Evaluation of a combination of sodium hypochlorite and polyhexamethylene biguanide as an egg wash for red-eared slider turtles (*Trachemys scripta elegans*) to suppress or eliminate *Salmonella* organisms on egg surfaces and in hatchlings

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Objective—To evaluate a combination of 2 nonantibiotic microbicide compounds, sodium hypochlorite (NaOCl) and polyhexamethylene biguanide (PHMB), as a treatment to suppress or eliminate *Salmonella* spp from red-eared slider (RES) turtle (*Trachemys scripta elegans*) eggs and hatchlings.

Sample Population—2,738 eggs from 8 turtle farms in Louisiana.

Procedures—Eggs were randomly sorted into 3 or, when sufficient eggs were available, 4 treatment groups as follows: control, pressure-differential egg treatment with NaOCl and gentamicin, NaOCl and PHMB bath treatment, and pressure-differential egg treatment with NaOCl and PHMB. Bacterial cultures were performed from specimens of eggs and hatchlings and evaluated for *Salmonella* spp.

Results—RES turtle eggs treated with NaOCl and PHMB as a bath (odds ratio [OR], 0.2 [95% confidence interval (CI), 0.1 to 0.3]) or as a pressure-differential dip (OR, 0.01 [95% CI, 0.001 to 0.07]) or with gentamicin as a pressure-differential dip (OR, 0.1 [95% CI, 0.06 to 0.2]) were significantly less likely to have *Salmonella*-positive culture results than control-group eggs.

Conclusions and Clinical Relevance—Concern over reptile-associated salmonellosis in children in the United States is so great that federal regulations prohibit the sale of turtles that are < 10.2 cm in length. Currently, turtle farms treat eggs with gentamicin solution. Although this has reduced *Salmonella* shedding, it has also resulted in antimicrobial resistance. Results of our study indicate that a combination of NaOCl and PHMB may be used to suppress or eliminate *Salmonella* spp on RES turtle eggs and in hatchlings. (*Am J Vet Res* 2007;68:158–164)

Reptile-associated salmonellosis remains a primary concern of public health officials in the United States.[1-5] The increased numbers of reptiles imported into the United States during the 1990s and the recent findings of illegal hatchling (< 10.2 cm in length) turtle and terrapin sales in major US markets have been blamed for the increased incidence of reptile-associated salmonellosis.[6] Currently, no federal or state regulations exist that are attributed to managing the risk associated with *Salmonella* spp in captive reptiles. Instead, the general consensus is that reptiles should be kept only by responsible adults and that households with young children or immunocompromised individuals should not keep reptiles.[7] Although these recommendations have been accepted as a method to minimize the likelihood of developing reptile-associated salmonellosis from imported wild reptiles and captive-bred nonchelonian reptiles by the Pet Industry Judicial Action Committee and the CDC, 1 segment of the pet reptile industry, the aquatic chelonian farmers, remains unable to participate in the market.

Aquatic chelonians have been raised in captivity on commercial farms for > 70 years. An increased incidence of turtle-associated salmonellosis in young children during the late 1960s and early 1970s led the FDA to enact a regulation that restricted the inter- and intrastate sale of chelonians < 10.2 cm in length.

Abbreviations

RES	Red-eared slider
PHMB	Polyhexamethylene biguanide
NaOCl	Sodium hypochlorite
DSE	Delayed secondary enrichment
CI	Confidence interval
OR	Odds ratio

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In response to the regulation, aquatic chelonian farmers from Louisiana initiated research to identify methods to eliminate *Salmonella* spp in hatchling turtles. Initial research focused on the use of common veterinary antimicrobials. Treatment of hatchlings with oxytetracycline in their tank water for up to 14 days reduced shedding in treated turtles, but did not affect enteric colonization.[8] Treatment of freshly laid eggs with oxytetracycline or chloramphenicol with a temperature-differential egg dip method was also successful at eliminating *Salmonella* spp in eggs < 1 day old, but did not clear eggs > 2 days old.[8] Large-scale experimentation on commercial turtle farms with surface decontamination and pressure- or temperature-differential treatment of eggs with gentamicin dip solutions for eggs > 2 days old, followed by hatching eggs on *Salmonella*-free bedding, substantially reduced *Salmonella* spp infections and shedding rates in hatchling turtles.[9] Forty percent of the eggs not treated with the gentamicin was found to harbor *Salmonella* spp, whereas only 0.15% of the treated eggs had positive results.

Unfortunately, the use of antimicrobials has led to the development of antimicrobial-resistant strains of *Salmonella* spp. One study[10] isolated *Salmonella* spp from 6 of 28 (21%) RES turtle (*Trachemys scripta elegans*) lots exported to Canada from Louisiana. Of the 37 *Salmonella* strains isolated, 30 (81%) were gentamicin resistant. Another study[11] collected environmental specimens and live hatchlings directly from 2 Louisiana turtle farms and found isolates of *Salmonella enterica* subsp *arizonae* and *S enterica* serotype Poona that were resistant to erythromycin, gentamicin, tetracycline, and sulfonamide. Findings of these studies suggest that the application of a single antimicrobial agent is not sufficient to suppress or eliminate *Salmonella* spp from captive-bred chelonians and that it leads to the development and dissemination of antimicrobial resistance in turtles and their environment.

Almost since the inception of the 1975 regulation, aquatic chelonian farmers have been in discussions with the FDA to determine possible methods to reverse the regulation and reopen the pet turtle market in the United States. The FDA's response to farmers has been that they must find 3 nonantibiotic methods to eliminate *Salmonella* spp from RES turtles. Recent research suggests that a PHMB compound may be used to suppress or eliminate *Salmonella* spp in the aquatic environment of captive RES hatchings.[12] This group of compounds may also prove valuable as a method to treat RES turtle eggs.

Polyhexamethylene biguanide is a sanitizing agent that is considered safe for human and animal use. This compound has been used as a mouth rinse for humans,[13] a microbicide for chicken eggs,[14] and a treatment for fungal keratitis.[15] The antimicrobial effect of this compound varies with concentration, being bacteriostatic at low concentrations and bacteriocidal at higher concentrations. Many derivatives of PHMB exist. One such derivative of PHMB (polyiminoimidocarbonylimino-hexamethylene hydrochloride)[a] is a commercially available swimming pool sanitizer and algistatic agent that can be used as a safe alternative to chlorine for swimming pools.

The purpose of the study reported here was to determine whether a combination of NaOCl (household bleach)[b] and PHMB could be used to treat RES turtle eggs and suppress or eliminate *Salmonella* spp in turtle eggs and hatchlings. The first hypothesis tested was that eggs treated with any of 3 treatment combinations would be less likely to have *Salmonella*-positive culture results than controls. The second hypothesis was that no difference would be found in the *Salmonella* status of the 3 treatment groups. The third hypothesis was that no difference would be found in *Salmonella*-culture results between the 3 geographic regions in Louisiana. The final hypothesis was that no difference would be found in *Salmonella*-culture results between specimen collection times.

Materials and Methods

Study design—This study was conducted in accordance with the regulations specified by the Louisiana State University Institutional Animal Care and Use Committee. Eight farms were selected for this study from the 3 major turtle-producing regions as follows: Ponchatoula, La (n = 2 farms); Pierre Part, La (3); and Jonesville, La (3). Specimens were collected from June to July 2004. Each farm was visited twice (June, July), and 1 farm was visited 3 times (farm 2, Pierre Part: June once and July twice).

Specimen collection—Individual turtle nests were randomly collected in the early morning after being deposited and immediately processed (< 1 hour). Eggs from each nest were randomly (with a random number generator) sorted into 3 or 4 treatment groups as follows: group 1, control treatment; group 2, NaOCl[b] and gentamicin pressure–differential treatment; group 3, NaOCl and PHMB[a] bath treatment; and group 4, NaOCl and PHMB pressure–differential treatment. Initially, we were concerned that insufficient eggs would be available to evaluate a control and 3 treatment groups; however, after the initial 2 visits, it was determined that it was possible and the fourth group was added. Group 3 was originally selected over group 4 because the farmers wanted to evaluate a nonpressure-differential method because it is less time-consuming. The control group was left completely untreated. This was done in an attempt to estimate the prevalence of *Salmonella* spp on eggs under natural conditions. Removing any of the nest soil, even by rinsing, would potentially bias the potential *Salmonella* spp exposure that eggs have under natural conditions. Sodium hypochlorite was used in combination with the gentamicin and PHMB treatments because the farmers currently use this compound as part of their treatment regimen. It would not have been possible to evaluate intranest variability by adding an additional NaOCl treatment group.

Tap water from each farm was collected, and the pH, alkalinity, hardness, and chlorine content were determined. Three samples of tap water were also collected from each farm on each visit and tested for the presence of *Salmonella* spp. All water sources on the 8 different farms were found to have *Salmonella*-

negative culture results. Tap water from each farm was used to mix the treatment solutions. The water temperature for all of the treatments was maintained between 32.2° and 34.4°C. Egg handling was all done by nest and treatment to minimize the potential for contamination. Eggs in the control group were not rinsed or treated with any antimicrobial. All remaining eggs were rinsed with chlorinated tap water to remove organic matter from the egg surfaces. Eggs were then placed into a plastic container with 18.9 L of NaOCl solution at 1,000 µg/L for 5 minutes. Water in the container was recirculated with a pump. After washing eggs in NaOCl, eggs were allowed to dry (15 to 30 minutes) in sterile containers. Once dried, group 2 eggs were treated with gentamicin (1,200 µg/mL) for a 10-minute soak and a 10-minute vacuum by use of the pressure-differential method.[8] Group 3 eggs were placed into a plastic container with 18.9 L of PHMB solution at 50 µg/L for 5 minutes, rinsed, and then treated again for an additional 5 minutes. Again, water in the container was recirculated with a pump. Group 4 eggs were treated with PHMB solution at 50 µg/L for a 10-minute soak and a 10-minute vacuum by use of the pressure-differential method. The water used to mix the PHMB solution was dechlorinated with sodium thiosulfate, and the pH was adjusted to 7.5 by use of a pH regulator[c] according to the manufacturer's recommendation.

One prehatch egg from the control group and each treatment group was removed for bacterial culture on-site immediately after collection and sorting. The surface of each egg was swabbed with a sterile cotton-tipped applicator that was premoistened with sterile saline (0.9% NaCl) solution. The swab was immediately placed into 7 mL of selenite enrichment broth.[d] An alcohol- (70% ethyl alcohol) soaked, cotton-tipped applicator was used to sterilize the external surface of the egg. A 16-gauge hypodermic needle fastened to a 3-mL syringe was used to collect 3 mL of egg contents. Egg contents were then immediately placed into 7 mL of selenite enrichment broth.

Remaining eggs were placed in sterile plastic containers (incubation chambers) on sterile perlite substrate (2.5-cm depth). The perlite substrate was mixed with chlorinated tap water at a 2:1 ratio. Containers were labeled with the appropriate identification information and placed into an incubation room on each respective farm. Eggs were incubated between 28.9° and 30°C. All specimens collected during the first visit were transported to the Louisiana State University School of Veterinary Medicine for processing.

Culture techniques—All specimens for bacterial culture were incubated under aerobic conditions at 37°C for 48 hours. After incubation, the enriched selenite cultures were mixed on a vortex agitator for 5 seconds. A heat-sterilized bacterial loop was used to transfer an aliquot of enriched broth to the surface of a Petri dish containing xylose-lysine-tergitol agar.[e] Streaked plates were incubated at 37°C for 48 hours under aerobic conditions. Presumptive *Salmonella* colonies were evaluated on indicator media including urea,[f] lysine iron agar,[g] and triple sugar iron agar.[h] A heat-sterilized bacterial loop was used to streak a portion of a suspect colony onto slants of urea, lysine iron agar, and triple sugar iron agar, and preparations were incubated aerobically at 37°C for 24 hours. The presence of *Salmonella* spp was denoted by a negative urea test result, production of hydrogen sulfide on lysine iron agar, and a basic (vs acidic) response with hydrogen sulfide production in triple sugar iron agar. Presumptive *Salmonella* colonies were further evaluated with test strips.[i] A heat-sterilized bacterial loop was used to transfer colonies from the triple sugar iron agar slant to 10 mL of 0.85% NaCl solution to attain a concentration equivalent to a 0.5-McFarland equivalence turbidity standard.[d] Bacterial cultures were placed into designated receptacles on the test strips[f] in accordance with the manufacturer's directions and incubated aerobically at 37°C for 24 hours. Bacterial reactions were interpreted with the appropriate key compiled by the manufacturer of the test strip.[i]

A 72-hour DSE was performed on all bacterial cultures to increase the likelihood of identifying those with low concentrations of *Salmonella* spp. The original selenite bacterial cultures were placed at room temperature (approx 22.2°C) for 72 hours. A 3-mL aliquot of the original selenite bacterial culture was added to 7 mL of selenite enrichment broth and incubated aerobically at 37°C for 48 hours. Bacterial cultures were processed with the techniques already described.

A second trip was made to each farm approximately 50 to 55 days after eggs were initially processed to collect posthatch eggshell remnants and hatchling specimens for processing. All specimens were processed at the Louisiana State University School of Veterinary Medicine. Eggshells collected on the second trip were removed from containers and placed directly into 7 mL of selenite enrichment broth. Eggshells were vortexed and macerated. Hatchlings were removed from containers and humanely euthanatized with an overdose of ketamine hydrochloride[j] (50 mg/kg, intracoelomic) and a barbiturate[k] (0.05 mL/turtle, intracardiac). A gross necropsy was performed by use of sterile techniques. Sterile scissors were used to cut bridges of the shell and facilitate removal of the plastron. The visceral pluck, including the yolk sac, liver, kidneys, spleen, and gastrointestinal tract, was excised, placed into 7 mL of selenite broth, and vortexed to macerate tissues. These specimens were used for primary and DSE bacterial cultures by use of the techniques described.

Six RES turtles were randomly selected during the study (farm 2, Ponchatoula, La) and necropsied to determine whether any negative adverse effects were associated with the different treatment methods. Tissues were processed routinely, sectioned (4-µm thickness), stained with H&E, and examined microscopically by a board-certified veterinary pathologist blinded to treatment groups.

Statistical analysis—The sample size used for this study was selected on the basis of the following assumptions and criteria: the proportion of RES turtles with *Salmonella*-positive culture results in the control group would be ≥ 0.3, the proportion of RES turtles with *Salmonella*-positive culture results in any of the

treatment groups would be ≤ 0.1, a value of $\alpha = 0.05$, and a value of power ≥ 0.99. The 95% binomial CIs were calculated for each of the proportion estimates. In instances where the prevalence estimate was 0, 95% CIs were estimated with the technique described by van Belle and Millard.[16]

If an egg, eggshell, or gastrointestinal tract specimen was found to have *Salmonella*-positive culture results, the treatment group for that nest was considered *Salmonella* positive (treatment failure) for analysis. This was done because if a single egg or hatchling in an incubation chamber had a positive culture result, the potential for contamination of other eggs or hatchlings existed.

A χ^2 test for homogeneity was performed to evaluate the homogeneity of the treatment groups (ie, control treatment, gentamicin pressure–differential treatment, PHMB bath treatment, PHMB pressure–differential treatment) with respect to *Salmonella* status. To test individual group differences, a χ^2 test for homogeneity was used to compare each treatment group with the control turtles. The Fisher exact test was used to compare the 3 geographic locations for gentamicin treatment failure. Separate logistic regression analysis was used to model risk and determine whether treatment (ie, control treatment, gentamicin pressure–differential treatment, PHMB bath treatment, or PHMB pressure–differential treatment), trip (June or July), or location (Ponchatoula, Pierre Part, or Jonesville) predicted *Salmonella* status. Main-effect variables were removed individually from full models to assess the affects on the model likelihood ratio statistics, magnitude of the coefficients for other variables, and Hosmer-Lemeshow goodness-of-fit statistics. Interactions between the main-effect variables were also evaluated in the models. Commercially available software[l] was used for analysis. A value of $P < 0.05$ was considered significant.

Results

A total of 2,738 eggs and hatchlings were processed for this study. Clutch size ranged from 8 to 23 eggs, and the mean nest contained 12.2 eggs. Of 2,728 specimens, 145 (5.3%) had *Salmonella*-positive culture results. The prevalence of *Salmonella* spp by treatment group, farm, and trip was determined (**Table 1**).

A significant difference was found in the prevalence of *Salmonella* spp in specimens of eggs and hatchlings between the control treatment (31.4% [95% CI, 25.3 to 37.5%]) group and gentamicin pressure–differential (5.4% [95% CI, 2.4 to 8.4%]; $\chi^2 = 24.8$; $P < 0.001$), PHMB pressure–differential (1.0% [95% CI, 0.0 to 2.3%]; $\chi^2 = 31.0$; $P < 0.001$), and PHMB bath (9.0% [95% CI, 5.2 to 12.8%]; $\chi^2 = 17.0$; $P < 0.001$) treatment groups. Also, a significant ($\chi^2 = 6.1$; $P = 0.025$) difference was found between eggs treated with PHMB by the pressure-differential method and bath method.

Salmonella spp were isolated from posthatch specimens (66% [95% CI, 56.8% to 75.1%]) more frequently than freshly laid and treated eggs (34% [95% CI, 24.0% to 43.1%]). However, when evaluating *Salmonella* spp recovery by individual specimen, and considering fewer specimens were collected before hatching, no significant ($\chi^2 = 0.7$; $P = 0.4$) difference was found in the likelihood of isolating *Salmonella* spp between pre- and posthatch specimens. Of the posthatch specimens, eggshells had *Salmonella*-positive culture results (60.2% [95% CI, 51.1% to 69.2%]) more often than the visceral pluck (39.8% [95% CI, 30.8% to 48.8%]).

The prevalence of gentamicin treatment failure by clutch was highest in Pierre Part (9/91 [9.9%]), compared with Ponchatoula (2/52 [3.8%]) and Jonesville (1/80 [1.2%]); the difference between Pierre Part and Jonesville was significant ($P = 0.01$). It is interesting to note that instances of *Salmonella*-positive culture results were limited to a single farm at each geographic location.

In the final logistic regression model, treatment ($\chi^2 = 78.0$; $P < 0.001$), location ($\chi^2 = 14.9$; $P = 0.001$), and trip ($\chi^2 = 8.1$; $P = 0.017$) were included in the model. No significant interaction terms were found. The final model was as follows:

$$\text{Salmonella status} = ([-1.46 + 0.264] \times \text{loc}_1) + (1.0 \times \text{loc}_2) - (2.21 \times \text{tr}_1)\ (1.65 \times \text{tr}_2) - (4.7 \times \text{tr}_3) + (0.52 \times \text{tr}_1) - (0.69 \times \text{tr}_2)$$

where loc_1 and loc_2 are locations 1 and 2, respectively, and tr_1, tr_2, and tr_3 are trips 1, 2, and 3, respectively. Specimens treated by gentamicin pressure–differential

Table 1—Prevalence of *Salmonella*-positive culture results (ie, treatment failures) in turtle clutches of farms at various sites in Louisiana.

			Treatment failures*			
Site	Farm No.	No. of trips to farm	Control	Gentamicin pressure–differential method	PHMB bath method	PHMB pressure–differential method
Ponchatoula	1	2	22.7% (5/22)	0% (0/22)	9.1% (2/22)	0% (0/12)
	2	2	30% (9/30)	6.7% (2/30)	6.7% (2/30)	3.3% (1/30)
Pierre Part	1	2	42.9% (9/21)	42.9% (9/21)	4.8% (1/21)	0% (0/21)
	2	3	42.5% (17/40)	0% (0/40)	22.5% (9/40)	0% (0/30)
	3	2	33.3% (10/30)	0% (0/30)	0% (0/30)	0% (0/30)
Jonesville	1	2	26.7% (8/30)	0% (0/30)	6.7% (2/30)	0% (0/30)
	2	2	28% (7/25)	4% (1/25)	8% (2/25)	4% (1/25)
	3	2	20% (5/25)	0% (0/25)	8% (2/25)	0% (0/25)

*Numbers and percentages on a per clutch basis.

Table 2—Turtle egg hatching rates by site and treatment.

Site	Farm No.	No. of trips to farm	Treatment failures*			
			Control	Gentamicin pressure–differential method	PHMB bath method	PHMB pressure–differential method
Ponchatoula	2	4	55.1% (43/78)	53.7% (44/82)	59.3% (48/81)	88% (44/50)
Pierre Part	3	7	76.9% (147/191)	70.6% (142/201)	74.8% (160/214)	73% (73/100)
Jonesville	3	6	67.2% (121/180)	74.1% (143/193)	61.9% (120/194)	66.9% (87/130)

See Table 1 for key.

(OR, 0.1 [95% CI, 0.06 to 2.0]; $P < 0.001$), PHMB bath (OR, 0.2 [95% CI, 0.1 to 0.3]; $P < 0.001$), or PHMB pressure–differential (OR, 0.01 [95% CI, 0.001 to 0.07]; $P < 0.001$) methods were significantly less likely to have *Salmonella*-positive culture results than controls. Specimens collected from Pierre Part were significantly ($P < 0.001$) more likely to have *Salmonella*-positive culture results (OR, 2.7 [95% CI, 1.6 to 4.7]) than those from Jonesville. Finally, specimens collected in July were significantly ($P = 0.03$) more likely (OR, 1.7 [95% CI, 1.04 to 2.7]) to have *Salmonella*-positive culture results than those treated in June.

Hatching success for each site by farm and treatment method was determined (**Table 2**). A significant difference was found in hatching success between control treatment (77.8% [95% CI, 73.5% to 82.0%]) and PHMB bath (69.5% [95% CI, 65.1% to 73.8%]; $P = 0.008$) and PHMB pressure-differential (67.4% [95% CI, 61.6% to 73.1%]; $P = 0.004$) treatments. No pathologic lesions of importance were associated with any of the treatments.

Discussion

Chelonian carriers of *Salmonella* spp are a concern for public health officials, and the propagation of *Salmonella*-free chelonians is a primary objective for aquatic chelonian farmers. Currently, chelonian eggs are sanitized by a combination of washing with water or NaOCl solution and temperature- or pressure-differential treatment with gentamicin. Although the apparent prevalence of *Salmonella* spp in these eggs is low, instances of antimicrobial-resistant *Salmonella* spp are reported.[10,11] To minimize the likelihood of introducing resistant strains of *Salmonella* spp, the focus of any research should be based on nonantibiotic microbicides.

Polyhexamethylene biguanide, a nonantibiotic microbicide, has been found to be effective at eliminating experimentally inoculated *Salmonella enterica* serotype Typhimurium from the surface of chicken eggs. In a study[14] to evaluate the efficacy of several different microbicides, including quaternary ammonium, peroxygen compounds, hydrogen peroxide, ethylene oxide, phenols, and sodium and potassium hydroxide, only a 0.035% solution of PHMB[m] was 100% effective at eliminating *Salmonella* organisms. Results of our study also indicated that PHMB products have some effect against *Salmonella* serotypes that infect reptiles.

Because the primary route of *Salmonella* contamination in RES turtle eggs occurs via a horizontal route during egg deposition[17] and hatchling chelonians serve as the primary source of infection for pet owners, neonates are a logical starting point for programs focused on suppressing or eliminating *Salmonella* spp. The primary objective of our study was to determine whether a combination of NaOCl and PHMB could be used as an egg wash to suppress or eliminate *Salmonella* spp on the surfaces of RES turtle eggs and to prevent the colonization of *Salmonella* in the gastrointestinal tract of RES hatchlings. These compounds were selected because they meet the FDA requirements for nonantibiotic compounds and would not pose any unnecessary health risks to humans if resistance developed. Although it might have been preferable to add additional treatment groups to evaluate the effect of each treatment compound separately (ie, NaOCl, PHMB, and gentamicin), this was not possible because of the limited number of eggs in a nest and our desire to minimize intranest variability. The findings of our study suggested that eggs treated with NaOCl and PHMB can suppress or eliminate *Salmonella* spp on egg surfaces and decrease the likelihood of recovering the organism from the visceral pluck of RES hatchlings. Although these products were not 100% effective, they did significantly reduce the prevalence of *Salmonella* spp on RES turtle eggs and in hatchlings, compared with controls. In addition, no difference was found in the prevalence of *Salmonella* spp between the gentamicin and PHMB treatment groups, suggesting that the compound has similar efficacy to the accepted standard.

In our study, the method used to treat RES turtle eggs with PHMB did affect the *Salmonella* status of eggs and hatchlings. Eggs that were bathed in PHMB for 10 minutes were significantly less likely to have *Salmonella*-positive culture results than control eggs; however, they were significantly more likely to have *Salmonella*-positive culture results than eggs treated with PHMB by use of the pressure-differential method. Pressure- and temperature-differential methods of egg washing have been used on poultry and aquatic chelonian farms for several decades. By use of these techniques, it is presupposed that the antibiotic or microbicide is carried through the protective barrier of the egg

and can eliminate pathogens within the egg prior to colonization of the embryo. Although not evaluated in our study, future studies are needed to measure PHMB concentrations in RES turtle eggs after treatment and also to evaluate the surface of RES turtle eggs by use of scanning electron microscopy to determine whether any negative effects occur on the eggshell surface associated with the treatment method.

A significant difference was found in the prevalence of *Salmonella*-positive culture results between the 2 specimen collection periods. The turtle egg-laying season in Louisiana generally begins in April and continues through early August. Historically, chelonian farmers report that they are more likely to fail bacterial culture tests for *Salmonella* organisms late in the season, and this had been anecdotally attributed to the warmer summer months. Results of a previous study[18] have established that eggs obtained early (April) in the season have lower numbers of total microbes ($< 7.5 \times 10^5$ microbes/g of egg), compared with those obtained late in the season (May; $> 2.1 \times 10^6$ microbes/g of egg). Warmer water temperatures, increased photoperiod, and increased biological activity in the ponds are all likely associated with this increase. Our study was performed late in the season (June and July) to increase the likelihood of encountering *Salmonella* spp, and our findings do confirm that the odds of encountering *Salmonella* spp increase over time. This can be important when planning biosecurity for Louisiana farms. Treatment plans may need to be altered on the basis of the time of season, with increased attention being paid to the latter months of the season.

A significant difference was found in the prevalence of *Salmonella*-positive culture results between turtle farms in Pierre Part and Jonesville. Pierre Part is located in southeastern Louisiana, whereas Jonesville is in northern Louisiana. Many of the farms from Pierre Part use bayou water in their ponds, whereas the facilities in Jonesville use well-water. By recirculating bayou water, it is possible that the farms are contaminating their ponds with *Salmonella* spp and exposing their turtles to higher overall burdens of microbes. Additional research is required to further elucidate the epidemiologic characteristics of *Salmonella* spp in Pierre Part so that appropriate recommendations can be made to minimize the potential for *Salmonella* exposure and contamination.

Gentamicin treatment failure was identified at least once in all 3 regions of the state and was highest in Pierre Part, compared with Jonesville. This affect appears to be a farm issue because only a single positive farm was found in each region. Farms that experience gentamicin treatment failure should reevaluate the methods they use to treat their eggs. It is possible that farms that experience these problems may need to further decontaminate eggs to ensure appropriate treatment contact time during the wash, reevaluate the dose of gentamicin administered, or change their solution more frequently. It is also possible that failure can be tied to gentamicin resistance; however, anecdotally, not all *Salmonella* isolates are gentamicin resistant. These findings do confirm that the current Louisiana regulation requiring chelonian farmers to treat eggs with a gentamicin pressure– or temperature-differential method is not 100% effective. Because of these limitations, additional compounds, such as PHMB, should be given serious consideration as part of potential treatment methods.

Although not rigorously tested in our study, antimicrobial resistance of bacteria to gentamicin was identified in a subset of *Salmonella* organisms and *Pseudomonas aeruginosa* isolates were cultured after application of the gentamicin pressure–differential method. The dissemination of antimicrobial resistance genes in microflora of RES turtles and into the surrounding environment may pose a special health risk for farm employees, other livestock, and wildlife in the area. These findings reinforce the importance of moving away from antimicrobials as a treatment method for turtle eggs.

Because the purpose of our study was to determine the prevalence of *Salmonella* spp on the egg surface or in the RES hatchling, an enrichment broth and DSE were used to increase the likelihood of isolating the organism. Attempts to isolate *Salmonella* spp without enrichment may result in misclassification (false-negative results). Results from the initial collection of specimens from each farm suggested that the prevalence of *Salmonella* spp in RES turtle eggs removed from the ground shortly after deposition is low. In a previous study,[11] no *Salmonella* spp were isolated from the surfaces of RES turtle eggs collected shortly after deposition at 2 Louisiana farms. The absence of *Salmonella* spp in the cultures performed in our study may have been attributed to the sensitivity of the assay. In our study, 61% (18/30) of the *Salmonella*-positive culture results for prehatch egg specimens were identified only on DSE. These results suggest that the number of *Salmonella* organisms on the surfaces of eggs may be low enough to avoid detection by use of a single culture or that the organisms are damaged and require time to regenerate. In future studies to characterize *Salmonella* spp on RES turtle eggs and in hatchlings, the use of DSE to maximize the likelihood of isolating the organism should be considered.

No significant histologic lesions were identified in the RES turtles selected for necropsy. The absence of lesions suggests that these compounds, NaOCl, PHMB, and gentamicin, can be safely used at the doses listed to decontaminate eggs. Future studies to determine the threshold of these compounds should be considered, especially because their bactericidal effects increase with dose.

The overall hatching rates in our study were considered slightly less than would normally be expected (> 80% preferred). These differences may have been attributed to the technique used to incubate eggs. For our study, eggs were held in small groups and placed on a perlite substrate to minimize the likelihood of cross-contamination. The incubation method used by aquatic chelonian farmers does not use a substrate, and a large number (ie, 70 to 80) of eggs are held in the same enclosure. It is possible that a fluctuation in the humidity of the incubation chambers may have affected hatching success or that a treatment effect existed. Unfortunately, the humidity was not evaluated in our study. In the future, eggs treated with various

compounds should be incubated by use of standard methods to determine whether treatment has an effect on hatching success.

Overall, results of our study are promising and suggest that the combination of NaOCl and PHMB may be used to suppress or eliminate *Salmonella* spp on egg surfaces and in hatchlings as efficiently as NaOCl and gentamicin. It is important for the aquatic chelonian industry to continue to evaluate new methods to suppress and eliminate *Salmonella* spp if they hope to reverse the current FDA regulation. In addition, chelonian farmers should maintain strict hygienic conditions in their processing facilities, use appropriate treatment doses for egg wash solutions, remove infertile eggs and eggshells from incubation chambers, and separate hatchlings to maintain a high degree of biosecurity and reduce the likelihood of exposing their turtles to *Salmonella* spp.

a. Baquacil, Avecia Inc, Wilmington, Del.
b. Clorox, Clorox Co, Oakland, Calif.
c. Proper pH 7.5, Aquarium Pharmaceuticals, Chalfont, Pa.
d. Becton, Dickinson & Co, Sparks, Md.
e. XLT4 agar base, Remel, Lenexa, Kan.
f. Eugon urea broth, Remel, Lenexa, Kan.
g. Lysine iron agar, Remel, Lenexa, Kan.
h. Triple sugar iron agar, Remel, Lenexa, Kan.
i. API 20E test strips, bioMeriux Vitek Inc, Hazelwood, Mo.
j. Ketaset, Fort Dodge Inc, Fort Dodge, Iowa.
k. Beuthanasia, Schering Plough Animal Health Care, Union, NJ.
l. SPSS 11.0, SPSS Inc, Chicago, Ill.
m. Cosmocil CQ, ICI Americas Inc, Wilmington, Del.

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