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Effects of challenge dose on crop colonization of *Salmonella* Enteritidis in experimentally infected chickens

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ABSTRACT

Salmonella enterica, subspecies *enterica* serovar Enteritidis (*S*. Enteritidis), is currently the main serovar causing frequent human illness associated with egg contamination. This study was conducted to determine the effects of a challenge dose of *S.* Enteritidis on crop colonization in experimentally infected chickens. Twenty-four specific-pathogen-free hens were divided into three groups of eight. The first and second groups were orally challenged with a dose of 1.3×10^8 and 1.3×10^4 colony forming units (cfu) of *S.* Enteritidis per hen respectively. The third group comprised uninfected controls. Crop lavage samples were collected weekly for 5 weeks and cultured for the presence of *S.* Enteritidis. *Salmonella* Enteritidis was isolated from the samples for 5 weeks and one week post-infection, from hens infected with 10^8 cfu/mL and 10^4 cfu/mL of *S.* Enteritidis respectively. Levels of *S.* Enteritidis recovered from the crops of hens infected with a dose of 10^8 cfu/mL were significantly higher ($P<0.05$) than from those infected with 10^4 cfu/mL. At week 1 post-infection, organisms recovered from the 10^8 cfu/mL group were greater than 4 logs and significantly higher ($P<0.05$) than in the 10^4 cfu/mL group. The rate of crop colonization of hens infected with 10^8 cfu/mL *S.* Enteritidis dropped from 100.0% to 62.5% and 25.0% at weeks 1, 3 and 5 post-infection respectively. Since the amount of organism colonizing a tissue is proportional to the level of antibodies produced, to ensure the protection of vaccinated chickens, *Salmonella* vaccines should contain an adequate vaccine dose.

Key words: *Salmonella* Enteritidis, chickens, crop colonization, infection, challenge dose

Introduction

Salmonella bacteria are a major problem in the poultry industry. This is largely the result of the entry of these bacteria into the human food chain through poultry. Human *Salmonella* infections and food-poisoning take the form of gastroenteritis, which can result in death in highly susceptible individuals (HERES et al., 2003). It is therefore important to control poultry infection and egg contamination, in order to reduce the worldwide

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salmonellosis problem. Apart from good hygiene and animal husbandry practices, other methods employed to reduce *Salmonella* on poultry farms include competitive exclusion by non-pathogenic bacteria, genetic selection of chicken strains for improved immune response and the development of *Salmonella* vaccines (LILLEHOJ et al., 2000). Both killed and live vaccines have been used to prevent *Salmonella* infection in birds. Various factors, including vaccine dosage, vaccine preparation, challenge bacteria, route of inoculation, age at immunization and individual vaccinated animals, have been reported to influence the results obtained (GAST et al., 1993; CORRIER, 1995; HASSAN and CURTISS, 1994, 1996, 1997). Live vaccines have been shown to offer better protection and are more effective than killed vaccines (BABU et al., 2004). Live attenuated strains of *Salmonella* can replicate, colonize and invade the intestinal and visceral organs of inoculated chickens, thereby leading to the induction of strong immunity (GERMANIER, 1972). Chickens vaccinated orally with a live attenuated vaccine of *Salmonella* Enteritidis at a dose of 10^9 cfu were shown to be protected against invasion by the wild strain of *Salmonella* Enteritidis; there was reduced colonization of the internal and visceral organs in vaccinated chickens, when compared to the unvaccinated group (CERQUETTI and GHERARDI, 2000). A dose of 1 mL, comprising 1000 organisms of an attenuated fowl typhoid vaccine, was shown to produce adequate humoral and cell mediated immunity (BEBORA, et al., 1998).

Generally, *Salmonella* infection is believed to start through ingestion of the organism, after which it traverses the upper alimentary tract, before reaching the lower intestine (HOLT et al., 2006). The internal organs of infected chickens are colonized by the organism (GAST, 2003). The crop (ingluvies) has now been identified as one of the first areas which *Salmonella enterica* subspecies *enterica* serovar Enteritidis (*S.* Enteritidis) will encounter on its way from the beak to the intestine. The crop is a sac-like organ in the cervical oesophagus and is proximal to the proventriculus or glandular stomach; it functions primarily as a food storage organ (DYCE et al., 1996). The crop, proventriculus and gizzard form the anterior parts of the gastrointestinal tract (GIT) of chickens, where the pH level is low, facilitating the initial inactivation of pathogens (FULLER, 1973; SCHNEITZ et al., 1993).

The crop has been earlier reported to have been colonized by *Salmonella* (HUMPHREY et al., 1993; HOLT et al., 2002; SEO et al., 2002; HOLT et al., 2006) and has also been identified as a source of *Salmonella* contamination of carcasses during poultry processing (HARGIS et al., 1995; CHAMBERS et al., 1998). Long term feed withdrawal has been reported to increase crop colonization with *S.* Enteritidis while humoral immunity involving the production of immunoglobulin A has been found to develop in the crops of chickens orally infected with *S.* Enteritidis (HOLT et al., 2006). Chickens are usually exposed to variable quantities of *Salmonella* organisms under natural conditions; however, the response of the crop to these variable quantities of *Salmonella* is not fully understood. This study

was therefore designed to study the effects of a challenge dose of *S.* Enteritidis on crop colonization, using experimentally infected chickens.

Materials and methods

Thirty-three-week-old single-combed White Leghorn laying hens 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 8. The hens in each group were housed in separate rooms in an environmentally controlled biosafety building, with each bird in an individual cage. Birds were fed rations of antibiotic-free pelleted layers and supplied with water *ad libitum*. A day before the weekly sample collection, birds were kept off feed for 12 hours in order to reduce crop bulk, thus expediting flushing of the crop. The hens were provided with sixteen hours of light daily.

In order to ascertain that the hens were *Salmonella*-free, each individual was screened prior to the commencement of the experiment (pre-challenge samples). Crop lavage (CL) samples were collected and 100 µL of the neat CL samples were spread-plated onto Brilliant Green agar (Difco Laboratories, Detroit, USA) containing 100 µL Novobiocin (Sigma Chemical Co., St. Louis, USA) (BGN) per mL. The plates were incubated at 37 ^{o}C overnight. *Salmonella* was not detected. Also, 1 mL of the neat CL sample per hen was added to 9 mL Rappaport Vassilliadis (RV) enrichment broth (Oxoid Inc. Basingstroke, U.K.); the crop lavage-RV mixture per sample was incubated overnight at 37 ^{o}C. One hundred microlitre (100 µL) of the incubated samples was then spread-plated on BGN plates and incubated overnight at 37 ^{o}C. No *Salmonella* was detected.

One group of 8 hens was challenged orally with 1 mL of a low dose of 1.3×10^{4} colony forming units (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. Each of the 8 hens in the second group was orally challenged with 1 mL of a high dose of 1.3×10^{8} cfu of the same *S.* 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) and incubated overnight at 37 ^{o}C. Single colonies were streaked onto Brilliant Green agar containing 100 µg/mL Novobiocin and 10 µg/mL nalidixic acid (Sigma) (BGNN) and incubated overnight at 37 ^{o}C. It was then inoculated into Tryptic Soy Broth (TSB, Difco) and incubated overnight at 37 ^{o}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 1.3×10^{8} cfu/mL and 1.3×10^{4} cfu/mL *S.* Enteritidis were used in orally challenging the hens in groups 1 and 2 respectively. Crop lavage samples were collected from the 24 hens before challenging them using the

crop lavage technique described by HOLT et al., (2002), starting from the control hens, followed by hens challenged with 10^4 cfu/mL and then the 10^8 cfu/mL dose group. The lavage technique comprises Tygon® (Fisher Scientific, USA) tubing and a 10 mL syringe containing a 5 mL glycine flush (1M Tris/glycine buffer with 0.25% Tween 20, pH 7 to 8) solution (lavage fluid). The crop lavage device tubing containing glycine flush solution was inserted down the hen's oesophagus into the crop. Lavage fluid was discharged into the crop and then immediately aspirated back into the syringe. It was then dispensed into a 15 mL sterile collection tube. All samples were transported immediately on ice to the laboratory for processing. Samples were collected at weekly intervals for 5 weeks post-infection.

One millilitre of each crop lavage sample obtained from the hens pre-challenge and post-infection was added to 9 mL RV enrichment broth and incubated at 37 °C overnight for selective enrichment. A 100 µL aliquot of each CL sample was also manually spread-plated onto BGN for pre-challenge samples, while in the case of 1, 2, 3, 4, and 5 week post-infection samples, a 100 µL CL sample was spread-plated onto BGNN.

One millilitre of crop lavage samples collected from the 10^8 cfu/mL dose group at 1 and 2 weeks post-infection was added to 9 mL RV broth and vortexed slightly in order to obtain a 1:10 dilution. Then, 100 µL of the 1:10 RV-diluted CL sample was manually spread-plated onto BGNN plates and incubated at 37 °C overnight. This was done to reduce the number of colonies of *S.* Enteritidis per plate. This dilution was later accounted for in the calculation of number of colonies per plate.

All BGN and BGNN plates were incubated at 37 °C overnight, after which *S.* Enteritidis counts were made using a Plate Q-counter (Spiral Biotech, Norwood, MA, USA). Any sample without detectable *S.* Enteritidis growth on the BGN or BGNN plate was streak-plated onto a fresh BGN or BGNN plate using 10 µL of the 24-hour RV enriched broth for that sample. These plates were then incubated for 24 hours at 37 °C and assessed for the presence of *S.* Enteritidis. 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 & Vi and *Salmonella* O Antiserum Group D1 Factors 1, 9, 12 (Difco).

Crop lavage with detectable *S.* Enteritidis colonies on incubated BGN or BGNN plates, 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) was regarded as positive and colonies were counted. Samples with no detectable growths at all, or with colonies not typical of *Salmonella*, which did not agglutinate when reacted with *Salmonella* O Antiserum poly A-I & Vi and *Salmonella* O Antiserum Group D1 Factors 1, 9, 12 (Difco) were regarded as negative. Incubated RV-enrichment (10 µL) of such negative samples were usually re-streaked onto fresh BGN or BGNN plates and

incubated overnight at 37 °C. Samples with no detectable growth (negative) on BGN/BGNN, but positive after plating its 24-hour RV enrichment broth, were given an arbitrary count of 9 (that is, 1, below the theoretical detection limit of 1×10^1). 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 crop samples, both at the 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 periods (1, 2, 3, 4 and 5 weeks post-infection) were analyzed via one-way analysis of variance (ANOVA) and pooled-variance *t*-test (SHOTT, 1990). The percentages of hens recorded positive per week and per group were also compared.

Results

Salmonella Enteritidis was not isolated from the crop lavage samples of any of the hens before challenge (or at any time from the uninfected control group); all birds remained clinically normal throughout the experiment. *Salmonella* Enteritidis was recovered from the crop lavage samples of infected hens by the culture method for 5 weeks and one week post-infection in hens infected with 10^8 cfu/mL and 10^4 cfu/mL of *S.* Enteritidis respectively. Levels of *S.* Enteritidis recovered from the crops of hens infected with a dose of 10^8 cfu/mL of *S.* Enteritidis were significantly higher ($P<0.05$) than from those infected with 10^4 cfu/mL. At week 1 post-infection, the level of *S*. Enteritidis recovered from the crops of hens challenged with 10^8 cfu/mL was greater than 4 logs (mean $\log_{10}$ 4.3632 with standard error +/- 0.1077) and significantly higher ($P<0.05$) than those in the 10^4 cfu/mL dose group (mean $\log_{10}$ 0.1357 with standard error +/- 0.1357). In the 10^8 cfu/mL dose group, the amount of organisms recovered then reduced to mean $\log_{10}$ 1.2094 with standard error +/- 0.2552 at week 3 post-infection and finally to mean $\log_{10}$ 0.9542 at week 5 (Fig. 1).

Crop colonization was only evident at week 1 post-infection in hens infected with 10^4 cfu/mL (low dose) *S*. Enteritidis and only 12.5% of the infected hens were culture positive for *S*. Enteritidis (Fig. 2). In the case of hens infected with 10^8 cfu/mL *S*. Enteritidis, crop colonization was at its peak by week 1 post-infection with 100% recovery of the organism from cultured crop lavage samples. The rate of isolation from crops reduced at week 2 to 25% but increased later, with *S*. Enteritidis being recovered from 62.5% of infected hens at weeks 3 and 4 post-infection, before dropping at week 5 (Fig. 2).

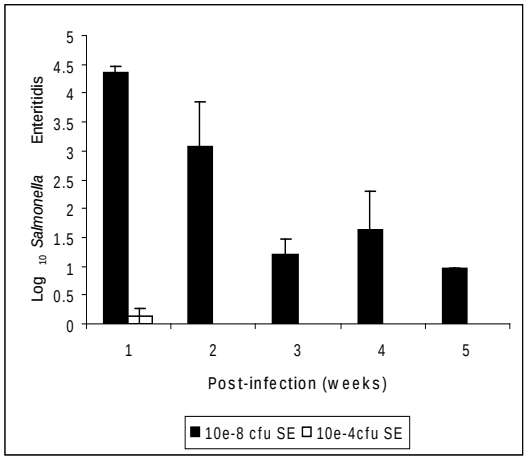

Fig. 1. Culture results of crop lavage samples (comparison of the mean with standard error of $\log_{10}$ *Salmonella* Enteritidis (SE) crop levels by hens infected with 10^8 cfu/mL SE and 10^4 cfu/mL SE). SE was only recovered at week 1 in the 10^4 cfu/mL SE dose group.

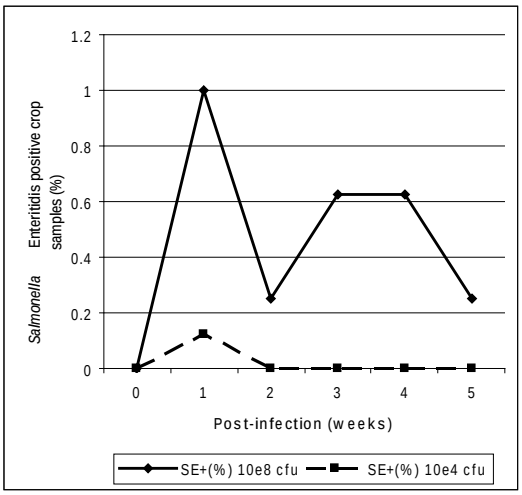

Fig. 2. Percentage of crop lavage samples positive for *Salmonella* Enteritidis post-infection in the 10^8 cfu/mL SE (high dose) and 10^4 cfu/mL SE (low dose) groups.

% positive /week was calculated as: $\frac{\text{Total number of hens positive per week}}{\text{Total number of hens sampled per week}} \times 100\%$

Discussion

The absence of clinical signs in infected hens is in agreement with the findings of previous authors, who reported that mature birds experimentally infected with *S*. Enteritidis have remained clinically normal, except for the possible occurrence of brief, mild diarrhea (HUMPHREY et al., 1989; TIMONEY et al., 1989). The crop has been reported to be readily colonized by *Salmonella* (DESMIDT et al., 1997; HOLT et al., 2006) and this is generating considerable attention as a source for *Salmonella* carcass contamination (CHAMBERS et al., 1998). Colonization of crops by *S.* Enteritidis in challenged hens were found to be challenge-dose dependent, since significant differences ($P<0.05$) were found in the proportion of hens that were culture positive from the two dose groups. The detection of more hens with crops colonized by *S*. Enteritidis in the 10^8 cfu/mL dose group compared to the 10^4 cfu/mL group may be due to the variation in the quantities of the organism to which the hens were exposed. Also, the initial inactivation at the upper GIT of birds and presence of mucosal immunity may contribute to the significant decrease in the levels of *S*. Enteritidis isolated between week 1 and week 2 post-infection in the 10^8 cfu/mL dose group. The presence of low pH in the anterior GIT tract comprising the crop, proventriculus and gizzard have been shown to be responsible for the initial inactivation of pathogens, including *Salmonella,* following ingestion (FULLER, 1973; SCHNEITZ et al., 1993; HERES et al., 2003). The presence of B and T lymphocytes in the upper GIT (VERVELDE and JEURISSEN, 1993; MATSUMOTO and HASHIMOTO, 2000), and anti-*S*. Enteritidis IgA in the crops of challenged hens (HOLT et al., 2006) have also been reported.

The highest detection of *S*. Enteritidis at week 1 post-infection in this study agrees with the findings of HOLT et al. (2006) who reported recovery of substantial amounts of *S*. Enteritidis from the crops of most hens experimentally challenged with either 9×10^6 or 5.6×10^6 cfu of *S*. Enteritidis by days 3 and 10 post-challenge. The finding of a decrease in the rate of crop colonization also agrees with a previous report of steady decline in the incidence of crop and intestinal colonization of *Salmonella*e by experimental infected chickens (GAST, 2003). The inability to detect *S*. Enteritidis in the cultured crop secretions of hens challenged with 10^4 cfu from weeks 2 to 5 post-infection may be due to the fact that they were exposed to too few *Salmonella* colonies to be able to establish a long term infection, or these were eliminated by the bird's innate immunity (HOLT et al., 2006). Hence the variation in the number of *S*. Enteritidis culture positive hens in the two dose groups. Mature chickens infected with paratyphoid *Salmonella*e usually have subclinical infection (GAST, 2003).

This study further confirms colonization of the crops of hens exposed to *Salmonella*; and these continue to present risk factors in terms of carcass contamination with *Salmonella* during processing. This is very important in chickens exposed to large infective doses of

Salmonella, while those exposed to few colonies are only involved in transient infection and crop colonization. Poultry farmers should therefore work towards minimizing the *Salmonella* contamination of the poultry environment, in order to reduce chickens' exposure. Also, since the amount of organism colonizing a tissue has been linked to the proportion of antibodies produced, in order to ensure protection of vaccinated chickens and reduce the risk of transmission of *Salmonella* to humans, *Salmonella* vaccines should contain an adequate vaccine dose. Hens vaccinated by spraying with an approximate dose of 1×10^8 cfu of live attenuated *Salmonella enterica* serovar Typhimurium vaccine organisms prior to *S*. Enteritidis experimental challenge and moulting have been reported to have reduced horizontal spread of *S*. Enteritidis infection and shed significantly less *S*. Enteritidis when compared to their unvaccinated, infected counterparts (HOLT et al., 2003). The duration of immunity to *Salmonella* vaccines has been reported to vary, according to vaccine dose, vaccine preparation, challenge bacteria, route of inoculation, age at immunization and individual vaccinated animals (GAST, 1993; CORRIER, 1995; HASSAN and CURTISS, 1994, 1996, 1997). Although, inactivated vaccines stimulate strong immune responses, they offer a relatively low degree of protection when compared with live attenuated organisms (BARROW et al., 1990; BABU et al., 2004).

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SAŽETAK

Salmonella enterica subspecies *enterica* serovar Enteritidis (*S*. Enteritidis) zasada je glavni serovar što uzrokuje česte zaraze u ljudi povezane s uzimanjem zagađenih jaja. Ovo istraživanje poduzeto je radi određivanja učinaka izazivačke doze *S.* Enteritidis na sposobnost naseljivanja u voljku pokusno zaraženih pilića. Ukupno su 24 SPF kokoši bile podijeljene u tri skupine po osam. Svaka kokoš prve skupine bila je zaražena per os dozom od $1,3\times10^8$, a druge skupine dozom od $1,3\times10^4$ kolonijotvornih jedinica (cfu) *S.* Enteritidis. Treća skupina bila je nezaražena, kontrolna skupina. Uzorci ispirka voljke bili su uzimani tjedno u razdoblju od pet tjedana te pretraživani na prisutnost *S.* Enteritidis. *Salmonella* Enteritidis bila je izdvojena tijekom pet tjedana nakon infekcije iz svih uzoraka kokoši zaraženih dozom od 10^8 cfu/mL, a samo tjedan dana nakon infekcije u kokoši zaraženih dozom od 10^4 cfu/mL. Broj bakterija *S.* Enteritidis izdvojenih iz voljki kokoši zaraženih dozom od 10^8 cfu/mL bio je značajno veći ($P<0,05$) nego u onih zaraženih s 10^4 cfu/mL. Prvi tjedan nakon infekcije, broj bakterija izdvojenih iz skupine zaražene s 10^8 cfu/mL bio je veći od 4 logaritma i značajno veći ($P<0,05$) nego u skupini koja je bila zaražena s 10^4 cfu/mL. Postotak naseljenja voljke kokoši zaraženih s 10^8 cfu/mL *S.* Enteritidis smanjio se sa 100% koliko je iznosio u prvom tjednu, na 62,5% u trećem tjednu te na 25.0% u petom tjednu nakon infekcije. Budući da je količina bakterija koje naseljavaju određeno tkivo razmjerna razini proizvedenih specifičnih protutijela, radi sigurne zaštite cijepljenih pilića, cjepiva protiv salmoneloze treba da sadrže odgovarajuću dozu bakterija.

Ključne riječi: *Salmonella* Enteritidis, pilići, kolonizacija voljke, izazivačka doza