 1 : 100 and 1 : 4 in PBS, respectively. The values for egg white and chick's plasma IgA and IgY in chick's plasma and egg yolk were

determined similarly except the samples were assayed at a dilution of 1 : 80 and 1 : 400 in PBS, respectively. The standard curve describing the relation between the concentration of standards and their absorbance value was generated, and the concentration of antibody for each sample was indicated as nanograms per milliliter (ng/mL). The absorbance of the developed color was measured at 492 nm. All assays were performed in duplicate.

### Lymphocyte proliferation assay

To examine the lymphocyte proliferation assay, the peripheral lymphocytes were separated from five randomly selected chickens per group using the gentle swirl technique [7, 18] until 6th week post prime immunization. The lymphocytes were washed twice with complete RPMI-1640 media supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 units/mL of penicillin, 50 μg/mL of streptomycin, and 2 μg/mL of fungizone. One hundred microliter of the cell suspension ($1 \times 10^5$ CFU) was incubated in triplicate in 96-well tissue culture plates with 50 μL of medium alone or medium containing 4.0 μg of APEC antigens such as PapA and IutA at 40°C in a humidified 5% $CO_2$ atmosphere for 72 h. The proliferation of stimulated lymphocytes was measured using adenosine triphosphate bioluminescence as a marker of cell viability with the ViaLight Plus Kit (Lonza, USA) according
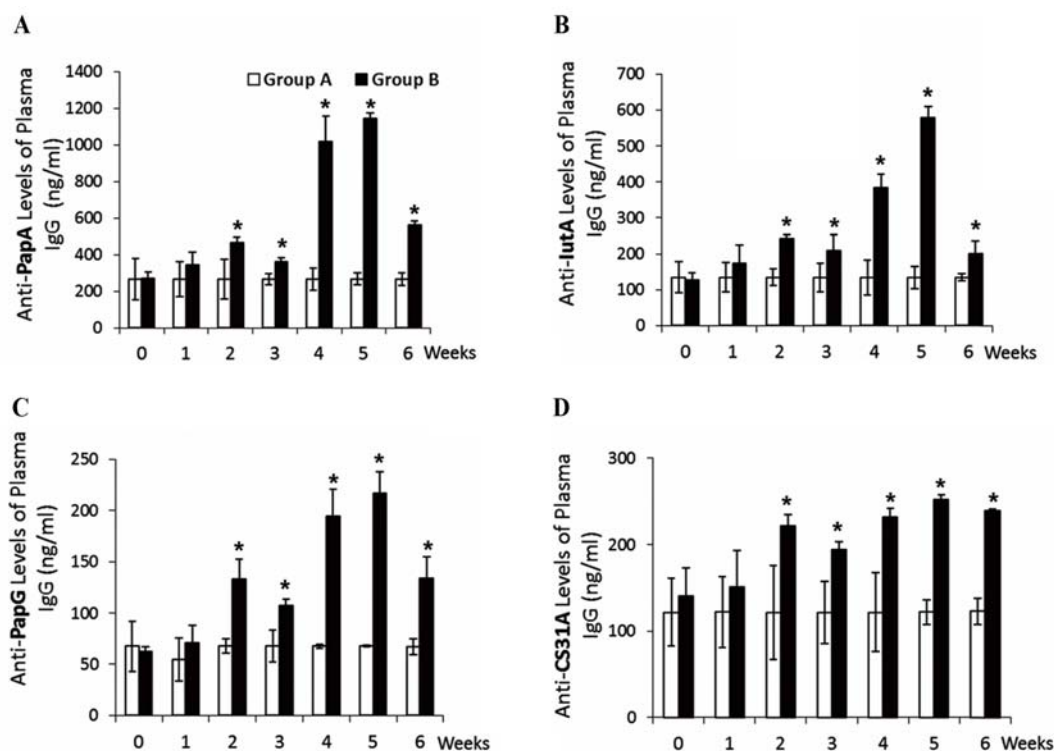


**Fig. 1.** Antigen specific plasma levels of IgG by indirect-ELISA in vaccinated and non-vaccinated hens. Plasma IgG concentration (ng/mL) was measured against the APEC antigens such as PapA (A), IutA (B), PapG (C) and CS31A (D) until six weeks post-prime immunization (PPI). The values are expressed as means ± SEM. Antibody levels were considered to be significant if $p \le 0.05$ or 0.01. *$p < 0.05$. Arrow indicates that the prime immunization was performed on the 15th week of hen's age and booster vaccination was performed at 3rd week PPI. Group A, non-immunized hens; Group B, immunized hens.

to the product information. The emitted light intensity was measured using a luminometer (TriStarLB941; Berthold Technologies, Germany) with an integrated program for 1 sec duration. The blastogenic response against the specific antigen was expressed as the mean stimulation index (SI) as previously reported [19].

### Statistical analysis

All data are expressed as means ± SEM unless otherwise specified. The analyses were performed with SPSS 16.0 (SPSS, USA). The levels of antibodies for ELISA and lymphocyte proliferative responses were compared using the Dunnett's test. Significant differences of lesion scores in chicks were determined by the Mann-Whitney $U$ test. Mortality was statistically compared using one-sided Fischer's Chi-squared test. The differences were considered significant at $p \leq 0.05$.

## Results

### Immunogenicity of live APEC vaccine in hens after immunization

**Systemic and mucosal immune responses induced by immunization of hens:** Antibody responses against four APEC antigens in the plasma and intestinal wash samples of the immunized and non-immunized groups were monitored for 6 weeks PPI. The immunized group manifested gradual

rise in plasma IgG levels that reached a plateau on the 5th week PPI (Fig. 1). The plasma IgG levels were approximately 1.9 and 1.7 times higher in group B than the control group at the 2nd- and 3rd-week PPI, respectively. The IgG response was significantly elevated after booster vaccination. After booster vaccination, the plasma IgG levels were approximately 4.8, 5 and 3.8 times higher in group B compare to the control group at the 4th-, 5th- and 6th-week PPI, respectively (Fig. 1). Mucosal secretory IgA (sIgA) levels were determined using intestinal wash samples. As shown in Figure 2, the immunized hens showed significant sIgA response with peak value at the second week PPI. The sIgA levels were 5 and 1.8 times higher in group B compared to the non-immunized group A at the 2nd- and 3rd-weeks PPI, respectively. The sIgA levels were significantly higher after booster immunization for group B. The sIgA levels were approximately 1.5, 3 and 1.9 times higher in group B than non-immunized control group at 4th-, 5th- and 6th-week PPI (Fig. 2).

**Cellular immune response:** To investigate the cellular immune responses, lymphocyte proliferation assay was carried out using PapA and IutA antigens. The proliferative responses are presented in Figure 3. Sequential monitoring of lymphocyte responses revealed a progressive increase in the SIs in the immunized hens (Fig. 3). As shown in Figure 3, there was a sharp rise in the lymphocyte proliferation response
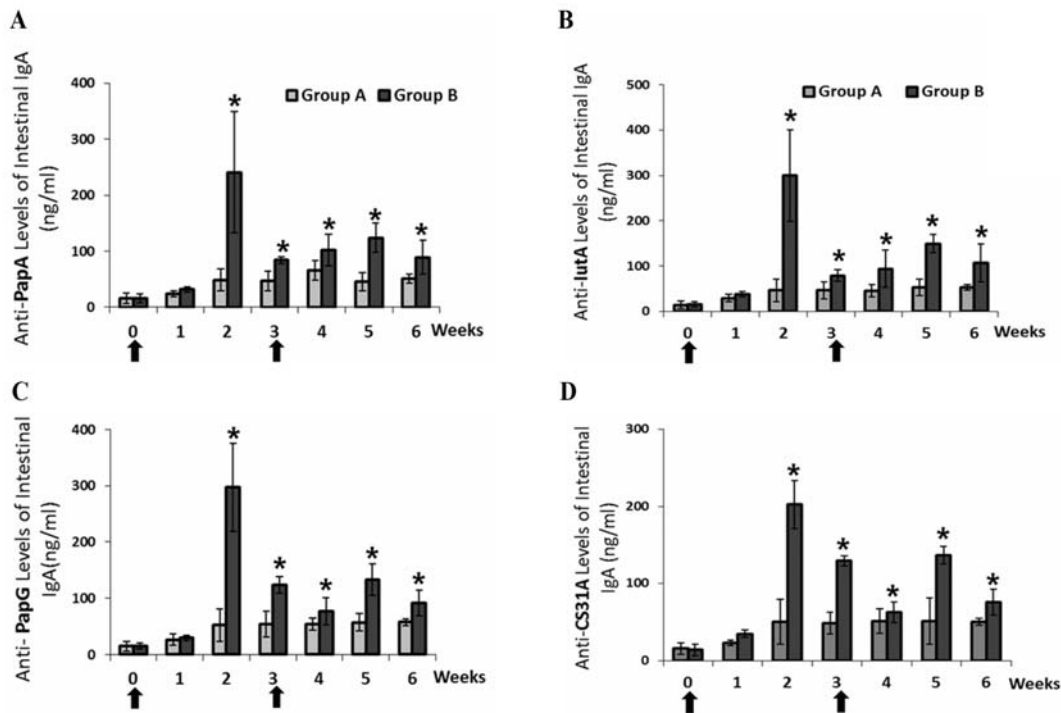


**Fig. 2.** Intestinal IgA antibody response was determined to specific antigens. The intestinal wash samples were examined for levels of IgA against the PapA (A), IutA (B), PapG (C) and CS31A (D) until six weeks PPI. The IgA antibody levels are expressed as means ± SEM. Antibody levels were considered to be significant if p ≤ 0.05 or 0.01. *p < 0.05. Arrows indicate that the prime immunization was performed on the 15th week of hen's age and booster vaccination was performed at 3rd week PPI. Group A, non-immunized hens; Group B, immunized hens.
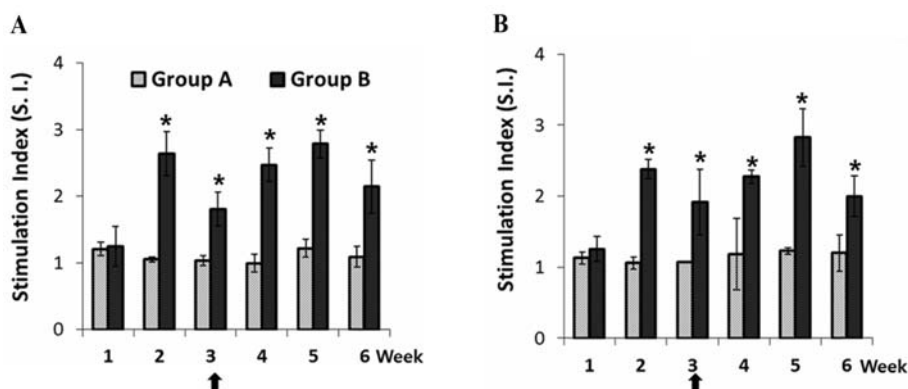
**Fig. 3.** Antigen-specific lymphocyte stimulation responses were measured in immunized hens. Lymphocyte stimulation responses against the APEC antigens such as PapA and Iut-A were determined until six weeks PPI. The stimulation index of lymphocyte sample from the chickens against PapA (A) and Iut-A (B) were indicated. *$p < 0.05$. Arrows indicate that the booster immunization was performed on the 3rd week PPI. Group A, non-immunized hens; Group B, immunized hens.
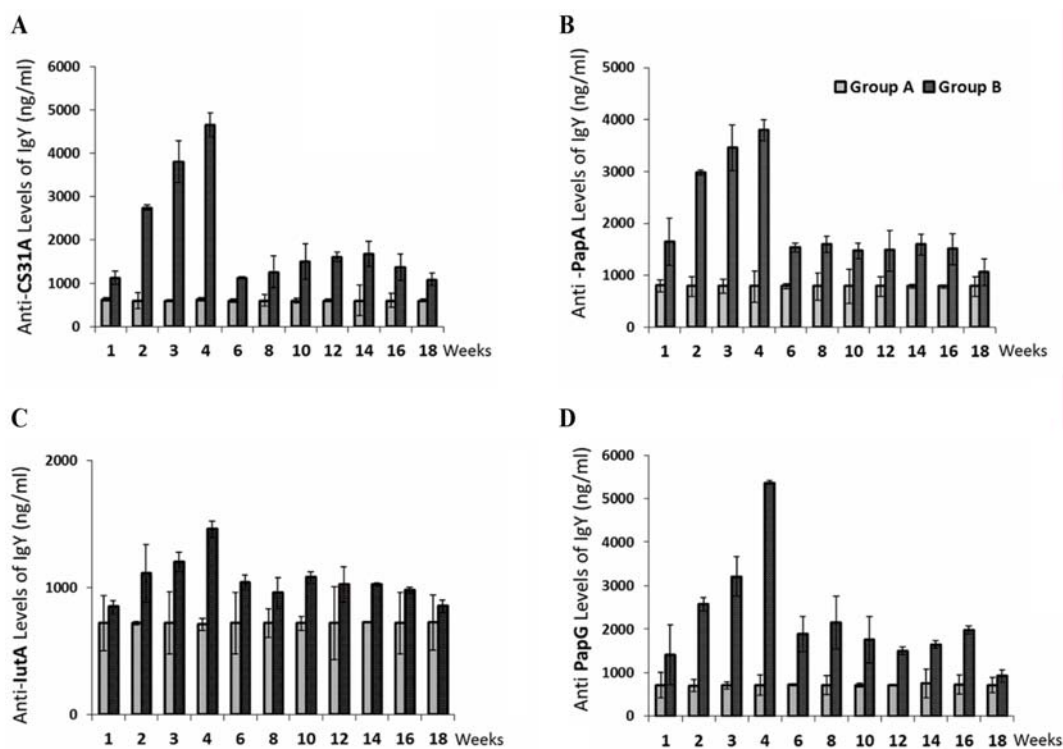


**Fig. 4.** Levels of IgY were determined in egg yolks of the immunized and non-immunized hens. The antibodies were extracted from egg yolks (5 eggs per group) and the IgY antibody levels were determined against the CS31A (A), PapA (B), IutA (C) and PapG (D) until eighteen weeks of laying. The IgY antibody levels are expressed as means ± SEM. Antibody levels detected in all eggs from the immunized hens were statistically significant ($p < 0.05$) compared to those from the control group. ◆ egg whites from non-immunized group A hens; ■ egg whites from vaccinated group B hens.

after 2 weeks PPI. The SIs for the immunized group were 2.6 and 2.3 at the second week PPI in responses to PapA and IutA antigens, respectively. There was a dramatic increase in lymphocyte proliferation at the 4th-, 5th- and 6th-week PPI probably as a consequence of booster vaccination. The SIs were 2.7 and 2.8 in group B in response to PapA and IutA antigens at the 5th-week PPI, respectively (Fig. 3).

**Evaluation of eggs for bacterial recovery and antibody presence in egg yolk and egg white**

**Bacterial recovery from the eggs of immunized hens:** To investigate the safety of the APEC recombinant vaccine to eggs, the vertical transmission of the vaccine strains to eggs were evaluated weekly. None of the vaccine strains was isolated from the eggs of immunized hens.

**Levels of egg yolk IgY and egg white IgA antibodies:** The values of egg yolk IgY and egg white IgA for group A (non-immunized hens) and group B (immunized hens) eggs against the APEC antigens are presented in Figures 4 and 5, respectively. The antigen-specific IgY levels were detectable in egg yolks of the immunized hens from the first week of laying. The IgY levels were approximately 2.5 times higher in group B eggs compared to the control group eggs at the first week and gradually increased for the subsequent four weeks (Fig. 4). The antigen-specific IgY levels in the egg yolks of the immunized hens reached significantly high concentrations at the 4th week of laying (Fig. 4). After peak antigen-specific concentrations of IgY in egg yolks of group B were observed at the 4th week, the egg yolk IgY levels were declined relatively but were significantly higher than the egg yolks of group A until the 16th week (Fig. 4). The antigen-specific IgA levels in egg whites of group B were significantly higher than the group A levels (Fig. 5). On subsequent weeks, the egg white IgA levels for group B were declined but were significantly greater than the group A IgA levels until the 8th week (Fig. 5). There were no significant differences between the IgA levels for group B and group A after the 8th week.

## Immunity and protection against an APEC virulent challenge in chicks

**Passive immunity in chicks:** The antigen-specific immune responses for group I (chicks of non-immunized hens) and group II (chicks of immunized hens) chicks were evaluated on days 3, 7 and 14 of age. The plasma IgY levels for group II were significantly higher on day 3 compared to group I (Fig. 6). The IgY levels were approximately 3 times higher in group II on day 3 compared to group I (Fig. 6). The plasma IgA levels were significantly higher for group II on day 7 of age (Fig. 7). The antigen-specific plasma IgY and IgA levels were declined for the group II on day 14 of age (Figs. 6 and 7).

**Protection assay in chicks:** Three day old chicks were challenged with an APEC virulent strain. Upon challenge, group I showed acute mortality (13.3%). Four out of thirty chicks died after the virulent APEC challenge in group I whereas group II did not show mortality (Table 2). The remaining chicks were sacrificed at day 10 post challenge. Group I showed significantly higher lesion score ($1.7 \pm 3.2$) compared to group II (Table 2). Eight out of twenty six remaining chicks in group I showed the typical APEC lesions whereas two among thirty birds in group II showed mild lesion score (Table 2).
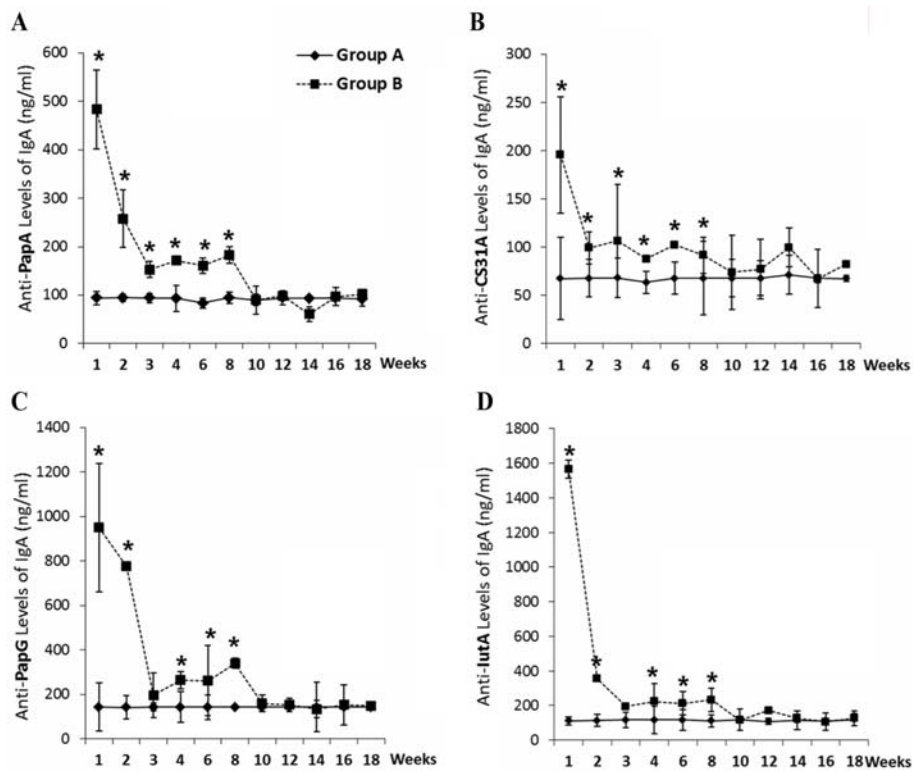


**Fig. 5.** The egg whites collected from the immunized and non-immunized hens were examined for the presence of IgA antibody levels. The antibodies were extracted from egg whites (5 eggs/group) and the IgA antibody concentration was determined against the PapA (A), CS31A (B), PapG (C) and IutA (D) until eighteen weeks PL. The IgA antibody levels are expressed as means ± SEM. Antibody levels were considered to be significant if $p \leq 0.05$. $*p < 0.05$. ◆ egg whites from non-immunized group A hens; ■ egg whites from vaccinated group B hens.
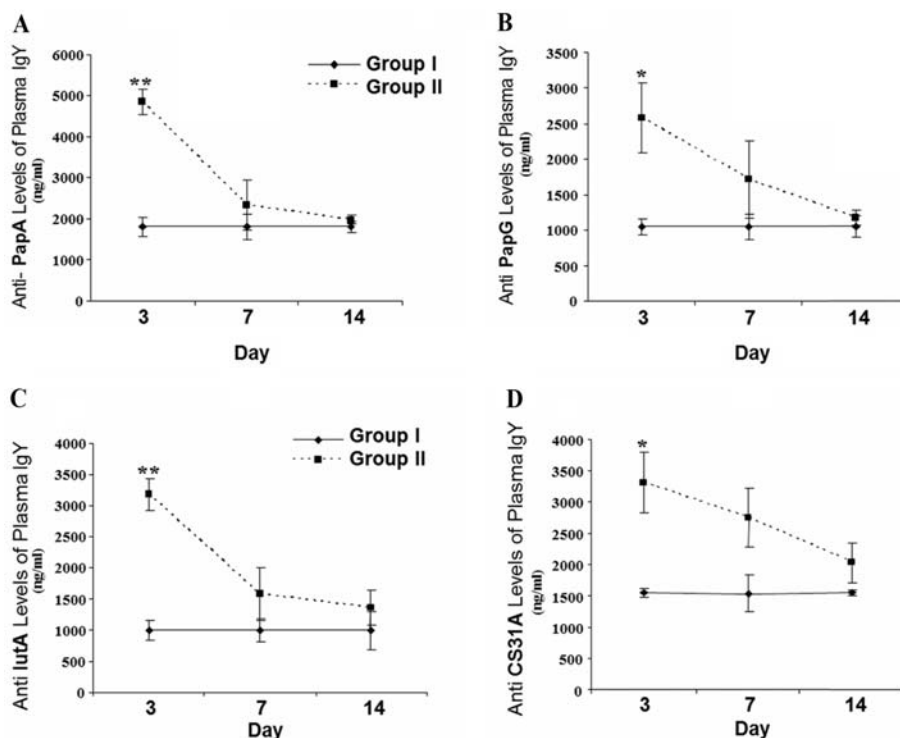
**Fig. 6.** Plasma Levels of IgY were determined in chicks of non-immunized hens and immunized hens. The IgY antibody levels were determined against the PapA (A), PapG (B), IutA (C) and CS31A (D) on 3, 7 and 14 day of age. The IgY antibody levels are expressed as means ± SEM. Antibody levels were considered to be significant if $p \leq 0.05$. *$p < 0.05$. Group I (◆), chicks of non-immunized hens; Group II (■), chicks of immunized hens.



**Fig. 7.** Levels of plasma IgA were examined in chicks of non-immunized hens and immunized hens. The IgA antibody concentration was determined against the PapA (A), PapG (B), IutA (C) and CS31A (D) on 3, 7 and 14 day of age. The IgA antibody levels are expressed as means ± SEM. Antibody levels were considered to be significant if $p \leq 0.05$. *$p < 0.05$. Group I (◆), chicks of non-immunized hens; Group II (■), chicks of immunized hens.
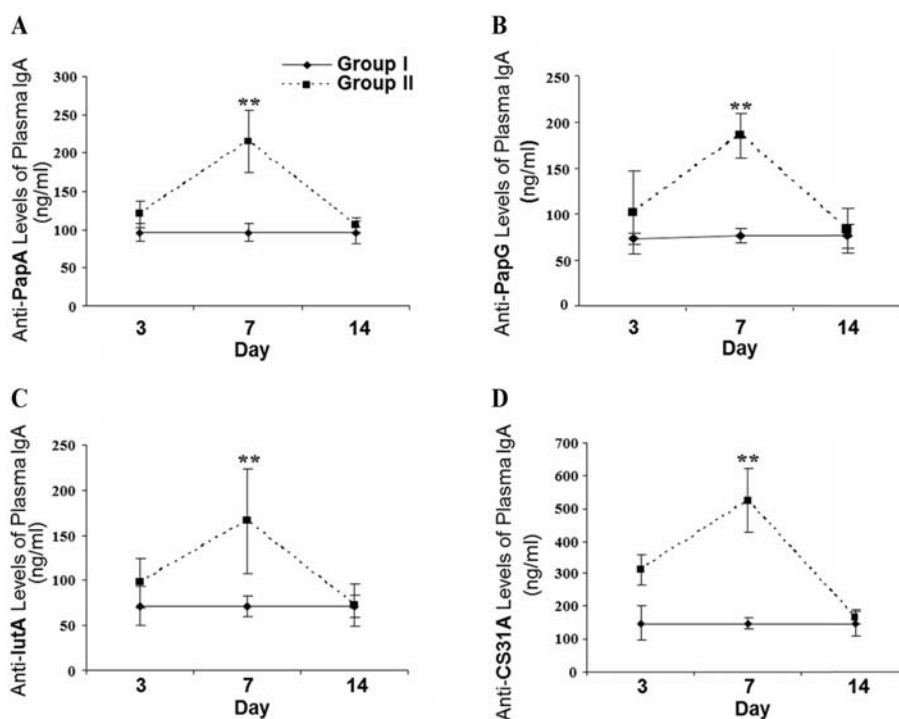
**Table 2.** Protections in hatched chicks

| Group* | Observations post challenge[†] | | |
|---|---|---|---|
| | Mortality[‡] (%) | Morbidity[§] (%) | Mean lesion score[‖] |
| I | 4 (13.3) | 8 (26.6) | 1.7 ± 3.2 |
| II | 0 (0) | 2 (6.6)[a] | 0.2 ± 0.76[b] |

*Each group contained 30 chicks. The groups were designated as group I, chicks of non-immunized hens and group II, chicks of immunized hens. [†]The APEC infection was performed by spray of JOL 718 with $1 \times 10^8$ CFU on 3rd day of age. [‡]The number of dead birds was expressed as mortality. [§]Morbidity referred to the number of birds with typical APEC lesions. [‖]A total lesion score was determined for each chicken and a mean lesion score per group was determined. Each acute mortality case was given a score as 3. Values were considered to be significant if $p \le 0.05$ or 0.01. [a]$p \le 0.05$, [b]$p \le 0.01$.

## Discussion

The objective of the present study was to examine whether the chicks, received passively transferred maternal antibodies from the dams immunized with a live recombinant APEC polyvalent vaccine set using a *Salmonella* delivery system, were resistant to APEC infection. This is an attractive approach as it obviates the need for immunization of individual chickens and it is well suited to the poultry industry, which seeks to minimize stresses to which the chickens are exposed during vaccination. Moreover, this approach, if feasible, would protect the new-born hatchlings and could be cost-effective since vaccination of a single hen would lead to protection of over 100 chicks. In the present study, hens were immunized with a *S.* Typhimurium delivery system, attenuated by deletions of *lon* and *cpxR* [14], expressing the various APEC virulent factors such as *papA*, *papG*, *iutA* and *clpG*. Being a facultative intracellular pathogen, *Salmonella* have a potential to induce systemic, mucosal and cellular immune responses [24]. *Salmonella* delivery system efficiently induces a strong cell-mediated immune response, which is more achievable with live vaccines and also targets the mucosal-associated lymphoid tissues in the gut and thereby improves the possibility of stimulating mucosal immunity [24]. We demonstrated that the immunization of hens with the live recombinant APEC polyvalent vaccine set using the ST delivery system induced significantly high mucosal, systemic and cellular immune responses against the specific APEC antigens in the hens (Figs. 1-3). The systemic and mucosal immune responses were higher after prime immunization and were further elevated after the booster dose of immunization. The cellular responses were also measured by using the lymphocyte proliferation assay, which is widely used to evaluate the cell-mediated immune responses [19]. The lymphocytes collected from the immunized hens showed significant proliferation. It has been reported previously that immunization of hens with an inactivated APEC vaccine induced antibody responses which were passively transferred to the progeny [9]. However, the immunization with the inactivated vaccine required adjuvant for maintaining the high antibody titers in hens to achieve the

long-lasting passive transfer [9]. In the present study, the live recombinant vaccine system was administered without the adjuvant and thus, provides an advantage of inducing long lasting immunity, as the live *Salmonella* system has a potential to robust the longevity of humoral and cellular immunity [21].

It is a well-known fact that the laying hens transfer large amounts of immunoglobulin (IgY) to the egg yolks [12], which may be transferred passively to the progeny. The pathogen specific IgY antibodies are transferred from the dam to her offspring via egg yolk [16] whereas IgA antibodies are transferred via egg whites [3]. However, wherein the passive immunization of chicks against the APEC infection was reported, no information is available about the persistence of the APEC-specific antibodies in egg yolks and egg whites. Therefore, we further investigated the time course persistence of the APEC specific IgY and IgA antibodies in the egg yolks and egg whites of the immunized hens, respectively. The antigen specific IgY levels were higher for the egg yolks of the immunized hens with a plateau on the 4th week and persisted until 16 weeks of laying whereas IgA levels were significantly higher until the 8th week with peak levels on the first week. In our present study, the passive transfer of these antibodies to the hatchlings was also investigated. The IgY and IgA antibodies in chick's plasma were examined on days 3, 7 and 14 of age against the APEC antigens. The plasma IgY and IgA levels for group II (chicks of the immunized hens) were significantly higher on days 3 and 7 of age, respectively. On day 14, the antigen specific antibody levels decreased. This decline in the levels of the pathogen-specific antibodies in hatchings may be due to the physiological breakdown of the antibodies [22].

There are several reports which demonstrated some protection of the hatchlings via passively transferred maternal antibodies against the virulent pathogen challenge [9, 22]. However, protection of the chicks was conferred by the extracted antibodies from immunized hens [22], which might cause inconvenience, and the protection offered with an inactivated vaccine was not efficient against the heterologous challenge [9]. The protection assay against the APEC virulent challenge in this study showed highly efficient protection for chicks. The challenge strain was an APEC bearing multi-virulence factor. Upon challenge, the chicks of the immunized hens did not show any mortality whereas non-immunized hen's chicks showed 13.3% mortality. The infection rate was higher for the control group chicks compared to the immunized hen's chicks. In addition, the clinical signs and lesion scores were moderate for the chicks of the immunized hens.

The main constraint of immunization of the parent flock with live vaccines is a risk of egg contamination with the vaccine strain [6]. Contamination of the edible contents of eggs by live vaccine strain has been a continuing international public health problem [6] and thus has a serious safety concern. The presence of the vaccine strain in eggs of the immunized group was also investigated in the present study. Fortunately, no vaccine strain was detected from the eggs of

the immunized hens which suggest that the usage of the live APEC vaccine set could be a safe measure for the parent flock immunization without vertical transmission of the vaccine to the eggs.

In conclusion, our results suggested that the immunization of hens with a live recombinant *E. coli* polyvalent vaccine set using a *Salmonella* delivery system could effectively induce the immune responses which were passively transferred to egg yolks, egg whites and chicks and thus offered protection of chicks against the APEC virulent infections. We also demonstrated the longevity of the APEC specific antibodies in the egg yolks and egg whites of the immunized hens. The knowledge about the time course persistence of the APEC specific antibodies in the hens and eggs may be useful information on the strategies for protecting chicks against the virulent APEC infection at young age.

## Acknowledgments

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