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Detection of Salmonellae in Different Turtle Species within a Headwater Spring Ecosystem

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ABSTRACT: Sediments and water from the slough arm of Spring Lake, the headwaters of the San Marcos River, Texas, USA, as well as swabs from biofilms on carapaces and from the cloacae of 18 common musk turtles (*Sternotherus odoratus*), 21 red-eared sliders (*Trachemys scripta elegans*), nine Texas river cooters (*Pseudemys texana*), one snapping turtle (*Chelydra serpentina serpentina*), and three Guadalupe spiny soft-shell turtles (*Apalone spinifera guadalupensis*), caught at the same site, were analyzed for salmonellae by culture and molecular techniques. Although enrichment cultures from sediment and water samples were negative for salmonellae in polymerase chain reaction (PCR)–based analyses, this technique detected salmonellae in the enrichments from both carapaces and cloacae of 11 musk turtles (61%), eight red-eared sliders (38%), and the snapping turtle. Salmonellae could also be detected in the enrichments from the carapaces of two additional red-eared sliders and two Texas river cooters; the remaining samples were negative. Further characterization of isolates obtained from the enrichment cultures of seven selected individuals that represented all turtle species with salmonellae confirmed the presence of *Salmonella enterica* subspecies *enterica*, with serovars Rubislaw, Newport, Gaminara, and Thompson identified. These results demonstrate the presence of different strains of potentially human pathogenic salmonellae naturally occurring on several turtle species with different life histories even within supposedly pristine environments.

Key words: Enrichment, PCR, rep-PCR, resuscitation, *Salmonella enterica*, Spring Lake.

Salmonellae have been detected in the gastrointestinal tracts of a large taxonomic variety of free-living and captive animals, including mammals, birds, and reptiles, throughout the world (Gray, 1995; Refsum et al., 2002; Briones et al., 2004). Although both free-living and captive animals have been identified as reservoirs for human-associated salmonellosis (Guard-Petter, 2001; Santos et al., 2001), the significance of free-living animals as potential carriers and vectors for salmonellae in human-associated salmonellosis is often less well established than for captive animals. Pet turtles, for example, are well-known reservoirs for salmonellae (Johnson-Delaney, 1996), and many studies have demonstrated their significance in human-associated salmonellosis (Shane et al., 1990; Anonymous, 1995; Sanyal et al., 1997; Anonymous, 1999; Geue and Löschner, 2002; Mermin et al., 2004; Nakadai et al., 2005). However, studies on the occurrence of salmonellae in free-living turtles are scarce, and the results are contradictory (Brenner et al., 2002; Briones et al., 2004; Richards et al., 2004; Chambers and Hulse, 2006; Saelinger et al., 2006). Although some studies failed to find any salmonellae in cloacal, fecal, or gastrointestinal mucosal samples of free-living turtles, and thus questioned the importance of free-living turtles as potential vectors for human-associated salmonellosis (Brenner et al., 2002; Richards et al., 2004; Saelinger et al., 2006), other investigations emphasized the role of reptiles, including turtles, as reservoirs for salmonellae, with salmonellae present in 41% of lizards, 54% of snakes, and 32% of turtles analyzed (Briones et al., 2004) or even in 100% of all turtles tested (Chambers and Hulse, 2006).

In a recent study, we demonstrated the presence of salmonellae in common musk turtles (*Sternotherus odoratus*) living in the headwaters of the San Marcos River,

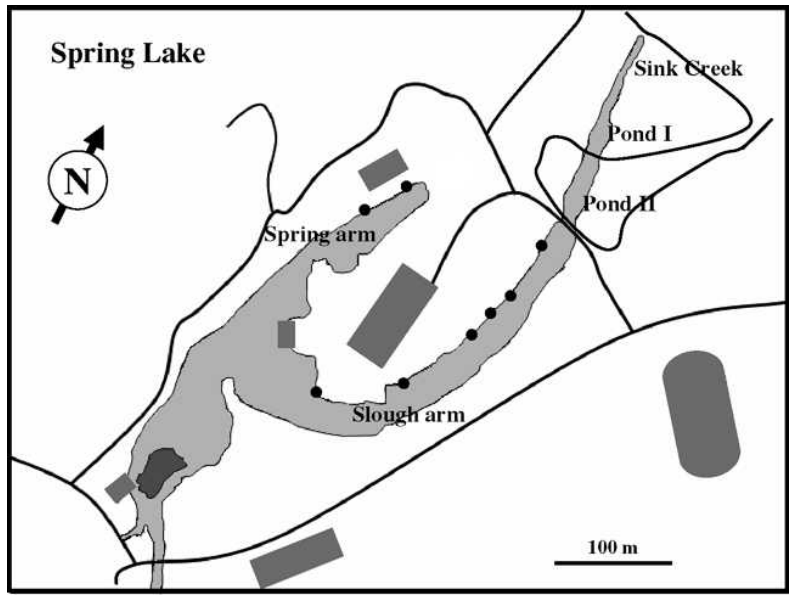

FIGURE 1. Schematic presentation of sampling sites (dots) in both spring and slough arms of Spring Lake, the headwaters of the San Marcos River, Texas, USA (79°53′N, 97°55′W). The rectangular shapes represent buildings; the lines are streets. Water flows south originating from the spring arm.

Texas, USA (Hahn et al., 2007). Although no salmonellae could be detected in sediment and water samples, about 50% of the musk turtles harbored salmonellae in both cloacal samples as well as in biofilms on the carapaces, supporting the idea that free-living turtles can serve as reservoirs for salmonellae. A generalization of this statement, however, requires additional data on the distribution of salmonellae in different taxa of free-living turtles in order to examine potential differences resulting from different life histories such as basking or foraging behavior.

The aim of this study was, therefore, to expand our previous survey and analyze additional turtle species for salmonellae in other areas of Spring Lake, the headwaters of the San Marcos River, Texas, USA (79°53′N, 97°55′W). In our previous study (Hahn et al., 2007), we examined the spring arm that is characterized by relatively constant environmental conditions in depth and throughout the year with high mineral nutrient availability regulated by the permanent supply of aquifer water through numerous springs and the resulting fast flow and exchange of the surface water (Groeger et al., 1997). Spring Lake also has a distinct slough arm, representing a more lentic environment with slow flow, large seasonal changes in temperature and redox conditions, and large deposition of organic material (Groeger, pers. comm.). We collected sediment and water samples as well as biofilms from the turtle carapaces and direct cloacal samples from turtles living in this habitat.

Water and sediment samples were retrieved from several sites at the upper slough arm of Spring Lake with a vertical point water sampler and a bottom dredge, respectively, at a distance less than half a kilometer from our previous sampling sites in the spring arm (Fig. 1). Water samples were taken just below the surface and at a depth of about 50 cm, just above the sediment. Turtles were collected with the use of a dip net or were caught in baited hoop nets. Fifty-two turtles were retrieved, 18 common musk turtles (*s. odoratus*, nine females, nine males), 21

red-eared sliders (*Trachemys scripta elegans*, nine females, 12 males), nine Texas river cooters (*Pseudemys texana*, all female), one snapping turtle (*Chelydra serpentina serpentina*, male), and three Guadalupe spiny soft-shell turtles (*Apalone spinifera guadalupensis*, two females, one male). Thus, we replicate our efforts from the spring arm (Hahn et al., 2007) by including a matching number of *S. odoratus* and *C. serpentina*, but significantly expand the overall taxonomic coverage and total number of turtles sampled.

Samples were taken with sterile cotton wool swabs from the carapaces and the cloacae of all turtle species (Hahn et al., 2007), except for the Guadalupe spiny soft-shell turtles, from which only cloacal samples were retrieved. Swabs, subsamples of sediment (about 100 mg wet weight), and pellets obtained from 1 ml of the water samples centrifuged at 14,000 rpm for 2 min, were incubated in 1 ml of buffered peptone water in 2-ml cryotubes at 37 C for 16–20 hr (International Standards Organization, 1993). Subsamples (100 µl) of these pre-enrichment cultures were transferred to 2-ml cryotubes that contained 1 ml of Rappaport-Vassiliadis (RVS) broth (Vassiliadis et al., 1981) and incubated at 43 C for 24 hr in order to enrich for salmonellae (Vassiliadis et al., 1981).

Pre-enrichment and enrichment cultures of all samples, that is, all sediment and water samples from the slough arm of Spring Lake as well as of all samples from carapaces and cloacae of turtles, showed significant increment in turbidity during incubation, suggesting microbial growth. Because enrichment conditions in RVS broth were only semiselective, the increase in turbidity in enrichment cultures did not presuppose enrichment of salmonellae, and thus their potential presence in enrichment cultures required confirmation (Hahn et al., 2007). This confirmation was based on polymerase chain reaction (PCR)–assisted detection of a 284–base-pair (bp) fragment of the *inv*A gene that encodes a protein of a Type III secretion system, essential for the invasion of epithelial cells by salmonellae (Suárez and Rüssmann, 1998; Khan et al., 2000). This assay detects all *Salmonella enterica* subspecies as well as *Salmonella bongori*, and was recently validated and proposed as international standard diagnostic method for quality-assurance laboratories in epidemiologic studies on *Salmonella* spp. (Malorny et al., 2003).

For the detection of salmonellae by PCR, 100-µl samples of pre-enrichment and semiselective enrichment cultures, as well as of cultures of *Salmonella typhimurium* ATCC 14028 that was always used as positive control, were centrifuged at 14,000 rpm for 1 min. The bacterial pellets were washed once in sterile distilled water, and bacteria lysed in 100 µl of 50 mM NaOH by incubation at 65 C for 15 min (Hahn et al., 2007). One microliter of this lysate was used as template for PCR amplification with primers 139 (5′GTG AAA TTA TCG CCA CGT TCG GGC AA) and 141 (5′TCA TCG CAC CGT CAA AGG AAC C) (Rahn et al., 1992). The PCR was carried out in a total volume of 50 µl containing 10× PCR buffer (500 mM KCl, 25 mM $MgCl_2$, 200 mM Tris/HCl, pH 8.4, 0.1% Triton 100), 1 µl deoxyribonucleotide triphosphates (dNTPs) (each 10 mM in 10 mM Tris/HCl, pH 7.5), 0.2 µl *Taq* polymerase (5 U μl^{-1}), and 1 µl of each primer (100 ng μl^{-1}). After an initial 10-min denaturation at 96 C, and subsequent addition of *Taq* polymerase (hot-start PCR), 35 rounds of temperature cycling were performed in a PTC-200 Thermocycler (BioRad, Hercules, California, USA) with denaturation at 96 C, primer annealing at 64 C, and elongation at 72 C, each for 30 sec (Malorny et al., 2003). This was followed by incubation at 72 C for 7 min (Hahn et al., 2007).

Agarose gel electrophoresis (2% agarose in TAE [Tris-acetate-ethylenediaminetetraacetic acid] buffer) (Sambrook et al., 1989) of 10-µl subsamples of the PCR did

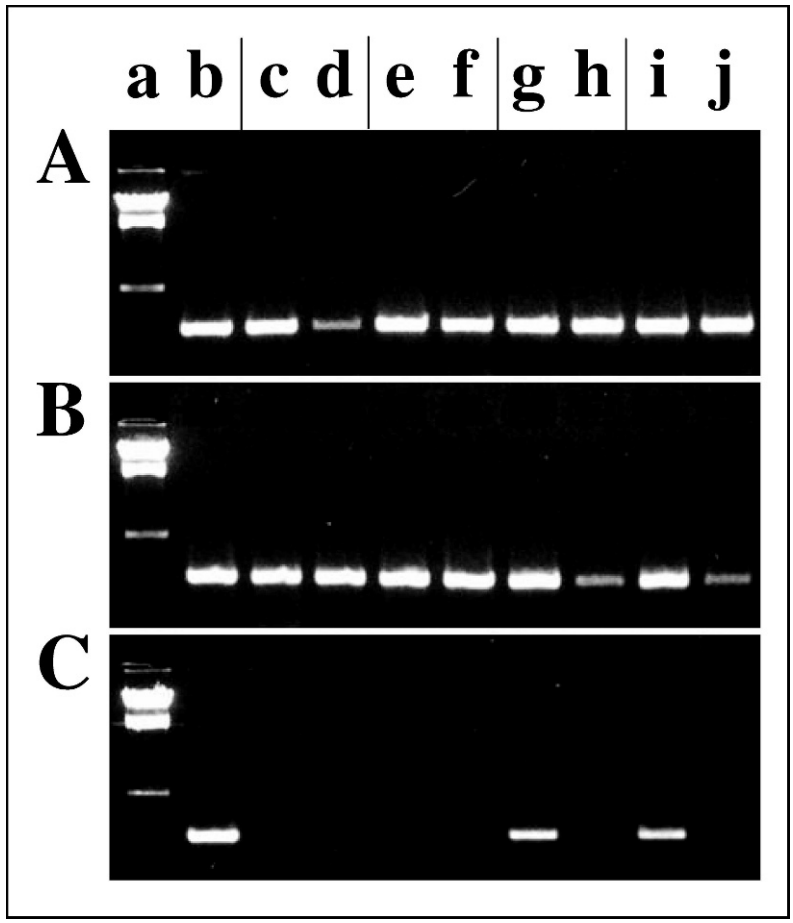

FIGURE 2. PCR amplification products showing the presence of *inv*A gene fragments in the enrichment cultures for salmonellae from swabs obtained from carapaces (lanes c, e, g, i) or cloacae (lanes d, f, h, j) of four individual musk turtles (*Sternotherus odoratus*) (a), red-eared sliders (*Trachemys scripta elegans*) (b), or Texas river cooters (*Pseudemys texana*) (c). Lanes marked a show a Lambda HindIII size marker; lanes marked b show the *inv*A gene fragment from *Salmonella typhimurum* ATCC 14028.

not display amplicons of *inv*A gene fragments in any of the environmental samples, that is, water and sediment samples. These samples remained negative even when inoculum sizes into preenrichment medium were increased, for example, from 100 to 500 μl of sediment, and from cells in 1-ml water samples to cells in 100-ml water samples, and two sequential enrichment steps in RVS broth were used. These results corroborate those of our previous study from the spring arm of Spring Lake, where none of the environmental samples were positive for salmonellae (Hahn et al., 2007). Although salmonellae had been recovered from rivers and streams in remote areas without any significant human impacts (Fair and Morrison, 1967; Hendricks and Morrison, 1967; Thomason et al., 1975), our results indicate that neither water nor sediments in both the lotic spring and the lentic slough arm of Spring Lake provide conditions that allow salmonellae to persist in densities that are detectable by our methodology.

In contrast to environmental samples, *inv*A gene fragments were detected in carapace and cloacal enrichment samples of 11 out of 18 musk turtles (i.e., in 61%,

five females, six males) (Fig. 2). Carapace and cloacal samples were also positive for eight red-eared sliders (38%, three females, five males) and the snapping turtle (male). Salmonellae could also be detected in enrichments from the carapaces of two additional red-eared sliders (two females) and of two Texas river cooters (two females); analyses of the cloacal samples of these animals remained negative (Fig. 2), as did analyses of samples from the three Guadalupe spiny soft-shell turtles. These results again corroborate our previous analysis on musk turtles of the spring arm of Spring Lake, Texas, USA, that demonstrated the presence of salmonellae in about 50% of the musk turtles analyzed (Hahn et al., 2007). These results are also comparable to percentages of detection, that is, 32% of the turtles analyzed, obtained in other studies (Briones et al., 2004).

The presence of salmonellae varied among the turtle taxa, but did not follow a trend affected by foraging or basking behavior. Musk and snapping turtles that are routinely found foraging along the bottom of the spring and slough arm are primarily opportunistic feeders and rarely bask in air (as opposed to the water surface). Soft-shell turtles bask infrequently and are piscivorous. Red-eared turtles are opportunistic feeders that take vegetative and animal matter, whereas the Texas river cooter is strictly herbivorous. The latter two species frequently bask in air for long periods (Ernst et al., 1994). Soft-shell turtles, because of the nature of their carapacial integument, do not support algal mats, which are frequently found on the other species. In the Spring Lake system, red-eared turtles and Texas river cooters shed the outer keratin layer (scutes) annually; snapping and musk turtles do so less frequently. Basking, thus, does not seem to eliminate salmonellae from the carapace.

Compared to samples from the carapace, cloacal samples often produced less visible amplicons than carapace samples or no amplicons at all (Fig. 2), suggesting lower amounts of template and thus smaller numbers of salmonellae in cloacal samples. Because cloacal swabs generally contained less material used as inoculum than carapace swabs, this assumption might reflect a sampling bias caused by different amounts of feces and thus numbers of salmonellae present before or after shedding (Kaufmann et al., 1967). Because detection of amplicons in carapace samples generally confirmed results from cloacal samples, except for two cases in which no amplicons were detected in cloacal samples (Fig. 2), carapace samples could probably serve as the sole resource for the analyses of salmonellae on turtles; this would circumvent potential sampling bias of cloacal samples. These results also demonstrate the ability of salmonellae to at least survive outside of the animals, which is in contrast to the general assumption that salmonellae live primarily in the intestinal tracts of animals (Foster and Spector, 1995).

In order to confirm the molecular identification of salmonellae in the cloacae and on the carapaces of the different turtle species, isolation attempts were undertaken from positive enrichment cultures of individuals of each species (i.e., two musk turtles, two red-eared sliders, two Texas river cooters, and the snapping turtle). From the semiselective enrichment in RVS broth, bacteria were plated onto RVS agar (i.e., RVS broth solidified with 15 g agar l^{-1}) and incubated at 43 C for 24 hr. Selected colonies ($n=10$ per sample) were subcultured in lysogeny broth (LB) medium (l^{-1}: 10 g tryptone, 5 g yeast extract, 5 g NaCl) and identified as salmonellae by PCR detection of the *inv*A gene as described above. This screening resulted in the detection of isolates of salmonellae in samples of all individual turtles analyzed, with usually all 10 isolates being positive except for the snapping turtle, where only five isolates produced amplicons, and thus resembled salmonellae.

All PCR-positive isolates were further analyzed by rep-PCR, a PCR-assisted fingerprinting technique targeting consensus motifs of repetitive elements common to prokaryotic genomes (Bennasar et al., 2000; Woo and Lee, 2006), with primer BoxA1R (5′CTA CGG CAA GGC GAC GCT GAC G) (Versalovic et al., 1998), in order to reduce redundancy of isolates as outlined in our previous study (Hahn et al., 2007). Profiles were analyzed by gel electrophoresis on 2% agarose gels in TAE buffer after staining with ethidium bromide (0.5 μg ml^{-1}) (Sambrook et al., 1989). Profiles were found to be identical for all isolates from carapace and cloacal samples for each individual, similar to results in our previous study (Hahn et al., 2007). Except for isolates from samples of the two red-eared sliders that produced identical profiles, all profiles between individual turtles differed from each other (Fig. 3). However, profiles of salmonellae from both musk turtles were identical to profiles from the Texas river cooters (M_1 to P_1, and M_2 to P_2) (Fig. 3), with one profile (M_1, P_1) being identical to those of salmonellae retrieved from musk turtles in the spring arm of Spring Lake in our previous study (Fig. 3; Hahn et al., 2007). These results again indicate that the different life histories of musk turtles and Texas river cooters with respect to basking or foraging behavior were not impacting the presence of salmonellae overall and of specific *Salmonella* strains in particular. In addition, the presence of the same strain established at both sites, that is, the spring and the slough arm of Spring Lake analyzed about 6 mo apart, and on different turtle species, that is, musk turtles and Texas river cooters, suggests that salmonellae persist in supposedly pristine environments like Spring Lake in, and on, different turtle species.

Characterization to the serotype level by slide agglutination with the use of *Salmonella*-specific antisera at the Texas Department of State Health Services (Austin, Texas, USA) resulted in the identification of serovar Rubislaw for those isolates showing identical rep-PCR profiles to the *Salmonella* isolate from the spring arm (Fig. 3). In our previous study on the spring arm all isolates had been identified as serovar Rubislaw (Hahn et al., 2007), and thus our serotyping results supports the idea that the same strain persisted in different habitats. In addition to serovar Rubislaw, however, other serovars were identified. Different strains of serovar Newport were found on a musk

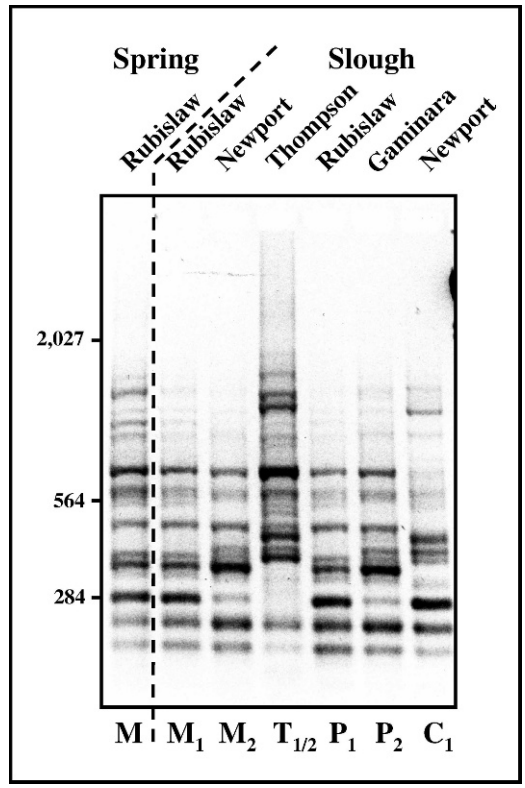

FIGURE 3. rep-PCR profiles of representative *Salmonella* spp. isolates from two individual musk turtles (*Sternotherus odoratus*) (M_1 and M_2), two red-eared sliders (*Trachemys scripta elegans*) ($T_{1/2}$), two Texas river cooters (*Pseudemys texana*) (P_1 and P_2), and the snapping turtle (*Chelydra serpentina serpentina*) (C_1) caught in the slough arm of Spring Lake. The left lane shows a representative rep-PCR profile from a *Salmonella* spp. strain isolated from a musk turtle in the spring arm (M) from our previous study (Hahn et al., 2007). The assignments on top of the profiles (i.e., Rubislaw, Newport, Thompson, and Gaminara) represent the corresponding serotyping results.

and the snapping turtle, and salmonellae of the red-eared sliders were identified as serovar Thompson, and those of the second Texas river cooter as serovar Gaminara (Fig. 3). All serovars have been identified in human-associated salmonellosis (Cook et al., 1998; Lyytikainen et al., 2000; Kimura et al., 2005), but were also detected in amphibians (Cook et al., 1998; Chambers and Hulse, 2006) and reptiles (Kaufmann et al., 1972; Johnson-Delaney, 1996; Chambers and Hulse, 2006). Our results support the idea that free-living turtles are reservoirs for salmonellae, which may be involved in human-associated salmonellosis. The failure to detect salmonellae in the environmental samples, however, suggests that turtles are not acting as a vector for large-scale dispersion of salmonellae into the environment, but are more likely reflecting isolated islands where salmonellae can persist as normal flora associated with these animals.

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