Destruction of *Salmonella enteritidis* by High pH and Rapid Chilling During Simulated Commercial Egg Processing

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ABSTRACT

Shell eggs were inoculated with a double mutant of *Salmonella enteritidis* (resistant to both nalidixic acid and streptomycin) by dipping the eggs into a sterile poultry fecal slurry inoculated with this organism. The inoculated eggs were washed with either pH 9 or pH 11 washwater at 37.7°C using a small-scale commercial egg washer. Both washed and unwashed eggs were subjected to either rapid or slow chilling. Both internal contents and external surfaces of eggs were examined immediately after: 1) inoculation; 2) washing; and 3) chilling and 30 days storage at 7.2°C. Washwater pH had a significant effect on the survival of *S. enteritidis* on the surface of the eggs (p <0.001). Significant cross-contamination occurred between inoculated and uninoculated eggs washed at pH 9; 75% of the uninoculated eggs became *S. enteritidis* -positive on their surface. No cross-contamination occurred at pH 11 between inoculated and uninoculated eggs, and only 8.3% of inoculated eggs were still surface-positive for *S. enteritidis*. Slow chilling increased the survival of *S. enteritidis* on the surface of the eggs (p < 0.01) and appeared to permit penetration of *S. enteritidis* into eggs washed at pH 9.

Key Words: *Salmonella enteritidis*, pH, simulated commercial egg processing

There are numerous ways eggs can become contaminated with salmonellae. In most instances, the oviduct of the hen is sterile and therefore the internal contents of the egg and the shell are sterile until the time of laying. However, several investigators have shown that the ovaries and oviducts of hens may become infected with salmonellae, which can be deposited inside the egg before it is laid ("vertical" transmission) (4,5,10,11,20,23). After the egg is laid, the shell can become contaminated with salmonellae by fecal material from the intestinal tract or other sources in the environment ("horizontal" transmission). Organisms on the surface of the shell can penetrate into the interior of the egg and contaminate the internal contents of the egg (17,18,22,24).

In the past 10 years there have been many changes in egg washing practices, including the use of high-speed, high-volume egg washers. These egg washers recycle washwater for up to 4 h, which leads to a build-up of bacteria and egg solids and variations in detergent concentration. With this system, washwater could easily become inoculated with *S. enteritidis* from feces on the surface of contaminated eggs. Since vertical transmission of *S. enteritidis* in eggs is rare (approximately 1 out of every 10,000 eggs produced by hens infected with *S. enteritidis* contain the organism) (9,11), the increase in salmonellosis from eggs seen lately may also reflect recent changes in washing procedures in the egg processing industry. In previous work (3), we determined that washwater pH had a profound effect on the survival of *S. enteritidis* in egg washwater. The purpose of the present study was to observe the effect of washwater pH and chilling rate on *S. enteritidis* contamination and penetration of shell eggs during simulated commercial processing.

MATERIALS AND METHODS

Cultures

Salmonella enteritidis phage type 8 (PS8), a strain from a Pennsylvania layer house, was isolated at the Veterinary Diagnostic Laboratory of the Pennsylvania State University and serotyped by the National Veterinary Services Laboratory (Ames, IA). During this study, PS8NSR, a mutant strain of *S. enteritidis* PS8 resistant to both 100 µg/ml nalidixic acid and 100 µg/ml streptomycin sulfate was isolated.

The PS8 strain was transferred monthly to fresh slants of Trypticase Soy Broth (TSB; BBL Microbiology Systems, Cockeysville, MD) supplemented with 0.6% Yeast Extract and 15.0% Agar (TSAYE), incubated for 24 h at 35°C and then stored at 3°C. The mutant strain, PS8NSR, was transferred monthly to fresh slants of TSAYE supplemented with 100 µg/ml of both nalidixic acid and streptomycin sulfate (Sigma Chemical Co., St. Louis, MO), incubated for 24 h at 35°C and then stored at 3°C.

Isolation of nalidixic acid and streptomycin sulfate resistant S. enteritidis

Salmonella enteritidis PS8 was grown in 30 ml of TSB supplemented with yeast extract (TSBYE) overnight at 37°C. After incubation, the culture was sedimented by centrifugation at $3000 \times g$ for 30 min. The supernatant was discarded and the pellet was spread with a sterile cotton swab onto a TSAYE plate containing 100 µg/ml of nalidixic acid. The pellet was also serial diluted in 0.1% peptone water (Difco Laboratories, Detroit, MI) and spread onto plates containing 100 µg/ml nalidixic acid. The plates were incubated for 24 h at 37°C. Large, isolated colonies (spontaneous mutants) were streaked onto fresh TSAYE plates containing 100 µg/ml nalidixic acid.

The nalidixic-acid resistant isolate was grown in 30 ml of TSBYE containing 100 µg/ml nalidixic acid overnight at 37°C. After centrifugation at 3000 × g for 30 min, the supernatant was discarded and the bacterial pellet was spread onto TSAYE plates containing both 100 µg/ml nalidixic acid and 100 µg/ml of streptomycin sulfate. The pellet was also serially diluted in 0.1% peptone water and spread onto TSAYE plates containing both 100 µg/ml of streptomycin sulfate. The pellet was also serially diluted in 0.1% peptone water and spread onto TSAYE plates containing the same antibiotics at the same concentrations. Plates were incubated for 24 h at 37°C and large colonies (spontaneous mutants resistant to both nalidixic acid and streptomycin) were streked onto fresh TSAYE plates containing 100 µg/ml of both nalidixic acid and streptomycin sulfate. The isolate was checked to confirm the presence of the original biochemical properties of *S. enteritidis* (*12*).

The isolate, PS8NSR, was also tested in synthetic washwater at pH 10 at temperatures of 32.2, 37.7 and 43.3°C to insure the mutant would behave as the original wildtype *S. enteritidis* culture under similar conditions.

Inoculation protocol

Fecal matter from leghorn hens was obtained at the Pennsylvania State University Poultry Farm. The fecal matter was sterilized in an autoclave at 121°C in plastic buckets for three 20-min time intervals. To insure the fecal material was sterile, a 25-g sample was removed from each bucket, placed in a sterile blender jar with 225 ml of 0.1% peptone and homogenized using a Waring blender on high speed for 2 min. Serial dilutions were made and pour plated using TSAYE. The plates were incubated for 48 h at 37°C. Samples having <1 × 10⁰ CFU/g est. were considered sterile.

A 10% fecal slurry was prepared by homogenizing 60 g of sterile fecal matter in 540 ml of 0.1% peptone in a Waring blender for 1 min on high speed. The slurry was poured into a sterile plasitc tub and held at 5°C until needed for inoculation. The fecal slurry was inoculated with an overnight culture grown in TSBYE containing 100 µg/ml of both nalidixic acid and streptomycin sulfate in yield a final concentration of 10^7 CFU of PS8NSR per milliliter of slurry.

All eggs used were nest run and freshly laid by white leghorn hens at the Pennsylvania State University Poultry Farm. The eggs were left at room temperature for 24 h prior to inoculation and washing so they would not develop thermal cracks when going through the washer. Room temperature eggs were labelled with plum pink Revlon™ nail enamel (Revlon, Inc., New York, NY) and inoculated by dipping them into a container holding the chilled fecal slurry (5°C) containing approximately 10^7 CFU/ml of PS8NSR. The eggs were then placed on plastic flats to be transported to the washer. The eggs were always handled using sterile gloves, and care was taken to avoid cross-contamination of uninoculated eggs.

Washing conditions

A small-scale commercial egg washer (Seymour W-161, Topeka, KS), designed to wash 1,800 eggs per hour, was employed. A total of 480 eggs were washed per trial; 5% of the washed eggs were externally inoculated with the fecal slurry containing PS8NSR as stated above. The eggs were washed at 37.7°C; three trials were performed using pH 9 washwater and three trials were performed using pH 11 washwater.

Cooling conditions

Unwashed-inoculated and unwashed-uninoculated eggs as well as washed-inoculated and washed-uninoculated eggs (from above) were subjected to both rapid and slow chilling. To simulate fast chill, individual flats of eggs were placed onto shelves in

a refrigeration unit (Model U-45, Mohawk Cabinet Co., Gloversville, NY) set at 7.2°C. To simulate slow commercial cooling of eggs (e.g., eggs inside boxes surrounded by boxes on pallets), eggs were placed into cardboard egg cases packed full of eggs. All holes were sealed with masking tape so that no ventilation could occur. These cases of eggs were then placed onto shelves in the above refrigeration unit. The internal temperatures of the slow and fast chilled eggs were monitored using thermocouples inserted into the centers of eggs at various locations in the cases and throughout the cooler. The air temperature in the refrigeration unit was also monitored using a thermocouple. Readings were taken using a 21× Micrologger (Campbell Scientific, East Brunswick, NJ).

Sampling procedures

Unwashed-inoculated and unwashed-uninoculated eggs, as well as washed-inoculated and washed-uninoculated eggs, were examined immediately after inoculation or washing. The external surface and internal contents of individual eggs were sampled for the presence of PS8NSR. The remaining eggs were stored for 30 days at 7.2°C. Eggs were removed 24 h prior to examination and placed in a 37°C incubator in order for numbers of *Salmonella* to increase, thereby increasing sensitivity of detection (*1*). The external surfaces of the eggs were sampled by placing each egg in an 18-oz. Whirlpack™ bag and immersing it in 50 ml of Buffered Peptone Water (BPW; Difco). The Whirlpack™ bag was closed, the egg was shaken in the BPW, and the contents were allowed to incubate for 18 to 24 h at 37°C. To sample internal contents, a procedure used by the Pennsylvania State University Veterinary Diagnostic Laboratory was employed. Shells were sterilized by rinsing with an iodine-ethanol solution consisting of 200 ml of a 1% (wt/vol) iodine solution in 2,800 ml of 95% ethanol. The pointed end of the eggs were then broken off using a sterile knife. The internal contents of each egg were placed into an 18-oz. Whirlpack™ bag, homogenized and incubated for 18 to 24 h at 37°C.

A 1-ml volume of BPW or whole egg was transferred into a tube containing 9 ml of TT broth base Hajna (Difco) for selective enrichment and incubated for 18 to 24 h at 42°C. A loopful of each selective enrichment broth was streaked onto Xylose Lysine Desoxycholate (XLD, BBL) and Brilliant Green with Novobiocin (BGN, BBL) plates containing 50 µg/ml of both nalidixic acid and streptomycin sulfate (the concentration of antibiotics found to inhibit other salmonellae, yet yield the greatest recovery of PS8NSR) and incubated for 24 to 48 h at 35°C. Suspect colonies were picked and used to inoculate Triple Sugar Iron agar (TSI; BBL) and Lysine Iron Agar (LIA; BBL) slants and TSAYE containing 100 µg/ml of both nalidixic acid and streptomycin sulfate. Group D factor 9 slide agglutination reactions were conducted on the colonies that had positive reactions in the TSI and LIA slants.

The number of eggs testing positive for PS8NSR either internally or externally was recorded. Since the dependent variable was dichotomous (presence/absence), logistic regression analysis was carried out using PROC CATMOD in Statistical Analysis System (SAS). The main effects and interactions were tested using chi-squared tests. The statistical significance of these effects was determined by successively fitting a series of models until a final model was obtained. Once the final model was obtained the data were tested to see if they fit the model.

RESULTS

Cooling study

The internal temperature of rapidly chilled eggs reached refrigeration temperature (7.2°C) within 2 to 4 h. In contrast,
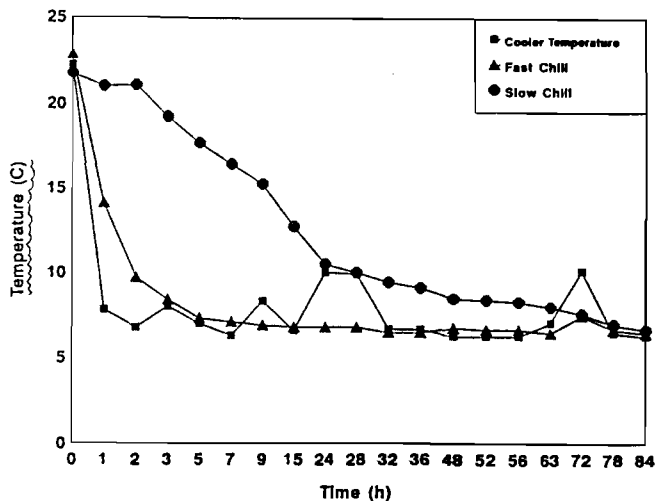
Figure 1. *Thermocouple temperature readings for rapid and slow chilling of eggs. All temperature readings, with the exception of cooler temperature, are internal temperatures of eggs.*
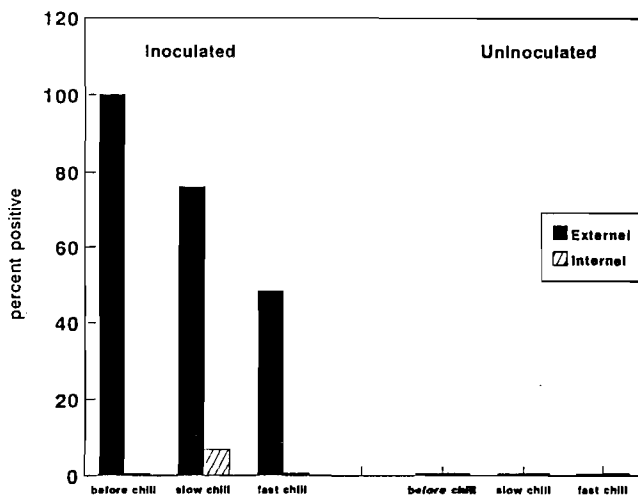

Figure 2. *Effect of chilling rate on the survival of* S. enteritidis *PS8NSR on/in unwashed eggs. Bars indicate the percentage of eggs testing positive for* S. enteritidis *PS8NSR.*

the internal temperature of slowly chilled eggs took 2 to 3 days to reach refrigeration temperature (7.2°C) (Fig. 1).

The effect of chilling unwashed eggs is illustrated in Fig. 2. Before chill, PS8NSR was isolated from the surface of 25 of 25 inoculated-unwashed eggs examined; no internal contents were positive. After slow chill, PS8NSR was isolated from the surface of 22 of 29 eggs and the internal contents of 2 of 29 eggs. After rapid chilling, PS8NSR was isolated from the surface of 14 of 29 eggs, but could not be isolated from the internal contents of any eggs (0 out of 29). There were significant reductions in numbers of eggs testing positive for PS8NSR after chilling; p <0.01 for slow chill and p <0.0001 for fast chill, compared with before chill. A significant reduction in the number of eggs testing positive for PS8NSR was seen with fast chill compared to slow chill (p <0.05).
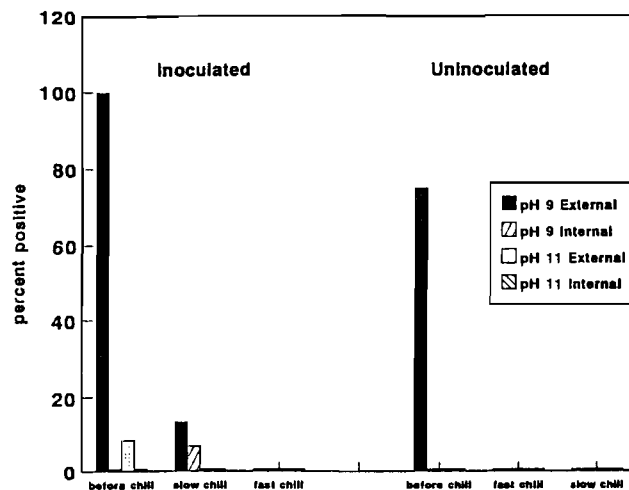
Washing and cooling study

Figure 3. *Effect of washwater pH and chilling rate on the survival of* S. enteritidis *PS8NSR on/in washed eggs. Bars indicate the percentage of eggs testing positive for* S. enteritidis *PS8NSR.*

Figure 3 shows the number of eggs positive for PS8NSR after washing in pH 9 and pH 11 washwater at 37.7°C. A significant difference (p < 0.001) in the reduction of external salmonellae was seen with pH 11 washwater, compared to pH 9 washwater. After washing in pH 9 water and before chilling, 12 of 12 eggs were positive for PS8NSR on the surface of the egg, but none were positive internally. It was determined that PS8NSR was found on the surface but not in the internal contents of 9 of 12 uninoculated eggs washed in pH 9 washwater and examined before chilling. Of the eggs washed in pH 11 washwater, PS8NSR was isolated from the surface of only 1 of 12 inoculated eggs examined before chilling. The PS8NSR was not detected in the internal contents of any of these eggs. No uninoculated eggs were positive for PS8NSR on the surface or internally after washing with pH 11 washwater.

After slow chilling, PS8NSR was found on the surface of 2 of 15 eggs and in the internal contents of 1 of 15 eggs washed at pH 9. After rapid chilling, PS8NSR was not isolated from the internal contents or the surface of any eggs washed in pH 9 washwater. The PS8NSR was not found on the surface or in the internal contents of any of the uninoculated eggs that were subjected to either rapid or slow chilling after being washed in pH 9 washwater. All eggs were internally and externally negative for the presence of PS8NSR when washed in pH 11 washwater and subjected to either rapid or slow chilling.

DISCUSSION

Cooling study

The rate of chilling appeared to have a significant effect on the number of eggs testing positive for PS8NSR (Fig. 2). Fast chill appeared to be most effective in reducing the numbers of microorganisms on the egg surface. It is possible that the eggs that tested internally positive for *S. enteritidis* had thin or cracked shells, which permitted penetration during slow chilling. The quality of the shell should be closely measured and controlled in future studies

to determine if penetration can occur in uncracked eggs.

Though not statistically significant, it is interesting that all of the positive internal samples occurred when eggs were slowly chilled. There are two main differences between fast and slow chilling: 1) moisture remaining on the shell and 2) egg temperature. Numerous studies have shown that there is an increase in bacterial penetration and egg spoilage with eggs that remain moist compared to those that have dry shells (6,7,16,18). Gillespie and coworkers (6,7) observed a greater amount of spoilage in eggs that were kept moist for 24 h and then cleaned, compared to eggs kept dry for 24 h before being cleaned. McNally (16) observed that moisture on the eggshell had a great effect on the incidence of bacterial infection of eggs. When eggs were packed wet after washing, as opposed to dry, the number of infected eggs more than doubled. Padron (18) demonstrated that water enhanced the penetration of Salmonella typhimurium. A greater percentage of membranes were positive from eggs inoculated by a (wet) spray method (100%) compared to those inoculated by a (dry) contact method (59%); a greater percentage of yolk sacs were positive after the spray method (83%) compared to the contact method (29%). The rapid chilling procedure used in this study could have caused the organism to dehydrate faster and become injured. Since there is no circulation of air in the containers of slow chilled eggs, the shells may have remained more moist than those undergoing fast chill and therefore more salmonellae would have remained viable. Moisture on the shell could also have allowed organisms to penetrate through the shell more easily. Also, the test organism might have reproduced on the moist shell, further increasing chances for penetration.

The rate of temperature drop would also have made S. enteritidis less physiologically active. The optimum growth temperature for salmonellae is between 35 and 37°C and the minimum has been observed to be as low as 6.2°C (15). Wolk et al. (24) found that the penetration and survival of bacteria in shell eggs were favored by elevated temperatures. After 21 days of storage, they observed a greater rate of infection in eggs stored at 25°C than those stored at either 5 or 35°C. Gillespie and coworkers (6,7) observed an increase in spoilage in machine-cleaned eggs that were held for several days at room temperature before being placed into cold storage. Slow chilling of eggs could therefore provide temperature and humidity conditions that maintain Salmonellae in a physiologically active state conducive to penetration.

Washing and cooling study

At pH 9, significant cross-contamination between inoculated and uninoculated eggs was observed (Fig. 3). Since the uninoculated-unwashed eggs were all negative for the presence of PS8NSR, cross-contamination was most likely the result of S. enteritidis being washed off the inoculated eggs and into the washwater, where it survived and contaminated the surfaces of uninoculated eggs. The pH 9 washwater did not eliminate the organism from the shell surface of the inoculated eggs because 100% of the inoculated eggs tested positive for the presence of PS8NSR after washing in pH 9 washwater. These results were

expected; pH 9 washwater permitted the growth of S. enteritidis at 37.7°C in earlier experiments (3).

A significant reduction of PS8NSR on the surface of eggs was observed with pH 11 washwater (p < 0.001). In contrast to pH 9 washwater, no cross-contamination was seen between inoculated and uninoculated eggs washed in pH 11 washwater. In addition, only 8.3% of inoculated eggs tested externally positive for the presence of PS8NSR after washing in pH 11 water. This finding also agrees with our previous results (3) and with the findings of others (8,13,14,19,21). In all cases, researchers found a decrease in cell numbers in solutions with a pH of 10.5 or above. Our findings demonstrate that high pH is effective in reducing the number of S. enteritidis on both egg shells and in washwater, and thus preventing cross-contamination during washing.

The results of these experiments indicate that the incidence of S. enteritidis on the surface of eggs can be significantly reduced by: 1) maintaining washwater conditions at pH 11 or above; 2) maintaining washwater temperatures at 37.7°C or above; and 3) rapidly cooling washed eggs to a temperature of 7.2°C or lower. In addition, those conditions may also impede the penetration of S. enteritidis (or other Salmonellae) into the egg. More research needs to be conducted to confirm this latter hypothesis.

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