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Evaluation of Methods for the Isolation of *Salmonella* and *Arizona* Organisms from Pet Turtles Treated with Antimicrobial Agents

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Turtles infected with and actively excreting *Salmonella-Arizona* organisms were treated with various concentrations of both Neo-Terramycin (N-Te) and Terramycin (Te) (Pfizer) for various periods of time and then tested for the presence of these pathogens by two methods, excretion and blending. Turtles treated with 200 μg of Te per ml of container water for 9, 12, or 14 days did not excrete detectable numbers of *Salmonella-Arizona* for 6 to 8 weeks, whereas when representative turtles from treatment groups were blended 72 h post-treatment these organisms were isolated from the whole turtle homogenate. *Salmonella* and *Arizona* could be recovered from homogenate prepared from turtles treated for 7 and 14 days with 400, 800, or 1,000 μg of Te or N-Te per ml. These findings suggest that the blending method is more sensitive than the excretion method for the detection of *Salmonella-Arizona* in the treated turtle.

It has been well established by others (1, 3, 5) that baby green turtles (*Pseudemys scripta-elegans*) carry and excrete *Salmonella* and *Arizona* organisms responsible for infections in humans, especially when these turtles are pets in the home. Some estimates suggest that 3×10^5 cases of human salmonellosis occur yearly and are turtle associated (2).

Because of this potential health problem both state and federal regulations have been implemented to prohibit importation and control interstate shipment of turtles carrying these pathogens. In 1972 the Food and Drug Administration (FDA) required that 60 turtles be removed from each lot of turtles being shipped to domestic markets and be tested at the state health laboratories (state of origin) for the presence of *Salmonella* and/or *Arizona*. This is presently being done by testing water in which the turtles have been held for 72 h (excretion method). Since the number of *Salmonella* isolates from turtle-associated salmonellosis did not decrease in the year 1973 after the adoption of the certification methods, new controls were proposed by the FDA in May 1974. Two proposals are presently under consideration: (i) a total ban on importation and interstate shipment of turtles with a carapace of 4 inches (ca. 9.2 cm) or less, or (ii) implementation of more sensitive testing procedures for examination of 72-h turtle container water for *Salmonella-Arizona* in the certification procedure.

Wells et al. (4) compared the currently used excretion method to assay of whole turtle and turtle organ homogenation (blending method) for recovery of *Salmonella* and *Arizona*. They reported that bacteriological assay of water was as sensitive as the blending method for detecting the presence of these organisms. This is probably true only if normal (nontreated) turtles are examined. However, the turtle raisers and shippers responded to the presence of *Salmonella* in their product and attempted to treat the newly hatched turtle with antibiotics dissolved in water, for various time intervals, in an attempt to eliminate these pathogens from the turtle. Therefore, the public health laboratories are not receiving 60 "normal" turtles to certify. In light of this information, we investigated the sensitivity of assaying treated turtle homogenates for *Salmonella* and *Arizona* with the presently used water assay. This report will show that homogenation of the whole turtle is superior to water assay for recovery of *Salmonella* from the treated turtle.

(A portion of this work was taken from P. M. N.'s M.S. thesis Louisiana State University, Baton Rouge, 1975.)

MATERIALS AND METHODS

Newly hatched red-eared turtles (*Pseudemys scripta-elegans*) were obtained from local turtle farmers (Jonesville, Pierre Part, and Ponchatoula, La.) as needed. During certification and treatment studies they were housed in groups of five at room tempera-

ture in 1,000-ml beakers covered with aluminum foil. These turtles were not fed, since for several months posthatching these animals received nutrients from a yolk sac which involutes through the plastron prior to hatching.

Bacteriology. During the course of this investigation both container water and whole turtle or visceral organ homogenates were tested for the presence of *Salmonella* and *Arizona*. Unless otherwise stated, 1.0 ml of container water was inoculated in 10 ml of tetrathionate broth (Difco) containing 10 mg of brilliant green dye per liter (TBG). Container water (25 ml) was also pipetted from each beaker into 225 ml of lactose broth (pre-enrichment) with the pH adjusted to 6.8, incubated for 24 h at 37 C, and then subcultured to duplicate tubes of TBG. After the tubes of TBG were incubated at 37 C for 48 h, a 5-mm loopful of broth was streaked onto brilliant green agar (Difco) containing 80 mg of sulfadiazine per liter and onto bismuth sulfite agar (Difco). The brilliant green and bismuth sulfite agar plates were incubated at 37 C for 24 h; three lactose-negative colonies were picked from brilliant green agar and three black colonies were picked from bismuth sulfite agar and inoculated into triple sugar iron agar and lysine iron agar (Difco). Subsequent inoculations were made from triple sugar iron agar to urea agar slants, motility-indole-ornithine deeps, and malonate broth (Difco). In addition to biochemical characterization, *Salmonella* were screened serologically in commercially available polyvalent and group-specific O antiserum (Difco). Suspected *Arizona* isolates were inoculated into Trypticase soy-tryptose broth (Trypticase soy [BBL] and 13 g of tryptose [Difco] per liter) and then screened serologically in phase 1 and phase 2 H antiserum (Difco). All TBG broth cultures which were negative for *Salmonella* and *Arizona* were subcultured to a second TBG (secondary enrichment) when the primary enrichment was 1 week old. These were processed as discussed above.

Homogenization procedure. Five turtles (approximately 30 g) were placed in a sterile, 200-ml capacity, stainless-steel cup with 20 to 30 ml of TBG. The turtles were blended at 16,000 rpm for 2 min at 4 C in a Sorvall Omnimixer. One milliliter of homogenate was then inoculated directly into duplicate TBG, and 25 ml was inoculated into 225 ml of lactose broth. Subsequent culturing was performed by the procedures described above.

Treatment of baby turtles with antimicrobial agents. Prior to treatment each lot of turtles were shown to be excreting *Salmonella-Arizona*, using the certification procedures set down in the Federal Registrar (18 November 1972). Since June 1974, the more sensitive certification procedure proposed in the Federal Registrar (28 May 1974) has been used. Only turtles shown to be excreting *Salmonella* and/or *Arizona* were used in the treatment studies.

Experimental treatment and control groups consisted of five to ten turtles. Neo-Terramycin (N-Te), and Terramycin (Te) (Chas. Pfizer Co., New York, N.Y.), and tylosin (Tylan-200) were obtained from a local livestock wholesale outlet. These three antibiotics were dissolved in water to give 20, 100, 200, 400, 800, 1,000, 1,200, or 1,500 μg/ml. Five turtles were placed in 100 ml or 10 turtles in 200 ml of antibiotic solution in 1,000-ml beakers, and the antibiotic solutions were changed daily for the duration of treatment. After treatment the turtles were transferred to sterile 1,000-ml beakers (five turtles per beaker) containing 50 ml of sterile water. Control group turtles (not treated with antibiotic) were treated with daily changes of water. The treatment times varied with each experiment (1 through 14 days).

In the first experiment, 160 turtles were brought to the laboratory. They had been treated with N-Te by the turtle farmer in the following way. Twelve groups of turtles consisting of 10 to 12 turtles/group had been treated with daily changes of 20 or 200 μg of N-Te per ml of container water for 1, 3, 6, 9, 12, or 14 days. These treated turtles (along with two nontreated control groups) were placed in sterile containers. Water samples were removed from each container once a week and tested for the presence of *Salmonella* and *Arizona*. At the time of testing turtles in each experimental group were transferred to sterile containers. The water was tested weekly until all turtles in a group had died. On some test dates bacteriological assay of water was not done; however, these animals were transferred to sterile containers. Nontreated control group turtles were included in this experiment to determine if *Salmonella-Arizona* were excreted throughout the duration of testing.

RESULTS

One hundred and sixty baby turtles were brought into the laboratory from a local turtle farm (Ponchatoula, La.) where they had been treated with N-Te. Twelve treated groups and two control groups were received. Each group consisted of 10 to 12 turtles which had been treated with 20 or 200 μg of N-Te per ml of container water for 1, 3, 6, 9, 12, or 14 days. Table 1 shows the results of weekly bacteriological assay of the container water for *Salmonella* and *Arizona* for each group through 26 weeks or until all animals in each group expired. As can be seen, control groups A and B and experimental groups treated for 1 and 3 days with either 20 or 200 μg of N-Te excreted *Salmonella* serogroups C_1 and C_2 and *Arizona* into the container water throughout the period of testing. Turtles treated for 6, 9, 12, and 14 days shed detectable numbers of *Salmonella-Arizona* only intermittently. Except for turtles treated with 200 μg of N-Te per ml of container water for 9 days (200-9) which shed *Salmonella* and *Arizona* the 4th week post-treatment, those groups treated with 200 μg of N-Te for 6, 12, and 14 days did not excrete detectable levels of these pathogens until the 8th week. The excretion of *Salmonella* and *Arizona* by turtles treated for 12 and 14

TABLE 1. *Treatment of baby green turtles with daily changes of 20 or 200 μg of N-Te per ml for 1, 3, 6, 9, 12, and 14 days*[a]

Experimental group[b]	Weeks post N-Te treatment[c]																								
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
Control A	+[d]	+	+	+	+	+	+	+	ND	ND	ND	ND	ND	ND	+	ND	−	−	−	−	−	−	−	−	−	−
Control B	+	+	+	+	+	+	+	+	ND	ND	ND	ND	ND	ND	+		−	−	−	−	−	−	−	−	−	−
20[e]-1 day	+	+	+	+	+	+	+	+	ND	ND	ND	ND	ND	ND	+	ND	+	−	−	−	−	−	−	−	−	−
200[e]-1 day	+	+	+	+	+	+	+	+	ND	ND	ND	ND	ND	ND	+	ND	+	−	−	−	−	−	−	−	−	−
20-3 days	+				+	+	+	+	ND	ND	ND	ND	ND	ND	+	ND		+				+			+	+
200-3 days				+	+	+	+	+	ND	ND	ND	ND	ND	ND	+	ND		+		+		+	−	−	−	−
20-6 days			+									+		ND	+	+	+	+	−	−	−	−	−	−	−	−
200-6 days									+	+	+	+	+	ND	+	+	+				+	+	−	−	−	−
20-9 days			+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
200-9 days				+										ND						+					+	+
20-12 days														ND							−	−	−	−	−	−
200-12 days									+					ND	+	+	+	−	−	−	−	−	−	−	−	−
20-14 days					+									ND	+	+	+									
200-14 days														−	−	−	−	−	−	−	−	−	−	−	−	−

[a] Treatment was followed by weekly assay of container water for *Salmonella-Arizona* organisms.

[b] Each experimental group consisted of 10 to 12 turtles, which were transferred each week on the day water was tested for the presence of *Salmonella* and *Arizona* to sterile jugs containing 100 ml of sterile water.

[c] −, indicates all turtles in the experimental group died; ND, not done.

[d] *Salmonella* (C_1, C_3) and/or *Arizona* isolates.

[e] N-Te (20 μg or 200 μg) per ml of container water.

days with 200 μg of N-Te per ml of container water was not detected when container water was tested using current FDA bacteriological assay procedures.

It was concluded from the above survey that infected turtles treated with 200 μg of N-Te per ml of container water for 3, 6, 9, 12, and 14 days could be certified "*Salmonella*-free" by the public health laboratory performing bacteriological assays on 72-h water samples using the current certification procedures.

The second experiment was performed to determine if *Salmonella* and *Arizona* could be recovered from turtle homogenates after these animals were treated for 14 days with daily changes of 200 μg of N-Te per ml of container water. Table 2 shows the results of this experiment. Three groups of 10 turtles, shown to be shedding *Salmonella* by bacteriological assay of container water, were treated with 200 μg of N-Te per ml for 14 days (jugs 55, 58, and 59). Seventy-two hours after treatment, five turtles from each group were blended, and *Salmonella* and *Arizona* isolates were recovered from the three treated group homogenates (Table 2). No *Salmonella* or *Arizona* organisms were isolated from water collected 72 h or 14 days post-treatment. *Salmonella* and *Arizona* were isolated from both turtle homogenate and water samples for each of the three control groups. These findings suggested that treatment of infected animals with 200 μg of N-Te for 14 days did suppress the excretion of *Salmonella-Arizona* to below detectable numbers but did not eliminate the systemic presence of these organisms. The systemic presence of these organisms was detected by bacteriological assay of blended turtles 72 h after treatment.

In the third experiment, groups of turtles known to be shedding *Salmonella* were treated with 100, 200, 400, and 800 μg of Te per ml for 7 or 14 days. There were four control groups treated with daily changes of water for 7 or 14 days. Seventy-two hours post-treatment all turtles in each group were blended. No water assays were done. Tables 3 and 4 present the results of this study. Table 3 shows the findings for turtles treated for 7 days. No *Salmonella* or *Arizona* organisms were isolated from homogenates prepared from turtles treated with 400 or 800 μg of Te per ml of container water, whereas these organisms were isolated from control groups and experimental groups treated with 100 and 200 μg of Te. The bacteriological assay of turtle homogenates prepared from turtles treated for 14 days (Table 4) shows *Salmonella* to be present systemically in all treated groups, however, only from TBG-enriched samples and

TABLE 2. *Treatment of infected baby turtles with 200 μg of N-Te per ml of container water for 14 days*[a]

Pretreatment certification[b]			Bacteriological assay for *Salmonella-Arizona* post-treatment		
Jug no.	Experimental group[c]	Serogroup	Turtle homogenate[d]	Container water[e] 72 h	Container water[e] 14 days
56	Control	F	D	D, F	F
57	Control	B, F	B	B	B
60	Control	B	*Arizona*	*Arizona*	*Arizona*
55	N-Te (200 μg)	C_2	D	0	0
58	N-Te (200 μg)	B	*Arizona*	0	0
59	N-Te (200 μg)	C_1	*Arizona*	0	0

[a] Treatment was followed by bacteriological assay of whole turtle homogenates and container water for the presence of *Salmonella* and *Arizona* 72 h post-treatment.

[b] Certification was done by bacteriological assay for *Salmonella-Arizona* in container water prior to treatment.

[c] Ten turtles in each experimental group.

[d] Five turtles were blended for 2 min at 4 C at 16,000 rpm in a Sorvall Omnimixer 72 h post-treatment.

[e] Five turtles were left in container water for 14 days post-treatment.

TABLE 3. *Treatment of infected baby turtles with 100, 200, 400, and 800 μg of Te per ml of container water for 7 days*[a]

Pretreatment certification[b]			7-day treatment group homogenates[c]	
Jug no.	Experimental group[d]	Serogroup	Direct TBG[e]	Lactose pre-enrichment[f]
9	Control	F	F	F
14	Control	C_2	F	F
10	Te (800 μg)	B, F	0	0
11	Te (400 μg)	E_1	0	0
12	Te (200 μg)	F	F	*Arizona*
13	Te (100 μg)	F	E_1	E_1

[a] Treatment was followed by bacteriological assay of whole turtle homogenates for *Salmonella* and *Arizona* 72 h post-treatment.

[b] Certification was done by bacteriological assay for *Salmonella-Arizona* in container water prior to treatment.

[c] Each group was blended for 2 min at 4 C at 16,000 rpm in a Sorvall Omnimixer 72 h post-treatment.

[d] Five turtles in each experimental group.

[e] Homogenate (1.0 ml) was inoculated directly into 10 ml of tetrathionate broth.

[f] Homogenate (25 ml) was inoculated directly into 225 ml of duplicate lactose broth.

not from lactose-pre-enriched samples.

In the fourth experiment, infected turtles were treated for 7 and 14 days with 1,000 μg of N-Te, Te, and Tylosin per ml of container water. Tylosin did not effectively suppress the *Salmonella-Arizona* systemic occurrence and, in addition, this agent was quite toxic to the baby turtle (Table 5). Neither N-Te nor Te was toxic for turtles through 14 days of treatment, and neither *Salmonella* nor *Arizona* was isolated from homogenates prepared 72 h post-treatment from turtles treated for 7 or 14 days with Te. However, *Salmonella* was isolated from turtle homogenate 14 days after treatment for 14 days with 1,000 μg of Te per ml.

TABLE 4. *Treatment of infected baby turtles with 200, 400, and 800 μg of Te per ml of container water for 7 days*[a]

Pretreatment certification			14-day treatment group homogenates	
Jug no.	Experimental group	Serogroup	Direct TBG	Lactose pre-enrichment
13	Control	E_1	*Arizona*	B, F
10	Control	B, F	B	B, F
12	Te (800 μg)	F	E_1	0
9	Te (400 μg)	F	E_1	0
14	Te (200 μg)	F	B	0
11	Te (100 μg)	E_1	B	0

[a] See footnotes to Table 3.

Before the 5th experiment was done, it was determined that 1,500 μg of Te per ml of treatment water killed 47 turtles out of 50 in 3 days. Thirty turtles were treated in 300 ml of water containing 1,200 μg of Te per ml for 7 days. On the 3rd, 10th, 16th, and 30th day post-treatment, five turtles were removed from treatment and control groups. The visceral organs were aseptically removed from each of the five turtles, pooled, weighed, and placed in a blended cup with 10 volumes of TBG (wt/vol). This pool of viscera was blended as described, incubated, and tested for the presence of *Salmonella* and *Arizona*. The visceral homogenates were uniformly negative for *Salmonella* and *Arizona* on each testing date, whereas control group visceral organ homogenates were positive (Table 6).

DISCUSSION

The purpose of this investigation was twofold. First, methods for detection of *Salmonella* in "treated" turtles were examined and compared, i.e., excretion versus homogenization. Secondly, attempts were made to treat infected turtles with various concentrations of Te to determine if it was possible to eradicate the *Salmonella-Arizona* carrier state with this antibiotic.

TABLE 5. *Treatment of infected baby turtles with 1,000 μg of Te, N-Te, and Tylosin (Ty) per ml of container water for 7 and 14 days*[a]

Pretreatment certification[b]			7-day treatment homogenate[c] (72 h)	14-day treatment homogenate	
Jug no.	Experimental group[d]	Serogroup		72 h	14 days
1	Control	D	C_1		
2	Te	E_1, *Arizona*	0		
3	N-Te	D	0		
4	Ty	D	C_1, *Arizona*		
5	Control	D		H, I	
6	Te	D		0	
7	N-Te	E_1, *Arizona*		E_1	
8	Ty	D, *Arizona*		*Arizona*	
9	Control	D			*Arizona*
10	Te	*Arizona*			D
11	N-Te	D			*Arizona*
12	Ty	D			Dead

[a] Treatment was followed by bacteriological assay of turtle homogenate for *Salmonella* and *Arizona* 72 h post-treatment and 14 days post 14-day treatment.

[b] Certification was done by bacteriological assay of 72-h container water prior to treatment.

[c] Each group was blended for 2 min at 4 C at 16,000 rpm in a Sorvall Omnimixer.

[d] Five turtles in each experimental group.

TABLE 6. *Treatment of 30 infected baby turtles with 1,200 μg of Te per ml for 7 days*[a]

Pretreatment certification[b]			Visceral organ homogenates[c] (days post-treatment)			
Experimental group	No. of turtles	Serogroup	3	10	16	30
Te (1,200 μg)	30	C_1 C_2, *Arizona*	0	0	0	0
Control	20	C_2, E_1	C_2, E_1	*Arizona*	*Arizona*	*Arizona*

[a] Treatment was followed by bacteriological assay of turtle visceral organ homogenates 3, 10, 16, and 30 days post-treatment for *Salmonella* and *Arizona*.

[b] Certification was done by bacteriological assay for *Salmonella-Arizona* from container water prior to treatment.

[c] All visceral organs from five turtles were blended in tetrathionate broth at 16,000 rpm for 2 min at 4 C.

When turtles which are shedding *Salmonella-Arizona* are treated for 12 to 14 days with N-Te at 200 μg per ml of container water these organisms are not excreted in detectable numbers for several weeks or months. The results from this study show that the current certification procedure that calls for the microbial assay of 72-h container water is unlikely to detect the presence of *Salmonella* and *Arizona*. Therefore, the use of the excretion method to certify turtles "*Salmonella*-free" is of questionable value, since shedding of *Salmonella* by treated animals may resume at a later time.

Wells et al. (4) compared the recovery of *Salmonella-Arizona* from container water (excretion method) with the recovery of these organisms from turtle homogenates (blending procedure). Both methods appeared to be equally sensitive in detecting overall infections with *Salmonella* or *Arizona*. However, our studies show that the two methods are not equally sensitive when applied to turtles treated with antimicrobial agents. Both *Salmonella* and *Arizona* were isolated by the blending method from turtles 72 h post-treatment with 200 μg of N-Te, but not from 72-h or 14-day container water, i.e., the excretion method. Because current regulations condemn lots of turtles found contaminated with either *Salmonella* or *Arizona* by microbial assay of container water, systemic *Salmonella-Arizona* infections would be missed. It would be more realistic to use the blending method to detect the systemic occurrence of *Salmonella-Arizona*, since it must be assumed that most lots of turtles presently being certified by the health laboratories have been treated in some manner. The use of high concentrations of Te, such as 400, 800, and 1,000 μg/ml, to treat turtles would surely reduce the chances for detecting *Salmonella-Arizona* by the excretion method. However, if these treated turtles are held for 14 days post-

treatment, these organisms can be isolated from whole turtle homogenates.

Apparently the baby turtle carries more than one *Salmonella* serogroup at a given time, since more often than not different serogroups are isolated from turtle homogenates than are isolated from the container water. Antibiotic treatment of the turtles may suppress the initially prominent serogroup and allow other serogroups to emerge by virtue of their resistance. *Arizona* is more frequently isolated from turtle homogenate than from container water, which suggests that it is not excreted. Wells et al. (4) reported similar findings in their blending experiments, and they suggest that *Arizona* has a greater tendency toward systemic involvement than do the salmonellae, for they recovered *Arizona* from kidney, liver, and ovary homogenates. Since localized systemic salmonellae or arizonae infections in the liver, kidney, and ovary may not result in active excretion of these pathogens by the infected turtle, the possibility does exist that such infections may progress to other tissue sites at some later date, which may result in active excretion. Thus, the blending method could detect the systemic presence of *Salmonella* and *Arizona*, whereas the excretion method might not.

On a small scale turtles were treated with 1,200 μg of Te per ml of container water, and bacteriological assay of visceral organ homogenates failed to detect the presence of *Salmonella* and *Arizona* through 30 days post-treatment. Whether these organisms have been eliminated or whether the tissues saturated with Te prevent recovery of these organisms remains to be determined. A serious problem could result with the emergence of antibiotic-resistant salmonellae either through mutation or acquisition of resistance transfer factors upon treatment with high concentrations of Te.

Presently, several turtle farmers are treating large lots of turtles with Te dissolved in water. Representative turtles from each treatment group are being closely followed in our laboratory by both excretion and blending to determine if they will resume excretion and to detect the systemic presence of these pathogens. In addition, representative turtles are being blended periodically to detect the systemic presence of these pathogens.

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