Research Note

Salmonella Penetration through Eggshell Associated with Freshness of Laid Eggs and Refrigeration

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ABSTRACT

Effects of egg age after laying and refrigeration on penetration of the eggshell by Salmonella enteritidis (SE) and Salmonella typhimurium (ST) were examined. Eggs 0.25 to 3 h, 3.25 to 6 h, 1 day, and 7 days old held at two temperatures were immersed in SE or ST suspensions containing 10^8 or 10^6 CFU/ml at 25°C for 10 min. After holding at 25°C for 2 h, the inner eggshell and egg contents were examined for Salmonella cells. The recovery rates of Salmonella cells from both the inner eggshell and egg contents of the 0.25- to 3-h-old eggs were significantly higher than those of other groups, especially at the high-exposure dose. There was no significant difference noted between SE and ST in ability to penetrate through eggshell. Salmonella penetration was significantly decreased by cooling the eggs at 4°C for 15 min prior to immersing them in SE or ST suspension. The data suggested that Salmonella cells readily penetrated through the shell of freshly laid eggs, but that this penetration was suppressed by cooling the eggs before they were exposed to Salmonella suspensions.

Outbreaks of human salmonellosis caused by Salmonella enteritidis (SE) have dramatically increased during the past 10 years and have become an important international public health and economic issue (5, 16). Epidemiologic analyses suggested contaminated eggs or egg products as the major source of the infection (9). Routes of SE contamination in the egg have not been clearly understood. Two possible routes of Salmonella contamination of intact chicken eggs in hens have been considered: direct contamination of yolk or albumen originating from Salmonella infection of the reproductive organs before the eggs are covered by the shell (vertical transmission) (3, 10, 12, 18, 23), and Salmonella penetration through eggshell after the eggs are covered by the shell (horizontal transmission) (1, 8, 11, 20). Several reports suggested that the vertical route would be important in SE contamination, and the horizontal route would be important in Salmonella spp. other than SE. However, no decisive evidence of the route of SE contamination of eggs has been obtained.

An understanding of the causes that are contributing to the dramatically increased food poisoning by SE relative to other Salmonella spp. is essential to reducing the public health risk associated with consumption of foods containing contaminated eggs. Many studies (2, 6, 14, 15, 17, 19, 20, 22, 24) have reported that Salmonella organisms are capable of penetrating the shell and multiplying within the contents of eggs. Sauter et al. (17) reported that eggs having specific gravity values of 1.070, 1.080, and 1.090 were challenged with 12 species of Salmonella, and eggshell penetration by the Salmonella spp. varied greatly depending on species and on shell quality. In their experiments, Salmonella typhimurium (ST) consistently penetrated a higher proportion of eggs of each shell quality than other species studied, but SE was not examined. The difference in permeability of eggshell to SE and ST has not been studied. It has been shown that there is rapid penetration through the outer egg structures by Salmonella cells with various exposure durations and incubation temperatures (24). Most of the penetration studies (15, 17, 19, 22, 24) have been performed with eggs several hours after laying. Only a few studies (7, 20) showed that the eggshell was susceptible to bacterial penetration within a very short time after laying because the cuticle was immature. Recently, much attention has been given to the fate of Salmonella cells that invaded eggs, but no detailed study has appeared on Salmonella penetration through the eggshell associated with the age of the hen’s eggs and development of a mature cuticle.

Catalano and Knabel (4) found that rapid cooling and subsequent storage at 7°C protected the eggs from SE penetration. On the other hand, Fajardo et al. (6) showed that rapid cooling of eggs was accompanied by an increase in the number and width of microscopic cracks in the shell and that the cooled eggs were more prone to penetration by SE. More information on the various factors influencing Salmonella penetration of the outer structure of eggs is needed to search for more effective methods for preventing the internal infection of eggs.

The present study was conducted to investigate the influence of post-lay age of egg and refrigeration on shell penetration by SE and ST, and to examine the relationship between eggshell permeability of SE and the increase in SE
food poisoning outbreaks relative to other Salmonella outbreaks.

MATERIALS AND METHODS

Experimental hens. White leghorn, Dekalb-TX35®, 50-week-old hens were used. No Salmonella spp. was found from cloacal swabs and feces of all birds before the study. Hens were raised in individual wire-floored cages in an air-conditioned isolation building with controlled artificial illumination and were provided with water and antibiotic-free layer-breeder ration (Nihonhaigo-shiryo Co., Ltd, Aichi, Japan) ad libitum.

Bacteria. SE phage type 4 and ST L-55, which were kindly supplied from the Osaka Prefectural Institute of Public Health and the National Institute of Animal Health in Tsukuba, respectively, were used. Bacteria were cultured in static Trypto soya broth (Eiken, Tokyo, Japan), homogenized, and incubated at 37°C for 24 h. After inoculation, eggs were held at 25°C for 2 h. The outside of the eggs was disinfected by dipping in 70% ethanol for 5 min and flaming over a burner until dry. Each egg was then cracked aseptically. The shell and its contents were placed in separate sterile WP bags (Nissui).

Eggs. Eggs were collected every 15 min from the cages. Cracked and feces-contaminated eggs were discarded. The eggs were handled aseptically during collection by using surgical gloves and sterile Nasco Whirl-Pak® (WP) bags. No Salmonella contamination was found from 50 eggs checked before the study.

Experimental design. Eggs were divided into 4 groups according to the egg age after laying. Eggs of groups 1, 2, 3, and 4 were kept at room temperature (about 22°C) for 0.25 to 3 h, 3.25 to 6 h, 1 day, and 7 days after laying. In order to induce horizontal contamination, 20 eggs of each group were immersed in SE or ST suspension containing 10^1 or 10^6 CFU/ml at 25°C for 10 min. After inoculation, eggs were held at 25°C for 2 h. The outside of the eggs was disinfected by dipping in 70% ethanol for 5 min and flaming over a burner until dry. Each egg was then cracked aseptically. The shell and its contents were placed in separate sterile WP bags containing 50 ml of Hajna tetrathionate (HT) broth (Eiken, Tokyo, Japan), homogenized, and incubated at 37°C for 24 h. After incubation, a loopful of broth culture was spread onto mannitol lysine crystal violet brilliant green (MLCB) agar plates (Nissui).

In the inner eggshell, 20 to 75% of eggs exposed to 10^1 CFU/ml of SE, 25 to 90% of eggs exposed to 10^6 CFU/ml of SE, 40 to 90% of eggs exposed to 10^1 CFU/ml of ST, and 40 to 100% of eggs exposed to 10^6 CFU/ml of ST were positive for SE or ST. The recovery rates of SE or ST from the inner eggshell in group 1 were higher than those in other groups. Especially, the recovery rates of SE or ST in group 1 exposed to 10^6 CFU/ml of SE or ST were highest.

RESULTS

Recovery rates of Salmonella spp. from inner eggshell and egg contents. Table 1 shows the recovery of SE or ST from the inner eggshell of eggs exposed to SE or ST. In the inner eggshell, 20 to 75% of eggs exposed to 10^1 CFU/ml of SE, 40 to 90% of eggs exposed to 10^6 CFU/ml of SE, 25 to 90% of eggs exposed to 10^1 CFU/ml of ST, and 40 to 100% of eggs exposed to 10^6 CFU/ml of ST were positive for SE or ST. The recovery rates of SE or ST from the inner eggshell in group 1 were higher than those in other groups. Especially, the recovery rates of SE or ST in group 1 exposed to 10^6 CFU/ml of SE or ST were highest.

Table 2 shows the recovery of SE or ST from the contents of eggs exposed to SE or ST. In the egg contents, 0 to 10% of eggs exposed to 10^1 CFU/ml of SE, 0 to 35% of eggs exposed to 10^6 CFU/ml of SE, 0 to 5% of eggs exposed to 10^1 CFU/ml of ST, and 5 to 40% of eggs exposed to 10^6 CFU/ml of ST were positive for SE or ST. The recovery rates of SE or ST in group 1 exposed to 10^6 CFU/ml of SE or ST were highest.

<p>| TABLE 1. Penetration of Salmonella enteritidis and S. typhimurium into the inner eggshell and effect of cooling the eggs before exposure to Salmonella suspensions on penetration |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Salmonella suspension (CFU/ml)</th>
<th>Cooling (+) at 4°C, 15 min or not (-)</th>
<th>Group 1 0.25-3 h</th>
<th>Group 2 3.25-6 h</th>
<th>Group 3 1 day</th>
<th>Group 4 7 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. enteritidis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10^3</td>
<td>-</td>
<td>15/20a (75%)</td>
<td>9/20ab (45%)</td>
<td>4/20a (20%)</td>
<td>9/20ab (45%)</td>
</tr>
<tr>
<td>10^6</td>
<td>+</td>
<td>4/20a (20%)</td>
<td>NT</td>
<td>1/20a (5%)</td>
<td>NT</td>
</tr>
<tr>
<td>S. typhimurium</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10^3</td>
<td>-</td>
<td>19/20a (95%)</td>
<td>8/20a (40%)</td>
<td>13/20a (65%)</td>
<td>12/20a (60%)</td>
</tr>
<tr>
<td>10^6</td>
<td>+</td>
<td>12/20a (60%)*</td>
<td>NT</td>
<td>11/20a (55%)</td>
<td>NT</td>
</tr>
<tr>
<td>a Values within rows with no common following letter differ significantly (P &lt; 0.05).</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>b An asterisk * indicates significant differences (P &lt; 0.05) compared with the recovery from the eggs exposed to same concentration of S. enteritidis and S. typhimurium without cooling.</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>c NT, not tested.</td>
<td></td>
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</tbody>
</table>

Effects of cooling on the recovery rates of *Salmonella* spp. from inner eggshell and egg contents. In group 1, the recovery rates of SE or ST from the inner eggshell after exposing the eggs to $10^3$ or $10^6$ CFU/ml of SE or ST with precooling were significantly lower than those without precooling (Table 1). In precooled eggs, no significant difference was found between groups 1 and 3 on the recovery rates of SE or ST from both inner eggshell and egg contents, when the eggs were exposed to the same concentration of each *Salmonella* sp.

**DISCUSSION**

The eggshell appeared to be more easily invaded by microorganisms immediately after the egg was laid (2, 14, 20). Two major factors affecting *Salmonella* penetration through eggshell may be involved when freshly laid eggs are exposed to *Salmonella* cells. One study (20) suggested that the cuticle of eggs does not mature for a short time after laying, and some pores in the shell may be open to *Salmonella* penetration. Another factor is that the natural cooling of freshly laid eggs may also assist bacterial penetration through the pores (2, 7). When eggs are placed in an environment cooler than the inside temperature of the egg, a negative pressure may develop (2). It is unknown if such a temperature differential would be enough to produce the negative pressure that allows the bacteria to penetrate through pores (2, 7, 14). In this study, the recovery rates of *Salmonella* spp. from both inner eggshell and contents of eggs within 3 h after laying were significantly higher than those from the eggs after 3 h of laying, especially in the high-exposure dose. Bacterial penetration through the eggshell occurs more readily in freshly laid eggs. The *Salmonella* penetration through the eggshell within 3 h after laying was significantly decreased by cooling the eggs before the inoculation of SE or ST. Cooling the eggs soon after laying appears to depress the *Salmonella* penetration thereafter. Since the refrigeration in the present experiment might decrease the temperature difference between the environment and eggs, one possible explanation for the increased penetration in fresh eggs might be the negative pressure caused by the temperature difference between the inside and outside of eggs. Once *Salmonella* cells have traversed the shell and membranes of laid eggs, it is very difficult to prevent their further invasion into the egg contents and to remove them thoroughly from the eggs. Fresh eggs should be protected from the exposure to *Salmonella* as soon as possible after laying, and nest sanitation is especially important. Cooling the eggs, which is initiated soon after the eggs are laid, is recommended.

To eliminate the hazard caused by microorganisms including *Salmonella* spp. several procedures for pasteurizing eggs in the shell have been developed (21). However, no pasteurizing method to eliminate the organisms from eggshell membranes without changes in flavor and functional properties has been developed. Most of the bacterial penetration studies (15, 17, 19, 22, 24) have been performed using eggs several hours after they were laid. Since contamination of the interior of eggs immediately after laying may occur more readily, experiments to evaluate the pasteurization of eggs should use eggs within 3 h of laying. Also the use of very fresh eggs will more closely resemble current practices.

Outbreaks of human salmonellosis due to SE have dramatically increased during the past 10 years, but the precise reason is unknown (5, 16). In the present experiments, no differences in the permeability of eggshell between SE and ST was observed. The permeability of eggshell to SE may not be related to the increased number of outbreaks directly. Several studies reported that the egg contents contamination before the egg is covered by the shell might occur as the result of infection in the reproductive organs, because SE was recovered from the reproductive organs in naturally (3, 12) or experimentally (13, 23) infected hens. However, Humphrey et al. (11) reported that the albumen was more frequently contaminated than the
yolk as evidence opposing the possibility of ovarian infection. In our previous experiments, vaginal inoculation with SE produced more contaminated eggs than cloacal inoculation (13). That SE adheres from the contaminated oviduct and/or cloaca to the egg surface and thereafter penetrates through eggshell cannot be ruled out. The dramatically increased food poisoning by SE relative to other SE spp. may be related to the SE infection of reproductive tissues. More research is necessary to confirm the causes for the increased food poisoning by SE and to reduce the public health risk from the consumption of eggs.

REFERENCES