

Eradication of *Salmonella* and *Arizona* Species from Turtle Hatchlings Produced from Eggs Treated on Commercial Turtle Farms

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On commercial turtle farms more than 40% of the hatchlings excrete detectable levels of *Salmonella* and *Arizona* spp. when hatched from nonsanitized eggs incubated in sawdust or dirt-filled chambers. Over a 3-year period on 10 farms, more than 10⁶ turtle eggs were treated in an attempt to hatch *Salmonella*-free turtles. Eggs were sanitized in disinfectant, treated by temperature- or pressure-differential dip methods in solutions containing 500 µg or more of gentamicin sulfate per ml, and hatched in sanitized plastic chambers free of bedding material. The *Salmonella* and *Arizona* spp. infection levels for turtles produced from treated eggs were 0 and 1.12% for years 1 and 2, respectively, whereas infection levels for hatchlings produced from nontreated eggs during these periods were 47 and 44%, respectively. During year 3, dip solutions were filtered daily, treated at 100°C for 15 min on a weekly basis to free the solution of microbial contaminants and egg protein, charged with gentamicin after 10,000 to 20,000 eggs had been treated to maintain antimicrobial activity at 500 µg/ml or more, and maintained at pH 6.0 to preserve optimal antimicrobial activity. The implementation of these measures in year 3 resulted in an infection level of 0.15% when the tissues of 3 of 1,959 hatchlings tested were positive for *Salmonella* and *Arizona* spp., whereas the tissues of 66 (49.0%) of 135 hatchlings produced from nontreated eggs were positive.

Twenty-four-hour-old turtle eggs artificially infected with *Arizona hinshawii* produced hatchlings which excreted detectable levels of *A. hinshawii* and harbored this organism in their visceral organs (7). Artificially infected eggs treated with 1,000 µg or more of gentamicin sulfate per ml by the pressure-differential (PD) method, on the other hand, did not harbor detectable *A. hinshawii* when shells and embryo-yolk homogenate of 12-day-old eggs were tested or when the gastrointestinal tract, kidneys, liver-gall bladder, and yolk of 50-day-old embryos were examined. Treated eggs produced hatchlings which did not excrete detectable *Arizona* spp. at 72 h or 30 days posthatching, nor were these organisms recovered from the visceral organs of these hatchlings. The findings obtained from this preliminary study formed the base upon which egg sanitization and egg dip treatment protocols were defined and then incorporated into the egg-hatching process on commercial turtle farms in Louisiana. Over a 3-year period, 10 commercial turtle farms installed both mechanized egg-washing machines and egg treatment tanks to sanitize and treat eggs in gentamicin baths by either the PD or temperature-differential (TD) procedure. During year 3, more than 33% of the annual crop of 4 × 10⁶ eggs was treated. In this paper, we report the findings of bacteriological studies done in each of the 3 years to determine the systemic and excretion incidence of *Salmonella* and *Arizona* spp. in hatchlings produced from commercially treated eggs.

MATERIALS AND METHODS

Egg treatment. Turtle eggs (*Pseudemys scripta-elegans*) were collected and treated daily (April through June) on each farm which participated in this study. The eggs were sanitized and then treated by the TD or PD method described previously (7, 9). On a commercial scale, eggs treated by PD were treated in a dip tank (Henry Kuhl Co.) which accomo-

dated 1,500 eggs per 15-min dip treatment cycle. Dip solutions were charged with gentamicin sulfate (Garasol; Schering Laboratories, Bloomfield, N.J.) to give 1,000 µg/ml. In year 3, dip solutions were filtered after each treatment cycle and pasteurized at 100°C for 15 min at weekly intervals. Dip solution samples were taken at periodic intervals, sent to Schering Corp. (Animal Health Research Div., Kenilworth, N.J.), and tested for gentamicin activity by methods previously described (1). Gentamicin-treated eggs were incubated in sanitized, sealed plastic chambers at 30 to 33°C for 55 to 65 days.

Follow-up studies. During years 1 and 2, eggs treated on a given day on each farm were assigned a chronological lot number. Upon hatching, 60 hatchlings from every seventh treatment lot were selected and brought to the laboratory where they were distributed in groups of five to a sterile, covered 1-liter vessel which contained 50 ml of water. Over the next 9 months, at intervals of 15 to 30 days, the water in each container was tested by inoculating 25 ml into 10 volumes of tetrathionate broth and the remaining 25 ml into 10 volumes of selenite-cystine broth (excretion assay). The enrichment broths were tested bacteriologically for the presence of *Salmonella* and *Arizona* spp. by methods previously reported (8-10). On each excretion assay date, the five turtles in one container were necropsied, and the yolk sac, liver-gall bladder, kidneys, and gastrointestinal tract (excluding stomach) were removed from each animal, minced, and tested bacteriologically (necropsy assay) for these pathogens by procedures previously described (7). The remaining animals were transferred to sterile containers.

In year 3, a lot number was assigned to all eggs treated in one dip solution preparation. In this approach, each time the dip solution was charged with gentamicin, a new lot number was assigned. Each lot was comprised of 10,000 to 25,000 eggs treated over a 5- to 10-day period. Five to ten hatchlings were selected from each day's treatment in each lot and placed in sterile containers as described above. Excretion

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assays were done 72 h and 14 days posthatching; on each excretion assay date, two or three animals from each vessel were necropsied; and their tissues were tested for *Salmonella* and *Arizona* spp.

RESULTS AND DISCUSSION

Year 1. During year 1, from 14 April through 9 June, 133,000 eggs were collected from two ponds on farm B and treated by the PD method. Table 1 summarizes the findings of excretion and necropsy assays done on 830 hatchlings selected from nine egg treatment lots. The first six lots (lots 1, 8, 15, 21, 26, and 31) represented eggs collected from both ponds. The eggs collected from each pond were treated and hatched separately; therefore, 60 animals were tested on each assay date from each of the two ponds. From the 1,820 eggs treated on 14 April (lot 1), 120 hatchlings were tested bacteriologically; over the 270-day test period, 186 excretion assays were done and 115 hatchlings were necropsied (five animals expired before completion of experiment). In the follow-up studies done in year 1 on hatchlings produced by treated eggs, 1,345 excretion assays and the visceral tissues from 830 hatchlings were uniformly negative when tested bacteriologically for these pathogens (Table 1).

On each of six dates (lots 8, 15, 26, 31, 43, and 49), 200 eggs were selected and not treated (NT). The NT eggs were hatched in separate chambers, and 40 to 45 hatchlings from each of the NT lots were tested in concert with hatchlings produced by treated eggs. Hatchlings produced from NT eggs collected on 29 April (lot 8NT; Table 1) did not excrete detectable levels of *Salmonella* and *Arizona* spp. for 150 days posthatching, nor were these organisms recovered from the visceral organs of 25 of these animals tested over the same time period. The *Salmonella* and *Arizona* spp. excretion rate for hatchlings in the remaining five NT lots was 90% (38 of 42, Table 1). *Salmonella* or *Arizona* isolates were recovered from the tissues of 36 animals in these NT lots (47% infection level).

Seven gallons (ca. 26.5 liters) of gentamicin solution (1,000 µg/ml), prepared on 14 April and recharged on 28 April with 4 liters of gentamicin solution after 30,000 eggs had been treated, was charged again on 17 May after an additional

30,000 eggs were treated. Bioassay of the dip solution on 17 May and 12 June showed gentamicin activities of 845 and 504 µg/ml, respectively.

Year 2. Three farms treated eggs by the PD method in year 2. The results of excretion and necropsy studies done on hatchlings from every 7th egg treatment lot from farms A and HL and every 14th lot from farm B are summarized in Table 2. A total of 1,680 turtle hatchlings from 28 egg lots were tested for 9 months posthatching. Out of 2,216 excretion assays done, 20 (0.9%) were positive for *Arizona* or *Salmonella* spp. Of the 1,512 turtles necropsied at 15- to 30-day intervals over the test period, the tissues of 17 (1.1%) of these animals were positive for *Salmonella* or *Arizona* spp. Typically, *Salmonella* or *Arizona* spp. were recovered from the water of a single container on successive assays, and these organisms were then isolated from the visceral organs of hatchlings housed in that container when they were necropsied. For example, *Arizona* sp. was isolated from the gastrointestinal tracts of three turtles produced by eggs treated in June (lot 87, farm B) on day 90 posthatching. The water tested from the container holding these turtles had been positive for *Arizona* sp. on the five previous assays, which accounted for 5 of the 8 positive excretion assays and 3 of the 11 positive necropsy assays detected in egg treatment lots on farm B (Table 2).

A total of 150 hatchlings tested from five lots of NT eggs were tested in parallel with follow-up studies done on treated egg hatchlings. The tissues of 66 (44%) hatchlings produced from NT eggs were positive for *Salmonella* or *Arizona* spp. when they were necropsied (Table 2).

Gentamicin levels in dip solutions used on farm HL during the second year dropped to 250 µg/ml during the last week in May, and eggs treated in this solution in the month of June were being treated under marginal conditions. It was determined during this time that gentamicin activity dropped ca. 100 µg/ml for every 10,000 to 20,000 eggs treated. In addition to low gentamicin levels, two related problems were recognized. Dip solutions became burdened with egg protein, due to cracked eggs, and exhibited excessive foaming during the filtration step, and large quantities of precipitate were generated in dip solutions when heated or autoclaved. The pH of

TABLE 1. Follow-up excretion and necropsy studies done on turtles hatched from treated and NT eggs on farm B

Date	Lot no.	No. of eggs		Excretion assay		Necropsy assay	
		Treated ^a	NT	No. of turtles tested	Positive/negative	No. of turtles necropsied ^b	No. of animals positive for <i>Salmonella</i> and <i>Arizona</i> spp.
14 April	1	1,820		120	0/186	115	0
21 April	8	2,550		120	0/183	120	0
	8NT		200	40	0/27	25	0
28 April	15	1,910		120	0/173	115	0
	15NT		200	45	7/9	5	5
5 May	21	2,240		120	0/155	115	0
12 May	26	2,660		120	0/187	102	0
	26NT		200	45	9/9	5	5
19 May	31	2,660		120	0/199	104	0
	31NT		200	40	8/8	5	2
26 May	38	1,820		60	0/88	53	0
2 June	43	2,800		60	0/86	51	0
	43NT		200	40	7/8	10	9
9 June	49	2,310		60	0/86	55	0
	49NT		200	40	7/8	5	5

^a Eggs treated by PD dip method in gentamicin baths (1,000 µg/ml).

^b Five to eighteen animals expired before completion of follow-up studies.

TABLE 2. Follow-up excretion and necropsy studies done on hatchlings produced by gentamicin-treated and NT eggs for *Salmonella* and *Arizona* spp.

Farm	Dates (inclusive)	No. of lots tested	Excretion assay ^a		Necropsy assay					
			No. done	No. positive	No. of hatchlings necropsied	Yolk ^b	Liver ^b	Kidney ^b	GI ^b	No. of hatchlings positive
A	24 April–17 June	9 ^c	693	6	504	1	0	0	1	2
HL	10 May–29 June	13	986	6	684	0	0	2	3	4
B	14 April–30 June	6 ^d	447	8	324	1	1	3	7	11
NT eggs ^e	14 April–15 May	5	82	36	150	4	9	15	61	66

^a Excretion assay, every 15 to 30 days over 270 days.

^b Data indicate number of tissues which were positive when tested. GI, Gastrointestinal tract.

^c Sixty hatchlings from each lot.

^d Farm B, in year 2 of treatment, every 14th treatment lot tested.

^e Every seventh lot tested for the first 5 weeks.

the dip solutions hovered at 8.0 through the last half of the egg treatment season. The manufacturer recommends using gentamicin at pH 6.0 to maintain optimum antimicrobial activity. At an elevated pH, dip solutions burdened with protein show diminished antimicrobial activity in that the gentamicin will bind to the protein, thereby rendering the antimicrobial agent inactive.

Year 3. In year 3, 10 farms treated eggs and participated in the follow-up studies. Each farm adhered to the formula that for every 10,000 to 20,000 eggs treated, a drop of 100 µg/ml in gentamicin activity should be anticipated. The dip solutions were assayed at the start and finish of each egg treatment lot, and gentamicin activity in dip solutions did not drop below 550 µg/ml during year 3. Dip solutions were filtered daily and pasteurized (100°C for 15 min) weekly to remove egg protein. The pHs of the dip solutions were monitored and adjusted accordingly on a weekly basis.

A total of 739,000 eggs were treated on 7 of the 10 farms (Table 3), and on 2 of these farms (LK and BK), 80,000 eggs were treated by the TD method. Excretion and necropsy follow-up studies were done on 1,839 hatchlings produced by 29 egg treatment lots. *Salmonella* serogroup C₁ was isolated from the water of one vessel (lot 1, farm BK) on both the 72-h and 14-day excretion assays; however, this organism was not recovered from the visceral organs of the five hatchlings which inhabited this vessel. *A. hinshawii* was isolated from the water of one container (lot 2, farm B) 14 days posthatching and from the livers of two and the gastrointestinal tracts of the three turtles remaining in this vessel, whereas the

excretion and necropsy assays done 72 h posthatching on the water and two hatchlings tested from this vessel were negative (Table 3).

Three farmers in northern Louisiana installed PD tanks in June of year 3 and were instructed verbally and by letter how to sanitize and treat eggs. A total of 25 hatchlings produced by eggs the day before treatment was instituted were selected by the farmers, and 19 were positive for both *Salmonella* and *Arizona* spp. by the necropsy assay (Table 4). A total of 120 hatchlings produced by eggs treated on day 1 of treatment were selected, and the follow-up excretion and necropsy assays were uniformly negative for *Salmonella* and *Arizona* spp.

A total of 1,959 hatchlings produced from treated eggs (Tables 3 and 4) were necropsied, and 3 were positive for *Arizona* sp. These three animals inhabited the same vessel, yielded 1 of 3 positive excretion assays of 761 done, and contributed the 0.15% infection level. The infection level for hatchlings produced by NT eggs was 42.7% in that 47 of 110 animals necropsied were positive for *Salmonella* and *Arizona* spp. This infection level is comparable to that seen in year 1 (47%) (Table 1) and year 2 (44%) (Table 2).

The eggs in the two treatment lots which produced positive excretion or necropsy assays were collected on days (28 April and 9 May) that were preceded by heavy rain. Eggs collected from dirt nests saturated with water appear to be pumped up, or swollen with water. Since the turtle egg is quite permeable, it will readily swell and shrink, depending on environmental conditions, within the boundary of an

TABLE 3. Follow-up excretion and necropsy studies done on hatchlings produced by gentamicin-treated eggs for the presence of *Salmonella* and *Arizona* spp.

Farm	Method of treatment	No. of eggs treated	No. of lots	Excretion assay ^a	Necropsy assay					
					No. of hatchlings necropsied	Yolk ^b	Liver ^b	Kidney ^b	GI ^b	No. positive ^c
LK	TD	61,618	6	0/142	349	0	0	0	0	0
BK	TD	18,892	2	2/70	177	0	0	0	0	0
NS	PD	78,830	5	0/102	255	0	0	0	0	0
JL	PD	72,000	3	0/88	220	0	0	0	0	0
WH	PD	192,000	7	0/152	410	0	0	0	0	0
A	PD	15,700	3	0/74	185	0	0	0	0	0
B	PD	300,000	5	1/95	243	0	2	0	3	3

^a Data indicate number positive of number tested. Of 723 samples tested, 3 (0.42%) produced positive excretion assays.

^b Same as in Table 2, footnote b.

^c Necropsy studies done on 1,839 hatchlings yielded 3 positive assays for a 0.16% infection level.

TABLE 4. Bacteriological follow-up studies done on hatchlings produced by NT and treated eggs on three farms which started PD treatment during the height of the egg-laying season

Farm	Gentamicin treatment ^a	Date	No. of hatchlings tested ^b	Excretion assay ^c	Necropsy assay				
					Yolk ^d	Liver-gall bladder ^d	Kidney ^d	GI ^d	No. positive
NWS	no	5 June	5	1/2	1	2	1	5	5
	yes	6 June	45	0/18	0	0	0	0	0
HE	no	31 May	10	3/4	1	2	2	8	9
	yes	1 June	25	0/10	0	0	0	0	0
JE	no	8 June	10	4/4	1	1	0	5	5
	yes	9 June	50	0/10	0	0	0	0	0

^a Eggs treated by PD method in gentamicin bath (1,000 µg/ml).

^b Of 145 hatchlings tested, 25 were products of NT eggs and 120 were products of treated eggs. In the necropsy assay, 19 of the 25 hatchlings from NT eggs, and 0, of the 120 hatchlings from treated eggs tested positive.

^c A total of 18 samples from NT eggs and 38 samples from treated eggs were used for the excretion assay. Of the 18 samples from NT eggs, 8 were positive and 10 were negative. Of the 38 samples from treated eggs, 0 were positive and 38 were negative.

^d Same as in Table 2, footnote b.

extremely flexible shell. Eggs swollen with water might be more difficult to treat in that neither PD nor TD may effectively drive the antimicrobial agent into the egg interior past or through the water barrier. A possible solution would be to recommend to the industry not to treat eggs harvested after a heavy rainfall or to institute a dehydration regimen.

Lamm et al. (5) estimated that 14% of all human salmonellae on the interstate shipment or sale of turtles with carapaces of 4 in. (10.2 cm) or less (June 1975) were turtle associated, which translated into an estimated 280,000 cases of turtle-associated salmonellosis annually. These data were generated by a retrospective epidemiological survey of households in six metropolitan areas in which an individual from whom a confirmed *Salmonella* isolate was recovered stated when interviewed that a turtle was in the home at the time of onset of illness. The authors stated that from the first report of Hersey and Mason (2) in 1943 to 1970, the year of this survey, there were ca. 100 documented cases of turtle-associated human salmonellosis. In each of the 3 years of our study, more than 40% of turtle hatchlings produced from NT eggs excreted *Salmonella* and *Arizona* spp. Therefore, in retrospect, it is not surprising that aquarium water samples taken from retail pet outlets were positive when tested for these pathogens during the 1970-to-1971 epidemiological survey (5).

Kaufmann et al. (3, 4) suggested that to achieve the goal of *Salmonella*-free turtles, a means to eliminate turtle breeding pond contamination must be found. This task, which would involve elimination of environmental *Salmonella* sources, identification and removal of *Salmonella*-infected breeders, and maintenance of a pond decontamination program, is paramount to the eradication of *Escherichia coli* from barn yards. With this aim in mind, a turtle pond in Louisiana was treated with copper sulfate in April 1970, just before the egg-laying season (3). The copper content in the pond water was maintained at 2 to 5 ppm (2 to 5 µg/ml) from mid-April to mid-September. The resulting reduction of *Salmonella* contamination in the breeding pond water did not affect the excretion rate of these organisms by hatchlings produced from eggs laid by breeders in the treated pond. In fact, excretion rates for hatchlings from the treated pond were greater than those for hatchlings produced from eggs gathered from NT ponds. In this same study (3), *Salmonella* and *Arizona* spp. were routinely isolated from NT breeder pond water, adjacent bayou and swamp water, adult female breed-

ers, egg nest soil, and hatchling excreta but not from eggs. These data suggest that *Salmonella* and *Arizona* spp. introduced into the egg via ovarian transmission (3) or penetration of the egg from contaminated nest soil (7, 9) are present in low numbers in contrast to the environment and subsequent hatchling. If *Salmonella* and *Arizona* spp. can be eradicated from the egg and a *Salmonella*-free hatchling can be produced from the treated egg, this precludes elimination of these organisms from the pond, soil bank, and breeders. In a previous report (9), it was shown that fresh turtle eggs treated by TD in terramycin or chloromycetin baths produced hatchlings which did not excrete *Salmonella* or *Arizona* spp. for the duration of the follow-up studies. For reasons delineated in a follow-up study (7), gentamicin sulfate was used to treat turtle eggs artificially infected with *A. hinshawii*. Such eggs, treated with 500 µg or more of gentamicin per ml of dip solution, produced hatchlings which did not harbor systemically or excrete *Salmonella* and *Arizona* spp.

An egg sanitization procedure, when implemented, produced a resultant decrease in aerobic mesophilic bacteria from 10⁶ to 10² organisms per g of egg homogenate (7). This sanitization step, coupled with gentamicin egg treatment when implemented on one farm, during year 1 produced a *Salmonella* and *Arizona* excretion-infection rate of 0.0% for hatchlings produced from treated eggs compared with 44% for hatchlings produced by NT eggs. In year 3, when 1,914 hatchlings selected from egg treatment lots from 10 farms were necropsied, only 3 animals were positive, yielding an infection rate of 0.1%. These three animals, which inhabited the same container, were hatched from a single egg lot treated on a single day (farm B; Table 4). One of the three positive excretion assays seen in year 3 was detected in 15-day-old water tested from this same vessel. The other two positive excretion assays were detected in a vessel holding hatchlings produced from eggs collected on farm BK. NT eggs in year 3 produced hatchlings showing excretion-infection rates of 42%. The success achieved in year 3 can be attributed to the implementation of measures which countered problems which arose during year 2. First was recognition of the fact that for every 10,000 to 20,000 eggs treated, a concomitant drop of 100 µg in antimicrobial activity should be anticipated. Second, it was found that filtration of dip solutions on a daily basis and pasteurization on a weekly basis freed the solutions of a protein burden. Third, a

periodic adjustment of the pH to 6 maintained optimal antimicrobial activity in the dip solution. Fourth, it was important to charge the dip solutions with gentamicin so as not to permit activity to drop below 500 µg/ml. The experience of year 3 alerted farmers not to treat eggs collected after a period of heavy rainfall, since it appeared that they became waterlogged and neither TD (on farm BK) nor PD (on farm B) could effectively drive the antimicrobial agent through waterlogged eggs.

All farms which participated in this study discarded the time-honored hatching procedures which entailed buying eggs in peat moss, sawdust, or dirt for the 60- to 65-day hatching period. Instead, eggs were incubated in sanitized, closed, plastic hatching chambers free of bedding material in a manner described previously (7). In this fashion, recontamination was effectively avoided and the hatching chambers were used again after proper disinfection. In addition, this permits egg treatment lots to be identified, segregated, and subjected to quality control.

McCoy and Seidler (6) reported the isolation of *Aeromonas*, *Citrobacter*, *Enterobacter*, *Klebsiella*, *Proteus*, *Salmonella*, and *Serratia* species from anal swabs and from aquarium water holding turtle hatchlings. At this point in our investigation, the fate of these bacterial genera, other than *Salmonella*, in hatchlings produced from treated eggs is not known. To our knowledge, during the extensive epidemiological surveys into turtle-associated enteric diseases, no evidence surfaced which implicated this animal as a reservoir for human infections produced by the above-mentioned microorganisms.

In a previous communication (7), we reported that resistance to gentamicin in *A. hinshawii* isolates recovered from the visceral organs of hatchlings produced by eggs treated with gentamicin was not significantly greater than that in the *Arizona* isolates used to artificially infect the eggs. The commercial treatment of turtle eggs in gentamicin dip solutions raises the specter of generating resistant *Salmonella* and *Arizona* strains and creating conditions which favor the emergence of other bacterial pathogens which may also be gentamicin resistant. At this time, as mentioned above, the presence or absence of pathogens other than *Salmonella* spp. in hatchlings produced from treated eggs is not known. The level of resistance of *Salmonella* and *Arizona* spp. to gentamicin and other antimicrobial agents is presently being studied.

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