Use of Luminescent Strains of Salmonella enteritidis To Monitor Contamination and Survival in Eggs

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ABSTRACT

Fresh and retail eggs were exposed to luminescent S. enteritidis cultures containing from $10^6$ to $10^9$ CFU/ml at either room temperature (approximately 21°C) for 3 days or 40°C for 16 h. The entry of S. enteritidis through egg shell was evidenced by luminescence in the eggs which was visualized using an Image Quantifier. The rate of contamination of the eggs increased with increasing inoculum size. Scanning electron microscopy was used to confirm the position of S. enteritidis cells in the eggs. The survival rate of the Salmonella cells in liquid eggs and whole shell eggs during storage at 4°C was investigated. Although S. enteritidis did not grow in eggs during storage at 4°C for up to 8 weeks, cells were able to survive. Under these storage conditions, the count was reduced by 1.7 to 2.5 log cycles per g in liquid egg and 0.8 to 1.4 log cycle per g in whole shell eggs. Similar trends were observed using both plate count and luminescence to monitor survival.

Key words: Salmonella, eggs, egg penetration, pathogen survival, bioluminescence

Salmonella enteritidis is one of the most important causes of human salmonellosis in Canada. Between the years 1983 and 1993, S. enteritidis was the third most common human isolate in this country (R. A. Khakhria, Health Canada, personal communication). Outbreaks caused by this organism in humans have been mainly associated with consumption of eggs and egg products (10, 11, 23–25, 36–38).

The incidence of S. enteritidis infection in humans has dramatically increased in recent years in several other countries. Reported human infection in the US increased from 6% before 1976 to 51% in 1987 (25). Among the 65 outbreaks reported from January 1985 to May 1987, 35 were associated with food. In 77% of these food-related outbreaks, grade A shell eggs were the vehicle for infection. This species became the most common cause of Salmonella infection in the US for the first time in 1991 (26). In Europe, increasing incidence of S. enteritidis contamination of eggs was also reported. In 1988, for instance, 50% of the 27,478 salmonellosis cases in the United Kingdom were caused by S. enteritidis, and at that time eggs became the most important source of Salmonella infections reported to the Communicable Disease Surveillance Centre (12). Outbreaks of human salmonellosis in Spain due to S. enteritidis increased from 8% in 1977 to 40% in 1984 (31).

As early as 1975, it was suggested that contamination of eggs was partially due to implantation of the microorganism within the hen’s ovary or oviduct (4). This was confirmed by several researchers in both naturally (20, 21) and artificially (34, 39) infected laying hens. In both cases, Salmonella cells could be isolated from many body parts, including the peritoneum, ovules, oviduct, and yolk and albumen of contaminated eggs. Borland (4) also suggested that eggs could become infected by fecal contamination through the pores of the shells after laying, confirming the much earlier work of Haines and Moran (17) and Pomeroy and Fenstermaker (32). More recent work has also demonstrated the ability of Salmonella cells to penetrate into eggs exposed to the organism (2). Indeed, these researchers go as far as to say that most contamination of eggs by Salmonella spp. occurs by this route. The incidence of penetration was affected by the integrity of the egg shell (3), which in turn can be affected by environmental factors such as the rate of cooling of eggs (14). Tung et al. (40) demonstrated by scanning electron microscopy that Pseudomonas fluorescens could invade the shell pores, but only after exposure to the culture for 72 h to allow enzymic breakdown of the cuticular barrier. A comprehensive review of the association between S. enteritidis and eggs has been published (20).

This paper describes how the availability of bioluminescent S. enteritidis phenotypes and the ability to visualize growth of the organism in whole shell eggs (7) can provide a powerful tool for studying the effect of environmental conditions on the contamination of food by potential pathogens.

MATERIALS AND METHODS

Bacterial culture and eggs

Bioluminescent S. enteritidis GCDE used in this study was constructed as described previously (7). Eggs used in the experiment were 2 days old and purchased from the Poultry Research Station, Guelph, Ontario, Canada. Eggs from a retail outlet in Guelph were also used in the experiment.
Penetration assay
Salmonella enteritidis GCDE was grown in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, MI) supplemented with 50 μg of kanamycin (Sigma Chemical Co., St Louis, MO) per ml at 37°C. An overnight culture was inoculated into fresh broth and incubated at 37°C until the optical density at 600 nm reached about 0.95. The culture was used as the source of contamination.

Fifty-six eggs from a retail outlet were washed with detergent, 5% (vol/vol) Liqui-nox (Alconox, Inc., NY). The washed eggs were exposed to S. enteritidis GCDE by immersing them in an overnight BHI culture containing approximately 10^8 CFU/ml. The eggs were incubated in the culture either at 40°C for 16 h (32 eggs) or at room temperature (about 21°C) for 3 days (24 eggs). Eggs were taken from the source of contamination, rewarshed with the same detergent and heat-sealed individually in plastic hybridization bags (BRL Bethesda Research Laboratories, Life Technologies Inc., Gaithersberg, MD) with 40 μl of 100% decanal (Sigma). Bioluminescence was observed using Biq Bioview Image Quantifier (Cambridge Imaging, Cambridge, UK).

The same experiment was repeated with 2-day-old eggs. Twenty-four eggs were exposed to S. enteritidis GCDE using the same procedure described above, but this time precontamination washing was eliminated. Half the eggs were incubated at 40°C for 16 h and half at room temperature for 3 days.

Fresh eggs were also exposed to cultures containing different cell concentrations. An overnight (BHI) culture was serially diluted with phosphate-buffered saline (PBS), pH 7.4, and bacterial dilutions containing between 10^4 and 10^6 CFU/ml were used as the inocula. A group of 6 eggs were placed in each bacterial suspension (500 ml) and incubated at 40°C for 16 h. After incubation, luminescence was measured using the method described above. The eggs were kept at room temperature (approximately 21°C) for six weeks to observe the growth, by plate count or bioluminescence, of the Salmonella cells inside the eggs and the number of infected eggs in each group was recorded.

Artificially inoculated chicken feces were also used as a source of S. enteritidis. Chicken feces were autoclaved at 121°C for 20 min to remove unwanted microorganisms and then inoculated with the luminescent S. enteritidis culture at a concentration of approximately 10^8 CFU/g. Eggs (n = 10) were placed in the chicken feces and incubated at room temperature for 3 days. The eggs were removed and treated in the same way as described above.

Scanning electron microscopy
The egg shell of contaminated eggs was broken into small pieces and placed into fixative solution (2% glutaraldehyde in 0.1 M sodium cacodylate buffer (SCB), pH 7.4) and kept at room temperature for 1.5 hours. The samples were then rinsed thoroughly with 0.1 M SCB pH 7.4, for 10 min. This washing step was repeated a further three times. Lipid fixation was performed at room temperature for 1 h in 1% osmium tetroxide followed by two rinses with SCB. The samples were further rinsed successively with 50%, 70%, 80%, 90%, 95% and 100% ethanol, each for 15 min, and stored at 4°C overnight in 100% ethanol. Critical drying was performed by flushing with CO2 the following day.

After the critical drying, the egg shell and membrane were carefully separated and mounted on a support. The samples were then placed in sputter coater (Anatech Hummer VII, Alexandria, VA) for further treatment to improve the conductivity and resolution. Egg shell and membrane were examined using a scanning electron microscope (Hitachi S-570, Tokyo, Japan) and Salmonella cells were located and photographed using an Ilford FP4 Plus 125 ASA camera. Egg shell and membrane from an artificially inoculated egg and a normal egg were prepared at the same time and used as controls.

Survival of luminescent S. enteritidis in liquid whole egg and whole shell eggs during storage at 4°C
Two-day-old eggs were infected in duplicate with luminescent S. enteritidis cells by injecting the bacterial cells through an artificially-made hole in the egg shells to give a population of between 10^3 and 10^4 CFU in each egg. The eggs were incubated at 37°C for 18 h. Following this incubation period, the eggs were opened and the contents placed into 250-ml sterile flasks and thoroughly mixed at room temperature. Contaminated egg contents were stored at 4°C for a period of 2 months. Both luminescence and population were examined weekly to determine the survival rate of S. enteritidis during the storage. At each week, serial dilutions of contaminated egg contents were made with PBS and the number of the S. enteritidis cells in each egg was determined by plating on both selective (BHI agar containing 50 μg of kanamycin per ml) and nonselective (BHI agar) media with incubation of the plates at 37°C for 24 h. Luminescence of the contents of the eggs was determined using a Multilite Luminometer (Biotrace Inc., Plainsboro, NJ). The experiment was repeated once.

Eighteen eggs were divided into three groups and each group (consisting of 6 eggs) was infected with either 10^3, 10^4, or 10^5 CFU of S. enteritidis GCDE. After overnight incubation at 37°C, infected whole shell eggs, instead of egg contents, were stored at 4°C for 8 weeks. Two of the 6 eggs infected at each inoculum level were pooled and examined for plate count and bioluminescence at 0, 28, and 56 days of storage.

RESULTS
Penetration of S. enteritidis into whole eggs
When retail and fresh eggs were exposed to the culture of luminescent S. enteritidis at a concentration of 10^5 CFU/ml at 40°C or room temperature, 100% (16 of 16) of the eggs tested became luminescent, indicating that they had been contaminated by the organism. At the initial stage of contamination, Salmonella cells seemed to be located between the egg shell and egg membrane since the luminescent images obtained varied depending on the position of the egg (Fig. 1A and 1B). Furthermore, the image from outside the broken shell was much brighter than that from the inside, suggesting that the luminescent organism had penetrated through the shell and was located on the inner surface of the shell (Fig. 2).

The conclusion that the microorganisms that migrated through the shell were initially located between the egg shell and membrane was supported by cultural studies. Bioluminescent colonies were recovered from inside the membrane-free shell but not from the outside when the egg shell was placed on a BHI plate and incubated at 37°C for 16 h. The luminescent Salmonella strain produced colonies beneath the egg membrane placed shell-side down on BHI agar. However, growth appeared on top of the membrane when the content-side was in contact with the medium. Evidence that the organism could eventually penetrate the membrane was produced when bioluminescence could be observed in egg contents, mainly in the yolk, after prolonged storage at room temperature (Fig. 3). Rates of contamination of eggs with S. enteritidis similar to those obtained by immersion in
FIGURE 1. Observation of luminescence emitted by the recombinant Salmonella cells in whole shell eggs from both sides. (A), image from one side; (B), image from the opposite side.

culture were observed when chicken feces containing the luminescent strain were used as the source of contamination (results not shown). In a further experiment, washing was eliminated to allow the cuticle of the egg to remain intact.

FIGURE 2. Luminescence observed from inside (A) and outside (B) of the broken egg shell. No light is emitted from the inside surface of the shell.

FIGURE 3. Luminescence observed from the content of an egg infected by recombinant S. enteritidis culture. Growth is associated with the yolk.

When unwashed eggs were exposed to cultures containing cell concentrations of $10^9$ CFU/ml at $40^\circ$C or room temperature, all eggs tested became contaminated as indicated by the luminescent image they produced when placed in the charge-coupled device (CCD) imaging system (results not shown).

After 6 weeks of storage, 100% of the eggs (12 of 12) exposed to the S. enteritidis culture containing between $10^8$ and $10^9$ CFU/ml exhibited luminescence. When exposed to cultures containing between $10^6$ and $10^7$ CFU/ml, 82% (9 of 11) of the eggs were infected; this value decreased to 27% (3 of 11) when the inoculum size was between $10^4$ and $10^5$ CFU/ml. Although a dose-response effect was observed, there was considerable variation in the intensity of luminescence from different eggs infected by the same dose.

Scanning electron microscopy

Scanning electron microscopy was used to confirm the location of the S. enteritidis cells inside the egg. The Salmonella cells were located on the inner side of the egg shell (Fig. 4A) and also the shell-side of the egg membrane (Fig. 4B).

Effect of storage on survival of S. enteritidis in eggs

The change in the number of luminescent Salmonella cells in egg contents tested during an 8-week storage period at 4°C is shown in Figure 5A. The number of bacterial cells, measured by plate count, decreased during the first 2 weeks and then remained fairly constant for the following 2-week period, after which it decreased gradually. The pattern of light reduction in the luminescent strain over the storage period paralleled the change in cell numbers determined by plate count. By the end of the 8-week storage period, a 1.7- to 2.5-log-unit reduction in the numbers of Salmonella cells and a 0.57- to 0.82-log-unit reduction in bioluminescence readings were obtained (Fig. 5B).

To confirm that the reduction in luminescence was due to a decrease in cell numbers and not to a loss of the plasmid bearing the luciferase and kanamycin resistance genes
the lux genes in the recombinant Salmonella strain not only were stable under heat treatment and acid treatment (7), but also were expressed well at low temperatures.

When luminescence was monitored in whole eggs during cold storage, plating was carried out on selective media as well as nonselective media. The comparison of the plate counts showed that the numbers of the bacterial colonies were similar on both media (Fig. 6), indicating that...
during an 8-week storage period, there appeared to be a reduction in the level of light emitted by the culture inside the eggs but it did not disappear completely (Fig. 7). Plate counts and luminescence from whole shell eggs after they had been stored for 2 months were similar to those obtained in liquid whole egg under the same storage conditions (Fig. 8). A 0.8- to 1.4-log-cycle reduction in the number of bacteria and a 0.16- to 0.24-log-cycle reduction in the bioluminescence readings were observed.

**DISCUSSION**

The present study indicates that luminescent strains of *Salmonella enteritidis* can play a useful role in studying factors influencing environmental contamination during food processing. The study confirmed that *S. enteritidis* has the ability to penetrate through the shell of eggs, but growth is limited to the region between the shell and the membrane at the initial stage of the invasion. In some cases, probably where there had been prior damage to the integrity of the membrane, the growth of the bacteria spread to the egg content, indicated by the appearance of luminescence in the egg content (Fig. 3). Other workers have stated that the inner shell membranes do not present a significant barrier to prevent penetration of eggs by either *E. coli* or *Salmonella* cells (16). Cason et al. (6) used a nalidixic acid-resistant strain of *S. typhimurium* to study the ability of the organism to penetrate eggs following immersion in a bacterial culture. They observed that all the shells and membranes of the eggs sampled were *Salmonella* positive 30 min after inoculation, but this value was reduced to 38% after 17 to 21 days of incubation. The organism could be isolated from 2% of the egg yolks, indicating that there was penetration of the membrane. In another study involving a nalidixic acid-resistant strain of *S. enteritidis*, Fajardo et al. (14) determined that 100 and 91.3% of eggs were penetrated by the organism when forced and natural convection, respectively, was used for cooling. The rate of penetration in uncooled eggs was 43%. In a study involving 1,440 eggs, no evidence was found that *S. enteritidis* could penetrate the shell (33). However, work by Barrow and Lovell (2) led them to suggest that most contamination of eggs by *Salmonella* spp. occurs by horizontal transmission; that is, from exposure to a contaminated environment.

The cuticle of the egg is thought to provide protection against microbial invasion for up to 96 h after the eggs are laid (41). Nascimento and Solomon (29) studied the ability of *S. enteritidis* to penetrate pieces of egg shell and concluded that the cuticular layer was rarely present as an even covering over the shell surface. The penetration did not depend on pore numbers; however, structural abnormalities of the shell were important for successful penetration. Bacterial movement across the shell was dependent on both temperature and humidity. The results support the conclusion that the cuticular layer is not a major barrier against bacterial penetration of the egg shell as similar rates of infection were observed in eggs that had been washed to remove the cuticle and in untreated eggs. Other evidence to support the importance of the structural integrity of the shell in determining the rate of contamination by *Salmonella* spp. has been provided by Fajardo et al. (14). They 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 *S. enteritidis*.

This study, using a real-time indicator of growth, confirms that fecal contamination of eggs is a route for *Salmonella* spp. entry. Clay and Board (9) have reported that, for eggs artificially contaminated with *S. enteritidis*, the incidence of contamination was greater when the inoculum was prepared in fecal extract rather than Ringer’s solution. The presence of water had a direct effect on the ability of *S. typhimurium* to penetrate eggs (30). However, there was no apparent difference in growth rate in eggs in this study when *S. enteritidis* inocula were prepared in BHI or feces.

The degree of environmental contamination appears crucial for infection of the egg by *Salmonella* spp. At high cell numbers (>10⁶ CFU/ml), all of the eggs used in the test became contaminated, but the frequency of contamination decreased with a reduction in inoculum cell number. However, varying degrees of infection among contaminated eggs
were also noticed. These results indicate that other factors, including the antimicrobial components of individual eggs, also play a role in the contamination process. The luminescence from the infected eggs varied independently of the degree of exposure to S. enteritidis. This phenomenon may relate to differences in the exterior structure of the egg, the amount of antimicrobial compounds such as lysozyme in an individual egg, and the chance of the bacterium coming into contact with the egg yolk. These results were consistent with those described by Clay and Board (8), who demonstrated that the extent and rate of contamination of S. enteritidis phage type 4 in artificially contaminated eggs was influenced by the cell number in the inoculum and the site of contamination relative to the position of the yolk. The importance of yolk movement relative to the site of contamination of eggs was also stressed by Dolman and Board (13).

From the luminescent images produced it was apparent that most of the growth of S. enteritidis was associated with the yolk. Several researchers have stated that yolk is a far better growth medium for the organism than albumen, which inhibited the organism (18, 19, 27, 28). However, Clay and Board (8) claimed that albumen did not exert an antimicrobial effect on S. enteritidis.

Results reported here support those of other researchers who showed that Salmonella spp. were unable to grow in eggs during prolonged storage at 4°C or refrigeration temperatures (5, 8, 9, 15, 18, 21, 27, 28), suggesting that storage of eggs at this temperature may have important public health benefits. There may be strain differences, however, as slight growth of S. enteritidis in eggs stored at 4°C has been observed (22). Since the initial inoculum size used in this study was high, significant numbers of Salmonella cells survived in liquid eggs and whole eggs at the end of the storage period. Survival of S. enteritidis in liquid egg at low temperatures has been described by other workers (27, 33).

The study of penetration of S. enteritidis in whole shell eggs was an attempt to simulate what may happen in nature. The eggs would be exposed to large numbers of S. enteritidis in feces, feed, etc., and initially the eggs would be at high temperatures. The prolonged length of exposure to high temperatures used may simulate conditions expected in a hatchery. The organism is capable of penetrating the eggs through the pores of the shell according to this study. If conditions permit, growth of the microorganism occurs between the shell and the membrane. Subsequently, egg contents and egg products could be seriously contaminated during breaking and processing.

Bioluminescent constructs of bacteria are being used increasingly to track organisms in the environment (1, 35). This study suggests that similar techniques will prove invaluable for determining potential sources of contamination of foods.

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REFERENCES

MONITORING CONTAMINATION OF EGGS WITH LUMINESCENT STRAINS OF SALMONELLA


25. Madden, J. M. 1989. Increase in the number of cases of Salmonella enteritidis in the United States due to whole chicken eggs and the implications to food handlers. J. Food Prot. 52:753.


