Salmonella Enteritidis Deposition in Eggs after Experimental Infection of Laying Hens with Different Oral Doses

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ABSTRACT

The continuing attribution of human Salmonella Enteritidis infections to internally contaminated eggs has necessitated the commitment of substantial public and private resources to Salmonella Enteritidis testing and control programs in commercial laying flocks. Cost-effective risk-reduction requires a detailed and comprehensive understanding of how Salmonella Enteritidis infections in hens result in deposition of the pathogen inside eggs. The present study sought to resolve some incompletely defined aspects of the relationship between Salmonella Enteritidis oral-exposure dose levels in experimentally infected laying hens and the frequency and location of subsequent egg contamination. In two trials, groups of specific-pathogen-free hens were experimentally inoculated with oral doses of 10⁵, 10⁶, or 10⁷ CFU of a phage type 4 Salmonella Enteritidis strain. Eggs were collected 5 to 23 days postinoculation, and the yolk and albumen of each egg were cultured separately to detect Salmonella Enteritidis contamination. Larger oral doses of Salmonella Enteritidis administered to hens were associated with significant increases in the frequencies of both yolk and albumen contamination. Moreover, Salmonella Enteritidis was found in the albumen of a far-higher proportion of contaminated eggs from hens given the largest dose than from the other two groups. Salmonella Enteritidis contamination was detected in 0.7% of yolk and 0.2% of albumen samples after inoculation of hens with 10⁷ CFU, 4.0% of yolk and 1.7% of albumen samples after inoculation with 10⁶ CFU, and 6.5% of yolk and 10.8% of albumen samples after inoculation with 10⁵ CFU. These results demonstrate that oral-exposure doses of Salmonella Enteritidis for laying hens can significantly affect both the frequency and location of deposition of this pathogen inside eggs.

The economic burden of foodborne salmonellosis in the United States could be as high as $11 billion annually (50). The overall incidence of human Salmonella infections did not change significantly between 1998 and 2010, but the incidence of infections with Salmonella enterica serovar Enteritidis increased during that interval (3). For more than 25 years, the vast majority of illness because of Salmonella Enteritidis around the world has been attributed to contaminated eggs (2, 32), even though the estimated prevalence of Salmonella Enteritidis in commercially produced eggs is very low (8, 9). Substantial public and private resources have been committed to testing and risk-reduction programs for Salmonella Enteritidis in egg-laying flocks (11, 52). Although persistent application of these programs has apparently led to progress in reducing the incidence of human illness because of Salmonella Enteritidis in several nations (46, 48), both epidemiologic calculations and active disease surveillance suggest that egg-transmitted Salmonella Enteritidis remains a significant public health concern (3, 4).

Environmental persistence of Salmonella Enteritidis in poultry houses creates extended opportunities for laying hens to be exposed and infected (5). Rodents and insects can introduce, transmit, and amplify Salmonella Enteritidis in laying houses to levels capable of surviving standard cleaning and disinfection procedures (7, 44). A more diverse assortment of Salmonella Enteritidis phage types has been detected in environmental samples from laying houses than in contaminated eggs, suggesting that the environment constitutes a reservoir from which strains with heightened abilities to cause systemic infection and egg contamination can periodically emerge (37, 38). The deposition of Salmonella inside eggs is a direct result of reproductive organ colonization (especially the ovary and upper oviduct) in a systematically infected laying hen (6, 20, 43). However, colonization of reproductive tissues at a high frequency or by large numbers of bacterial cells is not always correlated with a correspondingly high frequency of egg contamination (20, 22).

Colonization of different regions of the reproductive tracts of hens can lead to Salmonella Enteritidis contamination in either the yolk or albumen (or both) of developing eggs (6, 25, 41). Although Salmonella Enteritidis is more likely to be deposited in the albumen or on the outside of the vitelline (yolk) membrane than within the nutrient-rich yolk interior (17, 27), bacterial migration across this membrane can result in rapid and significant multiplication in the yolk.
contents during storage at warm temperatures (18, 21, 30). Because freshly laid eggs normally harbor no more than a few hundred Salmonella Enteritidis cells (25, 41), prompt refrigeration can prevent extensive bacterial multiplication during storage. Risk assessment studies have consistently identified refrigeration as one of the most effective mitigation options for protecting consumers against the threat of illness because of Salmonella Enteritidis contamination of eggs (45, 51). Most Salmonella Enteritidis risk-reduction plans, including federal regulations for shell egg producers in the United States, require egg refrigeration (46, 52).

The experimental administration of different dose levels of Salmonella Enteritidis to hens has been reported to influence several detectable parameters of infection including antibody responses, organ invasion, and both the frequency and duration of intestinal colonization (19, 23, 31). However, the effects of Salmonella Enteritidis infecting doses on the associated prevalence and characteristics of subsequently occurring egg contamination have been only incompletely described and evaluated in previous studies. In one prior investigation, both fecal shedding and antibody responses were found to be more strongly dose-dependent than egg contamination (39). The objective of the present study was to determine if (and how) experimental oral infection of groups of laying hens with three different exposure doses of a phage type 4 Salmonella Enteritidis strain affected the frequency and location of bacterial deposition inside eggs laid during the first few weeks after inoculation.

**MATERIALS AND METHODS**

**Experimental infection of laying hens with Salmonella Enteritidis.** In each of two replicate trials, 75 laying hens were obtained from a specific-pathogen-free flock of single-comb White Leghorn chickens (negative for antibodies to Salmonella in periodic routine monitoring) at the Southeast Poultry Research Laboratory in Athens, GA. These hens (30 and 35 weeks old at the beginning of the first and second trials, respectively) were distributed into four separately housed groups in a disease-containment facility, with 24 hens in each of three experimental groups, and a fourth group of 3 hens held as un inoculated negative controls. Each bird was kept in an individual laying cage and provided with water and pelleted feed ad libitum.

The three experimental groups of chickens in each trial were orally inoculated with different measured doses of Salmonella Enteritidis. For each trial, a lyophilized stock culture of phage type 4 Salmonella Enteritidis (originally isolated from the liver of an infected chicken by Dr. D. Munro at the Scottish Salmonella Reference Laboratory, Glasgow, UK) was resuscitated by transfer into tryptic soy broth (TSB; Acumedia, Neogen Corp., Lansing, MI) for two successive cycles of 24-h incubations at 37°C. After cell numbers in the incubated culture were estimated by determining the culture’s optical density at 600 nm, further serial 10-fold dilutions in 0.85% saline produced the desired final cell concentrations (confirmed by subsequent plate counts). The hens in one experimental group were each inoculated with 1-ml doses of diluted culture containing $1.6 \times 10^8$ CFU of Salmonella Enteritidis, the hens in a second group received doses of $1.6 \times 10^6$ CFU, and the third group of hens were each given $1.6 \times 10^4$ CFU.

**Fecal samples.** Immediately before inoculation, sterile cotton swabs were used to collect samples of freshly voided feces from polystyrene trays (food grade but not sterile) placed under each cage. These samples were transferred to 9 ml of tetrahionate broth (Acumedia, Neogen) and incubated for 24 h at 37°C. A 10-μl portion from each broth culture was then streaked onto brilliant green agar (Acumedia, Neogen) supplemented with 0.02 mg/ml novobiocin (Sigma Chemical Co., St. Louis, MO) and incubated for 24 h at 37°C. The identity of presumptive colonies of Salmonella was confirmed biochemically and serologically (53).

**Egg content samples.** All eggs laid between the 5th and 23rd days postinoculation were collected daily and stored at 4°C until sampled. This collection interval includes the period when most Salmonella Enteritidis–contaminated eggs were produced in prior experimental infection studies (16, 17). Each egg was cultured individually to detect internal contamination of the yolk or albumen with Salmonella Enteritidis. After eggshell surfaces were disinfected by dipping for 5 s in 70% ethanol, the shells were broken against a sharp edge covered by sterile foil strips. The liquid contents of each egg were poured through a sterile plastic separator, and the yolk and albumen were individually transferred to 30 ml of TSB supplemented with 35 mg/liter ferrous sulfate (Sigma), mixed by vigorous shaking for 15 s, and incubated for 24 h at 37°C. A 1-ml portion of each incubated sample was then transferred to 9 ml of Rappaport-Vassiliadis enrichment broth (Oxoid, Ltd., Basingstoke, UK) and incubated for 24 h at 37°C. A 10-μl aliquot from each of these incubated broth cultures was streaked onto brilliant green agar plus novobiocin and incubated for 24 h at 37°C. The identity of typical colonies of Salmonella Enteritidis was confirmed biochemically and serologically (53).

**Statistical analysis.** For each trial (and for both trials combined), significant differences ($P < 0.05$) between Salmonella Enteritidis inoculum doses or between contamination sites (yolk and albumen) in the mean frequencies of isolation from egg contents samples were determined by Fisher’s exact test. Data were analyzed with InStat biostatistics software (GraphPad Software, San Diego, CA).

**RESULTS AND DISCUSSION**

None of the fecal samples collected before inoculation were positive for Salmonella. For both trials combined, Salmonella Enteritidis was recovered from the contents (yolk, albumen, or both) of 0.7% of eggs laid between 5 and 23 days postinoculation by hens inoculated with an oral dose of $10^8$ CFU, 4.6% of eggs from hens inoculated with $10^9$ CFU, and 11.9% of eggs laid by hens inoculated with $10^8$ CFU (Table 1). The overall frequencies of Salmonella Enteritidis isolation from eggs laid by each of the three inoculation dose groups were significantly different from each other for both trials combined ($P < 0.001$) and in the two individual trials ($P < 0.0008$ for trial 1 and $P < 0.0145$ for trial 2). Contaminated eggs were produced by 25 of 48 hens inoculated with $10^8$ CFU of Salmonella Enteritidis (between 6 and 21 days postinoculation), 13 of 48 hens inoculated with $10^9$ CFU (11 to 19 days postinoculation), and 4 of 48 hens inoculated with $10^8$ CFU (11 to 17 days postinoculation).

For both trials combined, Salmonella Enteritidis was found in 0.7% of egg yolk samples from hens inoculated with the $10^4$-CFU dose, 4.0% of yolk samples from hens
Enteritidis infections over time

A (7.33) 25/232 %
(2.35) 14/255 B 0/273 (12, 28).

0.05) different.

Enteritidis was found significantly higher than after inoculation with 10^4 CFU in both trials 1 and 2 (P = 0.0034 and 0.0242, respectively) and for both trials combined (P = 0.0004).

Salmonella Enteritidis was recovered from the albumen (for both trials combined) in 0.2% of eggs laid by hens inoculated with 10^4 CFU, 1.7% of eggs from hens inoculated with 10^6 CFU, and 10.8% of eggs from hens inoculated with 10^8 CFU (Table 1). Significantly more albumen samples were contaminated by Salmonella Enteritidis among eggs from hens in the 10^8-CFU dose group than in either of the other two dose groups for both individual trials and for the two trials combined (P ≤ 0.0007 for the 10^6-CFU dose and P < 0.0001 for the 10^8-CFU dose). Salmonella Enteritidis was found significantly more often in albumen samples from the 10^8-CFU dose group than from the 10^6-CFU dose group only for both trials combined (P = 0.0098).

The frequencies of observed yolk and albumen contamination by Salmonella Enteritidis (for both trials combined) were not significantly different at the 10^4-CFU inoculation dose. In eggs from the 10^6-CFU dose group, the overall frequency of yolk contamination was significantly (P = 0.0399) greater than the frequency of albumen contamination. However, among eggs from the 10^8-CFU dose group, the frequency of Salmonella Enteritidis isolation from albumen was significantly (P = 0.0257) higher than from yolk.

Previous data from both experimentally and naturally infected poultry have shown that the initial oral-exposure dose can profoundly affect the progress and outcomes of Salmonella Enteritidis infection (19, 25, 31). In the present study, the frequency of Salmonella Enteritidis isolation from the contents (both yolk and albumen) of eggs laid by inoculated hens increased significantly with each successive 100-fold increase in the oral-exposure dose. The observed course of poultry Salmonella Enteritidis infections over time could be an interactive consequence of two contrasting effects of the initial bacterial-exposure dose. Larger doses are associated with a higher frequency and greater persistence of both intestinal and internal organ colonization, but they also stimulate stronger immune responses, which promotes the eventual clearance of infection from host tissues (12, 28). Both the incidence and kinetics of Salmonella Enteritidis deposition inside eggs laid by infected hens can be similarly modulated by exposure-dose effects (10). Experimental infection studies have generally reported Salmonella Enteritidis contamination inside eggs to occur rather infrequently, even after the inoculation of hens with very large oral doses (16, 25, 41). The prevalence of

<table>
<thead>
<tr>
<th>TABLE 1. Recovery of Salmonella Enteritidis from the contents of eggs laid by experimentally infected hens</th>
<th>No. of Salmonella-positive samples/total no. (%)</th>
<th>10^4</th>
<th>10^6</th>
<th>10^8</th>
</tr>
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<tbody>
<tr>
<td>Salmonella Enteritidis dose (CFU)</td>
<td>Trial 1</td>
<td>Trial 2</td>
<td>Both trials</td>
<td></td>
</tr>
<tr>
<td>Yolk</td>
<td>Any</td>
<td>Any</td>
<td>Any</td>
<td>Any</td>
</tr>
<tr>
<td>Albumen</td>
<td>Any</td>
<td>Any</td>
<td>Any</td>
<td>Any</td>
</tr>
<tr>
<td>0/273 (0)</td>
<td>0/273 (0)</td>
<td>0/275 (0)</td>
<td>0/278 (0)</td>
<td>0/273 (0)</td>
</tr>
<tr>
<td>0.265B (0.299)</td>
<td>0.265B (0.370)</td>
<td>0.265B (0.370)</td>
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<tr>
<td>167B (1.73)</td>
<td>167B (1.73)</td>
<td>167B (1.73)</td>
<td>167B (1.73)</td>
<td>167B (1.73)</td>
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<tr>
<td>a Collected for sampling between 5 and 23 days after inoculation, with three different oral doses of a phage type 4 strain.</td>
<td></td>
<td></td>
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<td>b Any, contaminated yolk, albumen, or both.</td>
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<td>c Values within columns sharing no common letters are significantly (P &lt; 0.05) different.</td>
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Salmonella Enteritidis contamination in eggs from naturally infected commercial laying flocks is usually even lower, perhaps because the overall prevalence of Salmonella Enteritidis infection within these flocks is also low, and because individual hens are typically only exposed to relatively small doses of the pathogen from her environment (9, 40). Experimental exposure of chickens by horizontal contact could provide an approximate simulation of naturally occurring transmission of infection. Among contact-exposed birds, intestinal colonization, organ invasion, and egg contamination have all occurred less often than among birds inoculated with large oral doses (13, 24).

Salmonella Enteritidis has been found in both the ovaries (sites of yolk maturation and release) and oviducts (sites of albumen secretion around the descending yolk) of infected hens. Invasion beyond the intestinal tract to reach internal organs such as the liver and spleen occurs within a few hours after initial oral exposure to Salmonella Enteritidis (36), creating opportunities for subsequent dissemination to reproductive tissues (10). The overall frequency of Salmonella Enteritidis colonization of internal organs characteristically undergoes a steep decline during the first few weeks after oral inoculation (22). However, extended Salmonella Enteritidis persistence in even a small fraction of the initially exposed birds can prolong the potential for subsequent horizontal transmission of infection to other hens within the flock and thus for the production of additional contaminated eggs (14). Environmental stressors such as heat, feed restriction, or water deprivation can heighten the susceptibility of laying hens to Salmonella Enteritidis infection (47).

The deposition of Salmonella Enteritidis in albumen in the present study occurred in a far-higher proportion of contaminated eggs after administration of the largest oral-exposure dose (10⁶ CFU) than for the lower dose levels (10⁴ and 10⁵ CFU). This difference could be attributable to either more frequent colonization of the oviduct (site of albumen secretion) after infection with the larger dose, more frequent deposition in albumen because of larger Salmonella Enteritidis numbers colonizing the oviduct, or more frequent survival in albumen after the deposition of larger numbers of Salmonella Enteritidis. The initial site of Salmonella contamination inside eggs affects opportunities for pathogen multiplication during storage. The abundant nutrients in yolk can support rapid and prolific Salmonella Enteritidis growth at warm temperatures (26, 35), whereas growth is slower or absent in the presence of bacterial inhibitors in albumen (42, 49). However, even after initial deposition outside of the yolk, Salmonella Enteritidis can penetrate the vitelline membrane to reach the nutrient-dense yolk contents (18, 30). Both Salmonella Enteritidis growth in egg yolks (26, 35) and migration across vitelline membranes (21, 29) are reduced or halted at refrigeration temperatures.

Salmonella Enteritidis strains can differ significantly from each other in their abilities to invade reproductive organs and contaminate eggs (25, 27, 34), but individual strains do not appear to possess any unique affinities for colonization of particular regions of the reproductive tract or deposition at particular sites within eggs (22). Variability within and between serovars complicates assessment of the pathological effects (including internal organ invasion and egg contamination) of Salmonella in chickens (22, 25, 27). For example, inoculation of hens with a high dose of a phage type 4 strain in the current study led to Salmonella Enteritidis deposition in albumen in a relatively high proportion of contaminated eggs, whereas prior studies with phage type 13a and 14b Salmonella Enteritidis strains yielded disparate results (16, 22). Experimental infection studies have generally reported higher egg contamination frequencies for Salmonella Enteritidis than for other serotypes (15, 21, 22). However, differentiation among Salmonella Enteritidis strains on the basis of defined genetic elements responsible for the ability to cause egg contamination has proven to be rather elusive, although patterns of accumulated single-nucleotide genomic changes have been more informative (1, 34). The coordinated expression of complementary phenotypic properties, relevant at different stages of infection in the avian host, could possible enable Salmonella Enteritidis strains to reach the contents of developing eggs (16, 33).

The present study determined that hens exposed to different oral doses of a Salmonella Enteritidis strain laid eggs that exhibited significant dose-specific variation in important parameters of internal contamination (frequency and location). The deposition of Salmonella Enteritidis inside eggs is responsible for an internationally prominent foodborne disease problem and constitutes the most critical confirmatory diagnostic criterion for identifying infected laying flocks that threaten public health. Accurate characterization of the genetic and phenotypic attributes that enable Salmonella Enteritidis strains to invade reproductive organs in systemically infected hens and contaminate the contents of developing eggs is essential for establishing epidemiological relationships between isolates, identifying the principal environmental reservoirs of the pathogen, and developing effective disease-mitigation strategies for egg-producing flocks.

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