Research Note

Effect of Refrigeration on In Vitro Penetration of *Salmonella* Enteritidis through the Egg Yolk Membrane

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ABSTRACT

Internally contaminated eggs have been implicated as leading sources of transmission of *Salmonella* Enteritidis (SE) to humans. Although SE is not often deposited inside the nutrient-rich yolks of naturally contaminated eggs, penetration through the vitelline membrane to reach the yolk contents could result in rapid bacterial multiplication. In previous studies, such penetration has been observed occasionally at warm temperatures during experiments with in vitro egg contamination models. The present study was conducted to determine whether refrigeration affects the frequency of in vitro SE penetration of the egg yolk membrane. After inoculation of small numbers of SE onto the outside of the vitelline membranes of intact yolks, immediate refrigeration of contaminated samples prevented the penetration of SE into the egg yolk contents during 24 h of storage. However, SE penetrated inside the yolk contents in 4% of contaminated egg samples refrigerated after 2 h of storage at 30°C, 15% of samples refrigerated after 6 h of storage at 30°C, and 40% of samples stored at 30°C for 24 h (48 samples per treatment group). These results highlight the value of prompt refrigeration for restricting the opportunities for SE to multiply to high numbers inside the yolks of contaminated eggs.

The contamination of eggs with *Salmonella* Enteritidis (SE) was responsible for an estimated 182,060 human illnesses in the United States in 2000 (28) and has been an important international public health issue for nearly two decades (8, 27). Refrigeration has been widely recommended as one of the pivotal strategies for minimizing the risk to consumers from SE in eggs (1, 5, 6, 13, 18, 22, 26). In experimentally and naturally infected hens, SE can lay eggs contaminated internally with SE (10, 14, 21). The contents of freshly laid eggs are reported to seldom harbor more than a few hundred SE cells (4, 11, 14, 23), so prompt refrigeration of contaminated eggs reduces the likelihood of SE multiplication within 7.2°C or lower. Nevertheless, even if the initial site of SE deposition is on the exterior surface of the yolk membrane or in adjacent regions of the albumen, bacterial penetration through the yolk membrane could still result in rapid and extensive multiplication in the yolk contents. Migration of SE through the vitelline membrane has been previously reported to occur at warm temperatures in an assortment of in vitro contamination models (3, 13, 16–18, 22, 26), although seldom at high frequencies. Declining vitelline membrane integrity during nonrefrigerated storage could facilitate bacterial penetration (5). However, the extent to which egg refrigeration affects the movement of SE through the yolk membrane has not been demonstrated in prior research. The objective of the present study was to determine the effect of refrigeration at 7°C on the ability of small numbers of SE to migrate through the egg yolk membrane in an in vitro contamination model. Different storage periods at 30°C before transfer to 7°C were employed to determine how rapidly bacterial penetration into the yolk occurred when eggs remained at a warm ambient temperature prior to refrigeration.

MATERIALS AND METHODS

Preparation of SE cultures. Two SE cultures (phage types 8 and 13a, both originally isolated from eggs laid by infected

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Preparation, inoculation, and incubation of egg samples. Freshly collected eggs obtained 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. Each yolk was then inoculated with SE with a pipette that dispensed 0.1 ml of the appropriate broth culture (containing approximately 100 CFU) onto the exterior surface of the vitelline membrane. After the inoculated yolk samples were held for 5 min at room temperature (approximately 24°C) to facilitate bacterial attachment to the external surface of yolk membranes, the albumen from a single egg was poured gently into each tube. Ninety-six egg samples were inoculated with the phage type 8 SE strain, and 96 were inoculated with the phage type 13a strain; 8 uninoculated samples were retained as negative controls. After preparation, 24 of the samples inoculated with each strain were stored for 24 h at 7°C, 24 samples were stored for 2 h at 30°C and then for 22 h at 7°C, 24 samples were stored for 6 h at 30°C and then for 18 h at 7°C, and 24 samples were stored for 24 h at 30°C.

Enumeration of SE inside egg yolks after incubation. Each incubated egg sample was poured out into a sterile plastic petri dish. A small area of the yolk membrane was seared with a flame-heated steel spatula to destroy any surface bacteria present in that region. A sterile syringe was then inserted through the seared area of the membrane to remove 5 ml of interior yolk contents (free of membrane material), typically representing approximately 35 to 40% of total yolk volume. The concentration of SE in the yolk contents was determined by making 10-fold dilutions of each sample in 0.85% saline and spreading 0.1-ml aliquots of the undiluted yolk and each dilution onto plates of brilliant green agar (Becton Dickinson, Sparks, Md.) supplemented with 0.02 mg/ml novobiocin (Sigma Chemical Co., St. Louis, Mo.). The agar plates were incubated for 24 h at 37°C, and typical SE colonies were counted. The identity of suspected SE colonies was confirmed biochemically and serologically (31). The detection threshold of this procedure was 10 CFU/ml.

Statistical analysis. Significant differences (P < 0.05) between treatment groups in the frequency of SE detection inside egg yolks after incubation were determined by applying Fisher’s exact test, and significant differences (P < 0.05) between groups in the mean concentration of SE cells inside egg yolks after incubation were determined by applying the Kruskal-Wallis test. Data were analyzed with Instat biostatistics software (GraphPad Software, San Diego, Calif.).

RESULTS AND DISCUSSION

Both SE strains were able to penetrate the egg yolk vitelline membrane into the interior of the yolk during as little as 2 h at 30°C following inoculation onto the exterior surfaces of the membrane (Table 1). No penetration through the yolk membrane was observed in samples that were held for 24 h at 7°C. The frequency of isolation of SE from yolk contents increased along with the duration of storage at 30°C before refrigeration, reaching a peak of 39.6% (for both strains combined) in samples held at 30°C for the entire 24-h storage period. Significantly less frequent penetration through the yolk membrane (P < 0.01) was observed for samples that were refrigerated for some portion of the storage period. Similarly, the mean log concentration of SE cells found inside egg yolks was significantly higher (P < 0.01) in samples held for 24 h at 30°C than for samples that were refrigerated for any part of the storage period. Nevertheless, even after 24 h of storage at 30°C, the mean concentration of SE (for both strains combined) only reached 1.367 log CFU/ml. None of the uninoculated negative control samples were positive for SE after storage.

Infected hens often have been reported to deposit SE in the albumen or on the outside of the vitelline membrane of eggs, but deposition of this pathogen inside the nutrient-rich contents of the yolk seems to occur much more rarely (12, 16). Although slow multiplication of SE has sometimes been noted in the albumen or on the outside of the vitelline membrane (5, 9), migration into the yolk contents could allow the bacteria to multiply extensively before growth-inhibiting internal temperatures were achieved by refrigeration. Penetration of SE through yolk membranes at warm temperatures has been observed previously in diverse in vitro egg contamination models (3, 13, 16–18, 22). Individual SE strains can differ significantly in both their growth properties in eggs (7, 15) and their ability to penetrate yolk membranes (17).

In the present study, the relatively modest degree of SE multiplication detected in the nutritionally abundant yolk contents (even after 24 h at 30°C) indicates that migration into the yolk proceeded slowly during this first day of storage. The typically small numbers of SE cells found inside freshly laid naturally contaminated eggs (23) likewise suggests that penetration into the yolk contents may

<table>
<thead>
<tr>
<th>SE strain</th>
<th>Storage temp and duration</th>
<th>No. of SE-positive yolk samples/total no. of samples</th>
<th>SE concn (log CFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phage type 8</td>
<td>7°C (24 h)</td>
<td>0/24 A</td>
<td>&lt;0.040 A</td>
</tr>
<tr>
<td></td>
<td>30°C (2 h) + 7°C (22 h)</td>
<td>1/24 A</td>
<td>0.062 A</td>
</tr>
<tr>
<td></td>
<td>30°C (6 h) + 7°C (18 h)</td>
<td>2/24 A</td>
<td>0.164 A</td>
</tr>
<tr>
<td></td>
<td>30°C (24 h)</td>
<td>10/24 B</td>
<td>1.542 B</td>
</tr>
<tr>
<td>Phage type 13a</td>
<td>7°C (24 h)</td>
<td>0/24 A</td>
<td>&lt;0.040 A</td>
</tr>
<tr>
<td></td>
<td>30°C (2 h) + 7°C (22 h)</td>
<td>1/24 AB</td>
<td>0.067 A</td>
</tr>
<tr>
<td></td>
<td>30°C (6 h) + 7°C (18 h)</td>
<td>5/24 BC</td>
<td>0.361 AB</td>
</tr>
<tr>
<td></td>
<td>30°C (24 h)</td>
<td>9/24 C</td>
<td>1.190 B</td>
</tr>
<tr>
<td>Both strains</td>
<td>7°C (24 h)</td>
<td>0/48 A</td>
<td>&lt;0.040 A</td>
</tr>
<tr>
<td></td>
<td>30°C (2 h) + 7°C (22 h)</td>
<td>2/48 AB</td>
<td>0.064 A</td>
</tr>
<tr>
<td></td>
<td>30°C (6 h) + 7°C (18 h)</td>
<td>7/48 AB</td>
<td>0.282 A</td>
</tr>
<tr>
<td></td>
<td>30°C (24 h)</td>
<td>19/48 C</td>
<td>1.367 B</td>
</tr>
</tbody>
</table>

* Within each column and each strain grouping, means followed by different letters are significantly different (P < 0.05).
not normally occur rapidly or at a high frequency. Entry into the yolk could become more likely over time as albumen viscosity and vitelline membrane integrity decline, especially at elevated temperatures (19, 22, 24, 25). The significant increase in SE numbers in the current study after 24 h of storage at 30°C (in comparison to shorter periods at the same temperature) suggests that more rapid bacterial growth had begun by that point.

Egg refrigeration has been identified in risk assessment studies as a pivotal tool for preventing eggborne transmission of SE to consumers (29). Mandatory egg refrigeration to an ambient temperature of 7.2°C is one of the cornerstones of a recently proposed regulatory plan for shell egg production in the United States (30). The effectiveness of refrigeration for limiting bacterial multiplication in eggs depends on the initial level and location of bacterial contamination, the movement of bacteria or nutrients within eggs during storage, and the rate at which growth-restricting temperatures are achieved. Rapid refrigeration of freshly laid eggs reduces opportunities for the expansion of small populations of microbial pathogens to more dangerous levels (4), but the possible effects of cold temperatures on the migration of SE into the nutrient-rich yolk contents have been unclear. In a recent experiment, refrigeration at 4°C led to better retention of yolk membrane integrity against physical rupture than was observed at warmer temperatures (5).

The results of the present study indicate that refrigeration can significantly reduce the migration of SE across the vitelline membrane to the interior contents of egg yolks. Neither SE strain used in this experiment was able to penetrate into yolks during storage at 7°C, and refrigeration apparently stopped any further penetration following initial storage at 30°C. Even after 6 h of initial storage at 30°C, refrigeration resulted in a lower frequency of SE penetration into yolks (and a correspondingly lower final concentration of contaminants inside those yolks) than was seen in eggs held at 30°C for 24 h. Thus, prompt refrigeration of eggs from infected laying hens is important for preventing extensive bacterial multiplication inside nutrient-rich yolks, even when the pathogen is initially deposited on the exterior surface of the vitelline membrane or in adjacent regions of the albumen.

REFERENCES

5. Chen, J., H. S. Thesmar, and W. L. Kerr. 2005. Outgrowth of salmonellae and the physical property of albumen and vitelline mem-


