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## Prevention of *Salmonella* Infection in Poultry by Specific Egg-Derived Antibody

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**Abstract:** *Salmonella enteritidis* (SE) colonizes the intestinal tract of poultry and causes food born illness in humans. Reduction of (SE) colonization in the intestinal tract of poultry reduces potential carcass contamination during slaughter. The purpose of this study was to investigate the effect of SE-specific yolk immunoglobulin (IgY) on prevention of SE colonization in orally infected broiler chickens. Commercial Single Comb White Leghorn (SCWL) hens were hyperimmunized with SE whole cell antigens. The presence of anti-*Salmonella* antibody, IgY and IgG in egg yolk and serum respectively, was monitored by Enzyme Linked Sorbent Assay (ELISA). Two hundred forty male "Ross 308" day old chicks were randomly assigned to 8 groups and 3 replications of 10 birds were grown for 42 days of experiment. Eight experimental groups identified with, S, P, A, SP, SA, AP, SPA, C. Four birds from four challenged groups (S), were orally inoculated with 1 mL of bacterial suspension that contained  $1 \times 10^6$  CFU mL<sup>-1</sup> *S. enteritidis* at 3 day of age. The groups that supplemented with antibody (A) received 15 mL of yolk contained antibody mixed per 3.84 mL of drinking water on day 1 and continuing for duration of the experiment. The probiotic treated groups (P) were received probiotic, 0.1% of feed and 0.5% of feed, until day 21 and 56 respectively. One group as control (C) did not received any treatment of probiotic and antibody. A-treated and A-P treated groups had significantly lower fecal shedding ( $p < 0.01$ ) and lower concentration of SE cecal colonization ( $p < 0.01$ ). These groups also had a lower isolation of SE from the liver, spleen and ileum. The use of *Salmonella enteritidis*-specific IgY combined with probiotic had a beneficial effect in reducing the colonization of *Salmonella* in market-aged broiler under the condition of this study.

**Key words:** *Salmonella enteritidis*, IgY, probiotic, ELISA, broiler

### Introduction

Passive immunity against gastrointestinal infections has recently been successfully applied as prophylaxis therapy in patients in a variety of virally and bacterially induced infections (Carlander, 2002). Prevention of bacterial infections, especially those due to *Salmonella* (Cooper *et al.*, 1994), *Campylobacter* and *Escherichia coli* (Fulton *et al.*, 2002), in meat animals have been of increased importance in the last few years due to highly publicized food borne illnesses in humans related to the consumption of meat products (Tsubokura *et al.*, 1997). The intestinal colonization of SE plays a significant role in carcass contamination during processing. The cecum is the main site of *Salmonella* colonization, therefore, reducing intestinal colonization of SE during the grow-out period is crucial in order to improve the carcass microbiological quality during processing (Wray, 2000; Michetti *et al.*, 1992).

The use of competitive exclusion organism and antibiotic have effectively prevented *Salmonella* from colonizing the chickens (Baba *et al.*, 1991). Providing newly hatched chicks with intestinal microflora from adult chickens decreases the incidence of *Salmonella* cecal colonization (Alvarez *et al.*, 2003). In addition,

chicken egg-derived antibodies, when given orally have been found to be effective in the prevention of clinical disease and infection with *Salmonella* and *E. coli* in broiler chickens (Stern *et al.*, 1990; Tini *et al.*, 2002) and other animals (Frendscho, 1994). Avian maternal antibodies are transferred to egg yolk (Sim *et al.*, 2001). Immunoglobulin Y (IgY) is the major antibody found in egg yolk from hyper immunized chicken (*Gallus domesticus*) (Hansen *et al.*, 1998). Simple collection of eggs instead of invasive blood sampling and bleeding has an advantage concerning the welfare of the immunized animals (Kruger, 2004; Moll *et al.*, 1976). Average volume of egg yolk (15 mL) contains 50-100 mg of IgY of which 2-10% can be of specific antibodies, this is much higher amount of immunoglobulin that could be obtained by bleeding the animal (Narat, 2003). Thus the eggs of immunized chickens are an economical and abundant source of polyclonal antibody (Michetti *et al.*, 1992). IgY can be used as an alternative to mammalian antibodies normally used in research and its use in immunotherapy has recently been proposed (Mine and Kovacs-Nolan, 1992). Immunoglobulin therapy may diminish the development of antibiotic resistant microorganisms (Shin *et al.*, 2002). The purpose of this

research was to investigate the possibility of IgY production against SE, recovered from hyper immunized hens and its potential application as prophylaxis in SE infected broilers. Birds were raised in a standard environmental condition which is recommended by the management guide.

## Materials and Methods

### Experiment 1

**Immunization of hens:** Four 33-week-old commercial Single Comb White Leghorn (SCWL) hens were hyper immunized intramuscularly in the breast and leg muscle with SE whole cell antigens were obtained by ultrasonication and administered at a protein concentration of  $500 \mu\text{g mL}^{-1}$  ( $1 \times 10^8$  CFU  $\text{mL}^{-1}$ ) after centrifugation. Primary immunization was performed with  $250 \mu\text{g}$  of the antigen emulsified in an equal volume of Freund's complete adjuvant. After the initial immunization the animals received two booster injections with 2 week intervals, using incomplete Freund's adjuvant (Lee *et al.*, 2002). Bleedings and egg collections were performed 20 and 25 days after first and subsequent injection, respectively. The eggs were kept at  $4^\circ\text{C}$  until use. The presence of anti-*Salmonella* antibody IgY and IgG in egg yolk and serum respectively, was monitored by ELISA, using bacterial whole cells extract as antigen, during the immunization period (about 117 days).

**IgY primary purification:** The eggs were broken out and the yolk was collected in an equal volume of PBS (0.1 M, pH = 7.6). This mixture was vortexed and the resulting mixture was centrifuged at 3500 rpm for 25 min. The superficial lipid layer was removed and the resulting supernatant was collected and stored at  $4^\circ\text{C}$  until it was used as the IgY source (Fulton *et al.*, 2002).

**IgY purification:** Egg yolk antibodies were purified following the method of (Polson, 1985) using polyethylene glycol (PEG) precipitation procedure with minor modification as follow. The yolk was separated from the white and carefully washed with phosphate buffer saline (PBS 0.1 M, pH = 7.6) in order to remove as much of the albumin as possible. The yolk membrane was cut and poured into a glass beaker and diluted with equal volumes of PBS. The mixture was homogenized for 20 min and filtered. To the homogenized solution of egg yolk was added 2.62 g of PEG 6000 in small quantities while stirring. The suspension was centrifuged at  $5000 \times g$  for 20 min at  $4^\circ\text{C}$ , precipitate was discarded and the supernatant was filtered and added with 1.08 g of PEG. The resulting suspension was incubated for 10 min at room temperature and centrifuged at  $5000 \times g$  for 25 min. The supernatant was discarded and precipitate was dissolved in 37.5 mL PBS. To this solution was added 4.5 g of PEG while

stirring, the resulting cloudy solution was centrifuged at  $5000 \times g$  for 25 min. The supernatant was discarded and precipitate was dissolved in 3.7 mL cooled ( $0^\circ\text{C}$ ) PBS. This clear solution was treated with 3.7 mL chilled ethanol ( $-20^\circ\text{C}$ ) and centrifuged at  $10000 \times g$  for 25 min. The supernatant was discarded and precipitate was dissolved in 3.7 mL PBS. Finally the product was dialyzed with three changes of PBS for 24 h at  $4^\circ\text{C}$ . Protein concentration of final solution was determined as  $60 \text{ mg mL}^{-1}$ .

**ELISA:** Flat bottomed polyvinyl chloride ELISA plates were coated overnight at  $37^\circ\text{C}$ , with  $100 \mu\text{L well}^{-1}$  SE whole cells antigens solution at a concentration of  $10 \mu\text{g mL}^{-1}$  in ELISA buffer (10 mmol PBS, pH = 7.4). Wells were washed three times using deionized water and blocked with 3% skimmed milk powder for 45 min at  $37^\circ\text{C}$  and finally was washed three times with deionized water. Different concentration of purified IgY ( $2.5, 5, 10, 20 \mu\text{g mL}^{-1}$ ) were prepared in EIA buffer. Serum samples were also diluted (1:1000) in EIA buffer. Finally  $100 \mu\text{L}$  of each dilution of IgY or serum samples were added to coated wells. In order to evaluate the non-specific binding reactions, normal serum sample of hen was also diluted (1:300) in EIA buffer and  $100 \mu\text{L}$  of this solution was added to coated well. The plate was incubated at  $37^\circ\text{C}$  for 2 h. At the end of incubation time, bound immunoglobulins (IgY and IgG) were detected by incubating with rabbit anti-chicken-IgY-horseradish peroxidase, for 1 h at  $37^\circ\text{C}$ , then plate was washed five times. Finally,  $100 \mu\text{L}$  tetramethyl benzidine (TMB), a horseradish peroxidase substrate was added. The reaction was stopped after 10 min by addition of HCl (0.5 M) and the plate was read at 450 nm in a ELISA-reader.

### Experiment 2

**Experimental animals:** Two hundred forty one-day-old male Hubbard chicks were obtained from a *Salmonella*-free parent flock and randomly assigned to three groups of 24 birds which were kept in floor pens on litter. The chickens were given feed and water *ad libitum* during the 39 days of experiment. The feed was conventional for broiler without antimicrobials and coccidiostats. The feed was analyzed for *Salmonella* content before experiment following an enrichment procedure (Barrow, 1991). Briefly, 10 g of feed samples were diluted 1:10 and pre-enriched in Buffered Peptone Water (BPW) overnight at  $37^\circ\text{C}$ ,  $100 \mu\text{L}$  were then transferred to 10 mL selenite F broth (Difco) and incubated overnight at  $37^\circ\text{C}$ . The selenite enrichment broths were analyzed for growth of SE on *Salmonella-Shigella* (SS) agar (Difco) after incubation of the plates for 24 h at  $37^\circ\text{C}$ .

**Bacterial strain and growth condition:** The strain used *Salmonella enteritidis* RITCC1695, was originally purchased from Razi Vaccine and Serum Research

Institute (Karaj, Iran). For the preparation of the inocula, bacteria were grown in nutrient broth at 37°C for 24 h. The cultures were centrifuged for 20 min at 5000 rpm and resuspended in fresh broth to a smaller final volume to produce a highly concentrated culture without bacterial extra cellular products. Tenfold dilution series for inoculation of chickens were made in sterile saline and the viable cell concentration of the inoculum was determined by counting the Colony Forming Units (CFU) on SS-agar plates, following a pour plate procedure (Bjerrum *et al.*, 2003).

**Experimental design:** Two hundred forty male "Ross 308" day old chicks were randomly assigned to 8 groups and 3 replications of 10 birds were grown for 42 days of experiment. Eight experimental groups identified by treatments: S, P, A, SP, SA, AP, SPA, C at the same order as described. Four birds from four challenged groups (S), were orally inoculated with 1 mL of bacterial suspension that contained  $1 \times 10^6$  CFU mL<sup>-1</sup> SE at 3 day of age. The groups that supplemented with antibody (A), received 15 mL of yolk contained antibody mixed per 3 mL of drinking water on day 1 and continuing for duration of the experiment. The probiotic treated groups (P) were received Primalac (Star Labs, Clarksdate, MO, USA, contained  $2.5 \times 10^7$  CFU g<sup>-1</sup> of *Lactobacillus acidophilus*, *Lactobacillus casei*, *Enterococcus durans*, *Bifidobacterium thermophilus*), 0.1% of feed and 0.5% of feed, until day 21 and 56 respectively. One group as control (C) did not receive any treatment of probiotic and antibody.

**Inoculation dose:** The dose for the experiment was chosen based on pre-experiments. The goal was to achieve an infection that lasted for the whole life of the chicken with counts of *Salmonella* as stable as possible. The birds received 500 µL of bacterial suspension contained  $2 \times 10^6$  CFU mL<sup>-1</sup> of SE.

**Bacterial culture:** During the experiment, one chicken were euthanized from each group on days 21, 28 and 35. Each bird was sampled and examined individually. The peritoneum was disinfected by flaming and opened. To screen *Salmonella*, spleen, ileum and liver from each chick were removed aseptically, mixed and 1 g of the tissue was pre-enriched in BPW, for 24 h at 37°C. The suspension was transferred to 9 mL selenite F broth, incubated 18 h at 37°C, streaked on SS-agar for 24 h at 37°C and examined for SE colonies. The confirmation of the cultured colonies was performed following serum agglutination test (Axelsson and Sorin, 1997).

To follow and quantify the infection after inoculation, *Salmonella* counts were determined as CFU from the content of the cecal samples. Briefly, approximately 1 g of cecal content from each bird was removed, weighed, diluted in 9 mL sterile saline and mixed thoroughly.

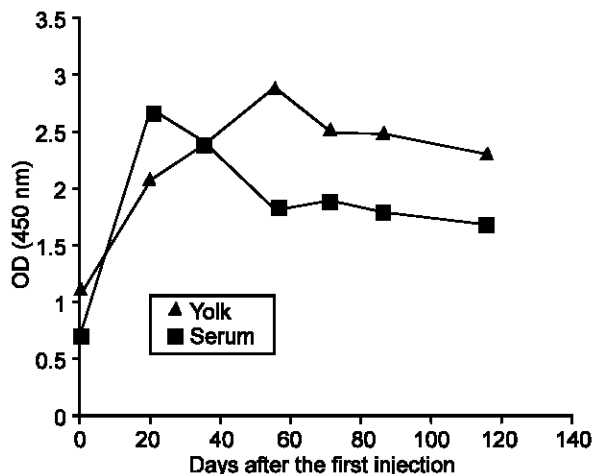


Fig. 1: Changes of antibody titers in serum and egg yolk during the immunization period. Antibody activities in sera (?) and egg yolk (?) from chickens were measured by ELISA and were expressed as absorbance at 450 nm. All experiments were performed at a dilution of 1:1000 serum or egg yolk

Tenfold dilution series were made in saline, thereafter plated on SS-agar and the number of CFU of SE in the original samples were calculated. Pre-enriched cecal samples in BPW (24 h at 37°C), also were prepared and *Salmonella* counts were determined (Clifton-Hadley *et al.*, 2003).

In order to check shedding of SE of the chickens after inoculation, cloacal swabs were obtained from inoculated birds on days 14, 21, 28 and 35 and were directly enriched in selenite F broth at 37°C for 18 h, mixed thoroughly and plated on SS-agar to screen positive SE samples.

**Determination of protection factor:** To evaluate the efficacy of antibody and probiotic treatment on resistance to *Salmonella* challenge, the protection factor (mean log<sub>10</sub> *Salmonella* control group/mean log<sub>10</sub> *Salmonella*-treated group) was calculated.

**Data analysis:** Differences between CFU of *Salmonella* were determined by SAS statistical program. Significant differences were further separated using Duncan's multiple-range test. Chi-square analysis was used to determine significant differences between groups in shedding of SE from feces.

## Results and Discussion

**IgY in yolk and serum:** The activities of anti-SE antibody were determined by ELISA in the serum and egg yolk from laying hens as shown in Fig. 1. The antibody activity in serum increased rapidly and reached a peak

Table 1: Shedding of SE following Challenge of 3 day old broiler chickens at several times post inoculation [number of isolates/total number of swabs (% positive cases)]

Group	Age (day)			
	14	21	28	35
S	14/30 (%46)	6/30 (%20)	4/27 (%14)	0/26 (%26)
SP	13/30 (%43)	4/30 (%13)	3/27 (%11)	0/27 (%0)
SA	9/30 (%30)	3/29 (%10.3)	0/26 (%0)	0/26 (%0)
SPA	8/29 (%27.5)	2/29 (%6)	0/26 (%0)	0/25 (%0)
	**	**	**	NS

\*\*Significant difference (p<0.01) NS non-Significant

Table 2: Effect of probiotic and antibody (IgY) on the mean concentration of SE from cecum in broiler chickens\*

Age	Log <sub>10</sub> <i>Salmonella</i> per gram cecal content **			
	S	SA	SP	SPA
Day 21	5.26 <sup>c</sup>	1.23 <sup>a</sup>	2.92 <sup>b</sup>	1.12 <sup>a</sup>
Day 28	3.98 <sup>c</sup>	0.27 <sup>a</sup>	1.98 <sup>b</sup>	0.38 <sup>a</sup>

\*Experimental condition are described in the " Materials and methods ", \*\*Log CFU SE per gram of cecal content (n = 3). A, b, c Means within a row lacking a common superscript differ significantly (p<0.01), using analysis of variance

on day 20. However the antibody activity in egg yolk was very weak on day 20 and gradually increased thereafter to reach the peak on day 55 after first injection.

**Clinical signs and mortality:** All birds which received 10<sup>6</sup> cells were dull and had diarrhea between days two and seven after SE administration, however all clinical symptoms were disappeared after this period. During these experiments no clinical symptoms of infection with SE were observed and the mortality rate for these chickens was not higher than that of the control group. At every sampling stages, chickens were selected from all groups. The control chickens were free of *Salmonella* throughout the experiment.

**Shedding of SE in feces:** There was a significant difference (p<0.01) in the isolation of SE from the swab samples when A and P-treated and control groups were compared. The cloacal swabs taken before inoculation were free of SE in all groups. The chickens receiving antibody alone or in combination with probiotic, were according to the direct plating, free of *Salmonella* on day 28 (Table 1), thus the declined in less than 4 weeks, whereas the SE was isolated from 4/27 (%14) swab samples of S group, on day 35. The total of positive swab samples of SA and SPA groups were lower than S group throughout the duration of the experiment (p<0.01). The average the time of shedding is longer in the S group than in the SA and SPA groups. Previous

studies have shown that cloacal swab samples could not be accurate estimate for *Salmonella* contamination of flocks, because of intermittent shedding of *Salmonella* from feces.

In this study, dose levels around 10<sup>6</sup> CFU bird<sup>-1</sup> yielded stable SE levels in 3-day-old chickens. Pre-experiments with lower doses did not result in 100% infection of the chickens (Barrow, 1991; Bjerrum *et al.*, 2003), which was our criterion for the infection. In contrast Methner *et al.* (1995) found that chickens inoculated with 10<sup>2</sup> CFU got an infection that lasted for several weeks post inoculation. However, in experiments with chickens kept on litter, reinfection may play a more important role in the persistence of the infection and, thus a lower dose may be needed (Beal *et al.*, 2004; Gast, 2000).

**Concentration of SE in intestinal contents:** There was a significant difference (p<0.01) in the mean number of SE in the cecal contents of the treated and non-treated challenged groups (Table 2). The groups receiving A alone or in combination with P, had a significantly lower concentration of SE colonization at days 21 and 28 when compared to the S group. Similar observations were made in broiler (Tellez *et al.*, 2001), ducklings (Fulton *et al.*, 2002), piglets and calves (Yokoyama, 1998). There was a considerable difference between the carriage of *Salmonella* in the cecal contents, indicating that broilers are able to clear systemic infections, but can remain intestinal carries (Bjerrum *et al.*, 2003). The intestinal carrier status is most important in control of contamination during transportation and processing of broilers, where cross contamination plays a major role (Gurtler *et al.*, 2004). From these results it appears that probiotics work synergistically with oral antibody to prevent infection, this has been previously reported in chickens (Coleman, 2000; Tellez *et al.*, 2001).

The investigations of Tusbokura *et al.* (1997) indicated that the antibody used did not possess any bactericidal or bacteriostatic effect *in vitro*, but led to altered growth characteristics with clumping of the bacteria, which may affect the degree of colonization. Similar data, that suggested the immune exclusion at the mucosal surface by monoclonal IgA antibodies, have also been recorded in model systems of gastrointestinal disease (Seo *et al.*, 2003; Hilpert, 1996). It is therefore likely that one of the major mechanisms of action in our experiment could be the blocking of bacteria to adhere to the intestinal wall, thus preventing its colonization (Ma *et al.*, 1990).

Various theories have been proposed as to the mechanisms by which probiotics protect their host against invading enteropathogens. Some of these include: competition for limiting nutrients, competition for attachment sites on the intestinal mucosa (Balevi *et al.*, 2001) and production of short-chain volatile fatty acids (Humphrey *et al.*, 1991).

Table 3: Efficacy of probiotic and antibody treatment on resistance to *salmonella* challenge in the cecal contents of broiler chickens

Age	PF*		
	SA	SP	SPA
Day 21	4.27	1.8	4.69
Day 28	14.77	2	10.47

• PF = mean Log<sub>10</sub>

Table 4: Isolation of *S. enteritidis* from internal organs at several times postinoculation: Spleen (S), Liver (L), Ileum (I). 0 = no chickens positive for SE, \* = one of three chickens positive for SE, \*\* = two chickens positive for SE, \*\*\* = all of three chickens positive for SE

Group	Age (day)								
	21			28			35		
	L	S	I	L	S	I	L	S	I
S	***	***	*	**	*	0	0	0	0
SP	***	**	0	**	*	0	0	0	0
SA	*	*	0	*	0	0	0	0	0
SPA	**	*	0	*	0	0	0	0	0

The PF were calculated to assess the efficacy of the antibody and probiotic (Table 3). The higher values of PF in the SPA and SA groups were indicated that A and P-treatment were more effective in preventing *Salmonella* colonization, when compared to the SP group. Probiotic alone was not effective for inhibition of *Salmonella* colonization in cecum which corresponded with results of *Salmonella* count in the cecum content (Tellez *et al.*, 2001).

**Organ invasion of SE:** Following oral challenge with  $1 \times 10^6$  CFU of SE, the SE bacterial infection was observed in livers, ileums and spleens of most birds until 3 weeks PI (Table 4). However as the time elapsed from the initial day of administration fewer and fewer positive samples from these organs were found. The SA and SPA groups had a marked lower number of positive cases at 28 day of age, when compared to the SP and control groups. However, all groups had showed no positive case, on day 35. Most samples from the ileum were negative for *Salmonella*, only one positive sample was found in S group at day 21. These results are in agreement with other studies, where *Salmonella* was followed in different organs postinfection (Bjerrum *et al.*, 2003; Heres *et al.*, 2003). The passage time of *Salmonella* in the ileum is very fast compared to the ceca where the bacteria will have more time to establish (Barrow, 1991).

Tellez *et al.*, 2001 demonstrated that avian-specific probiotics and anti-SE specific IgY, inhibited SE and *S. typhimurium* (ST) colonization and organ invasion of market-aged chickens.

Although this study was conducted in young birds, trials of the avian egg yolk antibody consumption in layers are needed especially as large scale incidences associated with SE and ST contaminated egg products have been

reported (Richardson *et al.*, 2003). IgY will be a useful tool in the control of *Salmonella* infection in the poultry industry and the use of SE-specific IgY combined with probiotic have a beneficial effect in reducing the colonization of *Salmonella* in market-aged broiler.

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