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Full Length Research Paper

Salmonella Enteritidis experimental infection in chickens: Effects of challenge dose on serum immunoglobulin G antibody response

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Salmonella enterica **serovar Enteritidis is a food borne pathogen of humans causing food-poisoning and sometimes deaths. In order to control egg-borne transmission of *Salmonella* Enteritidis to humans, prompt and accurate detection of infected poultry flocks is essential. This paper examined the effects of challenge dose of *Salmonella* Enteritidis on detection of specific immunoglobulin G (IgG) antibody in serum of experimentally infected chickens. An indirect ELISA technique based on lipopolysaccharide (LPS) antigen of *Salmonella* Enteritidis was used. Two groups of specific-pathogen-free chickens were infected orally with 10^8 and 10^4 colony forming units (cfu) of *Salmonella* Enteritidis. Serum samples were collected prior to challenge and at five subsequent weekly intervals. Levels of serum IgG antibody response observed in the chickens infected with 10^8 cfu of *Salmonella* Enteritidis were significantly stronger ($P<0.05$) than those of the 10^4 cfu group. Although considerable IgG response was seen with most of the chickens by 2 weeks post-infection, highest group mean optical density values of 2.177 and 0.984 were observed at 5 weeks for 10^8 and 10^4 cfu *Salmonella* Enteritidis infected groups, respectively. Chickens exposed to few *Salmonella* colonies during infection do not produce high IgG antibody and may continue to contaminate the flock.**

Key words: *Salmonella* Enteritidis, immunoglobulin G, serum, chickens, antibodies.

INTRODUCTION

Over the years, the incidence of human infection and food-poisoning by *Salmonella* has increased dramatically in Europe, USA and other parts of the world; and poultry are a major recognized source of infection (Angulo and Swerdlow, 1999; CDC, 2000; EFSA, 2007). *Salmonella enterica* serovars Enteritidis and Typhimurium are the two most frequently isolated serovars from humans and account for 57-67% of the world's total annual isolates (WHO, 2007). In poultry they have been isolated from broilers, breeders, and commercial egg-laying flocks (Chima and Ogbogu, 1998; Ogunleye et al., 2005). Some internally contaminated eggs are frequently implicated as sources of transmission of *Salmonella* Enteritidis to humans; detection of infected laying flocks is among the principal objectives of most control efforts (Hogue et al., 1997). Eggs from infected flocks could then be diverted for pasteurization (Gast et al., 2002).

It has been reported that bacteriological sampling does not always provide an accurate indication of infection within a flock because of low incidence of infection and the intermittent excretion of *Salmonella* organisms (Hassan et al., 1990). The delay of 3-5 days is also associated with bacteriological sampling before results can be obtained. Hence, serologic tests have been developed; slide or tube agglutination tests have been reported as not reliable (Olesuik et al., 1969). The enzyme-linked immunosorbent assay (ELISA) is recognized as a rapid and highly sensitive test for detecting a number of avian pathogens including *Salmonella* infections (Hassan et al., 1990; Ohore et al., 2002; Seo et al., 2003; Holt et al., 2006). ELISA based on LPS antigen of *Salmonella* Enteritidis had been used for serological detection of infection in chickens (Holt et al., 2002; Seo et

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al., 2003; Holt et al., 2006).

Since chickens may be exposed to variable quantities of *Salmonella* during infection or vaccination, this present study was therefore designed to evaluate the effects of challenge dose of *Salmonella* Enteritidis on serum IgG antibody response specific for *Salmonella* Enteritidis by experimentally infected chickens.

MATERIALS AND METHODS

Experimental Infection of Laying Hens

For this study, twenty-four single-combed White Leghorn laying hens were obtained from the specific-pathogen-free flock of the Southeast Poultry Research Laboratory, United States Department of Agriculture (USDA), Athens, Georgia, USA. These were divided into 3 groups of eight chickens and housed separately in climate controlled bio-containment building. The birds were 33-weeks old at the beginning of the experiment and were provided with water and pelleted feed *ad libitum*.

One group of 8 hens was challenged orally with a dose of 1.3 x 10^4 colony forming units (cfu) of a nalidixic acid resistant, phage type 13 strain of *Salmonella* Enteritidis originally isolated from chickens and obtained from the National Veterinary Service Laboratory, Ames, Iowa, USA. A second group of 8 hens was orally inoculated with a dose of 1.3 x 10^8 cfu of the same *Salmonella* Enteritidis strain. The remaining 8 hens served as the uninfected negative control group.

The organism was prepared from frozen stocks by sub-culturing it onto Nutrient agar (Difco Laboratories, Detroit, USA) and incubated overnight at 37°C. Single colonies were streaked onto Brilliant Green agar (Difco) containing 100 µg/ml Novobiocin (Sigma Chemical Co., St. Louis, USA) and 10 µg/ml Nalidixic acid (Sigma) (BGNN) and incubated overnight at 37°C. It was then inoculated into Tryptic Soy Broth (Difco).and incubated overnight at 37°C. The overnight culture was serially diluted in sterile normal saline from 10^{-1} to 10^{-7}, and plated on BGNN plates for enumeration. One millilitre each of dilutions 10^{-1} and 10^{-5} found to contain 10^8 and 10^4 cfu/ml *Salmonella* Enteritidis were used orally challenging the hens in groups 1 and 2, respectively.

Sample collection

Each chicken was maintained in lateral recumbency and using aseptic technique each was bled from the wing vein. The blood samples were allowed to clot, these were then centrifuged at 12,000 x g for 5 min, and the serum supernatant were dispensed in tubes and stored at -20°C until analysed. Samples were collected prior to experimental infection (pre-challenge) and at weekly intervals for 5 weeks post-infection.

Detection of specific immunoglobulin (Ig) G antibody by ELISA technique

The serum samples were thawed and serial two-fold dilutions using phosphate buffered saline (PBS) containing 0.05% Tween 20 (Sigma Chemical Co., St. Louis USA) (PT-buffer) of an initial 1:250 serum dilution was made for each sample. The ELISA protocol used was an adaptation of the procedure earlier described by Holt et al. (1999). Briefly, the serum serial dilutions were added to ELISA plates to which 10 µg/ml *Salmonella* Enteritidis lipopolysaccharide (LPS) antigen (Sigma) had been adsorbed and to which 3% polyvinyl pyrrolidone (PVP) blocker had been added to prevent unspecific reactions. Plates were incubated at room temperature for 60 min after which they were washed thrice using automatic ELISA plate washer (Bio-Tek ELX 405 microplate washer, Bio-Tek Instruments, Winooski, VT, USA).

Following room temperature incubation of the serum and antigen for 60 min, plates were washed three times. A 1:2000 of mouse anti-chicken IgG (Southern Biotech, USA) in PT-buffer containing 1% Bovine Serum Albumin (BSA) were added to plates and incubated at room temperature for 60 min. Plates were then washed after which a 1:2000 dilution of goat anti-mouse IgG-alkaline phosphatase conjugate (Calbiochem, Corp., La Jolla, CA, USA) in PT-buffer with 1% BSA were added and incubated at room temperature for 60 min. After washing the plates thrice, the substrate, *p*-nitrophenyl phosphate (Sigma) at 1 mg/ml in diethanolamine buffer was added and incubated in the dark for 30 min. Absorbance was read at 405 nm using an automatic ELISA reader (Bio-Tek EL311sx autoreader, Bio-Tek, USA).

Data analysis

Group mean optical density (OD) 405 readings of ELISA results at 1:250 serum dilution for each of the challenged and control groups was calculated. Titres were determined as the last dilution that gave an OD reading 1.5 times that of the negative control obtained previously from an uninfected chicken (Holt et al., 2002). Titre values were transformed to $\log_{10}$ titre and mean $\log_{10}$ titre calcuated. Percentage of sero-positive hens per group was also determined. Significant differences ($P<0.05$) between the two challenge doses (10^8 cfu and 10^4 cfu) and the control group, and between pre-challenge and the different post-infection weeks were examined using one-way analysis of variance (ANOVA) at 95% confidence level (Shott, 1990).

RESULTS

Specific serum (IgG) antibody response for *Salmonella* Enteritidis measured as the mean OD values of the group of hens challenged with 10^8 cfu of *Salmonella* Enteritidis increased from 0.289 at pre-challenge to 1.923 by week 2 and then to 2.177 at week 5 post-infection (Figure 1). The OD values increased steadily over the weeks except for a slight drop at week 4 post-infection. In the case of the group of hens challenged with 10^4 cfu, the mean OD increased from 0.287 at pre-infection to 0.506 by week 2 and then to 0.692 and 0.984 at weeks 4 and 5 post-infection (Figure 1). The overall antibody response of the 10^8 cfu dose group was found to be statistically significantly ($P<0.05$) stronger than those of the 10^4 cfu dose and the control hens.

There was no significant difference ($P>0.05$) in the antibody response of the 10^8 and 10^4 cfu dose groups from the uninfected control group at week 1 post-infection and also at pre-challenge. Although the antibody response of the 10^4 cfu dose group increased gradually over the weeks from 0.247 at pre-challenge to 0.984 at week 5, it did not differ significantly from those of the uninfected control hens throughout the experiment ($P>0.05$).

At one week post-infection, 12.5 and 0.0% of hens challenged with 10^8 and 10^4 cfu of *Salmonella* Enteritidis were sero-positive; the proportion however increased to 100.0 and 12.5% respectively by week 2 post-infection for the 10^8 and 10^4 cfu dose groups (Figure 2). Overall,

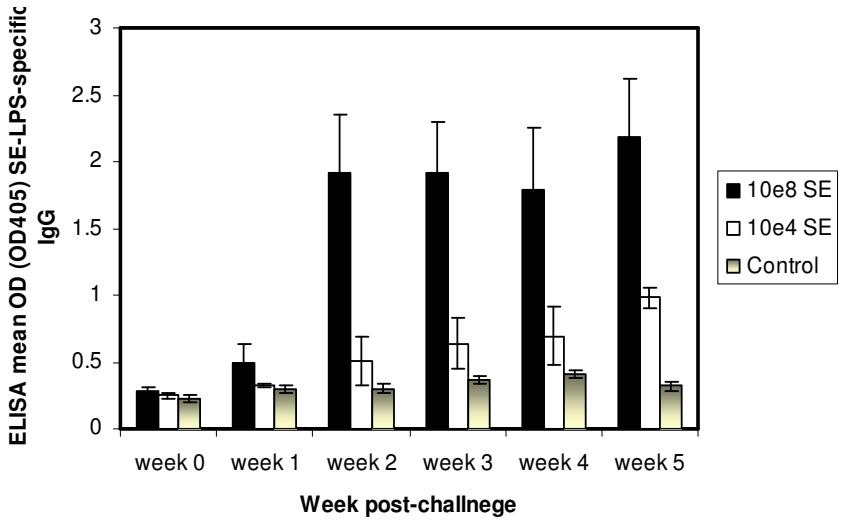

Figure 1. Level of *Salmonella* Enteritidis (SE)-LPS-specific IgG response (mean OD plus SDE of mean) detected in serum samples from White Leghorn hens infected with 10e8 SE (10^8 cfu/ml) Dose, 10e4 SE (10^4 cfu/ml) Dose and uninfected (Control). SE: *Salmonella* Enteritidis, Ig: Immunoglobulin, OD: optical density, SDE: Standard error.

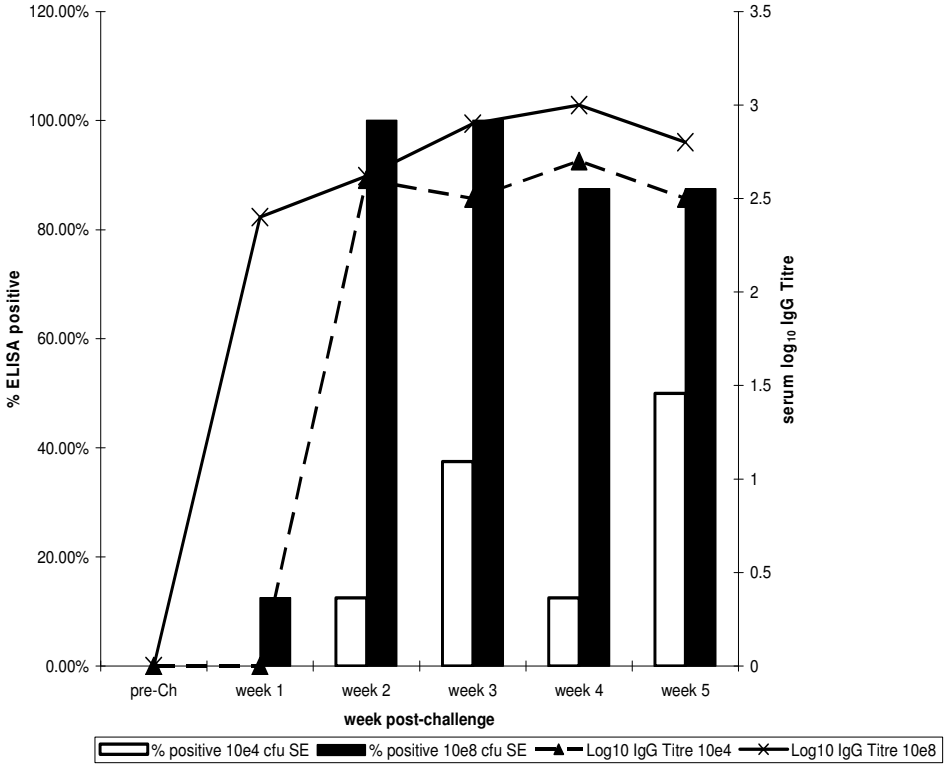

Figure 2. Comparison of percent sero-positive hens per group and means of *Salmonella* Enteritidis (SE)-IgG $\log_{10}$ titre in serum samples of White Leghorn hens infected with 10e8 SE (10^8 cfu/ml) Dose and 10e4 SE (10^4 cfu/ml) Dose. SE: *Salmonella* Enteritidis, Ig: Immunoglobulin.

for all the five post-challenge sampling intervals, more positive results were obtained at the 10^8 cfu dose level of *Salmonella* Enteritidis than the 10^4 cfu dose level. No positive titre was produced at one week post-infection by

the 10^4 cfu *Salmonella* Enteritidis infected hens while the highest mean $\log_{10}$ titre values of 3.0 and 2.7 were obtained at week 4 post-infection for the 10^8 and 10^4 cfu dose groups, respectively (Figure 2).

DISCUSSION

The marked increase in the level of anti-*Salmonella* Enteritidis IgG antibody as measured by high optical density and titre values in the laying hens examined in this study is in agreement with the findings of Gast and Beard (1990) who reported that when laying hens were orally infected with *Salmonella* Enteritidis, serum antibodies were produced by most birds by 1 week post-infection. High serum IgG titres have also been detected in laying hens after experimental oral inoculation with *Salmonella* Enteritidis (Barrow and Lovell, 1991).

In a similar study involving experimental oral inoculation of chickens with *Salmonella typhimurium*, increases were recorded in the IgG and IgM antibodies (specific for LPS antigen) between one and four weeks post-infection; high levels of IgG were detected up till 16 weeks post-inoculation even when faecal excretion of the organism had ceased (Hassan et al., 1990).

The presence of some levels of IgG antibody by week 1 post infection especially in the group infected with 10^8 cfu *Salmonella* Enteritidis agrees with the previous report where presence of serum antibodies by most birds that were orally infected with *Salmonella* Enteritidis, by one week post-infection had been observed (Gast, 2003). In a naturally infected broiler breeder flock, 70% of the birds were found to be positive for serum antibodies to *Salmonella* Enteritidis LPS at 35 weeks of age (Gast, 2003). Hence, ELISA has been said to be an appropriate method of indicating that infection (or exposure through vaccination) has occurred in the past and it can also detect infected chickens which are excreting *Salmonella* organism intermittently (Hassan et al., 1990). Detection of serum IgG has been reported as a very sensitive record of past exposure to *Salmonella* Enteritidis (Gast, 1997).

The poor anti-*Salmonella* Enteritidis IgG response of birds challenged with 10^4 cfu compared with the 10^8 cfu group could probably be due to lack of establishment of infection as a result of inadequate challenge dose or quick elimination of the organism from the system by the host's defense mechanism; thus stimulating fewer antibody-producing cells. Therefore, chickens exposed to too few *Salmonella* colonies during natural infection may not show evidence of infection and continue to spread the organism within the flock. The implication of this finding in vaccination programmes is that if inadequate vaccine dose is given to chickens during vaccination, they may respond with sub-optimal immune response which may not be strong enough to withstand challenge by a virulent field strain. Since serum IgG has been shown to be challenge-dose dependent, vaccine manufacturers are advised to incorporate adequate dose per vial during vaccine production.

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