ORIGINAL ARTICLE

Protection Against *Salmonella* Typhimurium, *Salmonella* Gallinarum, and *Salmonella* Enteritidis Infection in Layer Chickens Conferred by a Live Attenuated *Salmonella* Typhimurium Strain

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In the present study, we investigated the protection conferred by a live attenuated *Salmonella enterica* serovar Typhimurium (ST) strain against *Salmonella* Typhimurium, *Salmonella* Gallinarum (SG), and *Salmonella* Enteritidis (SE) infection in layer chickens. Birds were orally primed with the attenuated ST strain at 7 days of age and then boosted at 4 weeks post prime immunization (PPI). Sequential monitoring of plasma IgG and mucosal secretory IgA (sIgA) levels revealed that inoculation with ST induced a significant antibody response to antigens against ST, SE, and SG. Moreover, significant lymphoproliferative responses to the 3 *Salmonella* serovars were observed in the immunized group. We also investigated protection against virulent ST, SE, and SG strain challenge. Upon virulent SG challenge, the immunized group showed significantly reduced mortality compared to the non-immunized group. The reduced persistence of the virulent ST and SE challenge strains in the liver, spleen, and cecal tissues of the immunized group suggests that immunization with the attenuated ST strain may not only protect against ST infection but can also confer cross protection against SE and SG infection.

[Immune Network 2015;15(1):27-36]

Keywords: *Salmonella*, Vaccine, Cross immunity, Protection, Chickens

INTRODUCTION

Foodborne *Salmonella* infections in humans, occurring through the consumption of contaminated poultry meat and eggs, are a major public health concern. Therefore, the control of *Salmonella* within poultry breeding and rearing farms is an urgent issue (1). Intense international trade of animals and products of animal origin facilitates the spread of these bacteria (2), making salmonellosis an international public health concern that is responsible for serious economic losses to the poultry industry and governments worldwide (3,4). A variety of *Salmonella* strains are known to cause extra-intestinal infections in poultry (5), and the major species that cause infections in chickens include *Salmonella* Typhimurium (ST), *Salmonella* Enteritidis (SE), and *Salmonella* Gallinarum (SG). These serotypes can infect humans and are the main causes of food contamination (6). Although these infections do not cause severe symptoms in poultry, the eggs and meat of infected animals can become a reservoir for infection of human consumers. These asymptomatic bird carriers play a major role in *Salmonella* propagation and in food contamination (6). ST and SE infect chickens via the fecal-oral route, colonize the alimentary tract, invade internal organs such as the liver and spleen, and finally spread to the reproductive tract (7). SE bacteria can be transmitted to the

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Abbreviations: DPC, days post challenge; PPI, post prime immunization; SE, *Salmonella* Enteritidis; ST, *Salmonella* Typhimurium; SG, *Salmonella* Gallinarum

eggs, which can be transmitted to humans through consumption of these contaminated eggs, which is a major public health issue (8). In contrast, SG infection is host specific and causes fowl typhoid (FT), an acute disease that leads to septicemia in poultry (9). Even though SG has adapted to its avian host and rarely induces food poisoning in humans, FT outbreaks cause severe economic loses worldwide (10).

Vaccination is one of the best prophylaxes against diseases caused by infectious agents, which prevents infection via induction of innate and/or adaptive immune responses (11,12). There have been several attempts to prevent salmonellosis by vaccination (6,10,13-20), and in some studies, cross protective responses were shown to enhance pathogen clearance in an antigen non-specific way (12). More recently, cross-protective immunity among different *Salmonella* serovars has been reported in both animals and humans (21-23). Therefore, it appears that a vaccine constructed against one intracellular pathogen might provide cross-protective immunity against other similar infectious agents (24-27).

In recent years, the usage of genetically modified ST strains as immunization agents has gained remarkable popularity, as these attenuated ST strains have no ill effects (28-30) and may provide some protection against other related serovars (28). In one study, an attenuated ST vaccine strain was shown to offer protection against SE virulent challenge in chickens (31). One report also demonstrated the protection efficacy of SG vaccines against virulent SE infections in laying hens (32,33). In our previous studies, we constructed a live attenuated ST strain (34). As several other studies previously explored the cross-protection conferred by *Salmonella* vaccines against related serovars (31-33,35), immunization with this ST strain may confer protection against salmonellosis.

With these previous studies in mind, we first evaluated the safety of the live attenuated ST strain in chickens by monitoring fecal excretion and observing their general condition after administration. Next, we evaluated whether ST administration conferred protection against virulent ST, SE, and SG infections in layer chickens. Finally, the humoral and cellular immune responses against ST, SE, and SG antigens were evaluated.

MATERIALS AND METHODS

Bacterial strains and growth conditions

A live attenuated ST strain (JOL912) was constructed by as described previously (34). Wild-type SG (JOL422) and ST (JOL401) strains were obtained from the National Veterinary Research and Quarantine Service (Anyang, 430-016, Kyunggi 425-100, Republic of Korea). A wild-type SE strain (JOL1982) was obtained from Kyungpook National University (Daegu, Republic of Korea). All strains were grown at 37°C in Luria-Bertani media (Difco, Sparks, MD, USA). PBS (pH 7.0) was used to resuspend the vaccine and challenge strains.

Immunization and challenge experiment

The animal experiments were approved by the Chonbuk National University Animal Ethics Committee (CBU 2011-0017) and were performed in accordance with the guidelines of the Korean Council on Animal Care. One-day-old Brown Nick layer chicks were used for all the experiments and were provided with water and antibiotic-free feed *ad libitum*. The birds were divided into 2 groups (n=60/group), designated as group A (non-vaccinated controls) and group B (primed and boosted immunization via the oral route). At 7 days of age, the birds in group B (n=60) were primed with 1×10^7 cfu of the attenuated ST strain in 100 μl of PBS. The booster dose was administered at 4 weeks post prime immunization (PPI). Three weeks after booster administration (7 weeks post PPI), the birds (n=20/group of each serovar) were exposed to oral inoculations of wild-type virulent SG, SE, and ST strains. For the virulent SG challenge (n=20/group), the chicks were inoculated via the oral route with 100 μL of PBS containing 1×10^6 cfu of JOL422. The oral inoculations of the virulent ST and SE strains used contained 1×10^9 cfu.

Safety evaluation of the attenuated ST strain in chickens

To investigate the safety of the attenuated ST strain, the general condition of the birds was monitored daily for 2 weeks after immunization. The parameters evaluated included anorexia, depression, diarrhea, and mortality (29). Further, the presence of the delivered ST strain in fecal pellets was monitored at 3, 7, 10, and 14 days post-immunization. Animals were placed individually into clean empty buckets, allowed to defecate, and the feces were collected and weighed. The fecal material was homogenized in buffered peptone water (Becton Dickinson and Company) by mechanical disruption with a sterile wooden stick. To recover the bacteria, fecal samples were processed as described previously [18]. The positive samples were counted, and the number of positive cases for each group, with samples containing typical *Salmonella* colonies, was confirmed by PCR using an ST-specific primer set (36).

Observations of mortality, gross lesions, and bacterial recovery after SG, ST, and SE virulent challenge

After SG challenge, the mortality rate was assessed daily for 14 days. After the apparent recovery of all surviving animals, the birds were sacrificed 14 days post challenge (dpc) and were examined for macroscopic lesions and the presence of the challenge strain in internal organs such as the liver and spleen. The splenic and hepatic gross lesion exams and the challenge strain recovery were performed as previously described (18-20,37). To determine the presence of the ST and SE challenge strains in internal organs such as the liver, spleen, and caecum, 4 birds per group were randomly selected and sacrificed at 1, 4, 7, 11, and 14 dpc. PCR using specific primers (36) was performed using colonies recovered on BGA that were typical for the inoculated *Salmonella* strains.

Immune responses

Antibody responses: To examine the antibody responses, samples were collected from 5 birds per group every week after the prime and booster doses of the vaccine were administered. The levels of plasma IgG and intestinal secretory IgA (sIgA) against the outer membrane proteins (OMP) of ST, SE, and SG were determined by using the Chicken IgG and IgA ELISA Quantitation kits (Bethyl Laboratories, TX, USA) according to the manufacturer's instructions. Plasma IgG and intestinal wash samples were taken until 7 weeks PPI. To obtain plasma, peripheral blood samples were separated by centrifugation. Intestinal wash samples were collected as described previously (19,38).

Cellular response: At 3 and 7 weeks ppi, peripheral lymphocytes were separated from 5 randomly selected chickens per group using the gentle swirl technique (39). The lymphocytes were processed, and the proliferation assay was performed as previously described (18-20).

Statistical analyses

All statistical analyses were performed with SPSS 16.0 (SPSS Inc., Chicago, IL, USA). Student's t-test was used to analyze the statistical significance of differences in mortality, immune responses, gross lesion scores, and bacterial recovery among the vaccinated and unvaccinated groups. Data are presented as mean±standard deviation, and the differences were considered significant at p-values less than 0.05.

RESULTS

Safety evaluation of the attenuated ST

The general condition of the inoculated birds was observed for 2 weeks post inoculation. There was no evidence of mortality after oral inoculation. The animals were apparently healthy and did not show any signs of anorexia, depression, or diarrhea during the entire experimental period. Bacterial recovery from fecal shedding was assessed 3, 7, and 10 days PPI. Homogenized fecal samples from 5 chickens in the inoculated group were plated on BGA for direct colony counting, and no colonies were observed in the samples (Table I). However, bacterial colonies were recovered by the enrichment culture method. Bacteria were recovered from 3 of 5 inoculated birds at 3 days PPI, whereas bacteria were recovered from only 1 bird at 7 days PPI (Table I). No bacteria were recovered from feces at 10 and 14 days PPI and booster immunization.

Protection against SG challenge

Cross protection against virulent SG challenge was examined after prime-booster immunization with the attenuated ST strain. Upon challenge with the virulent SG strain, the immunized group (group A) showed significantly less acute mortality (4/20) than the non-immunized group (group B; 11/20). All surviving birds in group A showed anorexia, depression, and greenish-colored diarrhea, whereas the surviving birds in group B did not exhibit these symptoms. In addition, group B birds showed a milder average lesion score (0.1) than the group A birds (1.18). The presence of the challenge strain in hepatic and splenic tissues was examined by direct colony counting of homogenized tissues on BGA and from enrich-

Table I. Bacterial isolation from feces after immunization with the ST strain

Group[a]	Day[b]			
	3	7	10	14
A	0/5[c]	0/5	0/5	0/5
B	3/5	1/5	0/5	0/5

[a]The groups were designated as group A, non-immunized and group B, immunized. [b]The days post prime immunization. The bacterial recovery from feces was carried out at day 3,7, 10 and 14 post prime immunization. [c]Number of positive samples after enrichment culture

ment cultures. No colonies were observed on plates containing samples of direct cultures of homogenized liver and spleen tissues from group B (immunized) chickens (Table II). However, the challenge strain was recovered from the livers (4/9) and spleens (3/9) of group A (non-immunized) chickens.

Evaluation of protection against virulent ST challenge

The protection efficiency of the attenuated ST strain against virulent ST challenge was investigated. No acute mortality was observed after oral inoculation with the virulent ST strain. Examination of bacterial persistence in hepatic, splenic, and cecal tissues revealed consistently higher bacterial counts from chickens in group A than from chickens in group B from day 1 to day 14 post infection (Table III). Consistent bacterial recovery from liver tissues on days 1, 7, and 11 was observed in group A animals, which was higher than that detected in group B animals, whereas no significant differences were found on day 14 post-infection (Table III). Bacterial per-

Table II. Mortality, gross lesion and bacterial recovery in the chickens challenged with a wild type *Salmonella* Gallinarum

Group[a]	Mortality (%)	Challenge[b]					
		Gross lesion		Bacterial recovery[e]			
		Liver	Spleen	Liver		Spleen	
				Count	Positive No.	Count	Positive No.
A	11/20 (55)[c]	1.1±0.9[d]	1.1±1.0	0.4±0.5[f]	4/9[g]	0.3±0.5	3/9
B	4/20 (20)*	0.1±0.4**	0.2±0.5**	0.0±0.0**	0/16*	0.0±0.0[d]*	0/16*

[a]Each group contained 20 chicks. The groups were designated as group A, non-immunized and group B, immunized. [b]The SG infection was performed by oral inoculation at the 7th week post prime immunization. [c]The number of dead birds was expressed as mortality. [d]Group lesion score (Mean±Standard deviation). [e]The bacterial recovery from liver and spleen was carried out at the 14th day after challenge strain inoculation. [f]The bacterial count was determined and expressed as mean±Standard deviation Log_{10} cfu/g. [g]Number of positive chicken after enrichment culture. All values were considered to be significant if $p \leq 0.05$ or 0.01. *$p < 0.05$, **$p < 0.01$

Table III. Bacterial recovery in chickens after a virulent *Salmonella* Typhimurium and *Salmonella* Enteritidis challenge

Strain	Group[a]	Organ	Bacterial recovery post challenge[b]									
			Day 1		Day 4		Day 7		Day 11		Day 14	
			Count	Positive No.	Count	Positive No.	Count	Positive No.	Count	Positive No.	Count	Positive No.
Salmonella Typhimurium	A	Liver	0.5±0.5[c]	2/4[d]	0.5±0.5	2/4	0.5±0.5	2/4	0.2±0.5	1/4	0.0±0.0	0/4
		Spleen	0.5±0.5	2/4	0.6±0.9	2/4	1.2±1.2	3/4	0.7±0.5	3/4	0.2±0.5	1/4
		Caecum	2.5±1.7	3/4	1.4±1.6	3/4	1.0±0.8	3/4	0.8±1.0	1/4	0.2±0.5	1/4
	B	Liver	0.0±0.0	0/4	0.5±0.5	2/4	0.2±0.5	1/4	0.0±0.0	0/4	0.0±0.0	0/4
		Spleen	0.0±0.0	0/4	0.9±1.8	1/4	0.2±0.5	1/4	0.0±0.0	0/4	0.0±0.0	0/4
		Caecum	0.6±1.2	1/4	0.0±0.0	0/4	0.0±0.0	0/4	0.0±0.0	0/4	0.0±0.0	0/4
Salmonella Enteritidis	A	Liver	0.0±0.0	0/4	0.5±0.5	2/4	0.5±0.5	2/4	0.5±0.5	2/4	0.2±0.5	1/4
		Spleen	0.0±0.0	0/4	0.2±0.5	1/4	0.6±0.7	2/4	0.5±0.5	2/4	0.2±0.5	1/4
		Caecum	0.9±1.2	2/4	0.8±1.1	2/4	0.9±1.1	1/4	0.2±0.5	1/4	0.0±0.0	0/4
	B	Liver	0.0±0.0	0/4	0.0±0.0	0/4	0.0±0.0	0/4	0.0±0.0	0/4	0.0±0.0	0/4
		Spleen	0.0±0.0	0/4	0.2±0.5	1/4	0.2±0.5	1/4	0.2±0.5	1/4	0.0±0.0	0/4
		Caecum	0.0±0.0	0/4	0.0±0.0	0/4	0.0±0.0	0/4	0.0±0.0	0/4	0.0±0.0	0/4

[a]Each group contained 20 chicks. The groups were designated as group A, non-immunized and group B, immunized. [b]The bacterial recovery from liver, spleen and caecum was carried out on day 1,4,7,11 and 14 post *Salmonella* Typhimurium and *Salmonella* Enteritidis challenge strain inoculation. [c]The bacterial count was determined and expressed as mean±Standard deviation Log_{10} cfu/g. [d]Number of positive chicken after enrichment culture. All values were considered to be significant if $p \leq 0.05$ or 0.01. *$p < 0.05$, **$p < 0.01$

sistence in the spleen tissues of group A animals was higher on days 1 and 4 than that recovered from group B chickens, and this persistence further increased on days 7 and 11, with the highest bacterial counts on day 7, whereas bacteria were only recovered from 1 of 4 group B chickens at days 4 and 7 (Table III). The bacterial counts in the cecal tissues of group A animals were significantly higher on days 1, 4, 7, 11, and 14. Bacteria were only recovered from 1 bird in group B on day 1.

Protection against virulent SE challenge

To evaluate cross protection against SE infection, birds were sacrificed at 1, 4, 7, 11, and 14 days after SE challenge. The liver tissues of group A birds showed consistent bacterial persistence from days 4 to 14 post infection, with significantly higher bacterial counts than those in group B birds, which did not show bacterial persistence in liver tissues (Table III).

Higher bacterial counts were detected in the spleen tissues of group A birds at day 7 post infection, which was subsequently reduced on day 14. Although bacterial persistence was not observed in the cecal tissues of group B birds, bacterial persistence was detected in group A chickens on days 1, 4, 7, and 11.

Humoral responses

The plasma concentration of IgGs against ST, SE, and SG antigens was measured. As shown in Fig. 1, the plasma IgG concentrations against ST-, SG-, and SE-specific antigens were significantly higher in group B than in group A. The IgG response to ST antigens was significantly higher beginning at 1 week PPI. The response remained consistently high in subsequent weeks; it was further elevated after the booster was administered at 4 weeks PPI, and it then plateaued at 6 weeks PPI (Fig. 1A). Similarly, the IgG levels against SE antigen
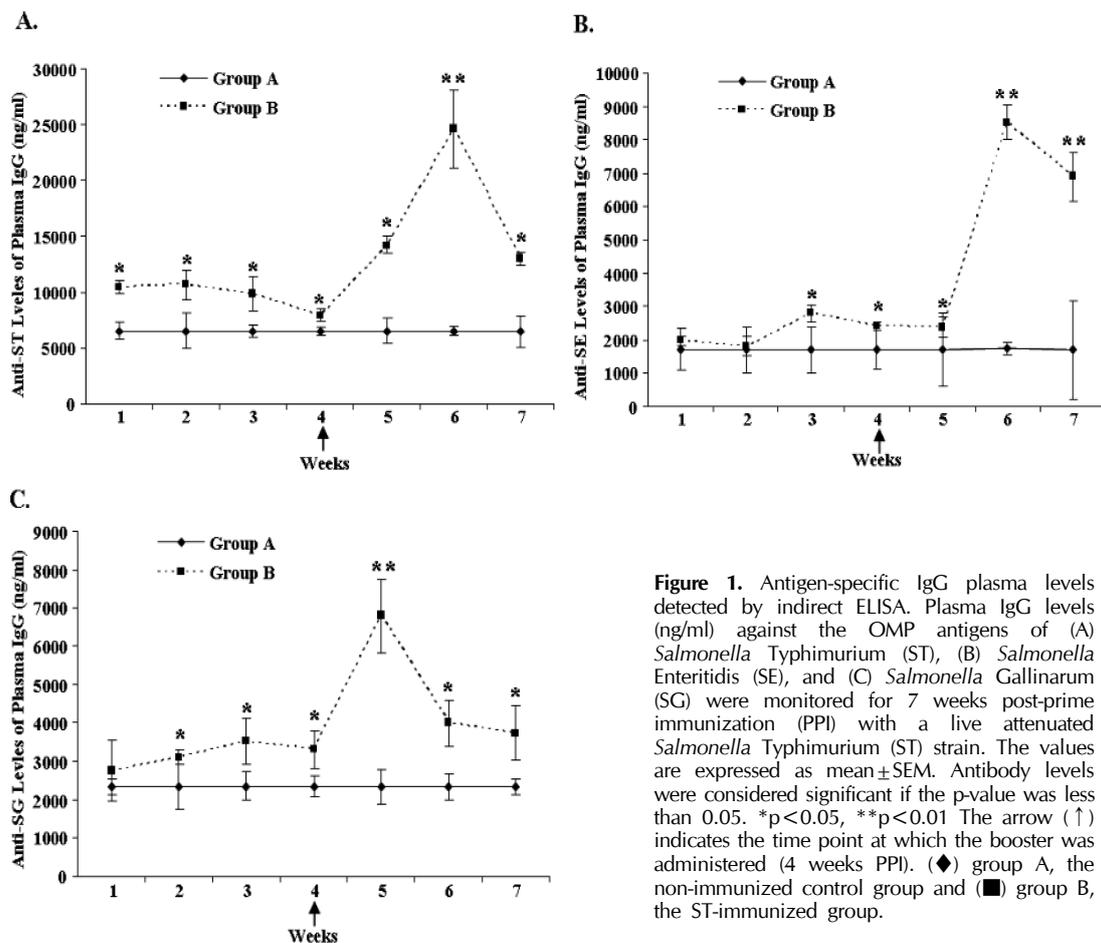

Figure 1. Antigen-specific IgG plasma levels detected by indirect ELISA. Plasma IgG levels (ng/ml) against the OMP antigens of (A) *Salmonella* Typhimurium (ST), (B) *Salmonella* Enteritidis (SE), and (C) *Salmonella* Gallinarum (SG) were monitored for 7 weeks post-prime immunization (PPI) with a live attenuated *Salmonella* Typhimurium (ST) strain. The values are expressed as mean±SEM. Antibody levels were considered significant if the p-value was less than 0.05. *p<0.05, **p<0.01 The arrow (↑) indicates the time point at which the booster was administered (4 weeks PPI). (◆) group A, the non-immunized control group and (■) group B, the ST-immunized group.

were significantly higher at 3 weeks PPI, and were further elevated at 6 weeks PPI, which was 2 weeks after the booster was administered (Fig. 1B). The IgG response to the SG antigen increased significantly beginning at 2 weeks PPI, and significantly higher values were detected 5 weeks PPI (Fig. 1C).

The sIgA response against the ST antigen was significantly induced in group B birds, with a plateau at 2 weeks PPI (Fig. 2A), and sIgA levels remained significantly higher until 7 weeks PPI. The sIgA levels against the SE antigen reached a peak at 3 weeks PPI, and further increased after booster administration (Fig. 2B). The IgA responses against the SG antigen were higher at 2 weeks PPI and then declined at 3 and 4 weeks (Fig. 2C). However, the IgG response increased significantly after booster administration and remained significantly higher until 7 weeks PPI (Fig. 2C).

Cellular response

To elucidate the cellular immune responses to ST, SE, and SG antigens following ST immunization, a peripheral lymphocyte proliferation assay was performed at 3 and 7 weeks PPI. Group B showed significant proliferative responses against ST-, SE-, and SG-specific antigens. Post prime vaccination, the stimulation indices against the ST, SE, and SG antigens for group B were 4.9, 2.3, and 3.7, respectively (Fig. 3A). Likewise, after booster administration, the stimulation indices against ST, SE, and SG antigens for group B were 5.6, 4.7, and 2.7, respectively (Fig. 3B).

DISCUSSION

A high incidence of livestock diseases due to infection with a wide array of *Salmonella* serovars and contamination of de-
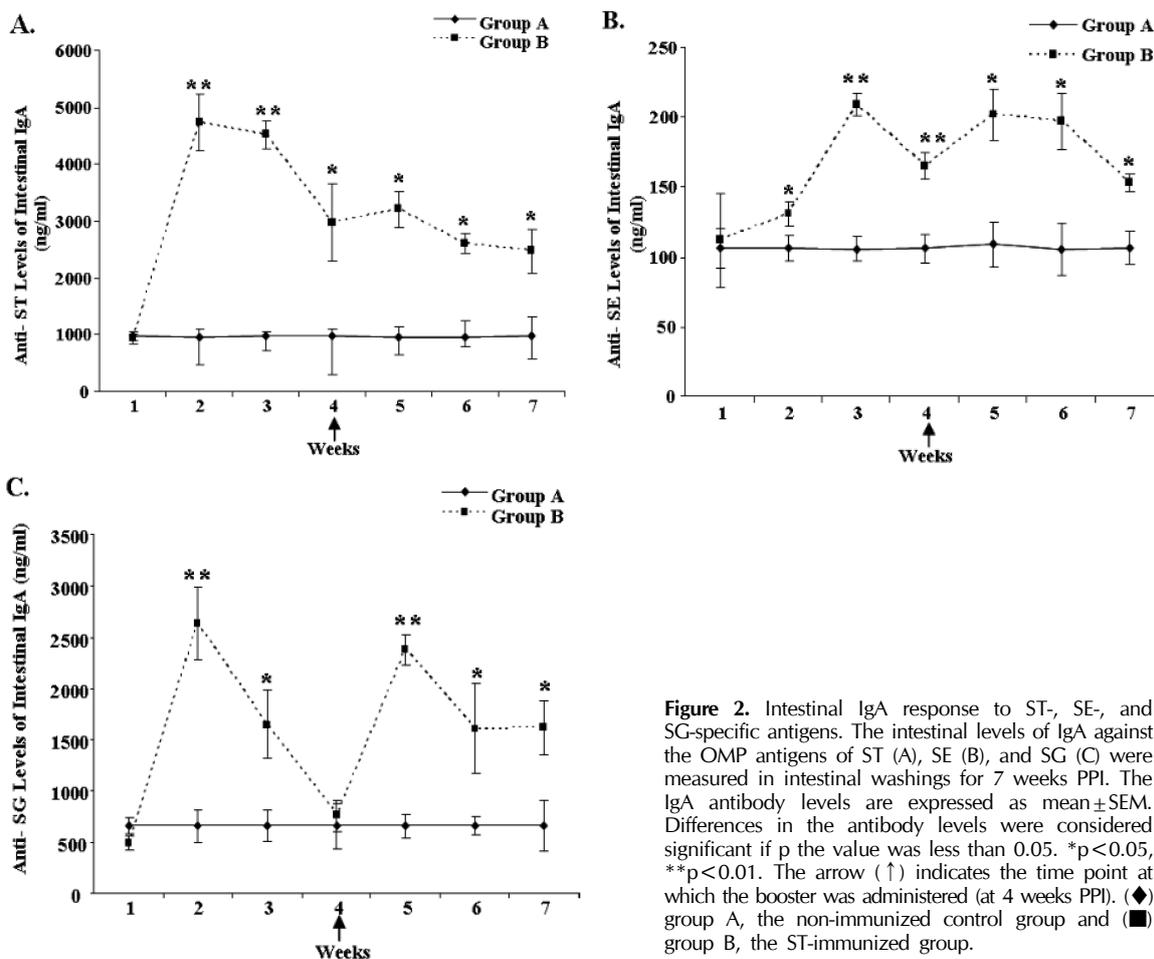

Figure 2. Intestinal IgA response to ST-, SE-, and SG-specific antigens. The intestinal levels of IgA against the OMP antigens of ST (A), SE (B), and SG (C) were measured in intestinal washings for 7 weeks PPI. The IgA antibody levels are expressed as mean±SEM. Differences in the antibody levels were considered significant if p the value was less than 0.05. *p<0.05, **p<0.01. The arrow (↑) indicates the time point at which the booster was administered (at 4 weeks PPI). (◆) group A, the non-immunized control group and (■) group B, the ST-immunized group.
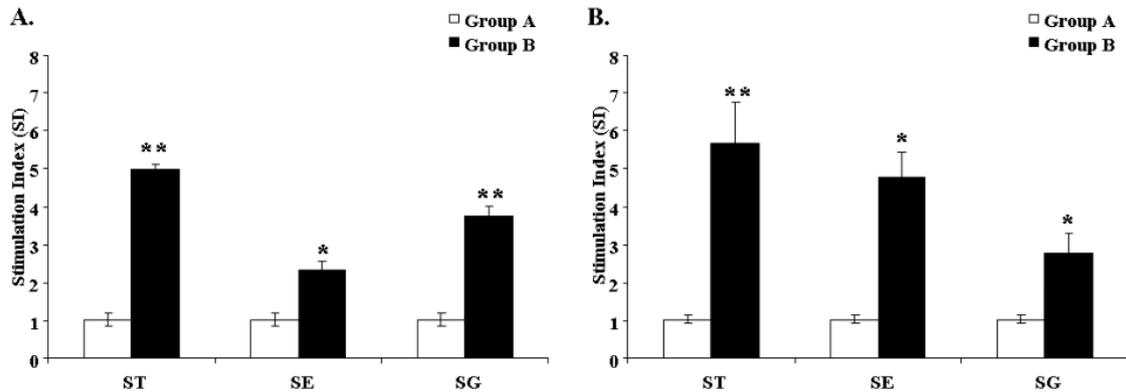
Figure 3. Antigen-specific lymphocyte stimulation responses were measured in ST-immunized and non-immunized chickens. Lymphocyte stimulation responses against ST, SE, and SG antigens were analyzed at 3 weeks ppi (A) and 3 weeks post booster immunization (B). The stimulation index of the lymphocyte sample from the chickens is shown. *p<0.05, **p<0.01. Group A, the non-immunized control group; group B, the ST-immunized group.

rived food products are associated with significant livestock production losses and public health risks, respectively (40,41). The present study demonstrated the cross-protective efficacy of a live attenuated ST strain (29,30) against three major *Salmonella* serovars in chickens. Vaccination using genetically-manipulated *Salmonella* strains is emerging as a popular strategy for effective control and prevention of *Salmonella* infections in commercial livestock production systems (11). However, the release of genetically modified microorganisms into the environment is associated with significant safety concerns (10,14,42,43). Although ST infection is common in chickens, it does not cause severe diseases in poultry (7). Therefore, attenuated ST strains have been successfully used to express foreign antigens and they have been suggested as safe strains (28). The results of this study indicated that oral inoculation of the attenuated ST strain (△*lon*, △*cpxR*, △ *asd-A16*) neither induces acute mortality nor yields any adverse effect in the vaccinated birds. In our previous study, we carried out an extensive safety evaluation of this *lon*, *cpxR* and *asd-A16*-deleted ST strain in chickens and reported its safe use as a delivery system to prevent colibacillosis (29). In addition, observations made in the present study showed that the strain was excreted in the feces of only a few vaccinated birds and that booster administration did not increase bacterial excretion in the feces. Only low level fecal excretion was observed during the monitoring period, which suggests that oral administration of the attenuated ST strain may be environmentally safe.

It has also been postulated that use of an attenuated ST

strain may confer cross protection against related serovars (28). This would provide an alternative use for the attenuated ST strain as a delivery system for immunizations. Although the protection afforded to immunized hosts by vaccination is generally highly specific, recently, vaccines have been developed that confer cross protection to multiple strains of the same species (28). Here, we further investigated the protections conferred by the attenuated ST strain against challenge with virulent ST, SG, and SE strains. Our results demonstrated that prime-boosted oral immunization of layer chickens with the live attenuated ST strain conferred protection against challenge with virulent ST, SG, and SE strains. Immunization with the ST strain significantly reduced the mortality and bacterial load in internal organs induced by SG virulent challenge (Table I), demonstrating the protective effect of the strain against SG infection. The only currently available SG vaccine has residual virulence in chickens. Therefore, the observation that immunization with the attenuated ST strain offers protection against SG virulent challenge is very important, and this ST strain may offer an alternative approach to prevent fowl typhoid in chickens. After SE and ST challenge, no acute mortality was observed. Bacterial recovery from internal organs of chickens immunized with the attenuated ST strain revealed its protective effects against ST and SE infections. Immunization with the ST strain significantly reduced the persistence of the ST and SE strains in the immunized host (Table III).

To gain insight into the underlying protective mechanisms, we monitored the immune response pattern in sequential weeks. The data showed that inoculation with the attenuated

ST strain induced antibodies against ST, SG, and SE antigens. The systemic IgG response against the *Salmonella* antigens was significantly higher after the booster was administered (Fig. 1), whereas the mucosal sIgA response was higher after the prime immunization. Plasma IgG levels against the ST and SE antigens were significantly higher at 6 weeks PPI, whereas significantly higher IgG levels against the SG antigens were observed at 5 weeks PPI, suggesting that administration of the booster elevated IgG levels against the SG antigen earlier than those against the ST antigen. In contrast, sIgA levels against the ST and SG antigens reached a plateau at 2 weeks PPI, whereas sIgA levels against the SE antigen increased significantly at 3 weeks PPI, which indicates that the sIgA response to the ST and SG antigens was induced earlier than the response to the SE antigen. Overall, these findings suggest that immunization with the attenuated ST strain not only induced a significant humoral immune response against the ST antigen but also offered cross-immunity to SE and SG antigens. However, the immune responses against the different *Salmonella* antigens observed in the present study require additional conformational studies. In addition, we also investigated cell-mediated immune responses against these antigens in the immunized and non-immunized groups using the lymphocyte proliferation assay, which is widely used for this purpose (44,45). Immunization with the attenuated ST strain induced significant lymphocyte stimulation to the ST, SE, and SG antigens. These significant lymphoproliferative responses observed in the immunized group are suggestive of T-cell mediated immune responses. The lymphocyte proliferation observed in response to the SE and SG antigens is interesting and suggests that immunization with the ST strain also confers cross immunity at the cellular level.

In conclusion, vaccination with an attenuated ST strain not only prevented ST infection after virulent challenge but also provided additional cross-protective immunity against infections with SE and SG serovars.

ACKNOWLEDGEMENTS

CONFLICTS OF INTEREST

The authors have no financial conflict of interest.

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