Differences in the Multiplication of *Salmonella enteritidis* Strains in Liquid Whole Egg: Implications for Detecting Contaminated Eggs from Commercial Laying Flocks

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ABSTRACT

Bacterial culturing of eggs for *Salmonella enteritidis* has become an important tool in efforts to identify laying flocks that potentially threaten public health. As pools of egg contents are generally incubated before culturing to allow *S. enteritidis* numbers to multiply to easily detectable levels, any differences in the multiplication of *S. enteritidis* strains in egg pools could result in similar differences in the likelihood of detection. To assess whether 12 *S. enteritidis* strains would multiply to reach different final levels in pools of egg contents, 100-mL samples of liquid whole egg were experimentally contaminated with <10 cfu of the various strains. After incubation for 24 h at 37 C, the number of colony-forming units of *S. enteritidis* in each pool was determined. Significant differences were observed between strains in the extent of expansion of the *S. enteritidis* population during incubation (some strains grew to levels more than a thousand times greater than others). Iron supplementation of the pools during incubation significantly increased *S. enteritidis* growth and reduced the extent of variation between strains.

(Key words: *Salmonella enteritidis*, chicken, eggs, culture, public health)

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INTRODUCTION

The contamination of eggs by *Salmonella enteritidis* has been identified as a significant cause of human disease outbreaks in recent years (Centers for Disease Control, 1993). In an investigation of three such outbreaks, the only *S. enteritidis* phage type that was found in eggs from the implicated laying flocks was also isolated from infected humans (Henzler *et al.*, 1994). Culturing eggs for *S. enteritidis* has thus been recommended as an important component in programs to detect laying flocks that potentially threaten public health (Mason, 1994). The selection and application of effective bacteriological culturing methods for this effort are constrained by the very low incidence at which eggs contaminated by *S. enteritidis* are produced and by the small numbers of *S. enteritidis* cells found in most contaminated eggs (Humphrey *et al.*, 1991; Gast and Beard, 1992). Under these circumstances, culturing to detect *S. enteritidis* in eggs can attain a satisfactory level of sensitivity only by sampling many eggs. To process such large sample sizes without overwhelming the resources of laboratory facilities, the contents of 10 or 20 eggs are often pooled together for testing (Gast, 1993a,b).

Pooling eggs for culturing, however, further dilutes the already small number of *S. enteritidis* cells initially present in contaminated eggs and thereby makes detecting these cells even more difficult. Incubating egg pools for 1 or more d before culturing to allow the *S. enteritidis* population to expand to more easily detectable levels has been shown to significantly improve the sensitivity of testing (Gast, 1993a). Some proposals for testing eggs have advocated using a relatively rapid and inexpensive method in which pooled samples, after a prelimi-
TABLE 1. *Salmonella enteritidis* isolates used to inoculate liquid whole egg samples

<table>
<thead>
<tr>
<th>Isolate designation</th>
<th>Phage type</th>
<th>Source (State)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>13a</td>
<td>Egg (PA)</td>
</tr>
<tr>
<td>B</td>
<td>13a</td>
<td>Human stool</td>
</tr>
<tr>
<td>C</td>
<td>13</td>
<td>Chicken viscera (TX)</td>
</tr>
<tr>
<td>D</td>
<td>8</td>
<td>Chicken environment (ME)</td>
</tr>
<tr>
<td>E</td>
<td>13</td>
<td>Chicken viscera (TX)</td>
</tr>
<tr>
<td>F</td>
<td>14b</td>
<td>Chicken environment (MD)</td>
</tr>
<tr>
<td>G</td>
<td>8</td>
<td>Chicken spleen (PA)</td>
</tr>
<tr>
<td>H</td>
<td>8</td>
<td>Egg yolk (PA)</td>
</tr>
<tr>
<td>I</td>
<td>13a</td>
<td>Chicken environment (ME)</td>
</tr>
<tr>
<td>J</td>
<td>14b</td>
<td>Chicken ovary (ME)</td>
</tr>
<tr>
<td>K</td>
<td>14b</td>
<td>Egg follicle (ME)</td>
</tr>
<tr>
<td>L</td>
<td>8</td>
<td>Chicken ovary (ME)</td>
</tr>
</tbody>
</table>

*Salmonella enteritidis* isolates were provided by C. Benson, University of Pennsylvania, Kennett Square, PA 19348, S. Glass, Texas Poultry Diagnostic Laboratory, Gonzales, TX 78629, H. Opitz, University of Maine, Orono, ME 04469, G. Stein, Maryland Department of Agriculture, Salisbury, MD 21801, and M. St. Louis, Centers for Disease Control, Atlanta, GA 30333.

Secondary incubation period to increase the concentration of *S. enteritidis* cells, are transferred directly onto plates of appropriate selective agar media without going through the conventional broth enrichment steps (USDA, 1993). However, the results of recent studies have indicated that this direct plating approach is far less sensitive than broth enrichment methods for detecting *S. enteritidis* in eggs (Gast, 1993c). Direct plating appears to be unreliable for detecting fewer than 10^5 *S. enteritidis* cells per milliliter of liquid egg contents (Gast and Holt, 1995). Supplementing incubating egg pools with a source of iron has been demonstrated to promote the rapid growth of small initial numbers of *S. enteritidis* to higher levels, and thereby to significantly improve the probability of detection by direct plating (Gast and Holt, 1994).

The extent of *S. enteritidis* multiplication during the incubation of egg pools appears to be the pivotal factor in determining the likelihood that contamination will be detected by culturing, especially when using relatively insensitive methods such as direct plating. However, if *S. enteritidis* strains differ in their growth rates in egg pools, the probability that a small initial level of contamination will grow to a consistently detectable level after incubation will not be the same for all strains. Incubation and culturing strategies for detecting *S. enteritidis* in eggs must account for any such differences in order to ensure that any strain that might be present will be detected. The objectives of the present study were to determine whether various *S. enteritidis* strains would multiply to reach different final levels during 24 h of incubation in liquid whole egg and to assess whether these differences could affect the sensitivity of detection of *S. enteritidis* in pools of egg contents.

**MATERIALS AND METHODS**

**Salmonella enteritidis Cultures**

All 12 *S. enteritidis* isolates tested (Table 1) were originally stored at -20 C on Protect cryopreservative storage beads. Each strain was prepared for use by transferring one frozen storage bead into 9 mL of tryptone soya broth and incubating the culture for 24 h at 37 C. Preliminary investigations indicated that broth cultures of all 12 isolates prepared in this manner consistently reached the early stationary phase of growth (data not shown). Each culture was adjusted by dilution in .85% sterile saline to contain approximately 5 cfu/mL.
Determining Differences in the Multiplication of Salmonella enteritidis Isolates in Liquid Whole Egg

Liquid whole egg samples (100 mL each) were prepared in four replicate trials by mixing the contents of eggs from our laboratory’s specific-pathogen-free Single Comb White Leghorn flock in a Stomacher Model 400 Lab Blender. For all replicate trials combined, 16 egg samples were inoculated with each of the 12 S. enteritidis cultures and 8 uninoculated egg samples were held as negative controls. Samples were inoculated by the addition of 1 mL of diluted broth culture containing approximately 5 S. enteritidis cfu. All samples were mixed by stomaching for 30 s and incubated 24 h at 37 C. The number of S. enteritidis colony-forming units present after incubation was then determined by performing serial 10-fold dilutions of each egg sample in sterile saline and spreading aliquots from each dilution onto plates of brilliant green agar supplemented with .02 mg/mL novobiocin. The agar plates were incubated for 24 h at 37 C and S. enteritidis colonies were identified (Mallinson and Snoeyenbos, 1989) and counted.

Three S. enteritidis strains (A, F, and K in Table 1) that multiplied to different levels in liquid whole egg were then employed to evaluate the effects of supplementing the incubating egg samples with iron on the patterns of S. enteritidis growth in two replicate trials. For both trials combined, 16 liquid whole egg samples were inoculated with each of the 3 S. enteritidis strains as described above. Two uninoculated samples were included as negative controls. All samples were supplemented with 6 mg/mL ferrous sulfate as an iron source (Gast and Holt, 1994) and then mixed, incubated, and tested for S. enteritidis as described above.

RESULTS AND DISCUSSION

Inoculum cultures of some S. enteritidis strains multiplied to reach significantly higher final levels than did those of other strains during incubation in liquid whole egg (Figure 1a). These differences, in some instances, were of more than 1,000-fold in magnitude. The mean log_{10} colony-forming units of S. enteritidis in inoculated egg samples after incubation without iron supplementation ranged from 3.9 for Strain A to 7.5 for Strain L. No S. enteritidis was recovered from uninoculated negative control samples. The postincubation level of S. enteritidis colony-forming units in samples inoculated with Strain A was lower (P < .05) than the corresponding levels in samples inoculated with Strains G, H, I, J, K, or L. In addition, the postincubation levels of S. enteritidis colony-forming units in samples inoculated with Strains B and C were lower (P < .05) than the corresponding levels in samples inoculated with Strains J, K, or L.

Rapid and extensive multiplication of S. enteritidis in egg yolks has been reported in previous studies (Bradshaw et al., 1990), but growth in samples of mixed yolk and albumen has generally been observed to be less prolific (Gast and Holt, 1995). The results of the present study suggest that the ability to grow rapidly in liquid whole egg is a characteristic that varies among S. enteritidis strains. If the various strains also grow at different rates in intact eggs or egg-containing foods, the likelihood that S. enteritidis multiplication will produce a high infecting dose before ingestion by humans may similarly depend on which strain is present. The extent of multiplication of particular S. enteritidis strains in egg samples in the present study could not be predicted on the basis of either their phage types or their original sources.

Statistical Analysis

Significant differences (P < .05) between the mean log_{10} colony-forming units of S. enteritidis in egg samples after incubation associated with different S. enteritidis strains or different incubation treatments were determined by a one-way analysis of variance (Snedecor and Cochran, 1980). As no significant variation was observed between replicate trials, the results were combined for analysis.
FIGURE 1. Mean \( \log_{10} \) colony-forming units per milliliter of various Salmonella enteritidis strains after incubation of inoculated liquid whole egg samples for 24 h at 37°C. a) For each of 12 S. enteritidis strains described in Table 1, 16 egg samples were inoculated with <10 cells. Standard deviations ranged from .8 to 1.5 \( \log_{10} \) cfu/mL. b) For each of three S. enteritidis strains described in Table 1, 16 egg samples were supplemented with 6 mg/mL ferrous sulfate and inoculated with <10 cells. Standard deviations ranged from .1 to .8 \( \log_{10} \) cfu/mL.

of isolation (Table 1). Humphrey (1990) reported that S. enteritidis isolates of several phage types grew at similar rates in eggs incubated at temperatures of 12 to 15°C.

When liquid whole egg samples in the present study were supplemented with iron, no differences between strains were observed in the number of S. enteritidis cells after incubation (Figure 1b). The final mean \( \log_{10} \) colony-forming units of S. enteritidis in such samples ranged only from 8.0 for Strain A to 8.8 for Strain F. The postincubation levels of S. enteritidis in iron-supplemented egg samples inoculated with Strains A, F, and K were all greater (\( P < .05 \)) than the comparable levels in samples inoculated with the same strains and incubated without iron supplementation in the earlier phase of the experiment.

During incubation without iron supplementation, the small initial numbers of S. enteritidis colony-forming units multiplied to reach the cell density (10⁵ per milliliter) necessary for efficient detection by direct plating in 44, 63, and 81% of samples inoculated with Strains A, F, and K, respectively. In contrast, during incubation with iron supplementation, this critical threshold level for detection was reached by 94% of samples inoculated with Strain A and all samples inoculated with Strains F and K. Previous investigations have similarly indicated that iron supplementation of inoculated egg samples can increase the growth of Salmonella (Clay and Board, 1991; Gast and Holt, 1995).

From the public health perspective, culturing methods should be capable of detecting any S. enteritidis strains present as contaminants in eggs. The multiplication of some S. enteritidis strains during incubation in liquid whole egg in the present study was insufficient to permit consistent detection of contamination by relatively insensitive methods such as direct plating. However, the supplementation of incubating egg pools with a surplus of iron significantly increased S. enteritidis multiplication and reduced the extent of variation in growth between S. enteritidis strains. Therefore, although iron supplementation during incubation was not necessary to ensure the efficient recovery of some S. enteritidis strains from egg samples, other strains would have seldom been detected without it.

ACKNOWLEDGMENT

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REFERENCES

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