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Enteric Bacteria in Natural Populations of Freshwater Turtles in Virginia

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ABSTRACT

We examined 174 freshwater turtles of seven species in six natural populations in central and southeastern Virginia for enteric bacteria. Five species were identified: *Arizona* spp., *Hafnia alvei*, *Citrobactor freundii*, *Escherichia coli*, and *Serratia liquefaciens*. No *Salmonella* was found. The total number of enteric bacteria genera now known for Virginia's freshwater turtles is eight. All have been found in *Chrysemys picta*, whereas six or fewer are known for the other five species studied. The low incidence of *Arizona* spp. and the lack of *Salmonella* in studies so far suggest that these pathogenic bacteria are uncommonly found in natural populations of freshwater turtles in Virginia. There appears to be little to no threat of turtle-associated bacterial pathogenecity, except when wild-caught turtles are placed under stressful conditions in captivity. We found *Salmonella enteritidis* (8 turtles), *S. java* (1), and *S. newport* (2), and *Arizona henshawii* (18) in a sample of 43 commercially obtained western painted turtles, *Chrysemys picta bellii,* used for classroom physiology studies. Because of the pathogenic threat and the decimation of turtle populations in the Midwest, we suggest that caution be taken when handling commercially sold turtles and that other models be sought to demonstrate physiological processes for which turtles are often used.

Key words: *Salmonella,* Bacteria, Turtles, Testudines, Epidemiology

INTRODUCTION

Some members of the bacterial family Enterobacteriaceae, particularly the genera *Salmonella* and *Arizona*, are human pathogens causing a variety of gastrointestinal and other symptomatic diseases. Their occurrence in reptiles is well-documented (Hinshaw and McNeil, 1947; Hoff and White, 1977; Marcus, 1980). Some reptilian species have also been found to be potential vectors of these pathogens (Oboegbulen and Isoghohimhen, 1985; Marcus, 1980; Chiodini and Sundberg, 1981). Turtles in particular were found to host these bacteria without apparent adverse affects (Boycott, 1962; Kaufmann, *et al*, 1967; Marcus, 1980). Turtle-associated salmonellosis was first reported in the United States human population in 1963 (Chiodini and Sundberg, 1981). Results of numerous epidemiological studies that followed (*e.g.*, Kaufmann and Morrison, 1966; Lamm *et al.*, 1972; Altman *et al*, 1972; Kaufmann *et al*, 1972) led to the 1975 ban in the United States on sales of turtle eggs and live turtles less than 10.2 cm (4 inches) carapace length that were not certified *Salmonella*-free (Chiodini and Sundberg, 1981; Hardy, 1988).

Few studies have surveyed for *Salmonella* and other bacteria in natural turtle populations. Baker *et al.* (1972) mentioned that three *Salmonella* serotypes were found in three wild-caught turtles in New Jersey and Behler (1973) noted that no *Salmonella* or *Arizona* were found in 60 turtles collected in the northeastern United States. However, neither provided data on turtle species, incidence of other bacteria, or methodology. Because so few published data are available on this subject, we examined turtles in six natural populations in Virginia for the presence of *Salmonella* and other enteric bacteria. For comparison, a group of turtles purchased for classroom study from a midwestern commercial dealer was examined. Our objective was to determine if potentially harmful bacteria occurred naturally in Virginia's freshwater turtles and if such bacteria were found in commercial turtles handled by students.

MATERIALS AND METHODS

Study Sites. - Freshwater turtles were captured in 1980 and 1981 from six sites in central and southeastern Virginia: (1) In Back Bay National Wildlife Refuge, 5 mi. S Sandbridge, Virginia Beach, turtles inhabited freshwater canals at the margins of migratory waterfowl management impoundments. These canals are 300-400 m east of the Atlantic Ocean and as close as 25 m to Back Bay. From these canals, 29 turtles of four species were sampled. (2) Seashore State Park, at the northern end of Virginia Beach, embodies several interdunal freshwater ponds. All ponds contain darkly stained water and deep organic sediments. Bald-cypress (*Taxiodium distichum*) dominate the vegetation in the ponds and oak (*Quercus* spp.) and alder (*Alnus serrulata*) line the dune slopes. Thirty turtles of six species were sampled at this site. (3) Grassy Swamp Lake, 5 mi. SW of Ashland, Hanover County, is a freshwater impoundment surrounded by a mixed hardwood-pine forest. It contains abundant aquatic and emergent vegetation. This lake is described in Mitchell (1985). Here we sampled bacteria from four turtles of two species. (4) Laurel Lake, 0.5 mi. W Laurel, Henrico County, is a warm polymictic urban impoundment supporting abundant emergent vegetation but little submerged aquatic vegetation. It is located in a former golf course and is described in Mitchell (1988). At Laurel Lake we sampled 47 turtles of five species. (5) Bryan Park Lake, in northern Richmond, is a shallow freshwater reservoir in an urban park. It contains abundant emergent vegetation but little submerged aquatic vegetation. The water is turbid and the substrate is clay covered with silt and other organic debris. Scattered hardwood occur on the surrounding slopes, along with several paved roads. Here we sampled 34 turtles of five species. (6) Westhampton Lake, on the University of Richmond campus, is an old urban reservoir with little aquatic vegetation of any sort. Most of the substrate is clay and silt and the water is always turbid. The western slope has a stand of mixed hardwood and pine trees but buildings and roads line all other slopes. We sampled 34 turtles of three species in this lake.

Field Methods.- Turtles were captured with one inch mesh, chickenwire funnel traps (Iverson, 1979) baited with canned sardines or chicken scraps. Traps were set in shallow water so that turtles were allowed to reach air and avoid drowning.

At sites where tampering with the traps by the public seemed likely, they were concealed with vegetation. Traps were checked each morning and evening, and all turtles caught were removed, processed and released.

Each specimen was marked for future identification by filing notches in the carapacial margin following Mitchell (1988). Straightline carapace length (CL, nearest mm), plastron length (PL), and weight (nearest gram) were taken on each turtle. The turtle's sex and any injuries or abnormalities were also noted. Turtles collected at Seashore State Park, Back Bay National Wildlife Refuge, and Laurel Lake were sampled, processed, and released at the site, whereas those collected at other sites were processed in the laboratory and released the following day.

We sampled for bacteria in all cases by gently inserting a sterile swab from a Cepti-Seal Culturette into the cloaca about 2.5-4.0 cm, rotating it gently, and then placing the swab into the Culturette. To keep the swab viable up to 72 h, the ampule containing 0.5 ml Modified Stewarts Bacterial Transport Media was crushed, releasing the media. For small specimens, a smaller, sterile swab made with a 2-3 mm dowel and roll cotton was used with the Culturette.

Commercial Turtle Samples- Forty three adult *Chrysemys picta bellii*, the western subspecies of the painted turtle, were obtained from commercial dealers in the midwestern United States in the fall of 1981. These were kept in laboratory sinks at the University of Richmond and fed lettuce ad libitum for about one week before being sacrificed. Upon sacrifice, the small intestine, upper and lower large intestine, and cloaca of each were swabbed as above for bacteria.

Laboratory Methods.- Within 72 h, exposed swabs were placed in 16 x 150 mm labeled, capped, sterile test tubes containing 10 ml Gibco Selenite Broth enrichment media and incubated at 37 C for 18 to 24 h. During incubation, caps on the test tubes were loosened to prevent gas build-up. Following incubation, a 2 mm nichrome innoculating loop was immersed in the enrichment media and streaked across a sterile disposable petri dish containing 5 ml Gibco Xylose-Lysine-Deoxycholate Agar (XLD), flaming the loop between samples. These labeled plates were incubated at 37 C for 18 to 24 h.

Colonies were identified with a Roache Diagnostics Enterotube II. The innoculating needle was pulled through the Enterotube II, partially reinserted, broken off and the unit capped, labeled, and incubated at 37 C for 18 to 24 h. Two subcultures of each suspect colony were made by touching an innoculating needle to them and stabbing and streaking the needle in Plain Nutrient Agar slants. These slants were incubated with the Enterotube IIs for 18 to 24 h.

The Enterotube II has 12 compartments with the following media: glucose, lysine-decarboxylase, ornithine-decarboxylase, an H_2S sensitive media, adonitol, lactose, arabinose, sorbitol, Voges-Proskauer media, dulcitol-phenylalanine-deaminase, urea, and citrate. In addition to reactions with these compounds, gas production is indicated by the separation of a wax overlay on the glucose media. Indole positive reactions are indicated by injecting 2 drops of Kovacs' reagent into the H_2S compartment. With each group of enrichment tubes, plates, and Enterotubes IIs, two control samples were tested, one sterile and one pure *Salmonella* spp. (from the Virginia State Laboratory). All culturing was done under a decontamination hood using Amway germicidal spray and ultraviolet radiation for asepsis before and after each group of cultures.

Colonies were identified by scoring the reactions in the Enterotube II scorecard, adding these numbers to obtain a five digit identification code. The code numbers were matched to bacteria genus and species in the Enterotube II computer Code Guide. If *Salmonella* spp. or *Arizona* spp. were indicated for a colony, their subcultures were sent to the Virginia State Laboratory-Enteric Section for positive identification. After the colonies were identified, all culturettes, swabs, enrichment tubes, plates, Enterotube IIs, and subcultures were destroyed by autoclaving.

RESULTS

Field Samples- A total of 174 freshwater turtles of eight species and subspecies were captured in the six locations studied (Table 1). The most abundant species and the only one captured at all sites was the eastern painted turtle, *Chrysemys picta picta*. This species, as well as red-bellied turtles (*Pseudemys rubriventris*), yellow-bellied sliders (*Trachemys scripta scripta*), and red-eared sliders (*Trachemys scripta elegans)* are basking turtles often seen on logs or banks. The latter subspecies has been introduced in many Virginia ponds and lakes by people who purchased them as juveniles at pet stores. It is found naturally in the Mississippi River drainage of central North America (Conant, 1975) and predominated in the sample from Westhampton Lake. The remaining four species, snapping turtle (*Chelydra serpentina*), striped mud turtle (*Kinosternon baurii*), eastern mud turtle *(Kinosternon subrubrum*), and stinkpot (*Sternotherus odoratus*), are all bottom-walkers (Berry and Shine, 1980) and bask less frequently than the turtles in the previous group. These species spend most of their lives on the bottom of shallow waters in contact with the substrate. *Kinosternon subrubrum* and *K. baurii* are the most terrestrial of the group and often spend considerable time in shallow burrows on land (Ernst and Barbour, 1972; Gibbons, 1983). The striped mud turtle has only recently been confirmed as a member of the Virginia herpetofauna (Lamb and Lovich, 1990). The majority of the turtles captured were adults, although juvenile *T. scripta elegans* comprised the largest fraction of the Westhampton Lake sample (Table 1).

No *Salmonella* spp. was found in any of the wild turtles, however, the closely related *Arizona* spp. and four other enteric bacterial genera were identified in our samples. Three genera were found at all six study sites, whereas *Serratia* and *Arizona* were found in three each.

The painted turtle, *Chrysemys picta*, contained the greatest diversity of bacteria identified (Table 2). Three turtles from three locations contained *Arizona* spp.

The sample of the slider turtle native to southeastern Virginia, *Trachemys scripta scripta*, all from the Back Bay site, contained four species of bacteria - *Hafnia alvei* (1 turtle), *Citrobacter freundii* (2), *Escherichia coli* (1), and *Serratia liquefaciens* (1). The introduced subspecies, *Trachemys scripta elegans*, found in three of the locations studied, contained three species of bacteria, *Citrobacter freundii* - one turtle each from Bryan Park Lake and Seashore State Park and 5 turtles from Westhampton Lake, *Escherichia coli* - three turtles from Westhampton Lake, and *Arizona* spp. - one juvenile from Westhampton Lake.

Pseudemys *rubriventris* contained two species of bacteria, *Hafnia alvei* - one turtle each from Bryan Park and Laurel Lake, and *Citrobacter freundii* - two turtles each from Bryan Park Lake and Laurel Lake.

TABLE 1. Composition of freshwater turtle samples in six freshwater lakes and ponds in Virginia. In each sample, the number of adults is the first number and the number of juveniles is the second. Abbreviations: BB = Back Bay National Wildlife Refuge, SSP = Seashore State Park, GSL = Grassy Swamp Lake, LL = Laurel Lake, BP = Bryan Park Lake, and WL = Westhampton Lake.

Species	BB	SSP	GSL	LL	BP	WL
Chrysemys picta	13/2	16/0	3/0	20/15	20/3	4/4
Chelydra serpentina		1/0		1/1	1/0	4/0
Kinosternon baurii		8/0				
Kinosternon subrubrum	5/0		1/0			
Pseudemys rubriventris	0/1			0/3	3/0	
Sternotherus odoratus		3/0		3/0	6/0	
Trachemys scripta scripta		3/6				
Trachemys scripta elegans					1/0	4/17

TABLE 2. Distribution of enteric bacteria in Virginia populations of *Chrysemys picta*. Abbreviations for sampling locations are in Table 1. The number represents number of turtles.

	BB	SSP	GSL	LL	BP	WL
Arizona spp.	1			1	1	
Hafnia alvei	2	1		2	1	
Citrobacter fruendii	2	6	1	10	5	3
Esherichia coli	2	2	2	3	1	
Serratia liquefaciens	1	1		2	1	

In the sample of *Chelydra serpentina*, *Hafnia alvei* was found in an immature female from Westhampton Lake and *Arizona* spp. occurred in an immature female from Bryan Park Lake. Three species of bacteria were documented from three populations of *Sternotherus odoratus*: *Hafnia alvei* - one turtle each from Seashore State Park and Laurel Lake, *Citrobacter freundii* - one turtle from Seashore State Park and two from Bryan Park Lake, and *Serratia liquefaciens* - one turtle from Laurel Lake. Two species of bacteria were found in two populations of *Kinosternon subrubrum*: *Hafnia alvei* - one turtle each from Back Bay and Grassy Swamp Lake and *Citrobacter freundii* - two turtles from Back Bay. *Kinosternon baurii* contained three species of bacteria, all from Seashore State Park: *Citrobacter freundii* (2 turtles), *Escherichia coli* (2), and *Hafnia alvei* (1).

Laboratory Samples.- Of the 43 adult *Chrysemys picta bellii* in the two commercial samples, 11 (26%) contained *Salmonella*. *Salmonella enteritidis* was isolated from eight turtles, *S. java* from one, and *S. newport* from two. Eighteen individuals contained *Arizona hinshawii*, ten contained *Citrobacter freundii*, one contained *Enterobacter cloacae*, four had *Escherichia coli*, one had *Hafnia alvei*, and two turtles contained *Serratia liquefaciens*. The eleven turtles that contained *Salmonella* also harbored *Arizona*.

Bacterial distribution in the large (upper and lower) and small intestine and in the cloaca revealed no consistent patterns. *Arizona henshawii, Citrobactor,* and *Salmonella enteritidis* were found in all anatomical locations. *Salmonella newport* occurred only in the lower large intestine, whereas *S. java* was found in all locations except the small intestine.

DISCUSSION

Salmonella and its close relative *Arizona henshawii* have been isolated from numerous wild animals under captive and natural conditions (*e.g.*, Morse and Duncan, 1974; Everard *et al.*, 1979). These bacteria have been found in over 275 species of reptiles, including 44 species of turtles (Hoff and White, 1977). Several of the serotypes commonly causing human health problems are among those listed, including *S. enteritidis* and *S. newport* (Altman *et al.*, 1972; Chiodini and Sundberg, 1981). Despite their occurrence in a variety of reptilian species, high incidence of human health problems in the United States has been linked only to the commercially sold red-eared turtle (*T. scripta elegans*). Although the 1975 ban may have significantly reduced the incidence of salmonellosis in humans from this source (Cohen *et al.*, 1980), the incidence of *Salmonella* in pet store and other captive reptiles remains high (Chiodini and Sundberg, 1981). These authors suggest, however, that knowledge of the potential health hazards and proper sanitation are sufficient to minimize human contamination.

How prevalent is *Salmonella* and *Arizona henshawii* in natural populations of freshwater turtles? The answer to this question is of interest because wild turtles are often caught and kept as pets by children and turtles of all sizes continue to be sold in pet stores (pers. obs.). We found only three published accounts on the incidence of *Salmonella* in natural populations. Baker *et al.* (1972) mentioned that a New Jersey study of 150 turtles revealed *S. newport* in one individual, *S. manchester* from a second, and *S. give* from a third. Behler (1973) reported that no *Salmonella* or *Arizona* were found in 60+ individuals of eight species from the northeastern United States. In neither of these papers were the species of turtles listed. Jackson *et al.* (1969) found the following bacteria in wild-caught turtles in Mississippi: *Salmonella miami* in *Sternotherus minor*; *S. infantis* in *Chelydra serpentina*; *Edwardsiella tarda* in *Chrysemys picta* and *Trachemys scripta scripta*; and *Citrobactor* spp. in *Kinosternon subrubrum* and *Pseudemys floridana*. The number of turtles infected was not given. Our study of 174 individuals of eight species and subspecies also revealed no *Salmonella* in their bacterial flora. We did, however, find *Arizona* spp. in *Chrysemys picta*, *Chelydra serpentina*, and *Trachemys scripta elegans*. Because the *Arizona* was not identified to species in these samples, we do not know if the pathogenic *A. henshawii* serotype was among them.

In an unpublished master's thesis, Gapp (1970) examined 16 adult freshwater turtles in an unpolluted Virginia lake (Chesterfield County) and 19 turtles in one contaminated with human wastes (James City County). He found six genera of enteric bacteria in five species of turtles, but no *Salmonella, Arizona*, or *Shigella*. The only qualitative difference between the two lakes was the absence of *Citrobactor* in the polluted system. In addition, turtles from the lake with the higher coliform

TABLE 3. Summary of occurrence of enteric bacteria in eight species and subspecies of freshwater turtles in Virginia. Data are from Gapp (1970) and this study. Abbreviations: CP = *Chrysemys picta*, CS = *Chelydra serpentina*, KB = *Kinosternon baurii*, KS = *Kinosternum subrubrum*, PR = *Pseudemys rubriventris*, SO = *Sternotherus odoratus*, TSE = *Trachemys scripta elegans*, and TSS = *Trachemys scripta scripta*. A plus sign represents a known occurrence and a negative sign indicates that the genus has not been recorded for a Virginia population.

	CP	CS	KB	KS	PR	SO	TSE	TSS
Arizona	+	+	-	-	-	-	+	-
Citrobacter	+	-	+	+	+	+	+	+
Enterobacter	+	+	-	+	+	+	-	-
Escherichia	+	-	+	+	-	-	+	+
Hafnia	+	+	+	+	+	+	-	+
Klebsiella	+	+	-	+	+	+	-	-
Proteus	+	-	-	+	+	+	-	-
Providence	+	-	-	+	-	+	-	-
Serratia	+	-	-	-	-	+	-	+

counts had fewer enteric bacteria than turtles in the lake with the lower coliform counts. Thus, the contamination level of the lake may not reflect the contamination level of the adult turtle (Kaufmann and Morrison, 1966). In turtles from the polluted lake, >50% of the isolates were *Escherichia*, *Enterobacter*, and *Klebsiella*, genera associated with human fecal contamination. These genera comprised only 15% of the enterics isolated in the unpolluted lake. Gapp concluded that the enteric flora of freshwater turtles is usually comprised of intermediate biochemical types reflecting the bacterial nature of the water and surrounding soil. Human fecal pollution alters the biochemical types but not the floral composition (Gapp, 1970).

Comparison of our study with Gapp (1970) is difficult because we did not score our samples for biochemical types. Of the three bacterial genera noted by Gapp (1970) to be commonly isolated with human pollution, only *Escherichia coli* was found in our study. It occurred in all of our study sites and in three species of turtles. Our study and that of Gapp (1970) provide the only list of bacterial flora for Virginia's freshwater turtles (Table 3). The low incidence of *Arizona* spp. (2.9%) and the lack of *Salmonella* in our studies suggests that bacteria pathogenic to humans are uncommonly found in natural populations of freshwater turtles in Virginia.

Given the diversity of bacterial flora in Virginia's turtles, do these bacteria pose health threats to these animals? *Citrobacter fruendii* is a Gram-negative rod normally found in soil and water and may cause septicemic cutaneous ulcerative disease (SCUD) in turtles (Kaplan, 1957). *Serratia* may cause clinical features similar to SCUD (Marcus, 1980). *Salmonella* is probably a saprophytic organism in reptiles causing diarrhea, anorexia, and listlessness (Chiodini and Sundberg, 1981) but individuals carrying these bacteria are usually asymptomatic (Marcus, 1980). One of us (JCM) has captured several thousand freshwater turtles in a variety of aquatic habitats in Virginia and fewer than five showed external signs of disease, *e.g.*, emaciation. We are aware, however, that turtles brought into captivity frequently show signs of disease after a period of time unless all aspects of husbandry are properly controlled. Thus, stress may allow an otherwise asymptomatic turtle to exhibit pathogenic symptoms (DuPonte *et al.*, 1978).

The identification of *Salmonella enteritidis*, *S. java*, *S. newport*, and *Arizona henshawii* in commercial painted turtles (*C. picta*) used for classroom physiology studies clearly warrents concern about handling and captive maintenance. None of these turtles showed obvious signs of disease, so they may have been asymptomatic prior to collection. The stress of shipment and being maintained on nutrient poor lettuce in a sink with only dripping water may have caused the bacteria to become active. We recommend that instructors and students be made aware of the potential for contamination and that they use proper sanitation procedures when handling and caring for classroom turtles. Because the number of wild turtles collected for commercial purposes has seriously reduced natural populations (Warwick, 1986; Warwick and Steedman, 1988), we suggest that other models be sought for the physiological processes usually demonstrated in the classroom with these animals.

Although we found no *Salmonella* in our samples, we cannot say conclusively that this bacterial genus does not exist in natural populations in Virginia. All of our turtles and those sampled by Gapp (1970) were examined by swabbing the cloaca only once. Because excretion rates vary within and among individuals, carrier identification is sometimes difficult (Chiodini and Sundberg, 1981). Studies of domestic animals indicate that 3-4 separate cultures taken from several grams of feces at different times must be taken to declare an animal *Salmonella*-free (Carter, 1986). A turtle that is a heavy shedder at one time may later have a negative stool culture (Kaufmann *et al.*, 1967). Thus, our study sets the stage for future research. Given that *Arizona*, *Enterobacter*, *Klebsiella*, *Citrobacter*, *Serratia*, and *Proteus* (Table 3), all potentially pathogenic to humans (McCoy and Seidler, 1973), have been found in Virginia's turtles, additional study of the bacterial flora of these animals, and perhaps certain captive reptiles as well, is clearly warranted.

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NOTE ADDED IN PROOF: Results in a new paper by D'Aoust *et al.* (1990, Am J. Epidemiology 132:233-238) found *Salmonella* in fertile eggs, packaging moss, and hatchlings in shipments of red-eared sliders from Louisiana. Their confirmation of antibiotic-resistant strains of bacteria demonstrate that the sale of red-eared sliders continues to pose a serious human health risk. Their results strengthen our argument that extreme caution should be taken when using live turtles for laboratory exercises.