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

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Effect of Prior Passage Through Laying Hens on Invasion of Reproductive Organs by *Salmonella enteritidis*

Richard K. Gast, Jean Guard-Bouldin, Rupa Guraya 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: The colonization of reproductive tissues in infected laying hens is a pivotal stage in the production of contaminated eggs that can transmit *Salmonella enteritidis* infections to humans. In an earlier study, a series of passages through infected laying hens increased the frequency at which an *S. enteritidis* isolate was deposited inside eggs. The present study evaluated the effect of *in vivo* passage of an *S. enteritidis* isolate on its ability to invade to internal tissues, including three different regions of the reproductive tract. In each of three trials, a group of laying hens was infected orally with a PT13a strain of *S. enteritidis* (prepared from a separate stock culture each time). After internal organ samples were removed from this first passage group for culturing at 7 days post-inoculation, an *S. enteritidis* isolate from the upper oviduct of an extensively infected hen was used to infect another (second passage) group of hens in each trial. The overall frequency of *S. enteritidis* isolation from internal organs increased between passages in only one of the three trials and no increases were observed between passages in the frequency of *S. enteritidis* recovery from any of the three reproductive tissue sites. Therefore, passage of *S. enteritidis* through infected chickens did not always select for either higher overall invasiveness or for a higher ability to colonize reproductive organs in the present study.

Key words: *Salmonella enteritidis*, chickens, egg contamination, ovary, oviduct, *in vivo* passage

INTRODUCTION

For more than twenty years, public health authorities have been reporting that eggs contaminated by *Salmonella enterica* serovar Enteritidis (*S. enteritidis*) are a significant source of food-borne human illnesses (Braden, 2006; Patrick *et al*., 2004). The persistence of this organism in poultry house environments poses a continuing threat of infection for laying hens (Davies and Breslin, 2003; Kinde *et al*., 2004; Lapuz *et al*., 2008). In the USA, voluntary testing and risk reduction programs for controlling *S. enteritidis* in egg-laying flocks have been implemented in numerous states (Mumma *et al*., 2004) and a national regulatory plan was proposed in 2004 (U.S. Food and Drug Administration, 2004). The deposition of *S. enteritidis* in the internal contents of developing eggs results from the colonization of reproductive tissues in systemically infected laying hens (De Buck *et al*., 2004; Gast *et al*., 2004). Experimental oral infection of hens with *S. enteritidis* has led to the invasion of a variety of internal organs, including the ovary and oviduct (Gast and Beard, 1990b; Gast *et al*., 2004) and produced sporadic egg contamination for several weeks (Gast and Beard, 1990a; Gast and Holt, 2000). The location (yolk or albumen) of *S. enteritidis* deposition in a developing egg is likely a consequence of which regions of the laying hen's reproductive tract are colonized (Bichler *et al*., 1996; Gast and Holt, 2000; Humphrey *et al*., 1991). Individual strains of *S. enteritidis* differ significantly from each other in the frequencies at which they invade reproductive organs and cause egg contamination (Gast and Holt, 2000, 2001b; Gast *et al*., 2007) and in their growth properties in yolk or albumen (Cogan *et al*., 2001; Gast and Holt, 2001a; Gast *et al*., 2005). However, no specific affinities of individual strains for particular regions of the reproductive tract have been identified that would produce distinctive patterns of deposition within eggs (Gast *et al*., 2007).

The ability of *S. enteritidis* strains to cause egg contamination has been attributed to phenotypic characteristics including growth to high cell density and the production of high-molecular-mass lipopolysaccharide (Guard-Petter, 1998; Guard-Petter *et al*., 1997; Parker *et al*., 2001, 2002). A biofilm-negative *S. enteritidis* phenotype, linked to specific single-nucleotide genomic changes, was associated with an increased propensity for deposition inside eggs laid by experimentally infected hens (Guard-Bouldin *et al*., 2004; Morales *et al*., 2007). Complementarity between subpopulations expressing different phenotypic properties has been postulated as necessary to facilitate the coordinated sequence of events that proceeds from intestinal colonization to deposition inside eggs (Gast *et al*., 2002). Selective pressures in the tissues of infected hens might promote the expression of these properties, as suggested by the increased egg contamination frequencies associated

with *S. enteritidis* strains that were re-isolated from eggs or tissues after repeated passages through hens in earlier experiments (Gast *et al*., 2003, 2005). The objective of the present study was to determine how a single *in vivo* passage affects the ability of an *S. enteritidis* isolate to invade internal organs (including specific reproductive tract sites) of orally inoculated laying hens.

MATERIALS AND METHODS

Experimental infection of laying hens: In each of three trials, 36 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, 44 and 52 wk old at the beginning of the first, second and third trials, respectively) were distributed among two 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*.

One group of chickens in each trial (designated as the first passage group) was inoculated with a phage type 13a strain of *S. enteritidis* (obtained from Dr. C. Benson, University of Pennsylvania, Kennett Square, PA, USA). Separate lyophilized stock culture vials (all originally prepared from the same broth culture batch) were used for the three trials. Each inoculum stock culture was resuscitated by incubation for 24 h at 37°C in 9 mL of Tryptone Soya (TS) broth (Oxoid Limited, Basingstoke, Hampshire, UK) and subsequent transfer by streaking onto plates of 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). After incubation of these plates for 24 h at 37°C, three typical *S. enteritidis* colonies were transferred to 50 mL of TS broth and incubated at 37°C for 24 h. Each hen was inoculated with a 1-mL dose of this culture, containing approximately 2.0×10^9 CFU of *S. enteritidis*.

After completion of the first passage portion of each trial, the other group of chickens (designated as the second passage group) was then inoculated with an *S. enteritidis* isolate obtained from a first passage hen in that trial as described below. The second passage inoculum culture was prepared by transferring three *S. enteritidis* colonies from a single BG agar plate into 50 mL of TS broth, incubating this broth at 37°C for 24 h and administering a 1-mL dose containing approximately 2.0×10^9 CFU of *S. enteritidis* to each hen.

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 BG agar supplemented with 0.02 mg/mL of novobiocin 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).

Internal organ samples: At 7 d post-inoculation in each passage of each trial, all hens were humanely euthanized to allow removal of internal tissues for bacteriologic culture. Portions (approximately 5-10 g) of the liver, spleen, 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 50 mL of tetrathionate broth and mixed by stomaching for 30 sec. Each broth culture was incubated for 40 h at 37°C and a 10-µL aliquot was then streaked onto BG agar plus novobiocin. After incubation of these plates for 24 h at 37°C, typical *S. enteritidis* colonies were subjected to biochemical and serological confirmation (Waltman and Gast, 2008). In each trial, a BG plate obtained by culturing an upper oviduct sample from the first passage was selected as the source of the inoculum culture for the second passage.

Statistical analysis: For each trial, significant differences ($p<0.05$) between passages in the frequency of *S. enteritidis* recovery from internal organs were determined by Fisher's exact test. Data were analyzed with Instat biostatistics software (GraphPad Software, San Diego, CA, USA).

RESULTS

None of the fecal samples collected prior to inoculation of the hens were positive for *Salmonella*. In each of the three trials, the *S. enteritidis* inoculum strain invaded to reach all five sampled tissues (Table 1). In trial 1, the frequencies of *S. enteritidis* recovery from internal organs ranged from 16.7% (upper oviducts) to 83.3% (spleens) for the first passage and from 0% (ovaries) to 33.3% (livers) for the second passage. The *S. enteritidis* recovery frequencies from spleens and ovaries declined significantly ($p<0.0001$ and $p = 0.0455$, respectively) between the first and second passages in trial 1. In trial 2, the frequencies of *S. enteritidis* recovery ranged from 5.6% (all reproductive tissues) to 61.1% (spleens) for the first passage and from 5.6% (ovaries) to 94.4% (livers) for the second passage. The frequency of *S. enteritidis* recovery from livers increased significantly ($p = 0.0178$) between the first and second passages in trial 2. In trial 3, the frequencies of *S. enteritidis* recovery ranged from 33.3% (upper and lower oviducts) to 100% (spleens) for the first passage and from 27.8% (lower oviducts) to 94.4% (livers and spleens) for the second

Table 1: Recovery of *Salmonella enteritidis* from internal organs of experimentally infected laying hens[1]

	S. enteritidis-positive/total (%)					
	Liver	Spleen	Ovary	Oviduct (U)[2]	Oviduct (L)[3]	All organ samples
Trial 1:						
First passage	9/18 (50.0)[a]	15/18 (83.3)[a]	5/18 (27.8)[a]	3/18 (16.7)[a]	4/18 (22.2)[a]	36/90 (40.0)[a]
Second passage	6/18 (33.3)[a]	2/18 (11.1)[b]	0/18 (0)[b]	1/18 (5.6)[a]	2/18 (11.1)[a]	11/90 (12.2)[b]
Trial 2:						
First passage	10/18 (55.6)[a]	11/18 (61.1)[a]	1/18 (5.6)[a]	1/18 (5.6)[a]	1/18 (5.6)[a]	24/90 (26.7)[a]
Second passage	17/18 (94.4)[b]	16/18 (88.9)[a]	1/18 (5.6)[a]	2/18 (11.1)[a]	2/18 (11.1)[a]	38/90 (42.2)[b]
Trial 3:						
First passage	17/18 (94.4)[a]	18/18 (100)[a]	8/18 (44.4)[a]	6/18 (33.3)[a]	6/18 (33.3)[a]	55/90 (61.1)[a]
Second passage	17/18 (94.4)[a]	17/18 (94.4)[a]	7/18 (38.9)[a]	6/18 (33.3)[a]	5/18 (27.8)[a]	52/90 (57.8)[a]

[1]In each trial, tissues were sampled 7 d after oral inoculation of a group of hens (first passage) with approximately 10^9 cfu of a phage type 13a *S. enteritidis* strain and 7 d after inoculation of another group of hens (second passage) with approximately 10^9 cfu of an oviduct isolate recovered from first passage hens. [2]Upper oviduct (centered on infundibulum/magnum junction). [3]Lower oviduct (centered on isthmus/uterus junction). [a,b]Values in columns (within trials) that share no common superscripts are significantly ($p<0.05$) different.

passage. No significant differences were observed between passages in trial 3 in the frequency of recovery of *S. enteritidis* from individual tissues.

DISCUSSION

Identifying the genetic and phenotypic characteristics that enable *S. enteritidis* strains to infect chickens and contaminate eggs is essential for controlling this organism in commercial poultry flocks (Gast, 2008). Individual strains of *S. enteritidis* can differ in the ability to colonize and invade cells of the gastrointestinal tract (Berndt *et al*., 2007), but persistent intestinal colonization and fecal shedding by *S. enteritidis* have not consistently predicted the probability of systemic infection and egg contamination (Gast and Holt, 2000; Humphrey *et al*., 1991). Bacterial deposition inside developing eggs (Keller *et al*., 1995) results from invasion of either the ovary (the site of yolk maturation and release) or the oviduct (the site of albumen secretion around the descending yolk). However, high frequencies of reproductive tissue colonization do not necessarily result in correspondingly high egg contamination frequencies (Barrow and Lovell, 1991; Methner *et al*., 1995). In a previous oral infection study, ovarian colonization occurred significantly more often than colonization of either the upper or lower portions of the oviduct for all three *Salmonella* isolates studied, but no corresponding difference was observed between the incidence of deposition in yolk or albumen (Gast *et al*., 2007). The initial location of *Salmonella* deposition in eggs has important consequences, as bacterial multiplication to dangerously high levels is far more likely in the nutrient-rich egg yolk than in the iron-restricted albumen (Chen *et al*., 2005; Kang *et al*., 2006). Naturally occurring infections in commercial laying flocks typically involve exposure to relatively low doses of *Salmonella* and are thus generally associated with lower frequencies of egg contamination than occur in experimental infection studies (Ebel and Schlosser, 2000; Humphrey *et al*., 1989, 1991). Intestinal colonization, organ invasion and egg deposition by *S. enteritidis* have all been reported to vary between lines of chickens (Beaumont *et al*., 1994, 1999; Berchieri *et al*., 2001).

Individual strains of *Salmonella* (within and across serotype boundaries) can differ very significantly from each other in the ability to cause egg contamination (Gast and Holt, 2000, 2001b; Gast *et al*., 2007) and to survive or multiply in yolk or albumen (Cogan *et al*., 2001; Gast and Holt, 2001a; Gast *et al*., 2005). Bacterial properties including growth to high cell density, production of high-molecular-mass lipopolysaccharide and the absence of biofilm formation have been linked to higher incidences of egg contamination (Guard-Bouldin *et al*., 2004; Guard-Petter, 1998; Guard-Petter *et al*., 1997). Selective pressures exerted in the tissues of infected hens may promote the expression of some of these attributes. Higher egg contamination frequencies have been obtained by experimental infection of hens with *S. enteritidis* and *S. heidelberg* strains that were re-isolated from eggs or tissues of infected hens than were associated with the original parent strains, suggesting that the interaction of *S. enteritidis* with reproductive tissues of chickens either induced or selected for the expression of microbial properties related to egg contamination (Gast *et al*., 2003, 2005). The expression of potential *S. enteritidis* virulence factors such as flagella, fimbria, outer membrane proteins and iron uptake systems can be influenced by environmental conditions such as pH and temperature (Chart *et al*., 1994; McDermid *et al*., 1996; Walker *et al*., 1999) or by growth in chicken tissues (Chart *et al*., 1993). Distinct *Salmonella* subpopulations, expressing attributes relevant to different environmental contexts within the infected avian host, may complement each other to result in egg contamination (Guard-Petter, 2001). A mixture of *S. enteritidis* strains expressing both properties associated with colonization and invasion of the intestinal tract and properties associated with colonization of reproductive tissues was used to

promote an increased frequency of egg contamination in a prior experiment (Gast *et al*., 2002).

In the present study, a single passage of *S. enteritidis* strains through reproductive organs of hens did not consistently select for higher invasiveness in a subsequent round of infection. The frequency of *S. enteritidis* isolation from an individual tissue site was significantly increased by passage in only one instance (livers in trial 2). The different results obtained in the three trials illustrate the complexity of the interaction between *S. enteritidis* and the multiple environments it encounters within the infected avian host. Accordingly, the consequences of *in vivo* passage of *S. enteritidis* through laying hens may depend on both the genetic and phenotypic characteristics of the original infecting bacterial population and the selective pressures exerted in the tissues of infected chickens.

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