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Continuing Multiplication of *Salmonella enteritidis* Strains in Egg Yolk During Refrigeration at 7.2°C

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Abstract: The continuing attribution of human illness caused by *Salmonella enteritidis* to the consumption of contaminated eggs has led to widespread implementation of risk reduction programs for commercial egg production, often emphasizing prompt refrigeration of eggs to prevent bacterial multiplication to dangerously high levels. However, microbial growth may not cease immediately inside warm eggs after transfer to refrigerated storage. The present study compared the abilities of 8 *S. enteritidis* strains (of 4 phage types) to continue multiplying in experimentally contaminated egg yolk during the first 24 h after transition from warm to refrigeration temperatures. After 15 mL samples of egg yolk were inoculated with 10 CFU/ml of *S. enteritidis*, they were incubated at 37°C for 16 h and then transferred into refrigeration at 7.2°C for 24 h. Bacterial cell concentrations were determined following 37°C incubation and again after both 8 and 24 h at 7.2°C. All 8 *S. enteritidis* isolates multiplied significantly during 16 h of incubation, reaching an overall mean of $\log_{10}$ 8.790 CFU/ml. After refrigeration, the observed mean values for cell concentrations in yolk samples were $\log_{10}$ 8.780 CFU/mL at 8 h and $\log_{10}$ 8.849 CFU/mL at 24 h. For 3 of 8 strains, a significant ($p<0.05$) increase in cell concentrations in egg yolk occurred during 24 h of refrigeration. These results support the importance of prompt egg refrigeration for minimizing the numbers of *S. enteritidis* in marketed table eggs, although refrigeration at 7.2°C may not immediately or completely arrest multiplication by all strains.

Key words: *Salmonella enteritidis*, egg yolk, multiplication, temperature, refrigeration

INTRODUCTION

The incidence of *Salmonella enterica* subspecies *enterica* serovar Enteritidis (*S. enteritidis)* infections of humans in the USA increased by 44% during the first decade of the present century, although the overall incidence of *Salmonella* infections remained relatively constant for that same period (Centers for Disease Control and Prevention, 2011; Chai *et al*., 2012). Since this issue emerged to international prominence in the 1980's, the largest proportion of human illnesses caused by *S. enteritidis* has been consistently attributed to the consumption of contaminated eggs (Braden, 2006; Greig and Ravel, 2009). Both public and private resources have been committed to programs for *S. enteritidis* testing and risk reduction in egg-laying flocks (Gast, 2007; U.S. Food and Drug Administration, 2009) and the sustained application of these efforts has yielded international progress in reducing the incidence of both egg contamination (Esaki *et al*., 2013) and human infections (Mumma *et al*., 2004; Poirier *et al*., 2008; O'Brien, 2013). Nevertheless, the ongoing association between the prevalence of *S. enteritidis* in laying hens and human salmonellosis remains strong (Havelaar *et al*., 2013) and both epidemiologic analyses and active disease surveillance efforts document a continuing public health threat from egg-transmitted salmonellosis (Centers for Disease Control and Prevention, 2011; Chai *et al*., 2012).

Epidemiological risk assessment has determined that refrigeration is among the most effective options for intervening to mitigate the public health consequences of *S. enteritidis* contamination in eggs (Schroeder *et al*., 2006; Latimer *et al*., 2008). Because the typical prevalence of *S. enteritidis* inside commercially produced eggs is very low (Ebel and Schlosser, 2000; DeWinter *et al*., 2011; Esaki *et al*., 2013) and freshly laid eggs rarely harbor more than a few hundred *S. enteritidis* cells (Humphrey *et al*., 1991; Gast and Beard, 1992; Gast and Holt, 2000a), prompt refrigeration is essential for preventing bacterial multiplication to higher levels (more likely to cause illness in consumers) during storage. Although poor egg storage practices are uncommon, they were recently implicated in nearly half of egg-associated illnesses in Canada (DeWinter *et al*., 2011). Most risk reduction plans for *S. enteritidis* include egg refrigeration requirements (Mumma *et al*., 2004). Federal regulations for commercial shell egg producers in the USA specify that eggs must be stored and

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transported at an ambient temperature of 7.2°C or lower, beginning within 36 h after laying (U.S. Food and Drug Administration, 2009).

The efficacy of refrigeration for preventing the expansion of small *S. enteritidis* populations in eggs depends on the initial level and location of contamination, the potential for movement of bacteria or nutrients within eggs during storage and the rate at which growth-restricting temperatures are attained. The initial deposition site for *S. enteritidis* in eggs laid by infected hens is more often associated with the albumen or vitelline (yolk) membrane than with the nutrient-rich interior contents of the yolk (Gast and Holt, 2001a; Gast *et al*., 2003). However, *S. enteritidis* can migrate across the yolk membrane to multiply in the yolk contents during storage at warm temperatures (Gast *et al*., 2005, 2007a, 2010a). Neither penetration into nor growth inside egg yolks occurs at refrigeration temperatures (Gast *et al*., 2006). When eggs are transferred to refrigeration at 7.2°C, their interior contents will begin to gradually cool toward this ambient temperature, but continuing bacterial growth in egg yolks during this transitional cooling period could reduce the protective value of refrigeration (Chen *et al*., 2002). The present study compared the abilities of 8 strains of *S. enteritidis* (representing 4 phage types) to continue multiplying in experimentally contaminated egg yolk samples during the first 24 h after a transition from warm to refrigeration temperatures.

MATERIALS AND METHODS

Preparation of *S. enteritidis* cultures: Eight *S. enteritidis* isolates were resuscitated by transfer into tryptone soya broth (Acumedia, Neogen Corp., Lansing, MI, USA) for two successive cycles of 24-h incubation at 37°C. Each culture was centrifuged for 10 min at 3,000 x g to concentrate cells, washed with 0.85% saline, centrifuged again and resuspended in saline. After the cell concentration of each resuspended culture was estimated by determining its optical density at 600 nm, further dilution in saline produced the desired final cell concentration for the inoculum. Plate counts to confirm these values yielded equivalent results using either non-selective trypticase soy agar (Acumedia) or selective (and differential) brilliant green agar (Acumedia). All *S. enteritidis* strains were originally isolated from contaminated eggs or from infected humans in egg-associated disease outbreaks. Isolates A and B were phage type 4, isolates C and D were phage type 8, isolates E and F were phage type 13a and isolates G and H were phage type 14b.

Preparation and inoculation of egg yolk samples: In each of 8 similar trials, 30 freshly collected eggs from the specific-pathogen-free flock of Single Comb White Leghorn chickens at the Southeast Poultry Research Laboratory (Athens, GA, USA) were aseptically broken, their contents (yolk and albumen) were separated and the pooled yolks were mixed together by vigorous stirring. Twenty-one samples were then prepared by transferring 15 mL aliquots of pooled egg yolk (approximating the typical volume of a single intact yolk) into sterile 50-ml plastic centrifuge tubes. Twelve yolk samples per trial were inoculated with 0.3 mL (containing 150 CFU) of one of the 8 diluted *S. enteritidis* broth cultures and mixed by vortexing. This initial inoculum level (10 CFU/mL) was intended to provide a realistic simulation of naturally occurring contamination. The remaining 9 samples in each trial were retained as uninoculated negative controls (3 for bacteriologic culturing and 6 for temperature monitoring).

Enumeration of *S. enteritidis* in egg yolk samples after incubation and refrigeration: All egg yolk samples were first incubated at 37°C for 16 h to encourage active bacterial multiplication and then transferred into refrigeration at 7.2°C for an additional 24 h. Immediately following 37°C incubation and again after both 8 and 24 h of 7.2°C refrigeration, a 1-mL aliquot was removed from each yolk sample and tested to enumerate *S. enteritidis*. The concentration of *S. enteritidis* in each yolk sample was determined by making 10-fold dilutions in 0.85% saline and spreading 0.1 mL of each dilution (including the undiluted yolk) onto plates of brilliant green agar. The agar plates were incubated for 24 h at 37°C and typical *Salmonella* colonies were counted. Biochemical and serological confirmation (Waltman and Gast, 2008) that randomly selected colonies (representing each positive sample) were always *S. enteritidis* validated the visual observation that only the inoculum strain was present on these agar plates. The detection threshold of this procedure was 10 CFU/mL. The temperature of 6 negative control yolk samples (not used for bacteriologic culturing) was determined after incubation and after both 8 and 24 h of refrigeration by the insertion of thermometers.

Statistical analysis: Significant differences ($p<0.05$) between isolates or sampling intervals in the mean concentration of *S. enteritidis* cells in yolk samples after storage were determined by applying the Kruskal-Wallis test and Dunn's multiple comparison post-test. Data were analyzed using Instat biostatistics software (GraphPad Software, San Diego, CA, USA).

RESULTS

All 8 *S. enteritidis* isolates multiplied from the initial inoculum level (10 CFU/mL) to more than $\log_{10}$ 8.0 CFU/mL during 16 h of incubation at 37°C in egg yolk (Table 1). Values for mean post-incubation cell concentrations ranged from $\log_{10}$ 8.370 to $\log_{10}$ 8.941 CFU/mL, with an overall mean of $\log_{10}$ 8.790 CFU/mL.

Table 1: Enumeration of *Salmonella enteritidis* strains from egg yolk samples[1]

Strains	Cell concentration in yolk samples[2] (mean log_{10} CFU/mL ± standard deviation: 16 h incubation at 37°C	8 h refrigeration at 7.2°C	24 h refrigeration at 7.2°C
A	8.784 ± 0.109ABa	8.852 ± 0.104ABab	8.893 ± 0.073ABb
B	8.770 ± 0.080BCa	8.565 ± 0.095CDb	8.830 ± 0.065BCac
C	8.941 ± 0.051Aa	8.948 ± 0.080Aa	8.956 ± 0.088ABa
D	8.573 ± 0.067CDa	8.611 ± 0.113BCDab	8.693 ± 0.095CDb
E	8.826 ± 0.052ABa	8.836 ± 0.057ABCa	8.846 ± 0.076BCa
F	8.370 ± 0.117Dab	8.403 ± 0.045Da	8.306 ± 0.099Db
G	8.840 ± 0.061ABa	8.802 ± 0.109ABCa	8.777 ± 0.119BCDa
H	8.930 ± 0.100ABa	8.938 ± 0.083Aa	9.078 ± 0.071Ab

[1]15-mL yolk samples (n = 12/strain) were each inoculated with approximately 150 CFU of *S. enteritidis*, incubated at 37°C and then refrigerated at 7.2°C

[2]Values within columns which share no common uppercase letters, or values within rows which share no lowercase letters, differ significantly ($p<0.05$)

After 8 h of subsequent refrigeration at 7.2°C, observed values for *S. enteritidis* cell concentrations in the yolk samples ranged from log_{10} 8.403 to log_{10} 8.948 CFU/mL, with an overall mean of log_{10} 8.780 CFU/mL. After 24 h of refrigeration of the yolk samples at 7.2°C, the observed *S. enteritidis* levels ranged from log_{10} 8.306 to log_{10} 9.078 CFU/mL, with a mean of log_{10} 8.849 CFU/mL. At each testing interval (after incubation and after refrigeration), significant ($p<0.05$) differences were observed between the cell concentrations of individual isolates, but a consistent rank-order of isolates was not maintained throughout the experiment. After 8 h of refrigeration, a significantly lower cell concentration was found for one strain (isolate B) than was observed prior to refrigeration and no significant changes occurred for the other 7 strains. Between 8 and 24 h of refrigeration, a significant increase in cell concentration was evident for 2 strains (isolates B and H), a significant decrease was detected for one strain (isolate F) and no significant changes were apparent for the other 5 strains. For 3 of the 8 *S. enteritidis* strains (isolates A, D and H), a significant increase in cell concentrations in egg yolk occurred over the course of the entire 24 h period of refrigeration. None of the uninoculated negative control samples were *Salmonella*-positive after incubation. The internal temperature of all negative control yolk samples was 37EC after incubation and 7.2°C after both 8 and 24 h of refrigeration.

DISCUSSION

The ability of *S. enteritidis* to colonize both the ovary and oviduct in systemically infected laying hens can lead to deposition of this pathogen in either the yolk or albumen of developing eggs (Gast *et al*., 2004, 2007b). The initial location of contamination inside eggs influences the efficacy of refrigeration for protecting consumers against egg-transmitted illness, because it determines how rapidly growth-inhibiting temperatures must be attained. Although *S. enteritidis* may survive or multiply slowly in egg albumen (Kang *et al*., 2006; Chen and Thesmar, 2008; Okamura *et al*., 2008), the abundance of nutrients found in egg yolk supports rapid and prolific bacterial growth (Humphrey and Whitehead, 1993; Gast and Holt, 2000b; Gurtler and Conner, 2009). Even if initially deposited outside of the yolk, *S. enteritidis* can grow actively on the vitelline membrane surrounding the yolk or migrate across this membrane to multiply extensively inside the interior yolk contents (Gast and Holt, 2000b; Gast *et al*., 2008, 2010a). Temperature is perhaps the most important parameter affecting *S. enteritidis* growth in egg yolks. Bacterial multiplication to high cell densities in yolk has been reported at 15°C or higher, but growth is slower at 10°C and altogether absent at 4°C (Schoeni *et al*., 1995; Gast and Holt, 2000b; Gurtler and Conner, 2009). At declining storage temperatures over a range of 10-30°C, both *Salmonella* penetration through yolk membranes and multiplication inside yolks have been found to decline significantly and neither penetration nor multiplication has occurred at 7.2°C (Gast *et al*., 2005, 2006, 2010a).

In the present study, all 8 *S. enteritidis* isolates multiplied to very high levels during 16 h of incubation of egg yolk samples at 37°C. Three of these strains also multiplied significantly during the first 24 h after transfer to refrigeration at 7.2°C. Although some further bacterial growth might have been anticipated as the temperature in the yolk samples declined steadily from 37° to 7.2°C during the first 8 h of refrigeration, two strains specifically showed significant growth between 8 and 24 h of refrigeration. This observation suggests the possibility of an accommodation to these temperatures by some *S. enteritidis* isolates that facilitated a continuation or resumption of multiplication (although at a vastly slower pace than during warm-temperature incubation).

Significant differences between individual *Salmonella* strains have been previously noted in both their growth properties in eggs (Gast and Holt, 2001b; Cogan *et al*., 2004) and in their ability to migrate across yolk membranes (Gast *et al*., 2007a; Gantois *et al*., 2008). In the present study, the observed population sizes in egg yolk samples differed among the 8 *S. enteritidis* strains after the initial incubation phase and after each measured interval of refrigeration. However, no direct relationship was evident between the apparent abilities

of individual strains to grow at 37 and 7.2°C. As in prior research (Gast and Holt, 2001b), the phage types of isolates did not consistently predict their growth properties in eggs. The deposition and growth of *S. enteritidis* in eggs are influenced by both the expressed phenotypic properties of this pathogen and by the susceptibility to infection of its avian host. In oral infection studies, the number of *S. enteritidis* cells administered to hens can significantly affect not only the frequency of resulting egg contamination, but also its location (Gast *et al*., 2013). Both *S. enteritidis* penetration into and multiplication inside egg yolks varied significantly between eggs from several genetically distinct commercial lines of laying hens (Gast *et al*., 2010b). Expression of very long O-antigen, perhaps by enhancing both reproductive tract colonization and survival in forming eggs, differentiates egg-contaminating *S. enteritidis* isolates from other environmental salmonellae (Guard-Bouldin *et al*., 2004; Coward *et al*., 2013). *S. enteritidis* strains which were sensitive to both acidic and oxidative stress were impaired both in their survival and growth properties in egg albumen and in their ability to infect chickens (Shah *et al*., 2012). Isolates of *S. enteritidis* were observed to survive in albumen more often than strains of other serotypes (De Vylder *et al*., 2012).

Although *in vitro* models may not exactly simulate naturally occurring egg contamination, bacterial growth behavior in these experiments documents a potential for similar outcomes (with corresponding public health consequences) in commercially produced eggs. The results of the present study support the vital importance of prompt egg refrigeration for protecting consumers by minimizing the number of bacterial contaminants in marketed table eggs, although refrigeration at 7.2°C may not immediately or completely arrest further multiplication by all *S. enteritidis* strains. Refrigerating eggs promptly after collection has been repeatedly recommended as one of the most effective practices for reducing the risk of egg-associated disease transmission (Mumma *et al*., 2004; Schroeder *et al*., 2006). The current national regulatory plan for commercial shell egg production in the USA requires refrigeration at an ambient temperature of 7.2°C within 36 h after laying, although eggs can later be equilibrated back to room temperature before processing (U.S. Food and Drug Administration, 2009). Some previous research has suggested that even a relatively brief interval of unrefrigerated storage may be sufficient for *S. enteritidis* to either multiply on the outside of yolk membranes or penetrate into the nutrient-dense yolk contents (Gast *et al*., 2007a, 2008).

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