Laying Season and Egg Shell Cracks on the Growth of Salmonella Enteritidis in the Egg Albumen during Storage

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ABSTRACT

We studied the effects of laying seasons and egg shell cracks on the ability of egg albumen to support the growth of Salmonella Enteritidis (SE) in eggs. Hens eggs used were those laid in February, June, and October in a farm in Japan and stored at 10, 20, and 30°C, and at 30°C after storage at 10°C, immediately after receipt or after cracking the shell. At several-day intervals during storage, the egg contents were poured into a dish, SE was inoculated into albumen, and then the growth of SE during 3 days incubation at 18°C was measured. The results demonstrated that storage temperature and laying season affected the growth of SE in the egg albumen. The proportion of eggs upon which albumen allowed the growth of SE was higher in the eggs stored at 30°C than those stored at 10°C. The growth of SE in eggs was lowest in the following order of laying: February, October, and June. SE grew preferably in albumen of cracked eggs than intact eggs.

Foodborne diseases caused by Salmonella Enteritidis (SE) have recently increased with eggs as a major vehicle (4, 6). Two types of contamination of shelled eggs with SE are known. One type, called external contamination, is the contamination on the shell surface with SE at or after egg laying (2, 5, 7, 12). The other, internal contamination, is the contamination of egg contents with SE taking place during formation of shelled eggs in reproductive organs of infected hens (3, 11, 18). Recent increases in SE contamination of eggs are thought to reflect the frequent occurrence of internal contamination (16). One of the practical and effective measures for reduction of the health risk associated with internal contamination may be the control of SE growth in the contents of eggs during storage. Humphrey et al. (9, 10, 14) reported that the number of SE in egg contents is suppressed below 20 CFU/egg at 21 days of storage at 20°C after laying, after which SE may grow rapidly to a concentration of 10,000 to about 100,000 CFU/egg in some eggs. Furthermore, the time period between the commencement of the rapid growth and egg laying has been shown to be shortened with the increase in storage temperature, indicating the importance of the time period and temperature of egg storage for the prevention of SE growth in egg contents. However, little is known about the effect of the factors other than the time period and temperature on SE growth in egg contents. To understand the effect of laying seasons and shell cracks on SE growth in egg contents, we studied the growth of SE in albumen of intact or cracked eggs laid at different seasons.

MATERIALS AND METHODS

Bacteria. A strain of H2S-producing SE, strain no. SE930448 isolated from a patient, was grown in tryptic soy broth (Difco, Detroit, Mich.) at 37°C for 18 h and diluted with phosphate-buffered saline (pH 7.2) to 10^-6 for inoculation into egg albumen. Each portion (0.1 ml) was plated onto 10 plates of tryptic soy agar (Difco) for counting inoculated bacteria.

Shelled eggs. Eggs were purchased commercially from a farm in Fukushima prefecture, Japan, within 2 days after being laid by White Leghorn hens in February, June, and October 1999. During transportation from the farm to laboratory, the eggs laid in June were kept in a refrigerator and the other eggs were kept at ambient temperature below 20°C. The farm had been confirmed to be free from Salmonella in the preceding year. For preparation of cracked eggs, a small crack of 3 to 4 cm length was made on the shell by hitting with a stick on the day of receipt, taking care not to destroy the shell membrane. The weight of the egg and thickness of the egg shell were measured with an electronic balance (Shimazu, Kyoto, Japan) and a dial pipe gauge (P-1, 0.01 to 10 mm; Ozaki Mfg. Co., Ltd., Tokyo, Japan), respectively. Approximately 80 eggs were confirmed not to be contaminated with SE in each of four series of experiments in the following manner. After the surface of each egg shell was sterilized with 70% propanol, the shell was cracked and egg contents were poured into a stomacher bag. The contents of each egg were homogenized with 60 ml of buffered peptone water (Oxoid, Hampshire, UK) with a stomacher (model 400; A. J. Seward, London, UK). After incubation at 37°C for 20 h, 0.5 ml of the culture was incubated with 10 ml of tetrahionate (Oxoid) and Rappaport-Vassiliadis (Oxoid) broths at 42°C for 20 h. Then, a loop of culture was streaked onto mannitol lysine crystal violet brilliant green (Nissui, Tokyo, Japan) agar. After incubation at 37°C for 24 h, suspected colonies...
were picked up and confirmed to be *Salmonella* with a latex agglutination kit (Oxoid) and fermentation of lactose, nonfermentation of lactose and sucrose, and production of H$_2$S with triple sugar iron agar (Nissui).

**Storage of eggs.** Immediately after receipt or cracking of the egg shell, intact or cracked eggs were placed in incubators at 10, 20, and 30°C for 4 to 46 days. A part of the eggs stored at 10°C for 19 days was transferred into an incubator at 30°C for further storage. The humidity of the incubator at 10, 20, and 30°C was kept at approximately 100, 85, and 55%, respectively.

**Ability of albumen to support growth of SE.** The method used was the same as that used by Humphrey and Whitehead (13) with some modifications. At intervals of 3 to 7 days, 14 to 30 eggs were removed from the incubators and examined for ability of albumen to support growth of SE. After the surface of each egg shell was sterilized with 70% propanol, the contents of each egg were poured into a sterilized dish (HS-dishes, 20 mm: Eiken Co., Tokyo, Japan). The albumen around the intact yolk was then inoculated with SE suspended in phosphate-buffered saline (10 μl) by a sterilized micropipette at a location of 1 to 2 mm from the yolk membrane. The number of inoculated SE was confirmed by culture of 0.1 ml of the SE suspension on tryptic soy agar. The inoculated egg contents were then kept for 3 days in an incubator at 18°C, rather than 5 days at 20°C, as originally used by Humphrey and Whitehead (13).

After keeping in an incubator, the egg contents were homogenized in a stomacher bag with the stomacher. Then 0.6 ml of each homogenate was plated onto two plates of mannitol lysine crystal violet brilliant green agar. After incubation at 37°C for 1 to 3 days, SE numbers on mannitol lysine crystal violet brilliant green agar were counted. In the preliminary experiment, we observed that SE placed in albumen near the yolk grew faster than that placed at the outer edge of the albumen, as has been reported by Humphrey et al. (14).

**Statistics.** Statistical differences in the number of SE in detected samples among eggs of different seasons, and between intact and cracked eggs, were analyzed by $\chi^2$ test.

**RESULTS**

*Salmonella* was not detected in any eggs tested for confirmation of natural *Salmonella* contamination. The number of SE inoculated to albumen was on average 13 CFU (the range: 8 to 20 CFU)/egg through all experiments. The weight (mean of 20 to 40 eggs) of eggs laid in February, June, and October was 69.0, 65.3, and 68.6 g, respectively. The shell thickness (mean of 60 eggs) of eggs laid in February, June, and October was 4.05, 3.47, and 3.94 mm, respectively.

Figure 1a through 1d shows the results of storage of eggs at 30, 20, and 10°C and storage at 10°C for 19 days followed by at 30°C, respectively. To compare the albumens of eggs under different conditions, findings were expressed as the number of SE in inoculated egg contents after 3 days incubation at 18°C and the proportion of the eggs allowing inoculated SE to grow to the detectable level (100 CFU/ml).

The proportion of the eggs from which albumen facilitated SE growth to detectable levels was 45% (9 of 20), 20% (4 of 20), 20% (4 of 20), and 25% (5 of 20) for intact eggs laid in February, June, and October and cracked eggs in October, respectively, before they were stored (see Fig. 1a through 1d, day 0). The proportion tended to decrease slightly during the early stages of storage at any temperature.

When the eggs were stored at 30°C, the ability of albumen to support SE growth remained relatively low (detection rate: ca. 20%) until day 25 for the eggs laid in February, while it was already high (detection rate: ca. 60%) at day 18 for the eggs laid in June (Fig. 1a). Intact eggs in October exhibited an ability lower than those in June but higher than those in February. The proportion of cracked eggs allowing SE to grow to detectable levels was significantly higher than that of intact eggs, when compared at day 14 of storage at 30°C ($P < 0.01$) (Fig. 1a). Most cracked eggs at day 14 supported SE growth in albumen, whereas fewer than 20% of intact eggs did so at the same day (Fig. 1a).

When the eggs were stored at 20°C, the growth of SE in albumen of intact eggs laid in February and October remained low (detection rate: ca. <30%) throughout the storage period, but that of intact eggs laid in June increased at days 21 and 25 of storage (detection rate: ca. >30%) (Fig. 1b). The growth of SE in albumen of cracked eggs was higher than that of intact eggs laid in February and October ($P < 0.05$), when compared at days 21 and 25 of storage (Fig. 1b).

Storage of intact eggs at 10°C did not increase the ability of albumen to support SE growth (Fig. 1c). However, the proportion of the eggs allowing SE growth to a detectable level at days 14 and 21 was higher in cracked eggs than in intact eggs (Fig. 1c).

Changes in the proportion of eggs allowing SE growth during storage at 30°C after 10°C storage resembled those observed in the eggs stored at 30°C from the time of receipt (Fig. 1d and 1a). The albumen of eggs laid in June facilitated SE growth to a larger extent than that of the eggs laid in October and February (Fig. 1d).

**DISCUSSION**

It has been reported that when SE internal contamination occurs naturally, SE exists at less than 20 CFU/egg at the time of laying (14). The principle site of contamination of eggs is regarded to be the albumen (14). The growth of SE in albumen is inhibited by ovotransferrin, which is a chelator of the iron needed for growth of the organism (1). However, when iron permeates into albumen from the yolk (15) or SE invades the yolk membrane (13) by weakness of the yolk membrane, the organism grows rapidly in eggs. In the present study, the ability of albumen to facilitate SE growth from less than 20 CFU/egg to more than or equal to 100 CFU/egg was used as an indicator for conditions of albumen in eggs.

The present method for assessing the ability of albumen to facilitate SE growth is the method originally used by Humphrey et al. (14) with modification. In the preliminary experiment, we confirmed that SE grew at 18°C for 3 days when inoculated into albumen near the egg yolk, although SE does not grow when inoculated into the outer edge of albumen as observed by Humphrey et al. (14). The
FIGURE 1. Growth of inoculated SE in egg albumen from shelled eggs stored at 30°C (a), 20°C (b), 10°C (c), and at 30°C after storage at 10°C for 19 days (d). Left columns show the number of SE in each egg after 3 days incubation at 18°C. ○ Eggs in which SE was detected; ● eggs in which SE was not detected. Horizontal bars indicate the mean of the number of SE for detected eggs. Right columns show the proportion of the eggs in which SE was detected. Arrows in d indicate the time point when storage temperature was changed from 10 to 30°C. The same letters, a through i, appearing in the right columns of a through d indicate significant differences in percentage of detected eggs between two groups of eggs on the same day: (a) P < 0.01: a, b, c, f, g; P < 0.05: d, e, h, i; (b) P < 0.01: c, e; P < 0.05: a, b, d, f; (c) P < 0.05: a, b; (d) P < 0.01: e, f, g, h, i; P < 0.05: a, b, c, d.
number of SE in egg contents after 3 days incubation at 18°C, and the proportion of eggs of which albumen facilitated SE to grow to detectable levels (the level higher than eight times the initial level) were used as indicators of the ability of the egg albumen to facilitate SE growth.

When SE exists in eggs, the storage temperature of the eggs is important for prevention of growth of SE. Gast and Beard (8) have shown that storage at 25°C for 7 days increases SE in naturally contaminated eggs, although storage at 7°C for 7 days does not alter the contamination level. Schoeni et al. (17) reported that SE grows during 1 day storage at 25°C but not at 10°C for 7 days. In the present study, we found that SE grew less frequently in the albumen of shelled eggs stored at 10°C than in that at 20 and 30°C from any of the laying seasons. The proportion of the eggs in which albumen facilitated SE growth to the detectable level decreased slightly during the early stages of storage, indicating that egg albumen enters a phase less favorable for SE growth during the early stage after laying. Then the proportion increased with storage time, depending on the storage temperature.

The proportion of eggs facilitating SE growth to the detectable level was higher in shelled eggs laid in June than those laid in February or October. It was also found that the weight and thickness of egg shells laid in June were less than those in February and October, possibly reflecting seasonal differences in physiological conditions of laying hens linked to ambient temperature. Mean ambient temperature in Fukushima prefecture, the location of the flock that laid the eggs used in this study, was −0.9, 17.5, and 12.6°C in February, June, and October, respectively. Also possible differences in the age of hens among seasons should be taken into account. In association with such changes of environmental temperature, the structure of the egg yolk membrane and chemical composition of egg contents might also change, which might directly affect the ability of albumen to facilitate or inhibit SE growth. The effect of condition of transportation of eggs from the farm to our laboratory may be excluded, because the eggs were kept below 20°C for less than 2 days during transportation.

There have been no studies on the differences in growth of SE in egg albumen between intact and cracked eggs. The present findings demonstrated that SE can grow more preferably in albumen of cracked eggs than intact eggs, indicating that the storage conditions should be considered more intensively for cracked eggs than intact eggs from the view of the ability of albumen to support SE growth. Likewise the seasonal differences in the ability of the albumen to facilitate SE growth may indicate that shelled eggs laid in a season of relatively high temperature should be consumed earlier than those laid in other seasons.

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REFERENCES