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*Full Length Research Paper*

# Effects of challenge dose on faecal shedding of *Salmonella enteritidis* in experimental infected chickens

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**Experimental infection of chickens with *Salmonella enteritidis* is often achieved by oral inoculation of live bacteria to caged chickens. Less information is available on influence of amount of *Salmonella* a chicken is exposed to during infection on the proportion that is eventually eliminated in faeces. This study evaluated the effects of challenge dose of *S. enteritidis* on faecal shedding by experimentally infected chickens. Twenty four specific-pathogen-free hens were divided into three groups of eight. The first two groups were orally challenged with a dose of 1.3 x $10^4$ and 1.3 x $10^8$ colony forming units (cfu) of *S. enteritidis* per hen, the third group was uninfected. Faecal samples were collected weekly for 5 weeks and cultured. Levels of *S. enteritidis* recovered from hens infected with $10^8$ cfu/ml *S. enteritidis* were significantly higher (P < 0.05) than from $10^4$ cfu/ml group. Rate of faecal shedding decreased from 100 to 75 and 37.5% at weeks 1, 2 and 4 respectively in $10^8$ cfu infected hens. Intermittent shedding pattern was observed in the $10^4$ cfu group. Hence, *Salmonella* contamination of poultry environment continues in sub-clinical infection. Multiple faecal sampling of flocks may be necessary to know their *Salmonella* status and in vaccine assessment.**

**Key words:** *Salmonella enteritidis*, chickens, faecal shedding, challenge dose, infection.

## INTRODUCTION

*Salmonella enterica* serotype *Enteritidis* established itself as a major poultry-industry problem for over two decades. Since then, an increase in the incidence of human *S. enteritidis* infections has been reported worldwide (CDC, 2000; Gast et al., 2002; FSIS, 2006). Human *Salmonella* infections and food-poisoning have been associated with consumption of contaminated poultry and eggs (WHO, 2007). Contamination of poultry meat commonly occurs following contamination of carcass with faecal material at slaughter (Barrow et al., 1988). Ingestion of *Salmonella* by chickens is followed by a process of movement of the organism through the alimentary tract which is associated with initial inactivation of the pathogen (Schneitz et al., 1993; Holt et al., 2006). Intestinal colonisation and systemic dissemination to internal organs have been recognized as the two consistently observed features associated with paratyphoid *Salmonella* infections in mature poultry (Gast, 2003). Evidence of colonisation of intestine by *Salmonella* organisms with isolation of *S. enteritidis* from intestinal contents and voided faeces have been reported (Barrow et al., 1988; Gast and Beard, 1990). Most infected chickens continue to shed *S. enteritidis* in their faeces for a variable length of time with the highest rate of shedding occurring within the first two weeks of infection (Gast, 2003). Chickens are usually exposed to variable quantities of *Salmonella* organisms under natural condition. However, the role played by the quantity of *Salmonella* a chicken is exposed to during infection, on the rate and amount of its faecal excretion still needs to be elucidated. This study was therefore designed to study the effects of challenge dose of *S. enteritidis* on faecal shedding of *S. enteritidis* by experimentally infected chickens.

## MATERIALS AND METHODS

### Chickens

Single-combed White Leghorn laying hens of thirty-three weeks ofage were obtained from the Specific-Pathogen-Free (SPF) flock of the Southeast Poultry Research Laboratory, United States Department of Agriculture (USDA), Athens, Georgia, USA. Twenty-four hens were divided into 3 groups of eight. The hens in each group were housed in separate rooms in environmentally-controlled biosafety building, each bird in individual cage. Birds were fed

antibiotic-free pelleted layers ration and supplied water *ad libitum*. Hens were provided with sixteen hours of light daily. On the day preceding the weekly sample collection, styrofoam trays were put under each of the hens to collect faeces from each hen.

In order to ascertain that the hens were *Salmonella*-free, each individual was screened prior to the commencement of the experiment (pre-challenge samples). Faecal samples were collected and 100 µl obtained from mixing 1 g faeces with 9 ml Rappaport Vassiliadis (RV) enrichment broth (Oxoid Inc. Basingstroke, U.K) were spread-plated onto Brilliant Green agar (Difco Laboratories, Detroit, USA) containing 100 µl Novobiocin (Sigma Chemical Co., St. Louis, USA) (BGN) per ml. Plates were incubated at 37°C overnight. *Salmonella* was not detected. A volume of 100 µl of each of the 24 h incubated faecal-RV enriched samples was spread-plated again onto BGN plates and incubated overnight at 37°C. No *Salmonella* was detected.

**Experimental Infection**

The first group of 8 hens was challenged orally with 1 ml of a low dose of 1.3 x $10^4$ cfu of a nalidixic acid resistant, phage type 13 strain of *S. enteritidis* originally isolated from chickens and obtained from the National Veterinary Service Laboratory, Ames, Iowa, USA. The hens in the second group were orally challenged with 1 ml of a high dose of 1.3 x $10^8$ cfu of the same *S. enteritidis* strain. The remaining 8 hens served as the uninfected negative control group. The *S. enteritidis* was prepared from frozen stocks by sub-culturing it onto Nutrient agar (Difco) and incubated overnight at 37°C. Single colonies were streaked onto Brilliant Green agar (Difco) containing 100 µg/ml Novobiocin (Sigma) and 10 µg/ml Nalidixic acid (Sigma) (BGNN) and incubated overnight at 37°C. It was then inoculated into Tryptic Soy Broth (TSB, 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 $10^{-5}$ and $10^{-1}$ found to contain 1.3 x $10^4$ cfu/ml and 1.3 x $10^8$ cfu/ml *S. enteritidis* were used in orally challenging the hens in groups 1 and 2 respectively.

**Sample collection and processing**

Using sterile cotton applicators, samples of voided faeces from each bird were removed from the center of individual styrofoam trays and dropped into sterile, labeled 50 ml collection tubes with caps. All samples were transported on ice immediately to the laboratory for processing. Samples were collected at weekly intervals for 5 weeks post-infection.

Rappaport Vassilliadis (RV) enrichment broth was added in a proportion of 9 x volume: weight to 1 g faecal sample (1:10 dilution) and vortexed. From the 1:10 faecal–RV mixture for each sample, 100 µl was spread–plated on BGNN agar for post–infection samples. At weeks 1 and 2 post-infection, 1 ml of 1:10 faecal–RV sample from the $10^8$ cfu/ml dose group was added to 9 ml RV broth and mixed thus giving a 1:100 dilution. 100 µl of the 1:100 faecal–RV mixture was then spread-plated onto BGNN agar in addition to spread–plating 1:10 faecal–RV mixture to BGNN agar. This was done to reduce the possibility of having plates overcrowded with too numerous *S. enteritidis* colonies.

All BGNN plates and RV-enrichment samples were incubated overnight at 37°C. Plates with no detectable *S. enteritidis* colonies were streak-plated onto fresh BGNN plates using 10 µl of the 24 h RV-enriched samples. Plates were incubated at 37°C for 24 h and thereafter evaluated for presence of *S. enteritidis*; colonies counted using Plate Q-Counter (Spiral Biotech, Norwood, MA, USA). Suspect *Salmonella* colonies on BGN and BGNN were confirmed culturally, biochemically using Triple Sugar Iron (TSI) and Lysine Iron Agar (LIA) slants, and serologically with *Salmonella* O Antiserum poly A-I and Vi (Difco) and *Salmonella* O Antiserum Group D1 Factors 1, 9, 12 (Difco).

**Data analysis**

Faecal samples with no detectable growths at all or that had colonies not typical of *Salmonella* and which did not agglutinate when reacted with *Salmonella* O Antiserum poly A-I and Vi and *Salmonella* O Antiserum Group D1 Factors 1, 9, 12 (Difco) were regarded as negative. Faecal samples with detectable *Salmonella Enteritidis* colonies on incubated BGNN plates and which agglutinated when subjected to slide agglutination with *Salmonella* O Antiserum poly A–I & Vi and *Salmonella* O Antiserum Group D1 factors 1, 9, 12 (Difco) were regarded as positive and colonies were counted.

Samples with no detectable growth (negative) on BGN/BGNN but positive after plating its 24 h RV enrichment broth were given an arbitrary count of 9 (that is, 1, below the theoretical detection limit of 1 x $10^1$). Samples with no growth on BGN/ BGNN either at direct plating or 1:10/1:100 plating and negative when re-plated following enrichment were given an arbitrary count of 0 (Holt et al., 2006). The number of *S. enteritidis* detected in the faecal samples both at low ($10^4$ cfu) and high ($10^8$ cfu) doses were transformed to $\log_{10}$; means and standard error of the means were calculated. Significant differences between mean $\log_{10}$ *S. enteritidis* per dose group and for different weeks post-infection were analysed via one-way analysis of variance (ANOVA) and pooled-variance t-test (Shott, 1990). Percentages of hens positive per week and per group were also compared.

## RESULTS

All the hens remained clinically normal throughout the experiment; *S. enteritidis* was not isolated from faecal samples of any of the hens before challenge (or at any time from the uninfected control group). Levels of *S. enteritidis* recovered from faeces of hens infected with $10^8$ cfu/ml were significantly higher ($P < 0.05$) than those from hens infected with $10^4$ cfu/ml. At week 1 post-challenge, the level of *S. enteritidis* recovered from the faecal samples of hens challenged with $10^8$ cfu/ml were greater than 2 logs and significantly higher ($P < 0.05$) than those in the $10^4$ cfu/ml dose group (Figure 1).

*Salmonella enteritidis* was isolated from faeces of hens infected with $10^8$ cfu/ml *S. enteritidis* throughout the five weeks post-infection. The rate of faecal shedding of the organism decreased gradually from 100% at week 1 to 75%, 50 and 37.5% at weeks 2, 3 and 4 respectively in infected hens. At week 5 post-infection however, *S. enteritidis* was isolated from 62.5% of the infected hens (Figure 1). The organism was isolated from 87.5% of hens infected with $10^4$ cfu at week 1 post-infection. The rate of faecal shedding then dropped to 25% at week 2 after which *S. enteritidis* could not be isolated from any of the infected hens at weeks 3 and 4 post-infection (Figure 2). Only one hen was culture positive for *S. enteritidis* at week 5 post-infection. ANOVA showed significant ($P < 0.05$) differences in the rates of recovery of *S. enteritidis* from faeces of hens challenged with $10^8$ cfu/ml *S. enteritidis* and those challenged with $10^4$ cfu/ml; and at week 1 compared to weeks 2 to 5 post-infection.

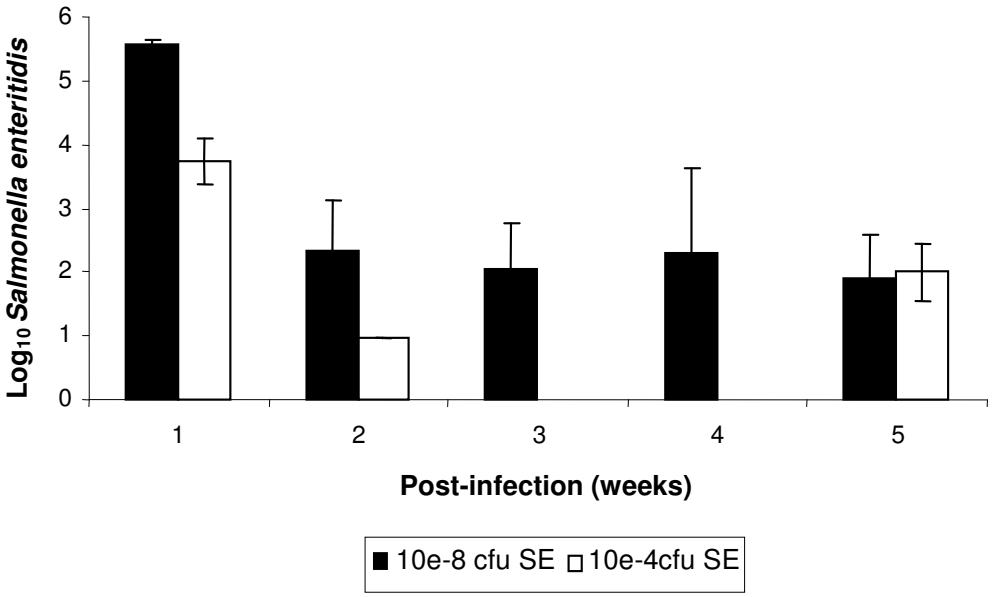


**Figure 1.** Comparison of the mean with standard error of $log_{10}$ *Salmonella enteritidis* (SE) faecal levels by hens infected with $10^8$ cfu/ml (10e-8 cfu) SE and $10^4$ cfu/ml (10e-4 cfu) SE. SE was not detected at weeks 3 and 4 in the $10^4$ cfu/ml SE dose group.

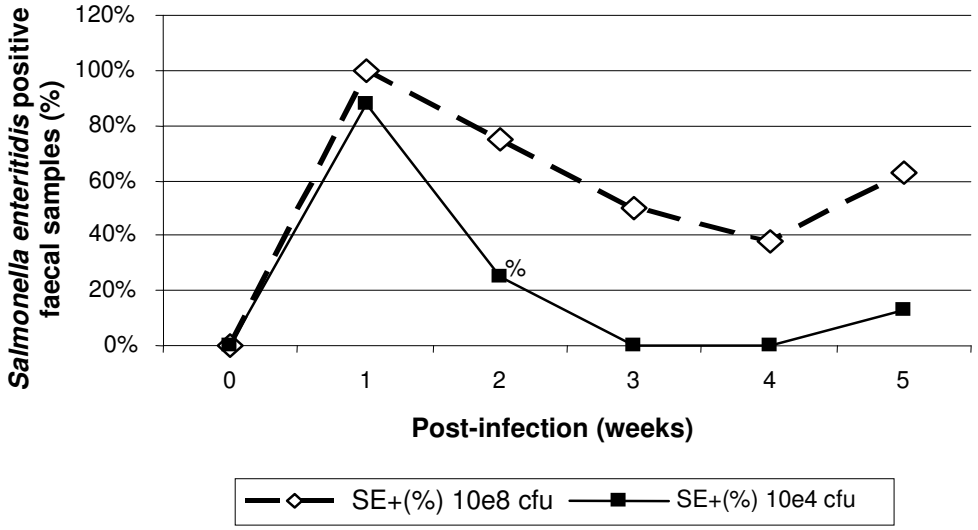


**Figure 2.** Percentage of hens with faecal samples positive for *Salmonella Enteritidis* (SE) post-infection in the $10^8$ cfu/ml SE (10e8 cfu) and $10^4$ cfu/ml SE (10e4 cfu) groups. % Positive / week = (total number of hens positive per week / total number of hens sampled per week) x 100%.

## DISCUSSION

Faecal shedding of *S. Enteritidis* in challenged hens were found to be challenge-dose dependent due to the significant differences ($P < 0.05$) observed in the proportion of hens that were culture positive from the two dose groups. Detection of more hens shedding *S. enteritidis* in faeces in the $10^8$ cfu/ml dose group compared to the $10^4$ cfu/ml dose was probably due to the variation in the doses given to these hens. Faecal shedding of a bacteria strain has been described as an indication that its presence in the alimentary tract is detected by culture of faeces or cloacal swabs. It has been reported that when an organism is detected through faecal shedding, colonisation of a part or the whole of the alimentary tract is implied. Therefore, an organism for which faecal shedding is of short duration is unlikely to have colonised the gut (Barrow et al., 1988), or probably had a transient colonisation.

The highest detection of *S. enteritidis* in faeces of infected hens at week 1 post-infection in this study agrees with the findings of Gast (2003) and Holt et al. (2006). Gast (2003) reported that during approximately the first 2 weeks following experimental oral infection of chickens or turkeys, paratyphoid salmonellae generally could be isolated from the intestinal tracts and voided faeces of a high percentage of inoculated birds. Holt et al. (2006) were able to recover substantial amount of *S. enteritidis* from faeces of most hens experimentally challenged with either 9 x $10^6$ or 5.6 x $10^6$ cfu of *S. enteritidis* by days 3 and 10 post-challenge. The finding of a gradual decline in the rate of faecal shedding also agrees with earlier reports. Gast (2003) reported a steady decline in the incidence of intestinal colonisation and faecal shedding of salmonellae by experimental infected chickens. Also, after oral inoculation of food-poisoning *Salmonella* serotypes, the number of chickens excreting salmonellae in their faeces has been reported to gradually decline over a period of at least 4 weeks (Barrow et al., 1988). The decline in rate of faecal shedding is an indication that birds were capable of reducing the level of systemic infection, probably through a humoral or cell-mediated immune response. Although both humoral and cell-mediated immune response may have been involved, the relative roles of each in the resistance of chickens to *Salmonella* infection are not fully established (Hassan et al., 1991; Muir et al., 1998). The increase in the percentages of hens shedding the organism at week 5 post-challenge could be due to gradual reduction in immune response.

The failure of isolation of *S. enteritidis* at weeks 3 and 4 post-infection followed by isolation at week 5 in the hens challenged with $10^4$ cfu confirmed intermittent shedding of *Salmonella* by these infected hens. This finding is consistent with earlier reports by Muir et al. (1998); these hens would continue to contaminate the environment (and infect other chickens) and may produce false negative culture results. Paratyphoid salmonellae infection in mature chickens has been reported to occur usually at sub-clinical level; infected chickens continue harbouring the organism in their organs and shed it in faeces for a variable period of time (Gast, 2003). This could make *Salmonella* control or eradication programs ineffective when it relies only on culture method. Multiple periodic sampling and the use of larger number of birds per sampling may therefore be necessary in such cases so as to know the true *Salmonella* status of birds (Muir et al., 1998).

Hens infected with *S. enteritidis* will continue to shed the organism in faeces and contaminate poultry environment for a variable period of time; and chickens carcasses during processing. Those exposed to few colonies of the organism may not be easily detected by culture technique due to intermittent shedding; there may also be failure of colonisation. Multiple (periodic) faecal sampling of flocks may be necessary to ascertain their *Salmonella* status and in vaccine assessment.

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### REFERENCES

Barrow PA, Simpson JM, Lovell MA (1988). Intestinal colonisation in the chicken by food-poisoning *Salmonella* serotypes: microbial characteristics associated with faecal excretion. Avian Pathol. 17: 571-588.

Centres for Disease Control (2000). Outbreaks of *Salmonella* serotype *enteritidis* infection associated with eating raw or undercooked shell eggs- United States, 1996-1998. Morb. Mortal. Wkly. Rep. 49: 73-79.

Food Safety Inspection Service (2006). Food Safety and Inspection Service, USDA. Progress report on *Salmonella* testing of raw meat and poultry products, 1998-2006. http://www.fsis.usda.gov/Science/Progress_Report_Salmonella_Testing/index.asp

Gast RK (2003). *Salmonella* Infections. In: Diseases of Poultry. Saif YM (Ed.) 11th ed. A publication of American Association of Avian Pathologists. Iowa State Press, Ames, Iowa, pp. 567-613.

Gast RK, Beard CW (1990). Isolation of *Salmonella Enteritidis* from internal organs of experimentally infected hens. Avian Dis. 34: 991-993.

Gast RK, Nasir MS, Jolley ME, Holt PS, Stone HD (2002). Serologic detection of experimental *Salmonella enteritidis* infections in laying hens by fluorescence polarization and enzyme immunoassay. Avian Dis. 42: 137-142.

Hassan JO, Mockett APA, Catty D, Barrow PA (1991). Infection and re-infection of chickens with *Salmonella* Typhimurium: bacteriology and immune responses. Avian Dis., 35: 809-819.

Holt PS, Vaughn LE, Moore RW, Gast RK (2006). Comparison of *Salmonella enterica* serovar *Enteritidis* levels in crops of fed or fasted infected hens. Avian Dis. 50: 425-429.

Muir WI, Bryden WL, Husband AJ (1998). Comparison of *Salmonella typhimurium* challenge models in chickens. Avian Dis. 42: 257-264.

Schneitz C, Nuotio L, Lounatma K (1993). Adhesion of *Lactobacillus acidophilus* to avian intestinal epithelial cells mediated by crystalline bacterial cell surface layer (_layer). J. Appl. Bacteriol. 74: 290-294.

Shott S (1990). Statistics for health professionals. W.B. Saunders Co. Philadelphia. 313-336.

World Health Organisation (2007). Drug resistant *Salmonella*. http://www.who.int/mediacentre/factsheets/fs139/en/