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Mob: +92 300 3008585, Fax: +92 41 8815544
E-mail: editorijps@gmail.com

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

Richard K. Gast, Rupa Guraya, Jean Guard and Peter S. Holt
United States Department of Agriculture, Agricultural Research Service,
Egg Safety and Quality Research Unit, Russell Research Center,
950 College Station Road, Athens, Georgia 30605, USA

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 egg-borne 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, 10^6, or 10^8 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% (10^4 CFU dose) to 90% (10^8 CFU dose) at 5d post-inoculation and from 0% (10^4 CFU dose) to 40% (10^8 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 10^8 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*.,

Corresponding Author: Richard K. Gast, United States Department of Agriculture, Agricultural Research Service, Egg Safety and Quality Research Unit, Russell Research Center, 950 College Station Road, Athens, Georgia 30605, USA

1991; Bichler *et al*., 1996; Gast and Holt, 2000). The typical incidence of internal egg contamination observed after oral inoculation of hens with *S. enteritidis* is relatively low and involves small initial numbers of bacterial cells, even following the administration of very large infecting doses (Humphrey *et al*., 1991; Gast and Holt, 2000).

Significant differences between *S. enteritidis* strains have been reported in their characteristic frequencies of both reproductive organ invasion and egg contamination (Gast and Holt, 2000; 2001a; Gast *et al*., 2007), but individual strains do not generally demonstrate specific affinities for defined regions of the reproductive tract that generate distinctive patterns of deposition inside eggs (Gast *et al*., 2007). The initial bacterial exposure dose has high potential significance for the progression and outcome of systemic *S. enteritidis* infections, as demonstrated by a strong association with the magnitude of both serum and egg yolk antibody responses (Gast and Beard, 1990a; Gast *et al*., 1997). However, prior research has not clearly documented the influences of bacterial dose levels on many important parameters of *S. enteritidis* infections in laying hens, including the colonization of internal tissues. The objective of the present study was to determine if (and how) experimental oral infection of groups of laying hens with three different doses of a phage type 13a *S. enteritidis* strain affected the bacterial cell numbers recovered from the livers of these birds at two different post-inoculation intervals.

MATERIALS AND METHODS

Experimental infection of laying hens: In each of two trials, 75 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, 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 1.7×10^8 CFU of *S. enteritidis*, the hens in a second group received doses of 1.7×10^6 CFU and the third group of hens were each given 1.7×10^4 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.

Table 1: Isolation and enumeration of *Salmonella enteritidis* in liver samples from experimentally infected laying hens[1]

S. enteritidis dose (CFU)	*Salmonella*-positive liver samples/total		*Salmonella* concentration in liver samples (log_{10} CFU/g)	
	5 d	20 d	5 d	20 d
10^4	6/20[a]	0/20[a]	0.307[a]	0[a]
10^6	12/20[ab]	2/20[ab]	1.017[a]	0.030[a]
10^8	18/20[b]	8/20[b]	3.109[b]	0.473[b]

[1]At 5 d and 20 d after oral inoculation with three different doses of a phage type 13a strain of *S. enteritidis*.
[a,b]Values within columns that share no common superscripts are significantly ($p<0.05$) different

Statistical analysis: For each trial (and for both trials combined), significant differences ($p<0.05$) between *S. enteritidis* inoculum doses or sampling dates in the mean frequencies of isolation from liver samples were determined by Fisher's exact test. Significant differences ($p<0.05$) between inoculum doses or sampling dates in the mean concentrations of *S. enteritidis* cells in liver samples were determined by Kruskal-Wallis analysis of variance followed by the Dunn multiple comparison test. Because the two replicate trials did not differ significantly, their results were combined for analysis and presentation. Data were analyzed with Instat biostatistics software (GraphPad Software, San Diego, CA, USA).

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% (10^8 CFU dose) at 5 d post-inoculation and from 0% (10^4 dose) to 40% (10^8 dose) at 20 d post-inoculation (Table 1). At both sampling dates, the frequency of *S. enteritidis* isolation from livers was significantly ($p\leq0.003$) higher following the administration of 10^8 CFU than 10^4 CFU. For all three inoculum doses, the frequency of *S. enteritidis* recovery from liver samples declined significantly ($p\leq0.020$) between 5d and 20 d post-inoculation. The concentration of *S. enteritidis* in liver samples ranged from 0.307 log CFU (10^4 dose) to 3.109 log CFU (10^8 dose) at 5 d post-inoculation and from 0 (10^4 dose) to 0.473 log CFU (10^8 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 10^8 CFU dose than for either the 10^6 or 10^4 CFU doses. For all three inoculum doses, the *S. enteritidis* concentration in liver samples declined significantly ($p\leq0.015$) 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 *Salmonella* isolation from reproductive organs do not necessarily ensure correspondingly high frequencies of egg contamination (Barrow and Lovell, 1991; Methner *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 *S. enteritidis* strains has often proven to be difficult and complex (Botteldoorn *et al*., 2010). The inherent capability of some *S. 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 *S. 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 *S. 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 *S. 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 *S. 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 *S. 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 *S. 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 *S. 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 *S. 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, 1990b,c; Nakamura *et al*., 1994; Gast and Holt, 1999). The overall course of *Salmonella* 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 *S. 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 *S. 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* 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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