Comparison between rinse and crush-and-rub sampling for aerobic bacteria recovery from broiler hatching eggs after sanitization

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ABSTRACT This study compared surface and deep eggshell aerobic bacteria recovered by the rinse and crush-and-rub sampling methods for commercial hatching eggs after treatment with sanitizers. Eggs were arranged into 5 treatments consisting of no treatment, water, and 3 sanitizers. The sanitizers were H2O2, phenol, and Q4B (a compound chemical containing 4 quaternary ammoniums and 1 biguanide moiety). Eggs were sprayed according to treatment and allowed to dry for 1 h before sampling. To collect samples for the eggshell rinse, each egg was massaged in a plastic bag with 20 mL of saline. Eggshells were then aseptically opened and their contents were discarded before being individually crushed into 50-mL centrifuge tubes containing 20 mL of saline. Aerobic bacteria were enumerated on Petrifilm after 48 h of incubation at 37°C. Aerobic bacteria recovered (log10 cfu/mL) from the eggshell rinse were highest and similar for the no-treatment (4.0) and water (3.7) groups, lower for the phenol (3.2) and H2O2 (3.1) groups, and lowest for the Q4B (2.4) group. Aerobic bacteria levels with the crush-and-rub method were similar for the no-treatment (2.5) and water (2.3) groups, lower for the phenol (1.6) group, intermediate for the H2O2 (1.2) group, and lowest for the Q4B (0.9) group. The overall correlation between the rinse and crush-and-rub sampling methods for individual egg aerobic bacteria counts was r = 0.71. The correlation within each treatment revealed the following r values: no treatment, 0.55; water, 0.72; H2O2, 0.67; phenol, 0.73; and Q4B, 0.38. A second experiment was designed to further examine the lower aerobic bacterial levels recovered by the crush-and-rub method (for previously rinsed eggs) than the levels recovered in the initial eggshell rinse sample. Eggs were either rinsed and then crushed and rubbed, or they were only crushed and rubbed without a prior rinse. Results confirmed a significant decrease (1.5 log10 cfu/mL) in bacteria levels between the initial rinse (4.4) and the subsequent crush and rub (2.9) for the same eggshell. For the crush-and-rub eggs with no previous rinsing, the bacteria recovery level (3.9) was not significantly different from levels for the rinse method. Therefore, either the rinse or crush-and-rub sampling methods can be used to recover similar levels of eggshell aerobic bacteria.

Key words: aerobic bacteria, crush and rub, eggshell sanitization, hatching egg, rinse

INTRODUCTION

Hatching egg sanitization remains a front-runner for the most important intervention point in preventing bacterial dissemination from broiler breeder flocks to their offspring. Because of the porous nature of the eggshells, bacteria can easily penetrate and potentially contaminate the developing embryos. Prior research has reported that nest-clean broiler eggshells typically contain aerobic bacteria levels between 3.2 and 5.5 log10 cfu/mL of rinsate (Cox et al., 1994; Berrang et al., 1997; Knape et al., 2002; Fasenko et al., 2009; Stephens et al., 2009). Salmonellae in particular are a major concern within the poultry industry as carcass prevalence goals are progressively lowered and sampling is more numerous because of the increased efforts to keep poultry products safe for consumers. Previous studies have shown that Salmonella spp. found in breeder flocks can be passed to offspring and traced through the life of the broiler, into the processing plant, and onto raw products, where Salmonella can become a food safety concern for consumers (Lahellec and Colin, 1985; Bailey

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et al., 1991). Reducing the eggshell contamination of broiler hatching eggs may greatly reduce the prevalence of *Salmonella* and other bacteria on the final processed carcass and products, and thereby reduce the instance of foodborne illness. Cason et al. (1994) showed that hatching eggs inoculated with *Salmonella enterica* serovar Typhimurium exhibited an 86% hatchability rate, indicating that eggs contaminated with *Salmonella* can readily hatch and potentially contaminate other chicks while in the hatching cabinet or on the grow-out farm. Previous research states that the ability of chemical treatments to completely eliminate bacteria from hatching eggs is greatly reduced (from 77 to 45%) after the egg has become contaminated and then cooled to room temperature (Cox and Bailey, 1991). After the eggshell and membrane complex have been penetrated by bacteria, preventing further contamination of the egg and chick is unlikely (Baxter-Jones, 1991). Although sanitizing eggs can sufficiently disinfect the eggshell surface, sanitizing does not effectively kill the bacteria that have already traversed the shell into the underlying shell membranes (Maclaury and Moran, 1959; Cox et al., 2000). Because of this, the application of chemical sanitizers should be accomplished as soon as possible after the eggs are laid and collected. According to a survey article by Grimes and Pardue (1996), early sanitization is already practiced in the turkey industry, with 67% of respondents reporting sanitizing eggs at the breeder farm. They also reported that most turkey hatching eggs (82%) were sanitized by spray washing. The vast majority of broiler hatching eggs are typically not sanitized before setting. Broiler hatching egg sanitation is limited to primary breeders, commercial egg marketers, and eggs for export. All hatching eggs for export are required to have been sanitized either by fumigation with formaldehyde or by spraying with or immersion in an eggshell disinfectant in accordance with the manufacturer’s instructions (OIE, 2003).

Hatching eggs are typically sampled for aerobic bacteria by the rinse or crush-and-rub methods (Musgrove et al., 2005; Chousalkar et al., 2010; Wells et al., 2010). Other methods used less frequently include swabbing the surface (Kawasaki et al., 2008; USDA 2010), rolling the egg onto agar plates (Wineland et al., 1992), or using sterile tape to remove bacteria from regions of the eggshell (Arheinbuwa et al., 1980). This study assessed 3 sanitizing chemicals to determine their efficacy against aerobic bacterial contamination on eggshells. The prevalence of *Salmonella*, *Escherichia coli*, and coliforms among nest-clean hatching eggshells is inconsistent; therefore, aerobic bacteria were selected for evaluating sanitizers (Hannah et al., 2011). The chemicals examined were *H₂O₂*, BioPhene (phenol; BioSentry Inc., Stone Mountain, GA), and Byotrol (Q₄B; Byotrol Inc., Spartanburg, SC). The study also assessed 2 eggshell bacterial recovery methods, the rinse and crush-and-rub methods, which were used for the recovery of aerobic bacteria.

**MATERIALS AND METHODS**

**Experiment 1**

**Sample Preparation.** For each of 4 trials, 50 nest-clean broiler hatching eggs were obtained from a commercial hatchery 2 d after lay. The eggs were from breeder hens at 58, 51, 28, and 51 wk of age (trials 1 to 4, respectively). The eggs were removed from the hatchery egg holding room on the morning of each trial and transported to the laboratory on cardboard flats, with each flat in an individual plastic bag. On arrival, the eggs were allowed to warm to laboratory room temperature for 2 h. Eggs had not been sprayed or otherwise sanitized before arrival or removal from the hatchery.

Ten eggs were placed into each of 5 treatment groups: a no-treatment control, a deionized water spray to account for the rinsing effects of water on bacteria recovery levels (Cox et al., 2007), and 3 sanitizer treatments: a 15,000-ppm (1.5%; 50 mL of 30% *H₂O₂*/L) solution of *H₂O₂* (CCI, Columbus, WI); a 794-ppm solution of BioPhene (phenol; 4 mL/L), composed of 7.92% o-phenylphenol, 9.97% o-benzyl-p-chlorophenol, and 1.95% p-tert-amylyphenol (BioSentry Inc.); and a 1,200-ppm solution of Q₄B (12 mL/L; not yet approved for use on hatching eggs), composed of 4 quaternary ammoniums and 1 biguanide biocide moiety attached to a polymer core (Byotrol Inc.). All initial chemical concentrations were at the levels recommended by the manufacturers, and *H₂O₂* (1.5%) was similar to the level used by Cox et al. (2007) in previous experiments and shown to be effective against *Salmonella* challenge. After trial 1, Q₄B proved to be the most effective chemical, so the concentration was reduced from 1,200 to 794 ppm (7.9 mL/L) to be directly comparable with the phenol concentration. In addition, previous research had indicated that Q₄B was effective against *Salmonella* on inoculated hatching eggs at concentrations as low as 2 mg/L (our unpublished data). After assignment and placement into clean plastic posted setting flats, the eggs were aseptically handled to prevent cross-contamination.

**Preparation of the Sanitizers.** Sanitizer treatments were mixed the day of treatment using deionized water as the solvent for the concentrated liquid chemicals. For each solution, 400 mL was mixed in 750-mL calibrated plastic spray bottles. Flats with eggs were moved using the stacking posts on the egg flat and placed into the sink for spraying. Each treatment was hand sprayed onto the eggs, with each bottle nozzle set to deliver 10 mL of solution to each egg. All eggs were allowed to dry on the laboratory bench for 1 h after spray sanitizing before sampling (Cox et al., 2007).

**Microbiological Sampling.** For the eggshell rinse, each egg was aseptically placed into a new 530-mL plastic bag with 20 mL of a sterile 0.85% saline-10% powdered milk solution. Powdered milk was included...
to deactivate any sanitizer residual on the eggshell that might affect remaining bacteria within the rinsate. Deactivation was required for the biguanide in the Q4B; therefore, powdered milk was added to all rinsates to ensure that all chemical activity occurred on the eggshell and not in the rinsate (Cox et al., 2007). Each egg was massaged in the saline by hand for 1 min before being aseptically removed and placed on a clean plastic flat. This process was repeated for all 10 eggs within each treatment group.

After rinsing, a modified crush-and-rub method was performed, similar to the procedures described by Berang et al. (1991) and modified by Musgrove et al. (2005). Briefly, each egg was aseptically opened and the internal contents (albumen and yolk) were discarded. The eggshell and membrane complex were then gently crushed in a gloved hand and forced into a sterile 50-mL centrifuge tube. A 20-mL quantity of the 0.85% saline-10% powdered milk solution was then pipetted into each tube. The eggshell and membrane complex was further mixed and crushed for 1 min by using a sterile glass rod.

The rinsates from both the rinse and crush-and-rub samples were serially diluted and 1 mL was plated onto Aerobic Count Plate Petrifilm (3M, St. Paul, MN). In trial 1, all samples were directly plated and were plated after dilutions of $10^{-2}$ and $10^{-4}$. After the results for the first trial were summarized, in the 3 subsequent trials, direct plating and a $10^{-2}$ dilution only were performed for the sanitizers (H$_2$O$_2$, phenol, and Q4B). For the water and no-treatment groups, only the $10^{-1}$ and $10^{-3}$ dilutions were plated. After plating, the Petrifilm were allowed to incubate at 37°C for 48 h.

**Experiment 2**

**Sample Preparation.** Experiment 2 examined the effects of prior eggshell rinsing on the subsequent crush-and-rub sampling procedure for aerobic plate count (APC) recovery. This was important because in experiment 1, there was an average 1.5-log decrease in bacterial recovery between the sequential use of the 2 methods on the same egg. Two trials were conducted using 20 eggs in each sample group per trial. This resulted in the same number of total eggs per sample group as in experiment 1 ($n=40$ eggs). Hatching eggs were gathered from the egg holding room of a commercial hatchery 2 d after lay. Eggs were transported and allowed to warm to laboratory room temperature for 2 h before sampling. Twenty eggs were placed into 1 of 3 sampling groups: a rinse group, a crush-and-rub following a rinse group, or a crush-and-rub with no prior rinsing group. Eggs were sampled as is from the hatchery and were not sprayed or otherwise sanitized before sampling. After the initial placement of eggs into plastic posted setting flats, eggs were aseptically handled to prevent cross-contamination. For the rinse, crush-and-rub after rinse, and crush-and-rub with no rinse groups, all sampling was done as described in the microbiological sampling section for experiment 1. After the rinse group was sampled, the same eggs were used in the crush-and-rub after rinse group.

**Sample Collection.** To collect samples for the rinse group, the same procedure as described above for experiment 1 was followed for rinsing, with the exception that powdered milk was not added to the rinse; only 20 mL of 0.85% saline was added. The powdered milk was not necessary in experiment 2 because none of the eggs had been treated with sanitizing chemicals. Once the rinse group samples were collected, the same eggs were used to sample for the crush-and-rub after rinsing group. Eggs were crushed-and-rubbed using the same procedure as in experiment 1. Last, the crush-and-rub with no prior rinsing group was sampled, also using the method described in experiment 1. Once rinsates for all 3 groups were collected, they were serially diluted and 1 mL was plated onto APC Petrifilm. After plating, the Petrifilm were allowed to incubate at 37°C for 48 h before enumeration.

**Statistical Analyses**

After incubation, the plates were enumerated for a total APC following the manufacturer’s instructions. Because no trial × treatment interactions were detected, trials were combined across all treatment groups within experiments 1 and 2. The positive averages for these data were compiled and converted to $\log_{10}$ cfu/mL of rinsate for statistical analysis. All data were analyzed using the GLM and Pearson’s correlation (CORR) programs of SAS (SAS Institute Inc., Cary, NC). With the GLM procedure of SAS, in experiment 1 (within rinse or crush-and-rub samples) the treatment means were separated using Tukey’s test at $P<0.05$.

**RESULTS AND DISCUSSION**

In experiment 1, the APC for the no-treatment rinse was highest and was similar to the water spray, with values of 4.0 and 3.7 $\log_{10}$ cfu/mL, respectively (Figure 1). A 0.5-log decrease was observed for the phenol rinse, which was similar to rinse values for H$_2$O$_2$, at 3.2 and 3.1 $\log_{10}$ cfu/mL, respectively. Last, the Q4B rinse exhibited the lowest bacterial recovery, at 2.4 $\log_{10}$ cfu/mL. For the crush-and-rub sampling method following the rinse, the APC for the no-treatment and water groups were again similar, at 2.5 and 2.3 $\log_{10}$ cfu/mL, respectively, before a decrease of 0.7 log to 1.6 $\log_{10}$ cfu/mL for phenol was observed. Phenol was not significantly different from H$_2$O$_2$ at 1.2 $\log_{10}$ cfu/mL, which was also not significantly different from Q4B at 0.9 $\log_{10}$ cfu/mL. However, the 0.7-log difference in APC between phenol and Q4B was statistically significant. When comparing the sampling methods within each treatment group, the no-treatment, water, phenol, H$_2$O$_2$, and Q4B groups all exhibited significant decreases...
es in APC between the rinse and the subsequent crush-
and-rub methods, ranging from 1.4 to 1.9 log10 cfu/mL.

To determine if there were relationships in APC re-
covery between the rinse and crush-and-rub sampling
methods (for each individual egg), Pearson correlation
coefficients were determined for both the overall ex-
periment and within each individual treatment group.
High correlations would indicate that the variability in
rinse APC recovery could predict identical variability
in crush-and-rub APC recovery for each individual egg.
Overall, the correlation between aerobic bacteria recov-
ery for the rinse and crush-and-rub sampling methods
was high, at $r = 0.71$ (Figure 2A). Within each group,
the no-treatment group exhibited a correlation of 0.55
(Figure 2B), the water group exhibited a correlation of
0.72 (Figure 2C), the H$_2$O$_2$ group exhibited a correla-
tion of 0.67 (Figure 2D), the phenol group exhibited a
correlation of 0.73 (Figure 2E), and the Q$_4$B group had
the lowest correlation, at 0.38 (Figure 2F). The correla-
tions for the water, phenol, and H$_2$O$_2$ groups were all
similar to the overall correlation ($r = 0.71$).

For the no-treatment group (Figure 2B), the fact that
no prior washing of the eggs had occurred may explain
the low correlation between the rinse and crush-and-rub
APC recovery values for this group. Because these
eggs were being sampled as is from the hatchery, this
presents the likelihood of wide variability in the eggshell
rinse numbers because of the wide differences in initial
eggshell aerobic bacteria contamination, even though
only nest-clean eggs were used. For Q$_4$B, not only was
the correlation the lowest, but the data points were also
shifted closer to zero for both the rinse and crush-and-rub
methods, as indicated in Figure 2F. For Q$_4$B, the
spread in data points ranged between 0.5 and 4.0 log$_{10}$
cfu/mL for the rinse method and between −0.3 and 3.3
log$_{10}$ cfu/mL for the crush-and-rub method. This was
a smaller range for both the rinse and crush-and-rub
methods compared with all other treatments (1.3 to 5.3
log$_{10}$ cfu/mL for rinse, and 0.0 to 4.6 log$_{10}$ cfu/mL for
crush-and-rub). This indicates that the low correlation
between the rinse and crush-and-rub methods for Q$_4$B
was the result of increased variability, which may be
explained by the enhanced killing or removal of bacte-
ria from the eggshell. As can be seen in Figure 2F, Q$_4$B
exhibited a bimodal distribution pattern. After data
from each of the 4 trials were separated, this pattern
was determined to be a trial effect, although none of
the trials was statistically different ($P > 0.05$) within
the Q$_4$B treatment.

Kawasaki et al. (2008) compared Salmonella recovery
by the swab, crush-and-rub after swabbing, and crush-
and-rub only methods for table eggs inoculated with 2
x 10$^7$ cells of Salmonella. They observed a difference of
3.2 log$_{10}$ cfu on 3.14 cm$^2$ eggshell in Salmonella recovery
between those eggs that were crushed-and-rubbed after
swabbing (3.3 log$_{10}$ cfu on 3.14 cm$^2$ eggshell) and those
eggs that were crushed-and-rubbed with no previous
swabbing (6.5 log$_{10}$ cfu on 3.14 cm$^2$ eggshell). Kawasaki
et al. (2008) also compared the rinse method with the
crush-and-rub method for APC recovery from separate
whole eggshells (processed table eggs) and yielded dif-
ferent results from the present study, which used nest-
clean broiler hatching eggs. When rinsed, the table eggs
had approximately 3.1 log$_{10}$ cfu/eggshell aerobic bac-
teria, whereas when eggs were crushed-and-rubbed with
no previous rinse, the recovery was greater, at 4.3 log$_{10}$
CFU/eggshell of aerobic bacteria. The 1.2 log_{10} CFU/eggshell lower recovery for the rinse method than for the crush-and-rub method may be attributed in part to the use of processed table eggs.

The second experiment aimed to assess whether sampling with a rinse before performing a crush-and-rub procedure would affect the bacterial recovery when using the same egg. In a previous experiment conducted by Stephens et al. (2009) in a commercial hatchery, no differences were observed between recovered aerobic bacteria levels from sanitized eggs using the rinse method or the crush-and-rub method following the rinse.

Figure 2. Pearson correlation between the rinse and crush-and-rub sampling methods. A) Overall correlation, where n = 200, r = 0.71, and y = 0.7074x − 0.6298; B) no treatment, where n = 40, r = 0.55, and y = 0.6118x + 0.0593; C) water treatment, where n = 40, r = 0.72, and y = 0.8024x − 0.7003; D) H_2O_2 treatment, where n = 40, r = 0.67, and y = 0.646x − 0.8203; E) phenol treatment, where n = 40, r = 0.73, and y = 0.6549x − 1.4233; and F) Q_4B, where n = 40, r = 0.38, and y = 0.2436x + 0.289. The sanitizers were H_2O_2 (CCI, Columbus, WI), phenol [BioPhene, composed of 7.92% α-phenylphenol, 9.97% α-benzyl-p-chlorophenol, and 1.95% p-tert-amylphenol (BioSentry Inc., Stone Mountain, GA)], and Q_4B (a compound composed of 4 quaternary ammoniums and 1 biguanide biocide moiety attached to a polymer core; Byotrol Inc., Spartanburg, SC).
purchased from a local supermarket in Japan.

However, experiment 1 in the present study indicated a consistent, significant decrease (1.4 to 1.9 log10 cfu/mL in APC (Figure 1) between the 2 sampling methods when used in sequence.

Results for experiment 2 indicate that there was an average decrease of 1.5 log10 cfu/mL in APC when an eggshell and membrane complex was crushed-and-rubbed after an initial eggshell rinse (Figure 3). This confirms that a significant decrease in aerobic bacteria recovery occurs for crush-and-rub if both sampling methods are used in sequence on the same egg. The results obtained by Stephens et al. (2009), in which no differences were detected in recovered aerobic bacteria levels when using the rinse or the crush-and-rub method following the rinse, may be attributed to the fact that the sanitized eggs remained in the commercial hatchery egg holding room for 1 h after sanitizing before removal for the setting buggy. There is a high probability for fan-blown aerobic bacteria recontamination of the sanitized eggs to occur within the hatchery egg holding room because of the large quantity of nontreated eggs (47 buggies) surrounding the single sanitized egg buggy, as well as the continuous transfer of additional eggs from farm buggies to setting buggies. A secondary goal of experiment 2 was to examine whether the sampling methods used could make a difference in the efficacy of recovery for aerobic bacteria if performed separately from one another, rinse vs. crush-and-rub without a prior rinse. In the current study, sampling eggshells with the rinse method or the crush-and-rub method without a prior rinse was statistically similar, with a difference of only 0.5 log10 cfu/mL of APC between the rinse and crush-and-rub methods. On untreated eggs, rinsing alone recovered 4.4 log10 cfu/mL, whereas performing an eggshell and membrane crush-and-rub method yielded a recovery of 3.9 log10 cfu/mL. These APC recovery levels per milliliter of eggshell rinsate for broiler hatching eggs exceed the values reported by Kawasaki et al. (2008) for the entire eggshell (3.1 to 4.3 log10 cfu/eggshell) and may be attributed to their purchase of table eggs from a local supermarket in Japan, where table eggs are required to be washed before sale (Ministry of Health, Labour and Welfare of Japan, 1998). Although there is no difference in using either recovery method for APC, it should be noted that an eggshell and membrane complex crush-and-rub method may be desirable, depending on the type of bacteria recovery necessary. Musgrove et al. (2005) examined the recovery of Salmonella from eggshells using both methods. For all the eggs sampled that had natural Salmonella, rinsing recovered Salmonella in only 23.5% of the samples, whereas the recovery increased 3-fold to 76.5% when crush-and-rub sampling was used. In addition, the study by Kawasaki et al. (2008) examined Salmonella recovery on inoculated eggs and found that swabbing alone recovered an average of 1.0 log less Salmonella than did the crush-and-rub procedure. This indicates that when looking for Salmonella on eggshells, it may be best to perform the crush-and-rub method, whereas when looking for aerobic bacteria, eggshell rinsing is as effective as the crush-and-rub method and is nondestructive.

Future research should aim to determine the lowest effective concentration of Q4B against eggshell Salmonella, to determine if that concentration affects hatchability, and to begin large-scale testing in a commercial hatchery environment to determine if Q4B can be used cost effectively for commercial hatching egg sanitization.

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