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[Article]

Detection of Salmonellae from Fish in a Natural River System

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Abstract.—Sediment, water, and fish gut samples taken at three sites near the headwaters of the San Marcos River, Texas, were analyzed for salmonellae *Salmonella* spp. by culture and molecular techniques. While enrichment cultures from sediment and water samples from the two uppermost sites were negative for salmonellae in polymerase chain reaction analyses, both sediment and water samples were positive at the downstream site. At all sites, salmonellae were present in the guts of different fishes (e.g., largemouth bass *Micropterus salmoides*, channel catfish *Ictalurus punctatus*, common carp *Cyprinus carpio*, and suckermouth catfish *Hypostomus plecostomus*). The highest percentage of detection (33% of analyzed fish) occurred at the downstream site, whereas detection percentages at the upper two sites were 18% and 17%. Detection of salmonellae was usually limited to one segment of the gut (i.e., upper or lower part). Serovars were highly variable among individuals and differed between the upper and lower gut in the only individual (a common carp) that had salmonellae in both gut segments. In situ hybridization demonstrated that salmonellae were normally associated with particulate material in the gut and occurred in highly variable numbers ranging from an occasional organism to a majority of the gut microbe population. These results demonstrate the presence of different serovars of potentially human pathogenic salmonellae among four ecologically distinct fishes within natural environments. They also suggest that salmonellae are not components of the indigenous microbial community in fish intestines but rather are ingested with particulate material.

Infection of humans with salmonellae *Salmonella* spp. is a serious public health problem presenting up to 1.3 billion cases worldwide (Pang et al. 1995), including 1.4 million cases in the United States alone (Mead et al. 1999). The majority of infections with salmonellae result from exposure to undercooked animal products or cross-contamination with fruits and vegetables (Pang et al. 1995; Tauxe 1997); however, salmonellosis can also result from direct contact with contaminated water (Foltz 1969; Harvey et al. 1969). Water is typically contaminated with salmonellae via the feces of many types of warm- and cold-blooded animals that might serve as a constant source of environmental contamination (Foltz 1969; Refsum et al. 2002). Salmonellae can, however, persist in the environment for extended periods of time (Hendricks 1971; Chao et al. 1987) and even occur in apparently pristine aquatic systems (Fair and Morrison 1967; Hendricks and Morrison 1967).

Although salmonellae are not considered to be part of the normal intestinal flora of fish (Janssen and Meyers 1968; Pal and Dasgupta 1991), fish exposed to salmonellae can become asymptomatic carriers of these pathogens (Brunner 1974; Bocek et al. 1992). Salmonellae may exist in the digestive tracts of fish in numbers up to one order of magnitude higher than those occurring in the water (Pal and Dasgupta 1991; Ampofo and Clerk 2003). Salmonellae have also have been shown to multiply in the intestine and to be shed in the feces (Morse et al. 1978a, 1978b; Lesel and Legac 1983). Consequently, fish constitute a potentially important source for the dissemination and persistence of salmonellae in aquatic environments (Lawton and Morse 1980). Studies on the occurrence of salmonellae in fish have been mainly limited to aquaculture systems, in which treatment with antibiotics or fertilization with sewage was thought to represent the mechanism of contamination with salmonellae and similar pathogens (Balasubramanian et al. 1992; Khalil and Hussein 1997; Pullela et al. 1998). Studies that evaluate the presence of salmonellae in wild fish of natural systems are lacking.

The purpose of this study was to investigate the potential for fish to disseminate salmonellae in a riverine ecosystem, the San Marcos River, Texas. Our previous study (Hahn et al. 2007) tested for the occurrence of salmonellae in the river's headwaters, Spring Lake. Spring Lake is fed by the second-largest spring system in Texas, encompassing more than 200 individual spring heads (Groeger et al. 1997). In the prior study, salmonellae were not recovered from water or sediments of Spring Lake; however, they were frequently detected in carapace biofilms and cloacae of common musk turtles *Sternotherus odoratus* (Hahn et al. 2007). In the present study, sediment, water, and

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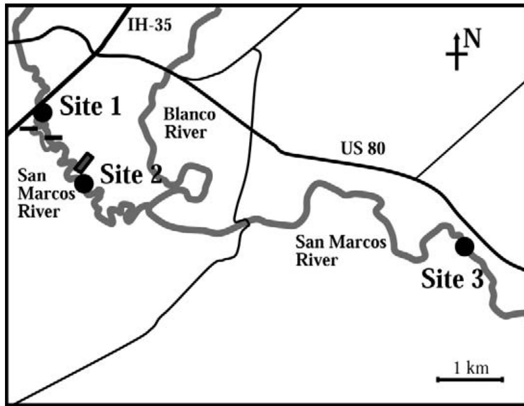

FIGURE 1.—Sampling locations (sites 1–3) along the San Marcos River, Texas. Site 1 is located about 5 km downstream from the river's headwaters and upstream from two low-head dams that prevent fish movement upstream during mean flow conditions. Site 2 is located downstream from the point of effluent releases by the City of San Marcos Wastewater Treatment Plant. Site 3 is located about 10 km downstream from site 2 and is downstream from the Blanco River confluence. Major roads (e.g., Interstate Highway [IH] 35 and U.S. Route 80) are shown.

fish gut samples collected at three sites downstream from Spring Lake were analyzed for salmonellae by use of traditional enrichment culture techniques in combination with molecular detection and identification tools, such as polymerase chain reaction (PCR) and in situ hybridization. Such tools were used successfully in our previous study. A more specific analysis of isolates was performed by repetitive sequence-based PCR (rep-PCR), followed by standard serotyping of representative isolates (Hahn et al. 2007).

Methods

Water samples, benthic sediments, and fish were obtained from three sites on the San Marcos River (Hays County; Figure 1) between April 1 and June 10, 2007. Site 1 (29°51′28.46″N, 97°55′52.19″W) was located about 5 km downstream from the river's headwaters and upstream from two low-head dams that prevented fish movement upstream during average flow conditions. Site 2 (29°51′59.99″N, 97°55′32.13″W) was located downstream from the point of effluent releases by the City of San Marcos Wastewater Treatment Plant. Site 3 (29°51′14.20″N, 97°51′40.49″W) was located about 10 km downstream from site 2 and was also downstream from the confluence with the Blanco River. At each site, four water column samples were taken with a vertical point water sampler, and four benthic sediment samples were taken with a dredge. Water and benthic sediment samples were placed on ice and processed within 4 h after sampling. Fish were captured by hook and line. Immediately upon capture, each fish was pithed and the abdomen was cut with sterile scissors, exposing the entire viscera. About 200 mg (wet weight) of gut contents were removed from each of two gut sections (upper and lower) by use of scissors and a small spatula. Gut contents were placed in separate 1.5-mL tubes and stored on ice until processing. The scissors and spatula were sterilized with ethanol and flaming between each use.

Salmonellae in pre-enrichment and enrichment cultures obtained from water samples, benthic sediments, and gut contents of fish were analyzed by the procedure outlined in Hahn et al. (2007). To obtain pre-enrichment cultures, approximately 100 mg (wet weight) of gut contents, benthic sediment samples, or pellets from centrifugation (14,000 revolutions/min for 2 min) of 50-mL water samples were transferred into 2-mL cryotubes that contained 1 mL of buffered peptone water (10 g of peptone/L of water, 5-g/L NaCl, 9-g/L Na_2HPO_4, and 1.5-g/L KH_2PO_4 [pH = 7.2]) and were incubated at 37°C for 16–20 h (ISO 1993). The first semiselective enrichment culture was created by transferring 100 μL of pre-enrichment culture into 2-mL cryotubes that contained 1 mL of Rappaport–Vassiliadis (RVS) broth (4.5-g/L peptone [soymeal], 29-g/L $MgCl_2 \cdot 7[H_2O]$, 8-g/L NaCl, 0.4-g/L K_2HPO_4, 0.6-g/L KH_2PO_4, and 0.036-g/L malachite green; pH = 5.2) and incubating at 43°C for 24 h (Vassiliadis et al. 1981). To enhance the sensitivity of the detection procedure, a second semiselective enrichment culture was made, which consisted of subjecting 100 μL of the first semiselective enrichment culture to RVS broth. Subsamples (100 μL) of pre-enrichment and first and second semiselective enrichment cultures were then used for molecular analyses. The remaining cultures were mixed with 600 μL of 60% glycerol in water and preserved at −80°C.

For molecular analyses, 100-μL subsamples of pre-enrichment cultures, first and second semiselective enrichment cultures, and cultures of *Salmonella typhimurium* ATCC (American Type Culture Collection) 14028 (control) were centrifuged at 14,000 revolutions/min for 1 min. Bacterial pellets were washed once with 1 mL of sterile distilled water, and bacteria were 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′-GTGAAAT-TATCGCCACGTTCGGGCAA-3′) and 141 (5′-TCATCGCACCGTCAAAGGAACC-3′; 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], and 0.1% Triton 100), 1 μL of each deoxynucleotide triphosphate (dNTP; each 10 mM in 10-mM tris HCl [pH = 7.5]), 0.2 μL of *Taq* polymerase (5 units/μL; enzyme code 2.7.7.7; IUBMB 1992), and 1 μL of each primer (100 ng/μL). 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) with denaturation at 96°C, primer annealing at 64°C, and elongation at 72°C (each for 30 s; Malorny et al. 2003). This was followed by incubation at 72°C for 7 min (Hahn et al. 2007). The presence of 284-base pair fragments was examined by agarose gel electrophoresis (2% agarose in tris–acetate–EDTA [TAE] buffer; Sambrook et al. 1989).

As in our previous studies (Hahn et al. 2007; Gaertner et al. 2008), second semiselective enrichments in RVS broth that tested positive for *Salmonella* invasion gene (*invA**) fragments were used for method verification. This verification was based on the isolation of salmonellae from these enrichments and their subsequent characterization. Bacteria were plated onto RVS agar (i.e., RVS broth solidified with agar at 15 g/L) and incubated at 37°C for 24 h. Selected colonies ($n = 10$ colonies/sample) were subcultured in Luria–Bertani medium (10-g/L tryptone, 5-g/L yeast extract, and 5-g/L NaCl) and identified as salmonellae by PCR detection of *invA** (Malorny et al. 2003) as described above. Lysates of all PCR-positive isolates were further analyzed by rep-PCR to reduce redundancy of isolates. The rep-PCR was performed in a total volume of 25 μL containing 5× Gitschier buffer (83-mM $[NH_4]_2SO_4$, 33.5-mM $MgCl_2$, 335-mM tris HCl [pH = 8.8], 33.5-μM EDTA, and 150-mM β-mercaptoethanol), 1.25 μL of each dNTP (each 100 mM; mixed 1:1:1:1), 2.5 μL of dimethyl sulfoxide, 0.2 μL of bovine serum albumin (20 mg/mL), 1.3 μL of primer BoxA1R (300 ng/μL; 5′-CTACGGCAAGGCGACGCTGACG-3′; Versalovic et al. 1998), 0.4 μL of *Taq* polymerase (5 units/μL), and 2 μL of lysate (Rademaker and de Bruijn 1997; Dombek et al. 2000). After an initial denaturation at 95°C for 2 min, 30 rounds of temperature cycling were performed in a PTC-200 thermocycler with denaturation at 94°C for 3 s and 92°C for 30 s, primer annealing at 50°C for 1 min, and elongation at 65°C for 8 min. These were followed by incubation at 65°C for 8 min (Rademaker and de Bruijn 1997; Dombek et al. 2000). Profiles were screened by gel electrophoresis on 2% agarose gels in TAE buffer (Sambrook et al. 1989). Representative isolates were subsequently characterized to the serotype level by slide agglutination using *Salmonella*-specific antisera; this work was performed by the Texas Department of State Health Services, Austin.

In contrast to PCR-based analyses, in situ hybridization allows visualization of individual cells in samples. For in situ hybridization, gut content samples (~100 mg) were fixed by mixing with 300 μL of 4% paraformaldehyde in phosphate-buffered saline (PBS; 0.13-M NaCl, 7-mM Na_2HPO_4, and 3-mM NaH_2PO_4; pH = 7.2) and subsequent incubation on ice for 16 h (Zarda et al. 1997). Fixed cells were washed with PBS twice, resuspended in 50% ethanol in PBS, and stored at −20°C until further use (Amann et al. 1990). Aliquots (1 μL) of the samples, as well as fixed cells of *S. typhimurium* ATCC 14028 used as positive controls, were spotted onto gelatin-coated slides (0.1% gelatin, 0.01% $KCr[SO_4]_2$ in distilled water), allowed to air dry, and subsequently dehydrated in 50, 80, and 96% ethanol for 3 min each (Amann et al. 1990). Hybridizations were carried out in 9 μL of hybridization buffer (0.9-M NaCl, 20-mM tris HCl, and 5-mM EDTA, and 0.01% sodium dodecyl sulfate [SDS]; pH = 7.2) in the presence of 10% formamide, 1 μL of cyanine-3 (Cy3) labeled oligonucleotide probe Sal3 (25 ng/μL), and 1 μL of 4′,6-diamadino-2-phenylindole (DAPI; 200 ng/μL) at 42°C for 2 h (Zarda et al. 1997). Probe Sal3 (5′-AAT CAC TTC ACC TAC GTG; *Escherichia coli* position 1713–1730; Nordentoft et al. 1997) binds to 23S ribosomal RNA of all *S. enterica* subspecies except IIIa but should not detect *S. bongori* (Fang et al. 2003).

After hybridization, slides were washed in buffer containing 20-mM tris HCl (pH = 7.2), 10-mM EDTA, 0.01% SDS, and 440-mM NaCl for 15 min at 48°C; slides were subsequently rinsed with distilled water and then air dried (Zarda et al. 1997). Slides were mounted with Citifluor AF1 immersion oil solution (Citifluor Limited, London, UK) and examined with a Zeiss Axiolab microscope (Zeiss, Oberkochen, Germany) fitted for epifluorescence microscopy with a high-pressure metal halide lamp, filter set F31 (AHF Analysentechnik, Tübingen, Germany; D360/40, 400DCLP, and D460/50) for DAPI detection, and filter set F41 (AHF Analysentechnik; HQ535/50, Q565LP, and HQ610/75) for Cy3 detection.

Results

At the three San Marcos River sites, we obtained upper and lower gut samples from 32 fish representing 6 species. Sixteen suckermouth catfish *Hypostomus plecostomus* and one largemouth bass *Micropterus salmoides* were taken from site 1. Three largemouth bass, one longnose gar *Lepisosteus osseus*, one common carp *Cyprinus carpio*, and one suckermouth catfish were taken from site 2. Three channel catfish

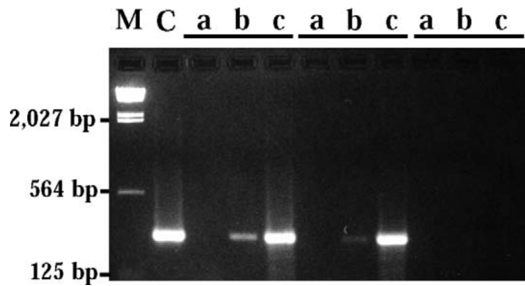

FIGURE 2.—Polymerase chain reaction analyses of *Salmonella* invasion gene (*invA**) fragments (base pairs [bp]) in preenrichment (lane a), first enrichment (lane b), and second enrichment (lane c) cultures from gut samples of three fish collected in 2007 from the San Marcos River, Texas, indicating the presence of salmonellae in two of three samples. Lane M represents a Lambda *Hin*d III size marker; lane C represents the *invA** fragment from *Salmonella typhimurium* ATCC (American Type Culture Collection) 14028.

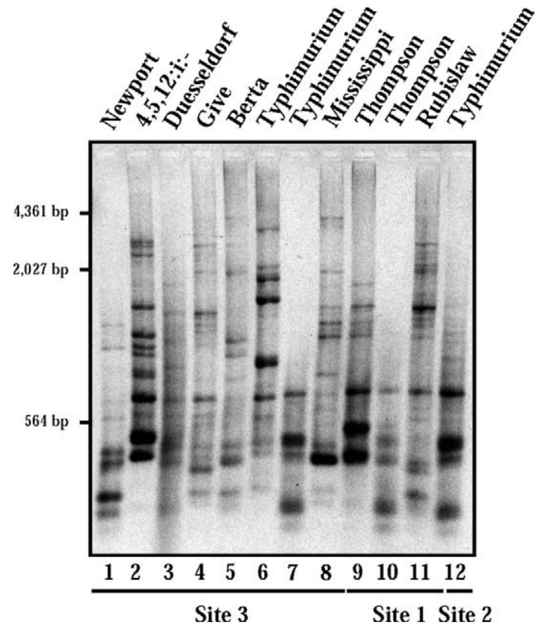

FIGURE 3.—Repetitive sequence-based polymerase chain reaction profiles of salmonellae isolates obtained in 2007 from water (lane 1), sediment (lane 2), and fish gut (lanes 3–8) samples from site 3 and fish gut samples from site 1 (lanes 9–11) and site 2 (lane 12) of the San Marcos River, Texas (Figure 1). Names at the top of the figure correspond to *Salmonella* serovar characterizations provided by the Texas Department of State Health Services, Austin. Representative fragment sizes (base pairs [bp]) of a Lambda *Hin*d III size marker are indicated.

Ictalurus punctatus, two longear sunfish *Lepomis megalotis*, one longnose gar, one American eel *Anguilla rostrata*, one common carp, and one largemouth bass were taken from site 3.

Amplicons were not detected in any of the preenrichment cultures with PCR analysis of the *invA** fragments. Instead, the first semiselective enrichment culture and occasionally the second semiselective enrichment culture were prerequisites for the detection of amplicons and, consequently, salmonellae (Figure 2). For water and benthic sediment samples collected at sites 1 and 2, *invA** amplicons were not detected in the first or second semiselective enrichment culture. However, *invA** amplicons were detected in both semiselective enrichment cultures from only the upper guts of three suckermouth catfish (18% of the 17 fish sampled) from site 1. For site 2, *invA** amplicons were detected in both semiselective enrichment cultures from the lower gut of one largemouth bass (17% of the 6 fish sampled). For site 3, *invA** amplicons were detected in one water sample (positive in both semiselective enrichment cultures) and one benthic sediment sample (positive in the second semiselective enrichment culture). In addition, *invA** amplicons were detected in both semiselective enrichment cultures from the lower guts of two channel catfish and one common carp (33% of the 9 fish sampled) and in the second semiselective enrichment culture from the upper gut of that same common carp.

The PCR-based detection of salmonellae in water, benthic sediments, and gut samples was confirmed for all samples by isolation of salmonellae from positive enrichment cultures and by subsequent physiological and immunological characterizations performed by the Texas Department of State Health Services. Among the four positive gut samples from sites 1 and 2, three serotypes were identified by isolation: *S. enterica enterica* serovar Thompson in two suckermouth catfish at site 1, serovar Rubislaw in a third suckermouth catfish at site 1, and serovar Typhimurium in the largemouth bass at site 2 (Figure 3). Among the five positive samples from site 3, at least seven serotypes were identified by isolation: *S. enterica enterica* serovar Newport from the water sample, *Salmonella* spp. 4,5,12:i:– from the benthic sediment sample, *S. enterica enterica* serovar Duesseldorf in the upper gut of the single common carp, serovar Give in the lower gut of that common carp, serovar Berta in the lower gut of one channel catfish, and serovars Mississippi and Typhimurium (with the potential of two Typhimurium strains) in the lower gut of the second channel catfish.

In contrast to PCR-based analyses that relied on semiselective enrichment of salmonellae, in situ hybridization allowed us to immediately detect salmonellae in samples of intestinal contents without

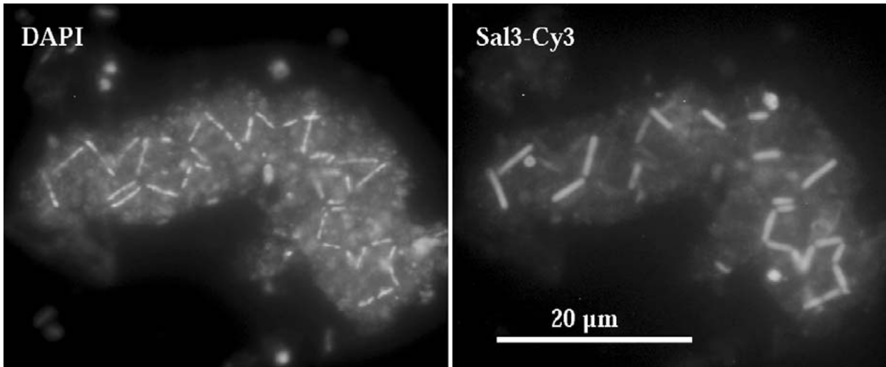

FIGURE 4.—Detection of salmonellae in fish gut samples from the San Marcos River, Texas (collected in 2007), after in situ hybridization with the cyanine-3 (Cy3) labeled oligonucleotide probe Sal3 and analysis by epifluorescence microscopy (right panel) and after staining with 4′,6-diamadino-2-phenylindole (DAPI), a DNA-intercalating dye used to detect all organisms (left panel).

enrichment. In situ hybridization results on these original samples confirmed results obtained by PCR on enrichment cultures, because salmonellae were only detected in original samples that had PCR-positive results. Salmonellae in the original samples were usually found in clumps of cells associated with particulate material (Figure 4). Numbers of cells were highly variable among clumps, ranging from an occasional cell to innumerable cells that dominated the surface of the particulate material.

Discussion

The percent occurrence of salmonellae ranged from 17% to 33% of fish sampled at three sites in the San Marcos River. These values are larger than those reported for fishes (0.0–2.3%) in circulating and noncirculating aquaculture systems, even those receiving treated wastewater effluents (Khalil and Hussein 1997; Pullela et al. 1998; Newaj-Fyzul et al. 2006). In a sewage-fed pond, however, all individuals (100%; $n = 7$) of six fish species contained high amounts of salmonellae in their intestines, and detritivorous species like common carp (198×10^2 cells/g of gut content) and Mozambique tilapia *Oreochromis mossambicus* (113×10^2 cells/g) exhibited higher numbers than zooplanktivorous species, such as rohu *Labeo rohita* (75×10^2 cells/g) and catla *Catla catla* (87×10^2 cells/g; Balasubramanian et al. 1992). In that study, the overall load of bacteria was reduced by up to 78% in the intestine when fish were transferred to freshwater and maintained there for 20 d. These results suggested that sewage (i.e., organic matter) affected bacterial loads in fish intestines in general and that particulate organic matter affected the presence of salmonellae in particular (Balasubramanian et al. 1992). Such results corroborate those of the current study, in that salmonellae were associated with particulate material in only certain locations of the intestine as opposed to being randomly distributed throughout the intestine.

The detection of salmonellae in fish was not related to the presence of salmonellae in water or sediments. Other studies have similarly observed that the presence of salmonellae in fish was not related to the abundance of salmonellae in water (Newaj-Fyzul et al. 2006). It has been proposed that salmonellae are part of the natural flora present in aquatic environments, at least in tropical regions (Reilly and Twiddy 1992). Our sampling sites were relatively close to the headwaters of the San Marcos River, where previous studies did not detect salmonellae in water and sediment samples (Hahn et al. 2007; Gaertner et al. 2008). However, as mentioned previously, salmonellae were frequently encountered in association with the common musk turtles in Spring Lake (Hahn et al. 2007; Gaertner et al. 2008). Serotypes retrieved from the common musk turtles (i.e., serovars Rubislaw, Newport, Gaminara, and Thompson) were largely different from those detected in fish at downstream sites, although serovar Thompson was also isolated from two fish at site 2, which is close to the headwaters. These data suggest that animals such as turtles provide habitats that allow salmonellae to persist in the environment but do not play a significant role in the dissemination of salmonellae.

In addition to interactions with animals, associations with biofilms (Armon et al. 1997; Barker and Bloomfield 2000) or sediments (Hendrick 1971; Marsh et al. 1998) were assumed to help salmonellae survive in the environment. Detection of salmonellae in biofilms on the carapaces of common musk turtles tends to support

the first assumption (Hahn et al. 2007; Gaertner et al. 2008). Results of the current study also support the second assumption even though salmonellae were not detected in sediments from two out of three sites. Both sites at which salmonellae were absent were relatively close to (i.e., about 5 km downstream from) the headwaters. The water of the headwaters is characterized by a high mineral nutrient availability, which supports a large macrophyte population (Groeger et al. 1997). These conditions provide optimal habitats for a wide variety of animals that feed on these plants and for saprophytic microorganisms that live on decaying plant material. Since salmonellae detected in the intestines of fish were associated with particulate material and were only found at certain locations within the intestine, one might speculate that salmonellae were taken up with detritus by feeding fish and were merely passing through the intestine. At the upper two sites, salmonellae were detected in only a few fish and were not detected in water or sediments. This is possibly due to the shallow depths and swift currents of this spring-influenced portion of the river, which is dominated by run-type geomorphic units that allow less particulate material to precipitate into the sediment. This is in contrast to the San Marcos River site located downstream of the Blanco River confluence; this site is characterized by more diverse riverine habitats, a greater number of backwater and pool geomorphic units, deeper water, and slower current velocities than are found at the other two sites. Salmonellae were present in a higher proportion of fish at site 3 than at sites 1 and 2 and were also detected in sediments and water at site 3. It is possible that higher pathogen loads entered the system through the Blanco River, which is part of a very different watershed with different overall quality than the San Marcos River. However, data supporting this hypothesis are not available.

The presence of salmonellae in fish in this natural river system raises questions of potential concern for fisherman. Although salmonellae were detected in high numbers in fish at certain sites, their presence in the intestine should not be a human health concern if proper evisceration procedures are followed. The ability to detect salmonellae directly in fish by in situ hybridization provides an additional opportunity for using fish in ecosystem health assessments and potential non-point-source pollution monitoring. To contain the spread of diseases and to eliminate nonpoint sources of contamination, continuous and accurate assessment of water quality is paramount for communities that are dependent on rivers for drinking water and agriculture. Surveillance for salmonellae and other pathogens in water used for recreation and bathing, however, is expensive and time consuming. It is only performed by a few countries and generally only in a small percentage of areas within those countries (Figueras et al. 1997). Water microbiology quality standards are typically based on the use of coliform bacteria as indicators of pathogenic organisms (Cabelli et al. 1982; Polo et al. 1998), and testing for salmonellae is performed only occasionally. The use of coliform bacteria as indicator organisms, however, often fails to detect threats posed by other pathogens such as salmonellae (Gales and Baleux 1992; Morinigo et al. 1992; Ferguson et al. 1996; Figueras et al. 1997). The use of fish in monitoring ecosystems for the presence of salmonellae could prove to be a useful alternative to coliform counts.

Acknowledgments

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