Frequency and Magnitude of Internal Organ Colonization Following Exposure of Laying Hens to Different Oral Doses of Salmonella enteritidis

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Abstract: Contaminated eggs produced by infected laying hens continue to pose a significant public health concern as a leading source of transmission of Salmonella enteritidis infections to humans. A recently implemented national regulatory program for egg-producing poultry in the United States seeks to control eggborne transmission of illness to consumers via a diverse program of mandatory risk reduction practices plus testing to detect infected flocks. However, many aspects of S. enteritidis infections in laying hens, including the precise relationship between the magnitude of oral exposure and infection parameters such as the numbers of bacteria that reach internal tissues, remain unresolved. In the present study, groups of laying hens were experimentally infected with oral doses of $10^4$, $10^5$, or $10^6$ CFU of a phage type 13a strain of S. enteritidis and the number of S. enteritidis cells in the livers of infected hens was determined at 5 d and 20 d post-inoculation. The frequency of S. enteritidis recovery from livers ranged from 30% (10^6 CFU dose) to 90% (10^5 CFU dose) at 5d post-inoculation and from 0% (10^4 CFU dose) to 40% (10^5 CFU dose) at 20 d post-inoculation. Significantly (p<0.05) greater numbers of S. enteritidis were isolated from livers at both 5 d and 20 d post-inoculation following inoculation with 10^6 CFU than after administration of either of the two lower doses. These results demonstrate that the oral exposure dose significantly affects important parameters of S. enteritidis infection in laying hens and could thereby influence the outcome of testing efforts. Interpreting the potential implications of testing results and improving the effectiveness of testing protocols are both contingent on understanding how different levels of exposure are likely to be detected by particular sampling methods.

Key words: Salmonella enteritidis, chickens, exposure dose, liver colonization

INTRODUCTION
Public health agencies throughout the world have focused their attention for more than two decades on the transmission of Salmonella enterica serovar Enteritidis (S. enteritidis) to consumers of contaminated eggs laid by infected hens (Braden, 2006; Greig and Ravel, 2009). Epidemiological calculations have attributed more than 100,000 annual illnesses in the United States to contaminated eggs (Schroeder et al., 2005) despite an estimated S. enteritidis prevalence in commercially produced table eggs of only 0.005% (Ebel and Schlosser, 2000). Accordingly, a national regulatory plan for S. enteritidis in egg-laying flocks was recently implemented in the United States (United States Food and Drug Administration, 2009). Intensive commitments of resources to testing and risk reduction programs by both governments and egg producers have led to reported reductions in the frequency of illness due to S. enteritidis in several instances (Mumma et al., 2004; Gast, 2008; Poirier et al., 2008). Continuing opportunities for laying hens to become infected with Salmonella are created by the environmental persistence of this pathogen in poultry houses. Sometimes able to survive cleaning and disinfection regimens, environmental contamination with S. enteritidis can be intensified by severe rodent or insect infestations (Carrique-Mas et al., 2009; Snow et al., 2010). Once introduced into poultry houses, Salmonella infection can rapidly spread horizontally throughout flocks (Gast and Holt, 1999; Thomas et al., 2009).

The deposition of S. enteritidis in the contents of developing eggs results from colonization of reproductive tissues (especially the ovary and upper oviduct) in systemically infected hens (De Buck et al., 2004; Gantois et al., 2009). However, high frequencies of colonization of reproductive tissue colonization have not always been associated with correspondingly high frequencies of egg contamination (Barrow and Lovell, 1991; Methner et al., 1995; Gast et al., 2007). Either the yolk or albumen (or both) of developing eggs produced by infected laying hens can be contaminated by S. enteritidis (Humphrey et al., 1991; Gast and Holt, 2000; De Buck et al., 2004), with the initial location of deposition determined by which regions of the laying hen's reproductive tract are colonized (Humphrey et al.,
MATERIALS AND METHODS
Experimental infection of laying hens: In each of two 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, USA. These hens (38 and 44 wk old at the beginning of the first and second trials, respectively) were distributed into four separately housed groups in a disease-containment facility, with 23 hens in each of three experimental groups and a fourth group of 6 hens held as uninoculated 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 *S. enteritidis*. For each trial, a lyophilized stock culture of phage type 13a *S. enteritidis* (originally isolated from a contaminated egg yolk by Dr. C. Benson at the University of Pennsylvania, Kennett Square, PA, USA) was resuscitated by incubation for 24 h at 37°C in tryptone soya broth (Oxoid Limited, Basingstoke, Hampshire, UK). After serial ten-fold dilution of this incubated broth culture in 0.85% saline, the hens in one experimental group were each inoculated with 1-ml doses of diluted culture containing $1.7 \times 10^8$ CFU of *S. enteritidis*, the hens in a second group received doses of $1.7 \times 10^6$ CFU and the third group of hens were each given $1.7 \times 10^4$ CFU.

Fecal samples: Immediately before inoculation, 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 h 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, USA) supplemented with 0.02 mg/ml of novobiocin (Sigma Chemical Co., St. Louis, MO, USA) and incubated for 24 h at 37°C. The identity of presumptive colonies of *S. enteritidis* was confirmed biochemically and serologically (Waltman and Gast, 2008).

Liver samples: At 5 d and 20 d post-inoculation in each trial, 10 randomly selected hens from each inoculated group and 3 negative control hens were humanely euthanized to allow the removal of internal tissues for bacteriologic culture. Portions (approximately 1-5 g) of the liver from each hen were aseptically removed, weighed and diluted 1:5 in cold tetrathionate broth. Each tissue sample was homogenized by stomaching for 2 min. The concentration of *S. enteritidis* in each liver sample was determined by making a series of ten-fold dilutions in 0.85% saline and spreading aliquots of each dilution (including a total of 1.0 ml of the original 1:5 sample dilution) onto plates of BG agar plus novobiocin. The agar plates were incubated for 24 h at 37°C and typical *S. enteritidis* colonies were counted. Biochemical and serological confirmation (Waltman and Gast, 2008) that at least 3 randomly selected colonies from each positive sample were always *S. enteritidis* validated the accuracy of the visual counts. The detection threshold of this procedure was 5 CFU/g. After completion of the dilution series for *S. enteritidis* enumeration, the remaining liver samples in tetrathionate broth were incubated for 40 h at 37°C and 10-µl aliquots were streaked onto BG agar plus novobiocin. After incubation of these plates for 24 h at 37°C, typical colonies of *S. enteritidis* were subjected to biochemical and serological confirmation (Waltman and Gast, 2008). When this enrichment procedure yielded positive results for *S. enteritidis* recovery in conjunction with negative enumeration results, the sample was arbitrarily assigned an *S. enteritidis* concentration value of 2 CFU/g. All samples (positive and negative) were included in the calculation of mean *S. enteritidis* concentrations.
RESULTS

None of the fecal or liver samples collected before inoculation or from uninoculated negative control hens were positive for Salmonella. The frequency of recovery of S. enteritidis from liver samples ranged from 30% (10^4 CFU oral inoculation dose) to 90% (10^6 CFU dose) at 5 d post-inoculation and from 0% (10^3 dose) to 40% (10^6 dose) at 20 d post-inoculation (Table 1). At both sampling dates, the frequency of S. enteritidis isolation from livers was significantly (p<0.05) higher following the administration of 10^6 CFU than 10^4 CFU. For all three inoculum doses, the frequency of S. enteritidis recovery from liver samples declined significantly (p<0.020) between 5 d and 20 d post-inoculation. The concentration of S. enteritidis in liver samples ranged from 0.307 log CFU (10^3 dose) to 3.109 log CFU (10^6 dose) at 5 d post-inoculation and from 0 (10^3 dose) to 0.473 (10^6 dose) at 20 d post-inoculation (Table 1). At both sampling dates, the number of S. enteritidis cells in livers was significantly (p<0.05) higher for the 10^6 CFU dose than for either the 10^3 or 10^4 CFU doses. For all three inoculum doses, the S. enteritidis concentration in liver samples declined significantly (p<0.015) between 5 d and 20 d post-inoculation.

DISCUSSION

The deposition of S. enteritidis in eggs is both a direct cause of food-borne human illness and the principal confirmatory diagnostic criterion for identifying infected commercial laying flocks. Efforts to control S. enteritidis in egg-laying chickens would benefit from refinements in the characterization of bacterial attributes (both genetic and phenotypic) that lead to systemic infection and egg contamination (Gast, 2008). The deposition of Salmonella inside developing eggs results from invasion of reproductive tissues (either ovaries or oviducts) in systemically infected hens (Thiagarajan et al., 1994; Keller et al., 1995), although high frequencies of S. enteritidis isolation from reproductive organs do not necessarily ensure correspondingly high frequencies of egg contamination (Barrow and Lovell, 1991; Metherrer et al., 1995). Invasion beyond the intestinal tract to internal organs such as the liver and spleen occurs within hours after initial oral exposure (He et al., 2010) and serves as the link to subsequent reproductive tissue involvement and egg contamination (Gantois et al., 2009). Persistent intestinal colonization does not consistently predict either systemic infection or egg contamination by S. enteritidis (Humphrey et al., 1991; Gast and Holt, 2000). The present study determined that both the frequency of S. enteritidis invasion to the livers of infected hens and the numbers of S. enteritidis cells recovered from these livers can vary significantly with different bacterial exposure doses at both 5 d and 20 d after oral inoculation. The frequency of internal organ colonization by Salmonella usually declines sharply during the first several weeks following oral inoculation, so testing at longer post-inoculation intervals is generally far less informative (Gast et al., 2007). However, pathogen persistence in the tissues of even a small fraction of infected birds could yield an opportunity for egg contamination (Gast et al., 2009). Environmental stressors such as feed or water deprivation may significantly influence the likelihood that egg contamination will occur in infected flocks (Okamura et al., 2010).

Differences in the ability to invade internal organs and contaminate eggs have been previously observed between Salmonella serotypes and even between strains of the same serotype (Gast and Holt, 2000; 2001a; Gast et al., 2007). However, some individual strains that were found to invade internal organs at high frequencies were associated with little or no deposition inside eggs (Gast et al., 2004; 2007). Higher egg contamination frequencies have often followed experimental infection with S. enteritidis strains in comparison to infection with strains of S. heidelberg or S. typhimurium, despite similar frequencies of isolation of all strains from reproductive tissues (Keller et al., 1997; Gast et al., 2004; 2005; 2007). The genetic differentiation of egg-associated and non-egg-
associated *Salmonella enteritidis* strains has often proven to be difficult and complex (Botteldoorn *et al*., 2010). The inherent capability of some *Salmonella enteritidis* strains to invade internal organs and contaminate eggs has been attributed to phenotypic properties including the production of high-molecular-mass lipopolysaccharide and growth to high cell density (Guard-Petter, 1998; Parker *et al*., 2001). Single-nucleotide genomic changes generated a biofilm-negative *Salmonella enteritidis* phenotype which had an increased propensity to contaminate the contents of eggs laid by experimentally infected hens (Guard-Bouldin *et al*., 2004; Morales *et al*., 2007). Another study identified specific genes that were highly expressed by *Salmonella enteritidis* isolates from both infected hens’ oviducts and from eggs (Gantois *et al*., 2008). Selective pressures exerted in the tissues of infected hens may affect the expression of critical bacterial virulence attributes, as demonstrated by an increased ability of *Salmonella enteritidis* strains to cause egg contamination after repeated passage through and re-isolation from groups of infected hens (Gast *et al*., 2003; 2005). Moreover, environmental conditions such as pH and temperature can influence the expression of potential *Salmonella enteritidis* virulence factors such as flagella, fimbria, outer membrane proteins and iron uptake systems (McDermid *et al*., 1996; Walker *et al*., 1999). Stress-induced factors have been hypothesized to support bacterial colonization of chicken oviducts and survival in egg albumen (Van Immerseel, 2010). Complementation by distinct bacterial subpopulations expressing phenotypic properties relevant to different environmental contexts in the infected avian host may help connect together the complicated series of events that occur between initial intestinal colonization and eventual deposition inside eggs (Guard-Petter, 2001; Gast *et al*., 2002a; Guard *et al*., 2010). Perhaps because of the intricacy of these interconnected events during the course of infection, efforts to select genetically distinct lines of chickens with globally heightened resistance to *Salmonella* have been only partially successful (Berchieri *et al*., 2001; Beaumont *et al*., 2009).

Prior studies have provided diverse perspectives about the significance of the initial oral exposure dose to the progress and outcomes of *Salmonella* infections in egg-laying chickens. Both organ invasion and egg contamination have been reported as significantly reduced at lower infection doses (Gast and Benson, 1996; Gantois *et al*., 2009). In one investigation, both the duration of *Salmonella enteritidis* shedding in feces and the serum antibody response were dose-related, but the frequency of egg contamination was not (Humphrey *et al*., 1991). Most experimental infection studies with *Salmonella enteritidis* have reported relatively modest incidences of egg contamination, even for very large oral doses (Humphrey *et al*., 1991; Gast and Holt, 2000; Gast *et al*., 2002a). Extremely low frequencies of egg contamination are typically associated with naturally infected commercial poultry, likely due to both a low prevalence of *Salmonella enteritidis* infection within flocks and the exposure of individual hens to relatively small bacterial doses (Humphrey *et al*., 1989; Ebel and Schlosser, 2000). Experimental horizontal contact transmission of *Salmonella enteritidis*, which presumably simulates naturally occurring infections, has led to intestinal colonization, organ invasion and egg contamination at lower incidences than are associated with large oral doses (Gast and Beard, 1990a,c; Nakamura *et al*., 1994; Gast and Holt, 1999). The overall course of *Salmonella enteritidis* infections observed over time (in terms of invasion and persistence in tissues) may be determined by an interplay between two opposing consequences of the initial exposure dose. Higher bacterial doses increase the severity of pathological effects, but also elicit a stronger immune response which promotes the clearance of infection (Gast and Beard, 1990a; Gast and Holt, 2001b). In the present study, the frequency of *Salmonella enteritidis* in liver samples decreased between 5 d and 20 days post-inoculation, even following inoculation with a very large (10^8 CFU) oral dose and the numbers of bacterial cells recovered from these samples declined even more steeply between the two sampling dates. One of the most prominent practical dimensions of different bacterial exposure levels is the demonstrated direct relationship between experimental oral inoculation doses and the sensitivity of both serologic and bacteriologic detection of infection (Gast, 1993; Gast *et al*., 2002b).

The results of the present experiment demonstrate that the oral exposure dose has significant effects on important parameters of *Salmonella enteritidis* infection in laying hens that could potentially influence the outcome of flock testing efforts. Understanding testing results and refining testing protocols requires an understanding of how different levels of exposure are likely to be detected by particular sampling methods. Further characterization of the genetic and phenotypic attributes of *Salmonella enteritidis* serotypes and strains which enable them to invade internal organs of laying hens, colonize reproductive tissues and contaminate developing eggs is vital for identifying and differentiating individual isolates, defining epidemiological relationships between isolates and developing effective testing strategies for detecting infected individuals and flocks.

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