AVIAN DISEASES 51:40–44, 2007

# Colonization of Specific Regions of the Reproductive Tract and Deposition at Different Locations Inside Eggs Laid by Hens Infected with *Salmonella* Enteritidis or *Salmonella* Heidelberg

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Received 8 June 2006; Accepted 14 September 2006

SUMMARY. Internal contamination of eggs by *Salmonella* Enteritidis has been a significant source of human illness for several decades and is the focus of a recently proposed U.S. Food and Drug Administration regulatory plan. *Salmonella* Heidelberg has also been identified as an egg-transmitted human pathogen. The deposition of *Salmonella* strains inside eggs is apparently a consequence of reproductive tissue colonization in infected laying hens, but the relationship between colonization of specific regions of the reproductive tract and deposition in different locations within eggs is not well documented. In the present study, groups of laying hens were experimentally infected with large oral doses of *Salmonella* Heidelberg, *Salmonella* Enteritidis phage type 13a, or *Salmonella* Enteritidis phage type 14b. For all of these isolates, the overall frequency of ovarian colonization (34.0%) was significantly higher than the frequency of recovery from either the upper (22.9%) or lower (18.1%) regions of the oviduct. No significant differences were observed between the frequencies of *Salmonella* isolation from egg yolk and albumen (4.0% and 3.3%, respectively). Some significant differences between *Salmonella* isolates were observed in the frequency of recovery from eggs, but not in the frequency or patterns of recovery from reproductive organs. Accordingly, although the ability of these *Salmonella* isolates to colonize different regions of the reproductive tract in laying hens was reflected in deposition in both yolk and albumen, there was no indication that any specific affinity of individual isolates for particular regions of this tract produced distinctive patterns of deposition in eggs.

RESUMEN. Colonización de regiones específicas del tracto reproductivo y deposición a diferentes sitios dentro de huevos puestos por gallinas infectadas con *Salmonella* Enteritidis o *Salmonella* Heidelberg

La contaminación interna de los huevos con *Salmonella* Enteritidis ha sido una fuente significativa de enfermedad en humanos por muchas décadas y es el objetivo de un plan regulatorio recientemente propuesto por la Administración de Alimentos y Drogas (FDA, por su sigla en Inglés). La *Salmonella* Heidelberg ha sido identificada como un patógeno que se transmite al ser humano por el huevo. La deposición de cepas de *Salmonella* dentro de los huevos es aparentemente consecuencia de la colonización del tejido reproductivo en gallinas ponedoras infectadas, pero la relación existente entre la colonización de regiones específicas del tracto reproductivo y la deposición en diferentes sitios dentro del huevo no está bien documentada. En el presente estudio, se infectaron experimentalmente grupos de gallinas ponedoras con grandes dosis orales de *Salmonella* Heidelberg, *Salmonella* Enteritidis fago tipo13a, o *Salmonella* Enteritidis fago tipo 14b. Para todos estos aislamientos, la frecuencia de colonización ovárica total (34.0%) fue significativamente mayor que la frecuencia de recuperación de las regiones superior (22.9%) o inferior (18.1%) del oviducto. No se observaron diferencias significativas entre las frecuencias de aislamiento de *Salmonella* de la yema o de la albúmina del huevo (4.0% y 3.3%, respectivamente). Se observaron algunas diferencias significativas entre los aislamientos de *Salmonella* en la frecuencia de recuperación a partir de los huevos pero no en la frecuencia de patrones de recuperación a partir de los órganos reproductivos. En consecuencia, aunque la capacidad de estos aislados de *Salmonella* para colonizar diferentes regiones del tracto reproductivo en gallinas ponedoras se reflejó en la deposición tanto en la yema como en la albúmina, no hubo ninguna indicación de que existiera alguna afinidad específica de estos aislados para regiones del tracto reproductor, produciendo patrones característicos de deposición en los huevos.

Key words: *Salmonella* Enteritidis, *Salmonella* Heidelberg, chickens, egg contamination, ovary, oviduct

Abbreviations: BG = brilliant green; TS = tryptone soya

Internal contamination of eggs by *Salmonella enterica* serovar Enteritidis (*Salmonella* Enteritidis) has been an internationally significant source of human illness for two decades (6,42). Although the overall prevalence of *Salmonella* Enteritidis in commercially produced table eggs in the United States has been estimated to be only 0.005% (9), one report suggested that as many as 182,060 human illnesses in 2000 alone were attributable to contaminated eggs (44). Public health agencies and the egg industry in the United States have already made substantial investments in testing and risk-reduction programs for controlling *Salmonella* Enteritidis (38), and a national regulatory plan for egg-laying flocks was proposed late in 2004 by the U.S. Food and Drug Administration (47).

The environmental persistence of salmonellae in poultry houses creates continual opportunities for laying hens to become infected by oral ingestion (7,27,36). Approximately 7% of the surveyed commercial laying flocks in the United States were found to be environmentally positive for *Salmonella* Enteritidis (10). Deposition of *Salmonella* Enteritidis inside eggs is a consequence of the colonization of reproductive tissues in systemically infected hens (8,13,41). Experimental oral inoculation of laying hens with *Salmonella* Enteritidis resulted in the invasion of a variety of internal organs, including the ovary and oviduct (12,13), and the production of contaminated eggs for a few weeks after infection (11,17). However, even when very large oral doses of *Salmonella* Enteritidis are administered, the reported incidence of resulting egg contamination is usually relatively low and involves small numbers of bacterial cells (15,17,30,33). *Salmonella* Heidelberg has also been recently identified as an egg-transmitted pathogen (4,26). For many years, *Salmonella* Heidelberg has often been among the most common serotypes found in North American laying flocks and their

housing environment (36,43). In experimental infection studies, oral inoculation of laying hens with *Salmonella* Heidelberg has caused reproductive organ colonization (13,45) and internal contamination of eggs (13,14).

Prompt refrigeration to limit *Salmonella* growth inside eggs before consumption has become a cornerstone of efforts to reduce the risks of disease transmission to humans (26,29). Because the rate of bacterial multiplication in yolk is far greater than in albumen (3,18,32), the initial site of deposition within eggs plays a critical role in determining whether small numbers of *Salmonella* contaminants have an opportunity to multiply to more dangerous levels before refrigeration restricts further growth. Infected hens have been reported to deposit *Salmonella* Enteritidis in either the yolk or albumen (or both) of developing eggs (2,11,17,30). However, the relationship between the colonization of specific regions of the reproductive tract and *Salmonella* contamination of different locations within eggs is not well understood or documented. The objective of the present study was to determine how the patterns of reproductive tissue colonization affected the sites of deposition in eggs following experimental oral infection of laying hens with isolates of *Salmonella* Enteritidis and *Salmonella* Heidelberg.

## MATERIALS AND METHODS

**Experimental infection of laying hens.** In each of three trials, 72 laying hens were obtained from the 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 (28, 38, and 47 wk old at the beginning of the first, second, and third trials, respectively) were distributed evenly among three separately housed groups in a disease-containment facility. Each bird was kept in an individual laying cage and provided with water and pelleted feed *ad libitum.*

The three groups of chickens in each trial were inoculated with different *Salmonella* isolates. One group of hens in each trial received a phage type 13a *Salmonella* Enteritidis isolate (provided by Dr. C. Benson, University of Pennsylvania, Kennett Square, PA), a second group received a phage type 14b *Salmonella* Enteritidis isolate (provided by Dr. M. Opitz, University of Maine, Orono, ME), and the third group was inoculated with an *Salmonella* Heidelberg isolate (provided by Dr. B. Swaminathan, Centers for Disease Control, Atlanta, GA). Each hen was given a 1-ml oral dose containing approximately $1.0 \times 10^9$ colony-forming units of the appropriate *Salmonella* isolate, prepared by incubation in tryptone soya (TS) broth (Oxoid Limited, Basingstoke, Hampshire, England) for 24 hr at 37 C.

**Fecal samples.** Immediately before inoculation and at 1 wk postinoculation, sterile cotton swabs were used to collect samples of voided feces from polystyrene trays (food-grade but not sterile) placed under each cage. These samples were transferred to 9 ml of tetrathionate broth (Oxoid) and incubated for 24 hr at 37 C. A 10-µl portion from each broth culture was then streaked onto brilliant green (BG) agar (Becton, Dickinson, and Co., Franklin Lakes, NJ) supplemented with 0.02 mg/ml of novobiocin (Sigma Chemical Co., St. Louis, MO) and incubated for 24 hr at 37 C. The identity of presumptive colonies of *Salmonella* Enteritidis or *Salmonella* Heidelberg was confirmed biochemically and serologically (48).

**Reproductive organ samples.** At 7 days and 17 days post-inoculation in each trial, eight hens were randomly selected from each treatment group and humanely euthanatized to allow removal of internal tissues for bacteriologic culture. Portions (approximately 5–10 g) of the ovary, upper oviduct (centered on the infundibulum–magnum junction), and lower oviduct (centered on the isthmus–uterus junction) from each hen were aseptically removed, transferred to 30 ml of tetrathionate broth, and mixed by stomaching for 30 sec. Each broth culture was incubated for 40 hr at 37 C, and a 10-µl aliquot was then streaked onto BG agar plus novobiocin. After incubation of these plates for 24 hr at 37 C, typical *Salmonella* Enteritidis or *Salmonella* Heidelberg colonies were subjected to biochemical and serologic confirmation.

**Egg content samples.** All eggs laid on the day before inoculation and between the eighth and 17th days postinoculation were cultured to detect internal contamination with *Salmonella*. Eggshell surfaces were disinfected by dipping for 5 sec in 70% ethanol, and the shells were then 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 then individually transferred to 30 ml of TS broth supplemented with 35 mg/l ferrous sulfate (Sigma), mixed by vigorous shaking for 15 sec, and incubated for 24 hr at 37 C. A 1-ml portion of each incubated TS broth culture was transferred to 9 ml of Rappaport–Vassiliadis broth (Oxoid) and incubated for 24 hr at 37 C. A 10-µl aliquot from each of these broth cultures was then streaked onto BG agar and incubated for 24 hr at 37 C. The identity of typical colonies of *Salmonella* Enteritidis or *Salmonella* Heidelberg was confirmed biochemically and serologically.

**Statistical analysis.** For each trial, significant differences ($P < 0.05$) among treatment groups, sampling days, egg fractions, or reproductive tissues in the mean frequency of recovery of *Salmonella* isolates were determined by Fisher's exact test. Data were analyzed with Instat biostatistics software (GraphPad Software, San Diego, CA).

## RESULTS

None of the preinoculation fecal or egg samples was positive for *Salmonella*. Oral inoculation consistently led to the establishment of intestinal colonization by the three *Salmonella* isolates because 96.7% of all fecal samples collected and cultured at 7 d postinoculation (and at least 91.7% in each infected treatment group) were positive for *Salmonella* Enteritidis or *Salmonella* Heidelberg.

All three *Salmonella* isolates were also recovered from each of the sampled reproductive tissue sites (ovary, upper oviduct, and lower oviduct) in every trial (Table 1). There were no significant differences ($P > 0.30$) among the three isolates in the frequencies at which they were found in any single reproductive tissue at 7 days postinoculation, 17 days postinoculation, or for both sampling days together. The frequency of *Salmonella* recovery (for all isolates and trials combined) declined significantly ($P < 0.001$) between 7 and 17 days postinoculation in samples from each reproductive tissue site. The overall *Salmonella* recovery frequencies (for all days and trials combined) ranged from 29.2% (SE14b) to 39.6% (SH) for ovary samples, from 20.8% (SE14b) to 25.0% (SE13a) for upper oviduct samples, and from 16.7% (SE13a) to 18.8% (SE14b and SH) for lower oviduct samples. The frequency of *Salmonella* recovery (for all isolates, sampling days, and trials combined) from ovary samples (34.0%) was significantly ($P < 0.05$) greater than from either upper (22.9%) or lower (18.1%) oviduct samples.

All three *Salmonella* isolates were recovered from both yolk and albumen of eggs laid by infected hens in each trial (Table 2). The frequency of *Salmonella* isolation from egg yolk (for all trials combined) ranged from 1.5% (SH) to 6.9% (SE13a), and the frequency of *Salmonella* isolation from egg albumen ranged from 1.9% (SH) to 5.2% (SE13a). There were no significant differences ($P > 0.50$) between the frequencies of yolk and albumen recovery for individual *Salmonella* isolates (or for all isolates combined). The frequencies of recovery of SE13a (for all trials combined) from both egg yolk and albumen were significantly ($P < 0.05$) higher than the corresponding values for SH. Likewise, the overall frequency of isolation of SE14b from yolk samples was also significantly ($P < 0.05$) greater than for SH.

Table 1. Recovery of *Salmonella* Enteritidis and *Salmonella* Heidelberg isolates from reproductive tissues of experimentally infected laying hens.[A]

| *Salmonella*[B] isolates | *Salmonella*-positive/total[C] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 7 days postinoculation | | | 17 days postinoculation | | | All samples | | |
| | Ovary | Oviduct (U) | Oviduct (L) | Ovary | Oviduct (U) | Oviduct (L) | Ovary | Oviduct (U) | Oviduct (L) |
| Trial 1 | | | | | | | | | |
| SE13a | 5/8 | 4/8 | 2/8 | 1/8 | 0/8 | 0/8 | 6/16 | 4/16 | 2/16 |
| SE14b | 3/8 | 3/8 | 2/8 | 1/8 | 0/8 | 0/8 | 4/16 | 3/16 | 2/16 |
| SH | 5/8 | 1/8 | 2/8 | 1/8 | 1/8 | 0/8 | 6/16 | 2/16 | 2/16 |
| Trial 2 | | | | | | | | | |
| SE13a | 5/8 | 4/8 | 2/8 | 1/8 | 0/8 | 0/8 | 6/16 | 4/16 | 2/16 |
| SE14b | 3/8 | 3/8 | 2/8 | 1/8 | 0/8 | 0/8 | 4/16 | 3/16 | 2/16 |
| SH | 5/8 | 1/8 | 2/8 | 1/8 | 1/8 | 0/8 | 6/16 | 2/16 | 2/16 |
| Trial 3 | | | | | | | | | |
| SE13a | 4/8 | 3/8 | 2/8 | 0/8 | 1/8 | 2/8 | 4/16 | 4/16 | 4/16 |
| SE14b | 5/8 | 3/8 | 4/8 | 1/8 | 1/8 | 1/8 | 6/16 | 4/16 | 5/16 |
| SH | 5/8 | 5/8 | 3/8 | 2/8 | 2/8 | 2/8 | 7/16 | 7/16 | 5/16 |
| All trials | | | | | | | | | |
| SE13a | 14/24 | 11/24 | 6/24 | 2/24 | 1/24 | 2/24 | 16/48 | 12/48 | 8/48 |
| SE14b | 11/24 | 9/24 | 8/24 | 3/24 | 1/24 | 1/24 | 14/48 | 10/48 | 9/48 |
| SH | 15/24 | 7/24 | 7/24 | 4/24 | 4/24 | 2/24 | 19/48 | 11/48 | 9/48 |
| All isolates | 40/72 a | 27/72 b | 21/72 b | 9/72 c | 6/72 c | 5/72 c | 49/144 a | 33/144 b | 26/144 b |

[A]U = upper oviduct, centered on the infundibulum–magnum junction; L = lower oviduct, centered on isthmus–uterus junction.
[B]After oral inoculation of hens with approximately $10^9$ colony-forming units of *Salmonella* Enteritidis (SE) phage type 13a, *Salmonella* Enteritidis phage type14b, or *Salmonella* Heidelberg (SH).
[C]Values in the bottom row (with identical denominators) that share no common lowercase letter are significantly ($P < 0.05$) different. No other significant differences were observed among tissues or among isolates.

## DISCUSSION

A variety of environmental reservoirs of *Salmonella* in poultry houses serve as sources for the transmission of infection to laying hens throughout the productive life of each flock (7,27). Following oral ingestion from environmental sources, *Salmonella* attachment to and invasion through mucosal epithelial cells of the gastrointestinal tract are often followed by systemic dissemination to diverse internal organs (including the ovary and oviduct). A temporary decrease in T-cell responsiveness to *Salmonella* at the onset of egg laying may promote spread to the reproductive tract in commercial hens (49). Invasion of the ovary (the site of yolk maturation and release) or the oviduct (the site of albumen secretion around the descending yolk) allows bacterial deposition inside developing eggs (34,46). However, high frequencies of colonization of reproductive tissues do not inevitably lead to similarly high frequencies of egg contamination (1,37). Distinct *Salmonella* subpopulations, expressing the specific attributes required for intestinal colonization or for invasion of reproductive organs, have been shown to complement each other and produce enhanced egg contamination by experimentally inoculated hens (19,28). Naturally occurring infections in commercial laying flocks may involve exposure to relatively low doses of *Salmonella* and are usually associated with lower frequencies of egg contamination than are typically reported in experimental-infection studies (9,28,31,33).

In the present study, the frequency of ovarian colonization was significantly higher than the frequency of recovery from either the upper or lower portions of the oviduct for all three *Salmonella* isolates. However, no corresponding difference was observed (for any of the three isolates) between the incidence of deposition in yolk or albumen. Accordingly, although the ability of these *Salmonella* isolates to colonize different regions of the reproductive tract in laying hens was reflected in deposition in both yolk and albumen, there was no indication that any specific affinity of individual isolates for particular regions of this tract produced distinctive patterns of deposition in eggs. The initial location of *Salmonella* deposition in

Table 2. Recovery of *Salmonella* Enteritidis and *Salmonella* Heidelberg isolates from the contents of eggs laid by experimentally infected hens.

| *Salmonella* isolates[A] | *Salmonella*-positive/total[B] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Trial 1 | | Trial 2 | | Trial 3 | | All trials | |
| | Yolk | Albumen | Yolk | Albumen | Yolk | Albumen | Yolk | Albumen |
| SE13a | 8/97 a | 5/97 a | 7/77 a | 5/77 a | 1/58 a | 2/58 a | 16/232 a | 12/232 a |
| SE14b | 6/134 a b | 6/134 a | 4/79 a b | 2/79 a | 2/62 a | 1/62 a | 12/275 a | 9/275 a b |
| SH | 2/121 b | 2/121 a | 2/118 b | 3/118 | 1/84 a | 1/84 a | 5/323 b | 6/323 b |
| All isolates | 16/352 | 13/352 | 13/274 | 10/274 | 4/204 | 4/204 | 33/830 | 27/830 |

[A]After oral inoculation of hens with approximately $10^9$ colony-forming units of *Salmonella* Enteritidis (SE) phage type 13a, *Salmonella* Enteritidis phage type14b, or *Salmonella* Heidelberg (SH). Eggs were collected for sampling between days 8 and17 postinoculation.
[B]Values within columns that share no common lowercase letters are significantly ($P < 0.05$) different. No significant differences were observed between yolk and albumen values.

eggs can have a very powerful influence on whether the refrigeration provisions of proposed risk-reduction programs (47) will be effective in preventing extensive bacterial multiplication from occurring in contaminated eggs after they are laid. Bacterial deposition in nutrient-rich egg yolk could lead to rapid growth to dangerously high levels before inhibitory internal temperatures are attained by refrigeration, whereas growth in albumen occurs far more slowly (3,18,32). Penetration of *Salmonella* across the vitelline membrane to reach the yolk contents is also possible at warm temperatures (23,39,40), but is apparently prevented by refrigeration (22). The results of the present study suggest that this potentially heightened risk to consumers posed by egg contamination inside or on the surface of yolks cannot be definitively attributed to any characteristic patterns of colonization in particular regions of the reproductive tract by individual *Salmonella* isolates or serotypes.

Nevertheless, this experiment did document some significant differences between *Salmonella* isolates in the frequencies at which they were found inside eggs. In general, the incidence of egg contamination associated with *Salmonella* Enteritidis infection was greater than that of *Salmonella* Heidelberg. Previous experimental infection studies have also reported higher levels of egg contamination for strains of *Salmonella* Enteritidis than for strains of *Salmonella* Heidelberg (13,14) or *Salmonella* Typhimurium (35), despite the absence of any corresponding differences in the frequencies of isolation from reproductive tissues. Individual strains of *Salmonella* (within and across serotype boundaries) have often been shown to differ very significantly from each other in their propensity for deposition inside eggs (17,21) and their growth properties in yolk or albumen (5,20,23). Bacterial attributes, such as the ability to grow to high cell density and the production of high-molecular-mass lipopolysaccharide, have been linked to a higher incidence of egg contamination (24,25). Selective pressures in the tissues of infected hens can apparently promote expression of these types of properties, as illustrated by elevated egg contamination frequencies associated with *Salmonella* Enteritidis and *Salmonella* Heidelberg strains obtained by reisolation from eggs or tissues after passage through hens in earlier experiments (14,16). Further elucidation of the precise characteristics of some *Salmonella* serotypes and strains that enable them to invade reproductive organs of laying hens and contaminate developing eggs is essential for developing epidemiologically relevant criteria for identifying, differentiating, and establishing relationships between individual isolates.

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## ACKNOWLEDGMENT

We gratefully express our appreciation for excellent technical assistance from Otis Freeman.