Contamination of eggs by *Salmonella* Enteritidis in experimentally infected laying hens housed in conventional or enriched cages

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ABSTRACT Both epidemiologic analyses and active disease surveillance confirm an ongoing strong association between human salmonellosis and the prevalence of *Salmonella enterica* subspecies *enterica* serovar Enteritidis in commercial egg flocks. The majority of human illnesses caused by this pathogen are attributed to the consumption of contaminated eggs. Animal welfare concerns have increasingly influenced commercial poultry production practices in recent years, but the food safety implications of different housing systems for egg-laying hens are not definitively understood. The present study assessed the effects of 2 different housing systems (conventional cages and colony cages enriched with perching and nesting areas) on the frequency of *Salmonella* Enteritidis contamination inside eggs laid by experimentally infected laying hens. In each of 2 trials, groups of laying hens housed in each cage system were orally inoculated with doses of $1.0 \times 10^8$ cfu of *Salmonella* Enteritidis. All eggs laid between 5 and 25 d postinoculation were collected and cultured to detect internal contamination with *Salmonella* Enteritidis. For both trials combined, *Salmonella* Enteritidis was recovered from 3.97% of eggs laid by hens in conventional cages and 3.58% of eggs laid by hens in enriched cages. No significant differences ($P > 0.05$) in the frequency of egg contamination were observed between the 2 housing systems.

Key words: *Salmonella* Enteritidis, chicken, egg, conventional cage, enriched cage

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INTRODUCTION

During the first decade of this century, the incidence of human *Salmonella* infections in the United States did not change significantly, and this disease was responsible for estimated annual costs of up to $11 billion (Centers for Disease Control and Prevention, 2011; Scharff, 2012). Moreover, the incidence of *Salmonella enterica* subspecies *enterica* serovar Enteritidis infections increased by 44% during the same period (Chai et al., 2012). *Salmonella* Enteritidis first emerged as a significant international public health problem in the 1980s, and the majority of human illnesses caused by this pathogen have been attributed to the consumption of contaminated eggs (Braden, 2006; Greig and Ravel, 2009). Significant resources from both government and private industry have been invested in *Salmonella* Enteritidis testing and risk reduction programs for egg-producing poultry (Gast, 2007; US Food and Drug Administration, 2009). After a sustained commitment to these efforts over time, international progress has been documented in regard to the incidences of both egg contamination (Esaki et al., 2013) and human infections (Mumma et al., 2004; Poirier et al., 2008; O’Brien, 2013). However, both epidemiologic analyses and active disease surveillance confirm an ongoing strong association between human salmonellosis and the prevalence of *Salmonella* Enteritidis in commercial egg flocks (Centers for Disease Control and Prevention, 2011; Chai et al., 2012; Havelaar et al., 2013).

The deposition of *Salmonella* Enteritidis inside the edible interior contents of eggs is a consequence of bacterial dissemination to reproductive tissues (ovary and oviduct) in systemically infected hens (Gast et al., 2004; Gantois et al., 2009). The number of *Salmonella* Enteritidis cells administered to hens can significantly affect both the frequency and location of resulting egg contamination (Gast et al., 2013a), but experimental infection with very large oral doses of *Salmonella* Enteritidis has typically only yielded low frequencies of egg contamination and small initial bacterial cell concentrations (Humphrey et al., 1991; Gast and Beard, 1992; Gast and Holt, 2000). Even lower frequencies of contamination are usually reported for commercially produced eggs, perhaps because laying flocks are sporadically exposed to environmental sources of *Salmonella* Enteritidis at relatively low doses (Ebel and Schlosser, 2013).
Although the frequency of *Salmonella* Enteritidis colonization of internal organs in mature chickens declines sharply during the first few weeks after oral inoculation (Gast et al., 2007, 2011a), persistent infections in individual birds might prolong the horizontal transmission of infection throughout flocks (Gast et al., 2009). Opportunities for laying hens to be exposed and infected can also be enhanced by extended bacterial survival in the housing environment, which is often sustained and amplified by rodent or insect vectors (Davies and Breslin, 2003; Dewaele et al., 2012b; Lapuz et al., 2012). The production environment may also serve as a reservoir for the periodic emergence of pathogen strains with heightened abilities to cause systemic infection and egg contamination (Henzler et al., 1998; Dewaele et al., 2012a).

Housing systems for egg-laying hens have been widely discussed and analyzed in recent years in a variety of contexts, including animal welfare and food safety. However, poultry housing conditions have diverse and complex influences on *Salmonella* contamination and infection, so no conclusive or authoritative consensus about their food safety implications has yet emerged from the published scientific literature on the topic (Holt et al., 2011). Several studies have reported an increased frequency of environmental *Salmonella* isolation from laying houses containing conventional battery cages (Huneau-Salain et al., 2009; Snow et al., 2010; Van Hoorebeke et al., 2010b), but other investigators observed lower incidences of *Salmonella* infection and egg contamination associated with cages than with floor systems (Kinde et al., 1996; Hannah et al., 2011). Enriched (furnished) cages have been considered as an intermediate alternative to conventional cages and cage-free systems. Although no clear or consistent pattern of differential effects on *Salmonella* prevalence has yet been found to distinguish the various cage systems (De Vylder et al., 2009; Van Hoorebeke et al., 2010b), significant higher frequency of invasion to internal organs occurred among experimentally infected hens housed in conventional cages than in enriched cages (Gast et al., 2013b). The objective of the present study was to determine the effects of 2 different housing systems (conventional and enriched cages) on the frequency of *Salmonella* Enteritidis contamination inside eggs laid by experimentally infected laying hens.

**Experimental Infection of Laying Hens with *Salmonella* Enteritidis**

In each trial, all laying hens were orally inoculated with a measured dose of *Salmonella* Enteritidis. Hens in trial 1 received a phage type 13a isolate, originally isolated from a contaminated egg yolk by C. Benson at the University of Pennsylvania, Kennett Square, Pennsylvania. Hens in trial 2 received a phage type 4 isolate, originally isolated from the liver of an infected chicken by D. Munro at the Scottish Salmonella Reference Laboratory, Glasgow, United Kingdom. Two different *Salmonella* Enteritidis phage types (both of which are epidemiologically important) were included in this study to minimize the strain specificity of results. The inoculum strains were resuscitated by transfer into tryptic soy broth (Acumedia, Neogen Corp., Lansing, MI) for 2 successive 24-h incubation cycles at 37°C. After cell numbers in each incubated culture were estimated by determining its optical density at 600 nm, further serial 10-fold dilutions in 0.85% saline produced a desired final cell concentration of approximately $1.0 \times 10^8$ cfu (confirmed by subsequent plate counts).

**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. A total of 30 samples were collected from each room, evenly distributed among all occupied cages. These samples were transferred to 9 mL of tetraphionate broth (Acumedia) and incubated for 24 h at 37°C. A 10-µL portion from each broth culture was then streaked onto brilliant green agar (Acumedia) supplemented with 0.02 mg/mL of novobiocin (Sigma Chemical Co., St. Louis, MO) and incubated for 24 h at 37°C. The identity of presumptive colonies of *Sal-
monella was confirmed biochemically and serologically (Waltman and Gast, 2008).

**Egg Content Samples**

All eggs laid on the day before inoculation and between 5 and 25 d postinoculation were cultured to detect internal contamination with *Salmonella*. Eggshell surfaces were disinfected by dipping for 5 s in 70% ethanol and the shells were then broken against a sharp edge covered by sterile foil strips. The entire liquid contents of each egg were transferred to 50 mL of tryptic soy broth supplemented with 35 mg/L of ferrous sulfate (Sigma), mixed by vigorous shaking for 15 s, and incubated for 24 h at 37°C. A 1-mL portion of each incubated tryptic soy broth culture was transferred to 9 mL of Rappaport Vassiliadis broth (Acumedia) and incubated for 24 h at 37°C. A 10-µL aliquot from each of these broth cultures was then streaked onto brilliant green agar and incubated for 24 h at 37°C. After incubation of these plates for 24 h at 37°C, typical *Salmonella* Enteritidis colonies were subjected to biochemical and serological confirmation (Waltman and Gast, 2008).

**Statistical Analysis**

For each trial (and for both trials combined), significant differences (*P* < 0.05) between housing systems in the mean frequencies of *Salmonella* Enteritidis isolation from egg contents were determined by Fisher’s exact test. Similarly, significant differences (*P* < 0.05) between *Salmonella* Enteritidis strains in their mean frequencies of recovery from eggs were determined by Fisher’s exact test for each housing system (and for both systems combined). Because the 2 replicate groups of hens for each housing system did not differ significantly within either trial in *Salmonella* Enteritidis recovery from eggs, their results were combined for analysis and presentation. Data were analyzed with Instat biostatistics software (GraphPad Software, San Diego, CA).

**RESULTS AND DISCUSSION**

None of the fecal samples or eggs collected before inoculation in either trial were positive for *Salmonella*. In trial 1, *Salmonella* Enteritidis (phage type 13a) was recovered from 3.77% of eggs laid between 5 and 25 d after oral inoculation by hens housed in conventional cages and from 3.78% of eggs laid by hens housed in enriched cages (Table 1). In trial 2, *Salmonella* Enteritidis (phage type 4) was recovered from 4.22% of eggs laid between 5 and 25 d postinoculation by hens housed in conventional cages and from 3.35% of eggs laid by hens housed in enriched cages. No significant differences (*P* > 0.05) were observed between housing systems in the frequencies of *Salmonella* Enteritidis isolation from eggs in either trial or for both trials combined. Likewise, no significant differences were observed between the 2 *Salmonella* Enteritidis strains in their associated frequencies of egg contamination in either housing system or for both systems combined.

*Salmonella* Enteritidis has often caused higher incidences of egg contamination than other serovars in oral inoculation experiments (Gast et al., 2005, 2007), perhaps as a consequence of stronger adherence to reproductive tract mucosa (Wales and Davies, 2011). For example, a recent study (Gast et al., 2011b) found *Salmonella* inside eggs at significantly different frequencies following experimental infection of hens with serovars Enteritidis (3.6%), Heidelberg (0.5%), or Hadar (0%). The deposition of *Salmonella* Enteritidis inside developing eggs may result from the sequential expression of bacterial virulence properties, which are relevant at different stages of infection in susceptible laying hens (Gast et al., 2002; Guard et al., 2010). The expression of very long lipopolysaccharide O-antigen enhances both reproductive tract colonization and survival in forming eggs, and may thus distinguish egg-contaminating *Salmonella* Enteritidis strains from other environmental salmonellae (Guard-Bouldin et al., 2004; Coward et al., 2013). The accumulation of single nucleotide changes within relevant genes can lead to divergence among isolates of the same phage type in their abilities to invade internal organs and eggs (Guard et al., 2011). Phage typing has been a useful tool for identifying the sources of outbreak-related strains, but no consistent patterns of expression of virulence properties have differentiated the various phage types of *Salmonella* Enteritidis (Gast and Benson, 1996; Gast and Holt, 2000; Gantois et al., 2009). Hens were infected with strains of 2 different phage types in the present study, but laid contaminated eggs at similar frequencies.

The environment of poultry housing facilities can serve as an important source of *Salmonella* Enteritidis introduction to laying flocks (Henzler et al., 1998; Dewaele et al., 2012 a,b). Different environmental reservoirs for *Salmonella* persistence have been associated with the

<table>
<thead>
<tr>
<th>Item</th>
<th>Trial 1</th>
<th>Trial 2</th>
<th>All trials</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional cage</td>
<td>18/478 (3.77)</td>
<td>17/403 (4.22)</td>
<td>35/881 (3.97)</td>
</tr>
<tr>
<td>Enriched cage</td>
<td>20/529 (3.78)</td>
<td>15/448 (3.35)</td>
<td>35/977 (3.58)</td>
</tr>
</tbody>
</table>

*Values sharing no common superscripts are significantly (*P* < 0.05) different.

*Egg Content Samples*  

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HOUSING AND SALMONELLA ENTERITIDIS IN EGGS

731

various commercial housing systems (Carrique-Mas et al., 2009b). A higher environmental prevalence of Salmonella has been reported in larger flocks (Mollenhorst et al., 2005; Namata et al., 2008; Huneau-Salaün et al., 2009), older flocks (Namata et al., 2008; Pitesky et al., 2013), multiple-age flocks (Mollenhorst et al., 2005; Huneau-Salaün et al., 2009; Snow et al., 2010), and older facilities (Van Hoorebeke et al., 2010a). Diverse flock management and housing parameters can also serve as risk factors for the transmission of Salmonella Enteritidis infection and for egg contamination. High stocking densities or unsanitary conditions can increase the susceptibility of chickens to infection (Asakura et al., 2001). Some comparative studies have reported a higher frequency of horizontal transmission of Salmonella infection in cage-free housing systems than for cage systems (De Vylder et al., 2011; Hannah et al., 2011), especially if outdoor areas allow Salmonella introduction from external environmental sources (Mollenhorst et al., 2005). Other studies have found a greater risk of Salmonella infection in caged-based housing, particularly when rodents are present in high numbers (Namata et al., 2008; Carrique-Mas et al., 2009a). However, several other investigators detected no significant differences between cage and cage-free systems (Siemon et al., 2007; Jones et al., 2012) or between conventional and enriched cage systems (De Vylder et al., 2009; Van Hoorebeke et al., 2011) in the environmental prevalence of Salmonella.

In one recent experiment, Salmonella Enteritidis was recovered at a significantly higher frequency from internal organs of infected hens housed in conventional cages than from hens in enriched colony cages (94 vs. 53% of spleens and 25 vs. 10% of ovaries), suggesting that parameters such as bird density or behavioral restriction might influence susceptibility to systemic dissemination of Salmonella Enteritidis (Gast et al., 2013b). Other environmentally mediated stressors, such as heat exposure, feed restriction, or water deprivation, can increase susceptibility to Salmonella infection (Humphrey, 2006). However, no comparable difference between these same 2 caging systems was observed for the incidence of egg contamination in the present study. Although systemic infection is a necessary component step in the egg contamination process, previous experiments have sometimes shown an imprecise relationship between the frequency or magnitude of organ invasion and the production of contaminated eggs (Gast et al., 2004, 2007, 2011c). Accordingly, environmental influences which affect systemic infection may not necessarily have a corresponding effect on egg contamination. Egg-borne transmission of Salmonella Enteritidis to consumers is epidemiologically linked to the presence of the pathogen in edible yolk and albumen (and the present study focused on this critical issue), but contaminated egg shells can also pose an indirect threat to food safety. Previous research has indicated that housing systems can affect the external microbiology of eggs. For example, a recent study reported lower levels of Enterobacteriaceae on the shells of eggs from hens in conventional cages than from hens in cage-free systems (Jones and Anderson, 2013).

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REFERENCES


