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Frequency and Persistence of Fecal Shedding Following Exposure of Laying Hens to Different Oral Doses of *Salmonella enteritidis*

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Abstract: Infection of egg-laying poultry with *Salmonella enteritidis* and the associated transmission of illness to consumers of contaminated eggs has been a prominent international public health concern for many years. Testing and risk reduction programs for laying flocks have been implemented in many nations with some success. However, several critical parameters of *S. enteritidis* infections in chickens, including the relationship between the magnitude of oral exposure and the frequency and duration of bacterial shedding in voided feces, remain incompletely defined or explained. 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 frequency at which the pathogen was shed in voided feces was determined at 8 weekly post-inoculation intervals. At 1 wk post-inoculation, the frequency of fecal shedding of *S. enteritidis* ranged from 23.8% for the 10^4 CFU dose to 87.5% for the 10^8 CFU dose. No fecal shedding was detected after 3 wk post-inoculation from hens inoculated with 10^4 CFU, but a small proportion (2.5% to 5.0%) of hens that received doses of 10^6 or more CFU of *S. enteritidis* were still shedding at 8 wk post-inoculation. The results of this study indicate that the oral exposure dose can significantly influence the frequency and duration of *S. enteritidis* fecal shedding into the environment by infected laying hens. A more complete understanding of how different levels of exposure are detected by particular sampling methods will support the effective application and interpretation of testing protocols for controlling poultry infections and preventing transmission to humans.

Key words: *Salmonella enteritidis*, chickens, exposure dose, fecal shedding, persistence

INTRODUCTION

The attribution of human illness to eggs contaminated with *Salmonella enterica* serovar Enteritidis (*S. enteritidis*) has been reported throughout the world for many years (Braden, 2006; Greig and Ravel, 2009). Governments and egg producers have committed substantial resources to testing and risk reduction programs for *S. enteritidis* infections in egg-laying flocks (Gast, 2008; Poirier *et al.*, 2008). These efforts have been associated with diminished incidences of human illness due to *S. enteritidis* in several nations (Mumma *et al.*, 2004; Gillespie *et al*. 2005), but epidemiological calculations and active disease surveillance both suggest that *S. enteritidis* contamination of eggs continues to pose a significant threat to public health (Schroeder *et al.*, 2005; Centers for Disease Control and Prevention, 2011).

The deposition of *S. enteritidis* inside developing eggs is a direct consequence of reproductive tissue colonization in systemically infected laying hens (Gantois *et al.*, 2009; Gast *et al*., 2011a). This pathogen can persist for very prolonged intervals in poultry houses (Davies and Breslin, 2003), thereby creating extended opportunities for hens to be exposed. One study concluded that commercial laying flocks most often became infected after transfer into contaminated laying houses (Van de Giessen *et al*., 1994). Environmental surveys of laying houses have reported the isolation of *S. enteritidis* from a wide assortment of samples, including manure, dust, rodents and insects (Garber *et al*., 2003; Kinde *et al*., 2005). Severe rodent or insect infestations can magnify *S. enteritidis* contamination of the environment, which can sometimes reach levels capable of surviving standard cleaning and disinfection regimens (Carrique-Mas *et al.*, 2009; Snow *et al.*, 2010). A greater diversity of *S. enteritidis* phage types has been found in poultry houses than in contaminated eggs (Henzler *et al*., 1994), suggesting that the environment may serve as a broad reservoir from which strains able to cause systemic infection and egg contamination can occasionally emerge (Henzler *et al*., 1998). No clearly significant advantage has been convincingly demonstrated for any particular poultry housing system in regard to the persistence of *S. enteritidis* in either infected chickens or their environment (Holt *et al*., 2011; De Vylder *et al*., 2011; Van Hoorebeke *et al.*, 2011).

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Because the presence of *S. enteritidis* in laying house environments is epidemiologically relevant but occurs far more frequently than egg contamination, many protocols for identifying infected flocks test environmental samples as an initial screening step (Gast, 2008). One of the principal sources of environmental contamination with *S. enteritidis* is fecal shedding of the pathogen by infected hens (Gast and Beard, 1990a). Immature poultry are especially susceptible to the establishment of *Salmonella* colonization in the intestinal tract, which can sometimes persist for many months (Gast and Holt, 1998; Nakamura *et al*., 1993). Fecal shedding of *Salmonella* was reported to peak just before commercial flocks commenced egg laying and then decline at later sampling intervals (Li *et al*., 2007). After introduction into poultry houses, *S. enteritidis* infection can rapidly spread horizontally throughout flocks (Gast and Holt, 1999; Thomas *et al.*, 2009). Airborne circulation of dust can disseminate both environmental contamination and infection (Gast *et al*., 1998). Stresses such as feed restriction and the onset of egg laying can increase the susceptibility of hens to *S. enteritidis* colonization (Holt, 1995) and their subsequent fecal shedding of the pathogen (Skov *et al*., 2002; Nakamura *et al*., 1994).

The initial bacterial exposure dose affects the progression and outcome of many aspects of *S. enteritidis* infections, including internal organ invasion, antibody responses, intestinal colonization and fecal shedding (Gast and Beard, 1990a; Gast *et al.*, 1997; 2011b). Even after the administration of very large oral doses of *S. enteritidis* to laying hens, the observed incidence of egg contamination is typically low and involves small initial numbers of bacterial cells (Humphrey *et al.*, 1991; Gast and Holt, 2000). Both fecal shedding and antibody responses have been found to be more strongly dose-dependent than egg contamination (Humphrey *et al*., 1991). The oral dose of *S. enteritidis* administered to chicks of different ages was reported to affect the observed frequencies of fecal shedding at different post-infection intervals, but not the long-term persistence of cecal colonization (Van Immerseel *et al*., 2004). Nevertheless, prior research has not clearly documented the influences of bacterial dose levels on many important parameters of *S. enteritidis* infections in mature laying hens, including the persistence of fecal shedding. This is an issue of considerable importance for the effective application of environmentally focused detection methodologies for *S. enteritidis* in egg-laying flocks. 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 frequency and duration of bacterial shedding in voided feces over a period of 8 wk post-inoculation.

MATERIALS AND METHODS

Experimental infection of laying hens: In each of two trials, 120 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 (27 and 44 wk old at the beginning of the first and second trials, respectively) were distributed into three separately housed groups of 40 hens each 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 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 1.1×10^8 CFU of *S. enteritidis*, the hens in a second group received doses of 1.1×10^6 CFU and the third group of hens were each given 1.1×10^4 CFU.

Fecal samples: Immediately before inoculation and at eight weekly post-inoculation intervals, 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).

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 fecal samples were determined by Fisher's exact 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 samples collected before inoculation were positive for *Salmonella*. All three inoculation doses

Table 1: Recovery of *Salmonella enteritidis* from voided feces of experimentally infected laying hens[1]

S. enteritidis dose (CFU)	*Salmonella*-positive fecal samples/total							
	1 wk	2 wk	3 wk	4 wk	5 wk	6 wk	7 wk	8 wk
10^4	19/80[a]	6/80[a]	1/80[a]	0/80[a]	0/80[a]	0/80[a]	0/80[a]	0/80[a]
10^6	46/80[b]	11/80[a]	5/80[a]	4/80[a]	3/80[ab]	3/80[ab]	1/80[a]	2/80[a]
10^8	70/80[c]	29/80[b]	22/80[b]	14/80[b]	9/80[b]	8/80[b]	4/80[a]	4/80[a]

[1]At weekly intervals 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

resulted in detectable fecal shedding of *S*. *enteritidis* at 1 wk post-inoculation, at frequencies of 87.5% for the 10^8 CFU dose, 57.5% for the 10^6 CFU dose and 23.8% for the 10^4 CFU dose (Table 1). The observed frequency of fecal shedding at all three inoculation doses had declined significantly ($p\leq0.008$) by 2 wk post-inoculation. None of the fecal samples collected from hens inoculated with 10^4 CFU were positive for *S*. *enteritidis* after 3 wk post-inoculation, but a small proportion of hens inoculated with either 10^6 or 10^8 CFU (2.5% and 5.0%, respectively) were still shedding *S*. *enteritidis* at 8 wk post-inoculation. The frequency of positive fecal samples for the 10^8 CFU dose was significantly ($p\leq0.022$) greater than for either of the other two doses at every sampling interval through 4 wk post-inoculation and remained significantly ($p\leq0.007$) higher than the 10^4 CFU dose through 6 wk post-inoculation. The 10^6 CFU dose also resulted in significantly ($p<0.001$) more fecal shedding of *S*. *enteritidis* than the 10^4 CFU dose at 1 wk post-inoculation.

DISCUSSION

The initial exposure dose of *S*. *enteritidis* (or other *Salmonella* serovars) can have significant and diverse effects on the progress and outcomes of the resulting infections in poultry. The incidences of both internal organ invasion and egg contamination have previously been shown to decrease significantly at lower experimental doses (Gantois *et al*., 2009; Gast *et al*., 2011b). The very low prevalence of egg contamination reported in commercial laying flocks may reflect both the correspondingly low prevalence of *S*. *enteritidis* infections in these flocks and the relatively low bacterial doses involved in most naturally occurring infections (Humphrey *et al*., 1989; Ebel and Schlosser, 2000). Experimental exposure to *S. enteritidis* via horizontal contact, simulating naturally occurring transmission of infection, has been associated with lower incidences of intestinal colonization, organ invasion and egg contamination than are typically observed following inoculation with large oral doses (Gast and Beard, 1990b; Nakamura *et al*., 1994; Gast and Holt, 1999). In the present study, incremental decreases in the experimentally administered oral dose of *S*. *enteritidis* resulted in fecal shedding at both a significantly lower frequency and of significantly shorter duration. The observed course of *S*. *enteritidis* infection of poultry over time may reflect an interplay between contrasting consequences of the initial exposure dose. Whereas higher doses may lead to a higher frequency and persistence of intestinal and internal organ colonization, they may also elicit stronger immune responses which enhance the clearance of infection (Gast and Beard, 1990c; Gast and Holt, 2001).

Meaningful conclusions about the consequences of *Salmonella* infections in chickens are inevitably complicated by the substantial degree of variability in pathological effects which is often observed between and within serovars. Definitive genetic differentiation of egg-associated and non-egg-associated *S. enteritidis* strains has been elusive (Botteldoorn *et al*., 2010). The complex series of events occurring between initial intestinal colonization and eventual deposition inside eggs may be linked together via the sequential expression of complementary phenotypic properties, relevant at different stages of infection in the avian host, by distinct bacterial sub-populations (Guard *et al*., 2010). For example, the expression of particular flagella and fimbria was reported as essential for the invasion of *S*. *enteritidis* to internal organs, but not for intestinal colonization (Dibb-Fuller and Woodward, 2000). The intricacy of these interconnected events during the course of infection has constrained efforts to select for lines of chickens with broadly defined genetic resistance against *Salmonella* (Beaumont *et al*., 2009).

In the present study, inoculation of laying hens with oral doses of at least 10^6 CFU of *S*. *enteritidis* led to fecal shedding that continued throughout the 8 wk of the experiment in a small proportion of hens. Although persistent intestinal colonization is known to sometimes be a prominent feature of *S*. *enteritidis* infections in egg-laying chickens, especially following exposure at very young ages (Gast and Holt, 1998), its epidemiological significance is uncertain. Bacterial shedding in voided feces is a direct consequence of intestinal colonization and is often the manifestation of *S*. *enteritidis* infections in poultry which can be detected at the highest frequency (Gast and Beard, 1990a,b). However, considerable variation over time has been reported in the observed prevalence of fecal shedding within commercial flocks (Wales *et al*., 2007). Moreover, persistent intestinal colonization and fecal shedding have not been consistently reliable predictors of the likelihood of either systemic infection or egg contamination by *S. enteritidis*

(Humphrey *et al.*, 1991; Gast and Holt, 2000; Gast *et al*., 2005). Nevertheless, persistence of this pathogen in even a small proportion of the hens in a laying flock could perpetuate opportunities for transmission to other hens and the subsequent production of contaminated eggs (Gast *et al.*, 2009), especially when the susceptibility of birds to infection is increased by environmental stressors such as heat, feed restriction, or water deprivation (Okamura *et al.*, 2010).

The results of the present study demonstrate the potential significance of the initial bacterial exposure dose for determining the duration of fecal shedding in infected flocks, but many other variables influence the outcome of environmental testing programs. The shedding of large numbers of *S*. *enteritidis* into the laying house by infected hens does not necessarily lead to a high probability of detection by environment sampling (Wales *et al*., 2006). Although the causal relationships between intestinal colonization, fecal shedding and contamination of the poultry house environment with *S*. *enteritidis* are superficially straightforward, testing for these different parameters can yield widely divergent results. Cloacal swabs taken directly from hens have been reported on different occasions to support either more sensitive or less sensitive detection of *S*. *enteritidis* infection than was obtained with fecal and environmental samples (Van Hoorebeke *et al*., 2009; García *et al*., 2011). Among samples taken from the laying house environment, combinations of feces and dust have often been recommended for *S*. *enteritidis* detection (Carrique-Mas and Davies, 2008; Arnold *et al*., 2010). Several investigators have indicated a preference for dust samples as particularly efficient sources of *S*. *enteritidis* (Huneau-Salaun *et al*., 2009; Arnold *et al*., 2011). Collecting airborne dust provided highly sensitive detection of *S*. *enteritidis* infection in groups of experimentally infected hens (Gast *et al*., 2004). Fans, egg belts and nest boxes are frequently targeted as likely reservoirs of *S*. *enteritidis* in laying houses, although no single sample type has been found to ensure detection of this pathogen (Davies and Breslin, 2001). Efforts to develop improved strategies for applying and interpreting diagnostic tests could benefit very substantially from an improved understanding of how testing results are influenced by parameters (such as exposure dose levels) which affect the course and outcomes of *S*. *enteritidis* infections in egg-producing flocks. Characterization of the genetic and phenotypic attributes of *Salmonella* strains which are responsible for intestinal colonization, organ invasion and egg contamination in infected laying hens is essential for both the differentiation of isolates according to their epidemiological significance and for implementing testing strategies that provide consistent and cost-effective detection of flocks with the potential to pose public health and economic risks.

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