Research Note

Multiplication of Salmonella Enteritidis in Egg Yolks after Inoculation outside, on, and inside Vitelline Membranes and Storage at Different Temperatures

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ABSTRACT

Prompt refrigeration to restrict bacterial growth is important for reducing eggborne transmission of Salmonella enterica serovar Enteritidis (SE). The nutrient-rich yolk interior is a relatively infrequent location for initial SE deposition in eggs, but migration across the vitelline membrane can result in rapid bacterial multiplication during storage at warm temperatures. The objective of the present study was to measure the multiplication of SE in yolks after introduction at three different locations and subsequent storage at a range of temperatures. Using an in vitro egg contamination model, approximately 100 CFU of SE was inoculated either inside yolks, onto the exterior surface of vitelline membranes, or into the adjacent albumen. After storage of samples from each inoculation group at 10, 15, 20, and 25°C for 24 h, SE was enumerated in yolks. For all three inoculation locations, the final SE levels in yolks increased significantly with increasing storage temperatures. At all storage temperatures, significant differences in SE multiplication were observed between inoculation sites (yolk inoculation > vitelline membrane inoculation > albumen inoculation). At 25°C, final log concentrations of 7.759 CFU of SE per ml (yolk inoculation), 2.014 CFU/ml (vitelline membrane inoculation), and 0.757 CFU/ml (albumen inoculation) were attained in yolks after storage. These results demonstrate that, even when the initial site of SE deposition is outside the egg yolk, substantial multiplication supported by yolk nutrients can occur during the first day of storage and the risk of bacterial growth increases at higher ambient storage temperatures.

Egg-associated human salmonellosis has been an important international public health problem for more than 20 years (2, 19, 32), even though substantial public and commercial resources have been invested in microbial quality assurance programs (28). Despite an estimated prevalence of contamination in commercially produced table eggs of only 0.005% (7), more than 100,000 annual human illnesses have been attributed to egg-transmitted Salmonella enterica serovar Enteritidis (SE) in the United States (36). Risk assessment studies by the U.S. Department of Agriculture have determined that refrigeration is one of the most effective intervention options for mitigating the consequences of SE contamination in eggs (26, 35). Freshly laid eggs typically contain no more than a few hundred SE cells (4, 8, 14, 23), so prompt refrigeration can prevent extensive bacterial multiplication during storage, which could increase the threat of illness for consumers. Most risk reduction plans incorporate provisions for egg refrigeration (28), including recently published federal regulations for shell egg producers in the United States, which require eggs to be cooled to an ambient temperature of 7.2°C or lower within 36 h after laying (37). The effectiveness of egg refrigeration for preventing the growth of small populations of SE 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 reached.

The initial site of SE deposition inside eggs is more commonly located in the albumen or on the outside of the vitelline (yolk) membrane than within the nutrient-rich interior of the yolk (10, 16). However, previous studies using in vitro egg contamination models have shown that SE can migrate across yolk membranes and begin to multiply inside the yolk contents during the first day of storage at warm temperatures (3, 11, 15, 18, 30). These prior investigations have not determined how storage temperature affects SE growth in egg yolks after initial deposition at different locations relative to the vitelline membrane. The objective of the present study was to measure the multiplication of SE in egg yolks after in vitro introduction at three different sites (inside yolks, on the exterior of vitelline membranes, and in the adjacent albumen) and subsequent storage at a range of temperatures.

MATERIALS AND METHODS

Preparation of SE culture. A phage type 13a SE isolate, originally isolated from the yolk of a contaminated egg, was resuscitated by transfer into tryptone soya broth (Oxoid Ltd., Basingstoke, Hampshire, UK) for two successive cycles of 24-h
incubation at 37°C. This culture was centrifuged for 10 min at 3,000 × g to concentrate cells, washed with 0.85% saline, centrifuged again, and resuspended in saline. After the cell concentration of the 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 this value yielded equivalent results when using either nonselective Trypticase soy agar or selective (and differential) brilliant green agar (BD, Franklin Lakes, NJ).

Preparation, inoculation, and storage of egg samples. Freshly collected eggs from the specific-pathogen-free flock of Single Comb White Leghorn chickens at the Southeast Poultry Research Laboratory (Athens, GA) were aseptically broken, their contents (yolk and albumen) were separated, and each yolk was transferred into the bottom of a sterile 50-ml plastic centrifuge tube. The egg samples were randomly allocated to three treatment groups and were each inoculated with 0.1 ml of SE in saline (containing approximately 100 CFU) by using a pipette and sterile tip. This inoculum size was intended to simulate a substantial (yet still realistic) level of contamination. In the first group, SE was injected inside the interior contents of each yolk and the previously removed albumen was discarded. In the second group, SE was inoculated onto the exterior surface of each vitelline (yolk) membrane, the yolks were then held for 5 min at room temperature (approximately 24°C), and the albumen of each egg was gently poured back into the corresponding yolk tube. In the third group, the albumen was added back to each yolk tube and the SE inoculum was then introduced into the albumen at an approximate distance of 3 mm from the vitelline membrane.

In each of two replicate trials, 48 egg samples were inoculated at each of the three locations. Equal portions of the samples from each inoculation group were then stored for 24 h at each of four temperatures: 10, 15, 20, or 25°C. Six un inoculated negative control samples in each trial were stored at 25°C for 24 h. An additional six samples per trial (inoculated onto the vitelline membrane) were tested immediately after preparation to serve as positive controls for prestorage SE concentration.

Enumeration of SE from egg yolks after storage. The albumen portion of each stored egg sample was poured out for disposal, and the remaining yolk was transferred into a sterile plastic bag and mixed by stomaching for 1 min. The concentration of SE in each yolk sample was determined by making 10-fold dilutions in 0.85% saline and spreading aliquots of each dilution (including a total of 1.0 ml of 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 (38) that randomly selected colonies (representing each positive sample) were always SE validated the visual observation that only the inoculum strain was present on these agar plates. The detection threshold of this procedure was 1 CFU/ml. All samples (positive and negative) were included in the calculation of mean SE concentrations.

Statistical analysis. Significant differences (P < 0.05) between treatment groups (inoculation location or storage temperature) in the frequency of SE isolation from yolk samples after storage were determined by applying Fisher’s exact test, and significant differences (P < 0.05) between treatment groups in the mean concentration of SE cells in yolk samples after storage were determined by applying the Kruskal-Wallis 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).

RESULTS AND DISCUSSION

For all three egg inoculation sites and at all four temperatures, SE was detected in association with yolks after storage for 24 h (Table 1). Initial bacterial introduction inside yolks resulted in 100% poststorage recovery of SE at all temperatures. Recovery of SE from yolks after introduction onto vitelline membranes increased significantly (P = 0.0006) from 58.3% after 10°C storage to 100% at 20°C or higher. Likewise, recovery of SE from yolks following introduction into albumen increased significantly (P = 0.0001) from 25.0% after 10°C storage to 83.3% at 25°C. The frequency of SE recovery from samples inoculated either into yolks or onto vitelline membranes was significantly greater (P ≤ 0.0016) than from samples inoculated into albumen after storage at 20°C or less, but SE recovery associated with yolk inoculation exceeded that obtained with membrane inoculation only at 10°C (P = 0.0006). None of the uninoculated negative control samples were positive for SE after incubation.

Among the preincubation positive control samples, the mean log concentration of SE in whole yolks immediately after inoculation was 0.338 CFU/ml. After introduction inside yolks, the mean log concentration of SE after 24 h of storage increased significantly (P < 0.0001) with each 5°C rise in temperature, ranging from 1.048 CFU/ml at 10°C to 7.759 CFU/ml at 25°C (Table 1). A similar pattern of consistently significant (P ≤ 0.0005) differences between temperatures was also observed following introduction of SE onto vitelline membranes, with mean log concentrations in yolks after storage ranging from 0.104 CFU/ml at 10°C to 2.01 CFU/ml at 25°C. After introduction into albumen, the mean log SE concentration in yolks increased significantly (P ≤ 0.0327) as storage temperatures increased above 15°C, with values ranging from 0.045 CFU/ml at 10°C to 0.757 CFU/ml at 25°C. The mean log concentration of SE detected after inoculation inside yolks was significantly (P < 0.0001) greater than for inoculation either onto vitelline membranes or into albumen at all storage temperatures. Also, the mean log concentration of SE in yolks following inoculation onto vitelline membranes was significantly (P ≤ 0.0004) greater than for inoculation into albumen at storage temperatures of 15°C and higher.

The initial site of bacterial deposition in eggs plays a major role in determining how quickly growth-inhibiting temperatures must be achieved by refrigeration in order to protect consumers. Contamination of the egg yolk or albumen by SE results from the colonization of different regions of the reproductive tract in systemically infected laying hens (1, 9, 13, 14). Although SE is able to survive or multiply slowly in the presence of inhibitory components in egg albumen (5, 6, 20, 24, 31), the abundant nutrients in egg yolks can promote rapid and prolific growth (3, 15, 21, 22). Even when SE is initially deposited outside the yolk, migration through the vitelline membrane to reach the yolk contents can eventually result in rapid multiplication (3, 15, 30). In the present study, the site of in vitro SE introduction
TABLE 1. Isolation and enumeration of Salmonella Enteritidis (SE) from egg yolk samples

<table>
<thead>
<tr>
<th>Inoculation site</th>
<th>No. of SE-positive yolk samples/total no. of samples at postinoculation storage temp of:</th>
<th>SE concn in yolk samples (mean log CFU/ml) at postinoculation storage temp of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10°C</td>
<td>15°C</td>
</tr>
<tr>
<td>Vitelline membrane</td>
<td>14/24 B</td>
<td>20/24 AB</td>
</tr>
<tr>
<td>Albumen</td>
<td>6/24 C</td>
<td>8/24 CD</td>
</tr>
</tbody>
</table>

*Samples were taken from entire yolks (including vitelline membranes) after inoculation of eggs with approximately 10^5 CFU and storage for 24 h. Egg samples were inoculated either into the interior yolk contents, onto the exterior surface of the vitelline membrane, or into the albumen (approximately 3 mm from the vitelline membrane). Values (within columns or rows) that share no common letters differ significantly (P < 0.05).*
storage and that higher storage temperatures increase SE growth in yolks regardless of the initial site of introduction. This information reinforces the critical importance of prompt egg refrigeration for protecting consumers. Minimizing the interval following oviposition during which eggs remain at warm temperatures can directly contribute to minimizing SE levels in marketed table eggs. Although in vitro egg contamination models are of uncertain predictive correspondence to naturally contaminated eggs, the occurrence of bacterial growth in experimental models documents the potential for similar outcomes with significant public health implications in commercially produced eggs.

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