DETECTION OF SALMONELLAE IN WILD TURTLES AND THEIR AQUATIC HABITATS

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DETECTION OF SALMONELLAE IN WILD TURTLES AND THEIR AQUATIC HABITATS

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TABLE OF CONTENTS

Page

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

ABSTRACT

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A sensitive and accurate methodology for the detection of salmonellae in wild turtles and in the environment was developed using a combination of traditional culture techniques and molecular tools. A combination of a semi-selective enrichment in growth media and subsequent detection by polymerase chain reaction (PCR) enhanced the sensitivity of detection and allowed us to detect salmonellae in sediments and wild turtles. The methodology was developed and tested on captive turtles, which are well-documented carriers of salmonellae. In order to determine the versatility and resolution of the proposed methods they were subsequently used to analyze environmental samples

collected from both wild turtles and sediments from a relatively undisturbed environment (Spring Lake, San Marcos, Texas) as well as heavily impacted environments (Rio Grande River, Big Bend National Park, Texas, and near Elephant Butte Reservoir, New Mexico). Sterile swabs were used to gather samples from sediments as well as the cloacae and carapace of turtles from Spring Lake. Captive turtles and turtles from the Rio Grande River were swabbed at five different locations on the body. Swabs were collected into 2-ml-cryotubes containing 1 ml buffered peptone water (pre-enrichment cultures). Samples were enriched in Rappaport-Vassiliadis (RVS) broth, which is semi-selective for salmonellae. Cultures were then tested for salmonellae using PCR, targeting the *inv*A gene that encodes a protein of a type III secretion system, essential for the invasion of epithelial cells by salmonellae. The presence of salmonellae was verified on selected samples using *in situ* hybridization with probe Sal3, which binds to the 23S rRNA of the vast majority of *Salmonella enterica* sub-species. Finally, individual colonies from selected turtle and sediment samples were isolated using traditional plating techniques and subjected to molecular, physiological and serological identification in order to verify the reliability of the methodology developed.

I. INTRODUCTION

Salmonellae are a large group of enteric bacteria with a broad range of hosts. Although not all cause disease, salmonellae are recognized worldwide as major zoonotic pathogens for both animals and humans (Humphrey 2000). Annually, infections with salmonellae account for 1.3 billion cases of gastroenteritis worldwide (Pang et al. 1995) and as many as 1.4 million cases in the United States alone (Mead et al. 1999). The majority of salmonellae infections are caused by contaminated food such as undercooked animal products and cross-contamination with fruits and vegetables (Pang et al. 1995, Tauxe 1997). The persistence of salmonellae is traditionally associated with the intestinal tract of animal reservoir hosts including birds, mammals, invertebrates and reptiles (Foltz 1969, Refsum et al. 2002). However, salmonellae are passed in the feces and can persist in an aquatic environment or in the soil for extended periods of time (Hendrick 1971, Chao et al. 1987). In addition, they have been isolated from aquatic environments and water sources (Cherry et al. 1972), which can also serve as bacterial reservoirs and aid in transmission between hosts (Foltz 1969).

Turtles are well-documented as carriers of salmonellae while in captivity (Sanyal et al. 1997) and may potentially be important vectors in the spread of salmonellae in the wild (Boycott 1962, Pasmans et al. 2002b). Studies on the occurrence of salmonellae in wild turtles, however, are scarce and results contradictory (Briones et al. 2004, Richards et al. 2004, Saelinger et al. 2006). While some studies did not find any salmonellae in

cloacal, fecal or gastrointestinal mucosal samples of wild turtles, and thus questioned the importance of wild turtles as potential vectors for human-associated salmonellosis (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).

Our hypothesis was that wild turtles –like many other vertebrates- are potential carriers of salmonellae, and that the failure to detect them was due to the lack of appropriately sensitive methodologies allowing researchers to retrieve information on the occurrence of salmonellae reliably. The aim of this study was therefore to develop a sensitive methodology for the detection of salmonellae that could be used to reliably assess the occurrence of salmonellae in turtles and their habitat. The methodology was developed and tested on captive turtles, and was then evaluated on turtles and sediment samples from environments with contrasting history, i.e., the relatively undisturbed Spring Lake in San Marcos, TX, and the heavily impacted Rio Grande in Big Bend National Park and near Elephant Butte Reservoir, New Mexico. The methodology development combined traditional culture techniques (Thomason et al. 1977, Vassiliadis et al. 1981, International Standard Organization 1993) with molecular techniques such as polymerase chain reaction (PCR) (Khan et al. 2000, Malorny et al. 2003) and *in situ* hybridization (Fang et al. 2003) for detection. Potential detection of salmonellae by these methods was confirmed by isolation and subsequent molecular and serological characterization in order to validate the methodology developed. Additional characterization to the strain level was based on rep-PCR, a PCR-based genomic fingerprinting technique (Bennasar et al. 2000, Woo & Lee 2006). This technique was

used to retrieve information on the diversity of salmonellae beyond the species and subspecies level as provided by PCR analysis and serotyping, respectively. Rep PCR and serotyping allowed us to retrieve valuable information on diversity in addition to absence/presence of salmonellae.

This study has several important implications. The cost in terms of monetary damages as well as that due to pain and suffering caused by infection by salmonellae is enormous. Of all the cases of salmonellosis reported in the USA, it is estimated that as much as 6% for all sporadic salmonellae infections and 11% among persons younger than 21 are attributable to reptile or amphibian contact (Mermin et al. 2004). Reptile-associated salmonellosis statistically tends to be more severe and likely to lead to hospitalization and/or death (Cieslak et al. 1994, Ackman et al. 1995). The understanding of the ecology of salmonellae and its interaction with hosts and the environment is therefore central to understanding the pathogen and limiting potential infections.

Furthermore, turtles are known to be carriers of salmonellae in captivity (Sanyal et al. 1997) and may represent a perfect indicator organism for long-term environmental effects that could include the accumulation and subsequent dissemination of salmonellae in various environments. Monitoring and early detection of dangerous levels of potentially infectious organisms could play a major role in limiting the extent or perhaps even preventing outbreaks of salmonellosis.

II. OBJECTIVES

Method development on captive turtles

The development of the methodology was based on samples from captive turtles in which previous studies have demonstrated a high prevalence of salmonellae (Sanyal et al. 1997). Habitats for captive turtles often contain high concentrations of unused food and fecal material and can quickly become perfect breeding grounds for bacteria. It is for this reason that bacteria from artificial environments can reach much higher densities than those in a natural environment. Turtles were obtained from the Water Life Collection and belonged to the species *Callagur borneoensis* (n=3 individuals), *Siebenrockiella crassicollis* (n=2), *Graptemys nigrinoda* (n=1), *Trachemys gaigeae* (n=2), and *Pseudemys gorzugi* (n=2). They were collected by hand and swabbed at five locations (on the carapace, the ventral base of the left rear leg, underneath one or more of the claws on the front feet, the ventral base of the tail, and in the cloacae). These sites were chosen to gain information about how salmonellae move on the body of the turtle as well as to collect data for use during future sampling. The samples were pre-enriched and subsequently enriched with a semi-selective media and tested for the presence of the *inv*A gene necessary for full virulence in salmonellae by PCR (detailed protocol in following section). This method was independently verified using *in situ* hybridization with probe Sal 3 and proved to be highly effective in detecting salmonellae.

Evaluation of detection methods on wild turtles and their habitat

Detection of salmonellae on turtles in the wild (Briones et al. 2004, Richards et al. 2004, Saelinger et al. 2006) has proved to be a much harder task than detection in captivity (Sanyal et al. 1997). In order to evaluate and verify the detection method developed on captive turtles, samples from wild turtles, sediments and water were collected from two habitats with contrasting history, i.e., the relatively undisturbed Spring Lake located in San Marcos, TX, as well as the heavily impacted Rio Grande in Big Bend National Park and near Elephant Butte Reservoir, New Mexico. These two sites were chosen for their unique qualities representing a relatively oligotrophic aquatic environment with limited resources available to salmonellae as well as one comparatively more eutrophic.

The Spring Lake study was conducted to determine if the techniques described would have the resolution necessary to detect salmonellae at presumably low levels in the environment. Spring Lake is fed directly by springs of the Edward's Aquifer and all turtles and sediment samples were taken within several hundred yards of its origin (Fig. 1). Aquifer water flows through more than 200 springs in this area resulting in fast flow and exchange of surface water (Groeger et al. 1997). The assumption was made that this area would show limited opportunities for growth and persistence of free-living salmonellae. Turtles were collected by net and sampled from both their carapace and cloacae. Turtles from the species *Sternotherus odoratus* (common musk turtle, n=17) and *Chelydra serpentina* (common snapping turtle, n=1) were tested.

Due to its high use and considerable amount of runoff from agriculture, the Rio Grande represents a relatively impacted system. Samples from the Big Bend and New

Mexico sites therefore represent perfect complements to the samples from Spring Lake. Turtles were collected from traps submerged at several sites along a 70 mile stretch of the Rio Grande River (Fig. 2) in Big Bend National Park and from the Rio Grande near Elephant Butte Reservoir, New Mexico. Turtles from the species *Trachemys gaigeae* (n=36), *Apalone spinifera* (n=43) and *Chrysemys picta* (n=1) were swabbed at five locations on the body (on the carapace, at the ventral base of the right rear leg, underneath the claws, on the ventral base of the tail, and from inside the cloacae) for testing.

Verification of any salmonellae in enrichment cultures was based on isolation, and subsequent identification by the molecular tools to the genus (i.e., by PCR detecting the *inv*A gene) and species (i.e., *in situ* hybridization that will only detect *S. enterica*). Additional confirmation on selected samples was obtained by physiological (i.e., genus level identification) and serological (i.e., subspecies and serotype level) analyses performed at the Department of State Health Services, Austin, TX. Selected samples were also subjected to genomic fingerprinting using rep-PCR to assess diversity in the system on the strain level of resolution.

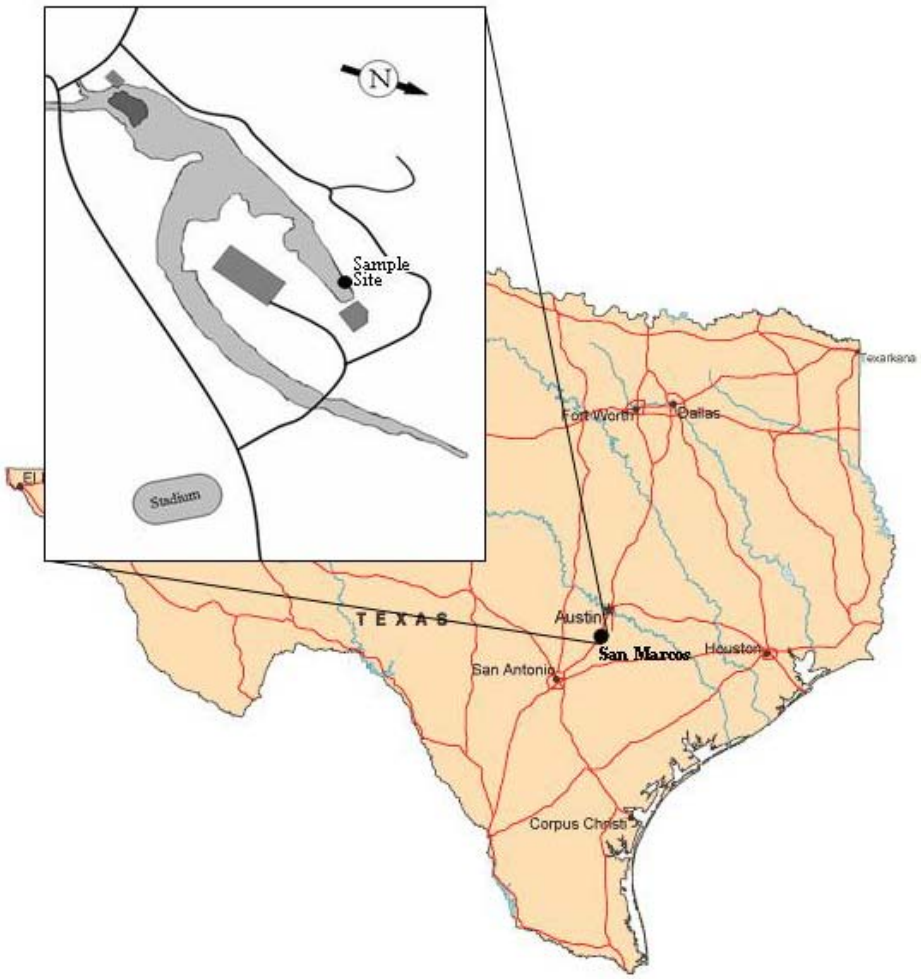

Fig. 1: Sample sites on the spring arm of Spring Lake, San Marcos, Texas that were used in this study for the collection of salmonellae samples from turtles captured included in the species *Sternotherus odoratus* (n=17) and *Chelydra serpentina* (n=1) as well as sediment samples.

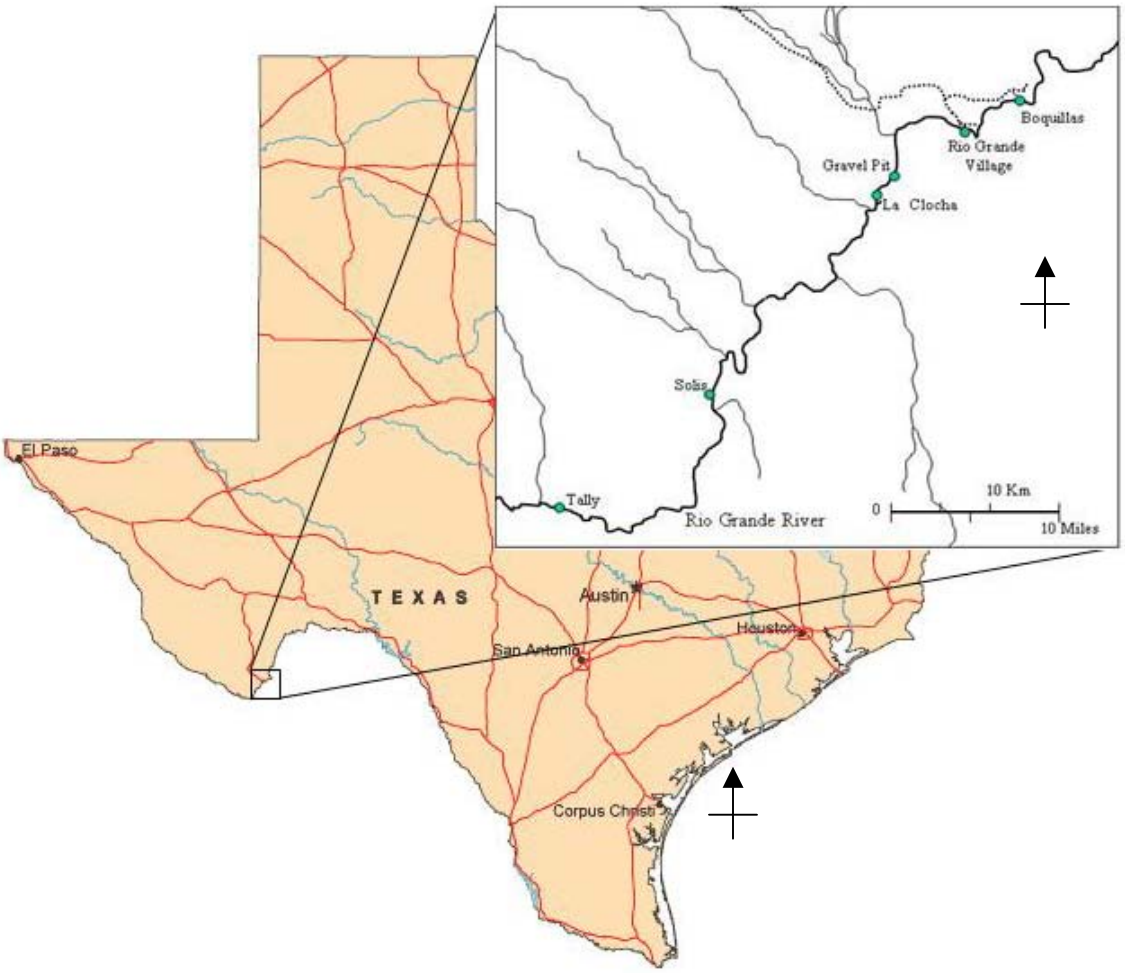

Fig. 2: Sample sites used in this study for the collection of samples from turtles captured included in the species *Trachemys gaigeae* (n=36), *Apalone spinifera* (n=43) and *Chrysemys picta* (n=1) as well as sediments along the Rio Grande River in Big Bend National Park. Tally, Solis, La Clocha, Gravel Pit, Rio Grande Village, and Boquillas. Flow of the river is from Tally to Boquillas.

III. METHODS

Turtles were collected from the wild or from captivity using live traps or the use of nets. Samples from turtles and sediments were then collected using sterile swabs. The swabs were placed directly into 2-ml cryotubes containing 1-ml of Buffered Peptone Water. The samples were then incubated for 16-20 hours at 37°C. A 100-µl aliquot was removed and placed into another 2-ml cryotube containing 1 ml of Rappaport-Vassiliadis (RVS) Broth and incubated for 24 hours at 43°C. Aliquots of the enrichment media were removed and processed for use as template for PCR using salmonellae specific primers. The presence of salmonellae was confirmed on selected samples using *in situ* hybridization. Selected enrichment samples were then plated on RVS agar and colonies obtained. Isolated colonies were again tested for salmonellae using PCR and subjected to genetic profiling using rep-PCR. Isolates representing a unique rep-PCR profile were sent to the Department of State Health Services, Austin, TX, for serotyping.

Sample collection and processing

Samples were taken from turtles captured in nets or in live traps submerged in the river. Sterile cotton wool swabs were used to collect samples on various locations of the turtle. Turtles from captivity, as well as those caught in the Rio Grande, were swabbed on the carapace, the ventral base of a rear leg, underneath the claws, the ventral base of the tail, and from within the cloacae. Turtles collected from Spring Lake were only sampled from the carapace and the cloacae. Swabs were also used to collect sediment

(about 100 mg wet weight) corresponding with sampling sites for turtles from both the Rio Grande and Spring Lake. The samples were transferred into 2-ml cryotubes containing 1 ml of Buffered Peptone Water (l^{-1}: 10 g peptone, 5 g NaCl, 9 g Na_2HPO4, 1.5 g KH_2PO_4, pH 7.2) (Thomason et al. 1977, International Standard Organization 1993). Samples were kept at 4°C for future processing. Samples were then removed from 4°C and incubated at 37°C for 16-20 hours (International Standard Organization 1993). 100-µl-samples of the pre-enrichment cultures were transferred to 2-ml cryotubes containing 1 ml of Rappaport-Vassiliadis (RVS) Broth (l^{-1}: 4.5 g peptone (soymeal), 29 g $MgCl_2$ x 7 H_2O, 8 g NaCl, 0.4 g KH_2PO_4, 0.036 g malachite-green, pH 5.2) (Vassiliadis et al. 1981) and incubated at 43°C for 24 hours (Vassiliadis et al. 1981). Two 100-µl aliquots were removed from the pre-enrichment and enrichment cultures for DNA extraction and cell fixation (described below). The remaining cultures were then mixed with 600 µl of 60% glycerol and stored at -80°C for future processing.

PCR amplification

The detection of salmonellae was performed by amplification of the *inv*A gene essential for the triggering and initialization of salmonellae invasion of epithelial cells resulting in full virulence (Khan et al. 2000, Malorny et al. 2003). 100 µl of liquid cell culture was removed and centrifuged at 14,000 rpm for 2 minutes. The supernatant was discarded and the pelleted cells washed in distilled water and re-suspended in 100 µl of 50 mM NaOH. The cell mixture was lysed by incubation at 65°C for 30 minutes. One µl of the 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) (Table 2) (Khan et al. 2000, Malorny et al. 2003).

PCR was performed in a total volume of 50 µl containing 10 x PCR buffer (500 mM KCl, 25 mM $MgCl_2$, 200 mM Tris/HCl, pH 8.4, 0.1% Triton 100), 1 µl dNTPs (each 10 mM in 10 mM Tris/HCl, pH 7.5), 0.2 µl *Taq* polymerase (Promega or GeneScript, 5 U μl^{-1}), 1 µl of each primer (100 ng μl^{-1}), 8 µl bovine serum albumin (BSA, 30 mg ml^{-1}), and 1 µl lysate (Widmer et al. 1999). After an initial 10-min-denaturation at 96°C, and subsequent addition of *Taq* polymerase (hot-start PCR), thirty-five rounds of temperature cycling were performed in a PTC-200 Thermocycler (MJ Research) with denaturation at 96°C, primer annealing at 54°C, and elongation at 72°C, each for 30 seconds (Malorny et al. 2003). This was followed by incubation at 72°C for seven minutes. The PCR product was examined by gel electrophoresis (2% agarose in TAE buffer) (Sambrook et al. 1989). DNA of *Salmonella typhimurium* ATCC 14028 and sterilized distilled water were used as positive and negative controls, respectively.

In situ hybridization

For the detection of salmonellae by *in situ* hybridization, 100 µl of cell culture was centrifuged at 14,000 for 2 minutes. The supernatant was discarded and the pelleted cells re-suspended in 100 µl of phosphate buffered saline (PBS; 0.13 M NaCl, 7 mM $Na_2H_2PO_4$, pH 7.2). Cell were fixed by mixing with 300 µl of 4% paraformaldehyde in PBS, and incubation at 4°C for 16 hours (Zarda et al. 1997). Fixed cells were washed twice with PBS, re-suspended in 50% ethanol in PBS, and stored at -20°C (Amann et al. 1990).

Two µl of the fixed cell solution was added to a gelatin-coated slide (0.1% gelatin, 0.01% $KCr(SO_4)_2$ in distilled water). The sample was allowed to dry on the slide at 42°C for 20 minutes and was subsequently dehydrated by passing through successive

washes of 50, 80, and 96% ethanol for 3 minutes each (Amann et al. 1990). Hybridizations were performed in 9 µl of hybridization buffer (0.9 M NaCl, 20 mM Tris/HCl, 5 mM EDTA, 0.01% SDS; pH 7.2) in the presence of 10% (Sal 3) or 30% (EUB338) formamide, 1 µl of Cy3-labeled oligonucleotide probe (Sal3 or EUB338), and 1 µl of a solution of DAPI (200 ng μl^{-1}) at 42°C for 2 hours (Zarda et al. 1997). Probe EUB338 (Table 2) ($^{5'}$GCT GCC TCC CGT AGG AGT, *Escherichia coli* position 338-355) (Amann et al. 1990) binds to 16S rRNA of nearly all members of the domain Bacteria, and was used to verify that all cells present were permeable for probes and thus accessible for *in situ* hybridization with the more specific probe. Probe Sal3 (Table 2) ($^{5'}$AAT CAC TTC ACC TAC GTG, *E. coli* position 1713-1730) (Nordentoft et al. 1997) binds to 23S rRNA of all *Salmonella enterica* subspecies tested so far (except for subspecies IIIa), but should not detect *S. bongori* (Fang et al. 2003).

After hybridization, the slides were gently washed with distilled water and immersed in buffer containing 20 mM Tris/HCl, pH 7.2, 10 mM EDTA, 0.01% SDS and 440 mM NaCl for 15 minutes at 43°C. Slides were removed from buffer, rinsed with distilled water, and air-dried (Zarda et al. 1997). The slides were then mounted with Citifluor AF1 immersion oil solution (Citifluor Ltd., London, UK) and examined with a Nikon microscope (Nikon, Lewisville, TX) fitted for epifluorescence microscopy with a high-pressure metal halide lamp and filter sets F31 (AHF Analysentechnik, Tübingen, Germany; D360/40, 400DCLP, D460/50, for DAPI detection) and F41 (AHF Analysentechnik; HQ535/50, Q565LP, HQ610/75, for Cy3 detection), respectively. Fixed cells of *Salmonella typhimurium* ATCC 14028 and *Escherichia coli* DH5α were always used as positive and negative controls, respectively.

Isolation and characterization of salmonellae

From selected enriched cell cultures in RVS Broth, 100 µl was removed and plated onto RVS Agar (i.e., RVS Broth solidified with 15 g agar l^{-1}). Plates were incubated at 43°C for 24 hours. Selected colonies were sampled and cultured in Luria-Bertani medium (LB; l^{-1}: 10 g tryptone, 5 g yeast extract, 5 g NaCl) (Sambrook et al. 1989) and identified as salmonellae by PCR detection of the *inv*A gene (Malorny et al. 2003) as described above. All isolates positively identified as salmonellae by PCR were further analyzed by rep-PCR to reduce redundancy of isolates.

For rep-PCR analysis, samples of the cultures were harvested and lysed as descried above. Two µl of the lysate was used as template for PCR amplification using primer BoxA1R (Table 2) ($^{5'}$CTA CGG CAA GGC GAC GCT GAC G) (Versalovic et al. 1998) targeting the BOX element (Martin et al. 1992). PCR was performed in a total volume of 25 µl containing 5 x Gitschier buffer (83 mM $(NH_4)_2SO_4$, 33.5 mM $MgCl_2$, 335 mM Tris/HCl, pH 8.8, 33.5 µM EDTA, 150 mM ß-mercaptoethanol), 1.25 µl dNTPs (100 mM each, mixed 1:1:1:1), 2.5 µl di-methyl-sulfoxide (DMSO), 0.2 µl bovine serum albumin (BSA, 20 mg ml^{-1}), 1.3 µl of primer (300 ng $µl^{-1}$), 0.4 µl *Taq* polymerase (5 U $µl^{-1}$) (Rademaker & de Bruijn 1997) and 2 µl lysate (Dombek et al. 2000). After an initial denaturation at 95°C for 2 minutes, thirty rounds of temperature cycling were performed in a PTC-200 Thermocycler with denaturation at 94°C for 3 seconds and subsequent 92°C for 30 seconds, primer annealing at 50°C for 1 minute, and elongation at 65°C for 8 minutes. This was followed by incubation at 65°C for 8 minutes (Rademaker & de Bruijn 1997, Dombek et al. 2000). Profiles were screened by gel electrophoresis on a 2% agarose gel in TAE buffer (Sambrook et al. 1989).

Selected isolates representing a unique rep-PCR profile were sent to the Department of State Health Services, Austin, TX, for physiological characterization and serotyping.

Statistical methods

In order to determine differences in the proportion of positive samples, the 95% confidence intervals were calculated. Proportions with overlapping confidence intervals were assumed to be the same. Differences in the percentage of positive samples from different sites on the Rio Grande as well as the percent positive of total samples from the Water Life Collection, Spring Lake, and the Rio Grande localities were all evaluated in this manor. In addition to confidence intervals, samples from the Rio Grande were compared and differences in species and sex were determined using a logistic regression. The statistical program R was used for this analysis with a p value of 0.05 for significance.

Table 1: List of primers and probes used during the study. The primers 139 and 141 target the *inv*A gene necessary for full virulence of salmonellae and is specific for *Salmonellae* spp. Probe EUB338 binds to the 16S rRNA of nearly all members of the domain Bacteria and was used to verify that all cells present were permeable for probes. Probe Sal3 binds to the 23S rRNA of all *Salmonella enterica* subspecies tested with exception of subspecies IIIa. The Box A1R primer targets the BOX element present in most bacterial genomes.

Primer	Sequence	Target
139	5'GTG AAA TTA TCG CCA CGT TCG GGC AA	*inv*A gene
141	5'TCA TCG CAC CGT CAA AGG AAC C	*inv*A gene
EUB338	5'GCT GCC TCC CGT AGG AGT	16S rRNA
Sal3	5'AAT CAC TTC ACC TAC GTG	23S rRNA
BoxA1R	5'CTA CGG CAA GGC GAC GCT GAC G	BOX element

IV. RESULTS

Method development on captive turtles

Pre-enrichment cultures of samples from captive turtles all showed increase in turbidity suggesting microbial growth. Upon transfer to the semi-selective RVS media only about 80% of the samples showed signs of growth (Table 3). Detection of the *inv*A gene fragments by PCR relied on the enrichment step as no signals were obtained on pre-enrichment cultures. An increase in turbidity of the enrichment cultures, however, did not presuppose detection of salmonellae. Several of the enrichment cultures showing significant increases in level of turbidity tested negative for salmonellae by PCR (Table 4).

Of the ten turtles sampled, five tested positive for salmonellae by PCR in at least one of the five samples taken (Table 4, Fig 3). Turtles testing positive for salmonellae generally showed a high percentage of positive samples with four of the turtles (CB01, CB02, CB03, and SC01) testing positive in all five samples and one turtle (SC02) testing positive in three of the five samples taken. Analysis of samples from the remaining five turtles did not detect salmonellae.

In situ hybridization of selected samples using the Sal3 probe corroborated the PCR results. Hybridization with probe EUB338, which binds to 16S rRNA of nearly all members of the domain Bacteria (Amann et al. 1990), detected all cells that were stained

with the DNA intercalating dye DAPI demonstrating that the cells were permeable to probes and therefore good candidates for *in situ* hybridization. The more specific probe Sal3 (Nordentoft et al. 1997), which binds to 23S rRNA of all *Salmonella enterica* subspecies tested so far (excepting only subspecies IIIa) (Fang et al. 2003), was then used to verify the presence of salmonellae. Samples that tested positive for salmonellae using PCR showed approximately 30-80% of the DAPI-stained cells to also be marked with the Sal3 probe. Samples testing negative for salmonellae by PCR generally showed positive signals using probe EUB338 but lacked any cells stained with probe Sal 3.

Evaluation of detection methods on wild turtles and their habitat

I. Spring Lake

Sediment and water samples as well as seventeen turtles from the species *Sternotherus odoratus* (common musk turtle) and one turtle from the species *Chelydra serpentina* (common snapping turtle) were collected near the headwaters of Spring Lake, San Marcos, Texas. Pre- and subsequent enrichment cultures from sediment and water samples remained negative for salmonellae using PCR detection of the *inv*A gene fragment whereas enrichment cultures from both carapace and cloacal samples of 9 of the turtles showed amplification products (Fig. 4). Amplicon yield was usually higher in samples from the carapace when compared to those of samples from the cloacae (Fig. 4). All positive samples were from musk turtles while the snapping turtle tested negative.

Again, *in situ* hybridization using probe EUB338 or probe Sal3 confirmed the PCR results (Fig. 5). Enrichment cultures from carapace samples showed up to 60% of the DAPI-stained cells to also be marked with probe Sal3 whereas only a few cells were typically shown to be salmonellae in cultures from cloacae samples. Only one exception

(F X) showed high detection rates of salmonellae in both the carapace and cloacal samples.

Isolation attempts from cultures testing positive showed large numbers of colonies from each sample. Screening of 10 colonies picked at random for the *inv*A fragment showed two individual turtles from which all isolates from both the carapace and cloacae displayed amplicons (individuals F IX and M III), whereas only nine or eight isolates from the carapace and three or zero from the cloacae of other individuals (F X and F VIII respectively) were positive. Rep-PCR demonstrated that isolates from carapace and cloacal samples were identical for each individual (Fig. 6), and identical for individuals M III and F X (Fig. 7). Salmonellae were far more diverse in samples from the carapace of individual F VIII with five different rep-PCR profiles retrieved. One of these resembled those found in isolates from M III and F X (Fig. 7). From this individual, no isolates could be obtained from cloacal samples even though PCR detected the *inv*A gene.

Serotyping of the seven isolates representative for each rep-PCR profile by the Texas Department of State Health Services confirmed the molecular identification as salmonellae and identified all isolates as *S. enterica* subspecies *enterica* serotype Rubislaw.

II. Rio Grande

Samples from 80 turtles included in the species *Trachemys gaigeae* (Big Bend slider), *Apalone spinifera* (spiny soft shell turtle) and *Chrysemys picta* (painted turtle) were collected using submerged traps at six different locations along a 70 mile stretch of the Rio Grande in Big Bend National Park as well as a site in New Mexico near Elephant

Butte Reservoir. Sediment samples were also collected at each of the seven sites for a total of 27 (Table 1).

Pre-enrichment and enrichment cultures from sediment samples all showed an increase in turbidity during incubation, suggesting microbial growth. Tests for salmonellae using PCR amplification of the *inv*A gene revealed four sediment samples with amplicon production. All positive sediment samples were from the Big Bend area and included one sample from the Rio Grande Village site (RGV70S), one sample from Boquillas (Boq76S), and two samples from Solis (Sol82S and Sol83S). All showed relatively low amplicon yields and the detection pattern did not change with higher concentrations of template (i.e., 5 or 10 µL of lysate instead of the normal 1 µL). Attempts to isolate salmonellae from samples by plating enrichment cultures were unsuccessful.

When possible, samples from all five locations on the body of turtles captured in the Rio Grande were taken from each turtle as described above for a total of 387 individual samples. Each enrichment culture from those samples was tested by PCR for the *inv*A gene and in total 87 samples (22%) showed amplification products. Of the 80 turtles sampled in the Rio Grande, 41 individuals tested positive for salmonellae in at least one of the five samples taken. The fraction of total turtles testing positive for salmonellae from site to site ranged from 40 to 75% (Fig. 8). When broken down by species 20/43 *A. spinifera* (46%), 20/36 *T. gaigeae* (56%) and the only *C. picta* all tested positive for salmonellae.

The infection rate of samples taken from the carapace, tail, cloacae, and claw were all about 20% with the range being 18% to 22%. The leg, however, showed

considerably more positive samples with about 33% of total samples positive. Eighteen percent of carapace samples (14/80), 33% of samples from the leg (26/79), 22% of samples from the tail (17/78), 18% of claw samples (13/71) and 22% of cloacae samples (17/79) tested positive for the *inv*A gene indicating salmonellae. These numbers varied only slightly within the species collected (Fig. 9).

Isolation attempts were made on cultures from seven positive samples. Enriched cultures from selected samples were plated and ten colonies subsequently selected at random from each plate and tested by PCR for the *inv*A gene. All but one sample (LaC5.5C) had at least one colony testing positive by PCR, identifying the colony as *Salmonella* spp. Samples 22.1P and 11.2P both had only one colony testing positive for salmonellae (profiles in Fig. 10). Samples 22.1T, 26.1L, and 13.4T had six, nine, and ten colonies respectively testing positive for salmonellae. After rep-PCR it was determined that of the samples with multiple positive colonies samples 13.4T and 26.1L (Fig. 11 and 12) each yielded two unique genetic profiles and 22.1T yielded three (Fig 10). Genetic profiles were not shared between samples.

Four colonies from samples were sent to the Texas Department of State Health Services for serotyping. Two colonies isolated from samples taken from the leg of a turtle (26.1 - *A. spinifera*) from the La Clocha sample site were identified as *Salmonella* spp. of an undetermined serotype within group -:y:1,7. Two additional isolates collected from the cloacae and tail of turtles 13.4 and 22.1 (*A. spinifera*) were identified as serotypes Newport and Assen, respectively.

Table 2: Overview of samples taken from turtles trapped in the Rio Grande. "Site" refers to 6 sample sites from within Big Bend National Park corresponding with campsites near the river as well as a site in New Mexico on Elephant Butte Reservoir.

	Number of Samples			
Site	***Apalone spinifera***	***Trachemys gaigeae***	***Chrysemys picta***	**Sediment**
Tally	3	1	0	3
Solis	2	3	0	3
La Cloacha	11	6	0	4
Gravel Pit	24	14	0	5
Rio Grande Village	3	2	0	3
Boquillas	1	4	0	4
New Mexico	0	5	1	5

Table 3: Enrichment cultures from samples collected from captive turtles from the species *Callagur borneoensis* (CB), *Siebenrockiella crassicollis* (SC), *Graptemys nigrinoda* (GN), *Trachemys gaigeae* (TG), and *Pseudemys gorzugi* (PG) from the Water Life Collection. A "+" denotes obvious increase in turbidity suggesting microbial growth.

	Captive Turtle Enrichments				
	Carapace	**Leg**	**Claw**	**Tail**	**Cloacae**
CB-01	+	+	+	+	+
CB-02	+	+	+	+	+
CB-03	+	+	+	+	+
SC-01	+	+	+	+	+
SC-02	+	+	+	+	-
GN-01	-	+	-	+	-
TG-01	+	+	-	+	+
TG-02	-	-	+	+	+
PG-01	+	+	-	+	+
PG-02	+	+	+	-	-

Table 4: PCR amplification products showing *inv*A gene fragment in enrichment culture from samples collected from turtles of the species *Callagur borneoensis* (CB), *Siebenrockiella crassicollis* (SC), *Graptemys nigrinoda* (GN), *Trachemys gaigeae* (TG), and *Pseudemys gorzugi* (PG) from the Water Life Collection. A "+" denotes detection of *inv*A fragments suggesting the presence of salmonellae.

	Captive Turtle PCR Product				
	Carapace	**Leg**	**Claw**	**Tail**	**Cloacae**
CB01	+	+	+	+	+
CB02	+	+	+	+	+
CB03	+	+	+	+	+
SC01	+	+	+	+	+
SC02	+	+	-	+	-
GN01	-	-	-	-	-
TG01	-	-	-	-	-
TG02	-	-	-	-	-
PG01	-	-	-	-	-
PG02	-	-	-	-	-

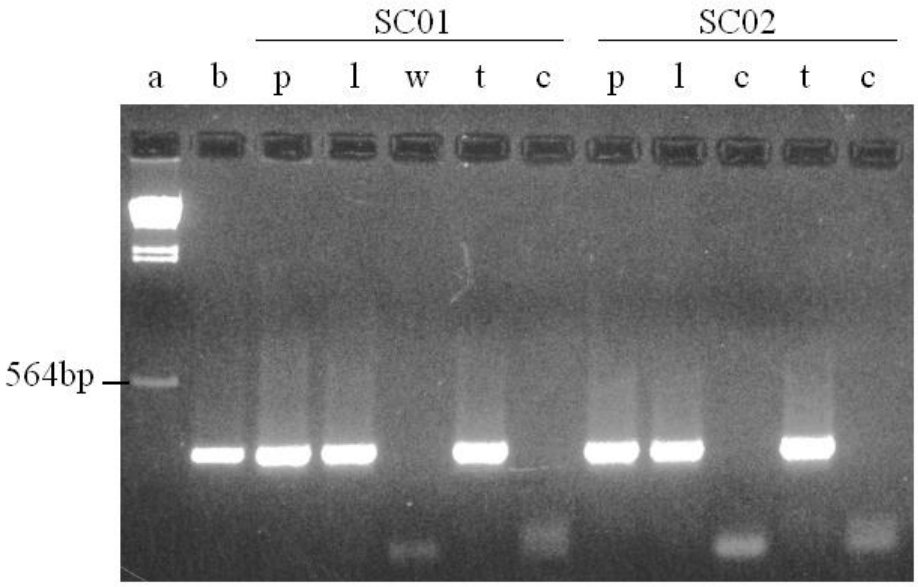

Fig. 3: PCR amplification products showing *inv*A gene fragment in enrichment culture of samples taken from the carapace (p), ventral base of the right rear leg (l), the underside of a claw (w), the ventral base of the tail (t) and from inside the cloacae (c) of two black mud turtles (*Siebenrockiella crassicollis*) from the Water Life Collection. Lane marked a shows the Lambda HindIII size marker and lane b contains the positive control *S. typhimurum* ATCC 14028.

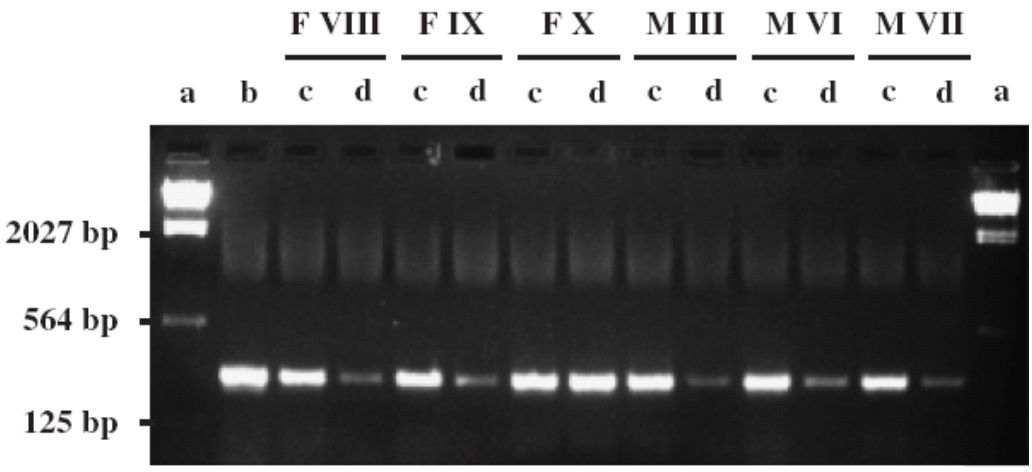

Fig. 4: PCR amplification products showing *inv*A gene fragment in enrichment cultures of samples taken from the carapace (c) or inside the cloacae (d) of musk turtles (*Sternotherus odoratus*). Lane a shows the Lambda HindIII size marker and lane b contains the positive control *S. typhimurum* ATCC 14028.

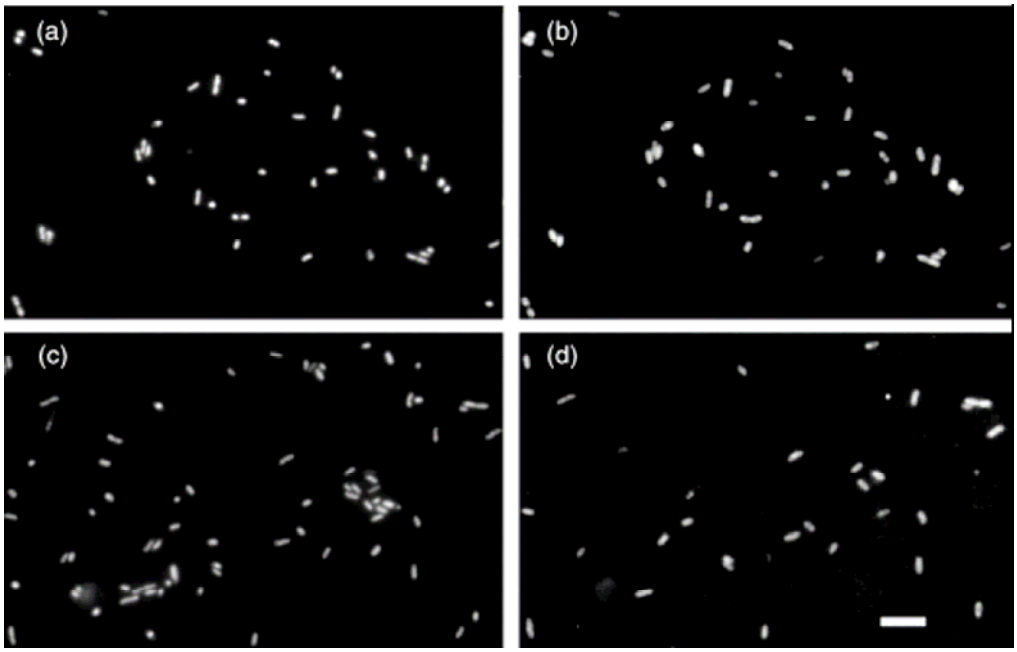

Fig. 5: Epifluorescence microscopy on enrichment cultures for salmonellae from the carapace of a musk turtle (*S. odoratus*) after DAPI-staining (a, c) and *in situ* hybridization with Cy3-labeled probes EUB338, targeting all bacteria (b), and Sal3 targeting *Salmonella enterica* (d). Scale bar represents 5µm.

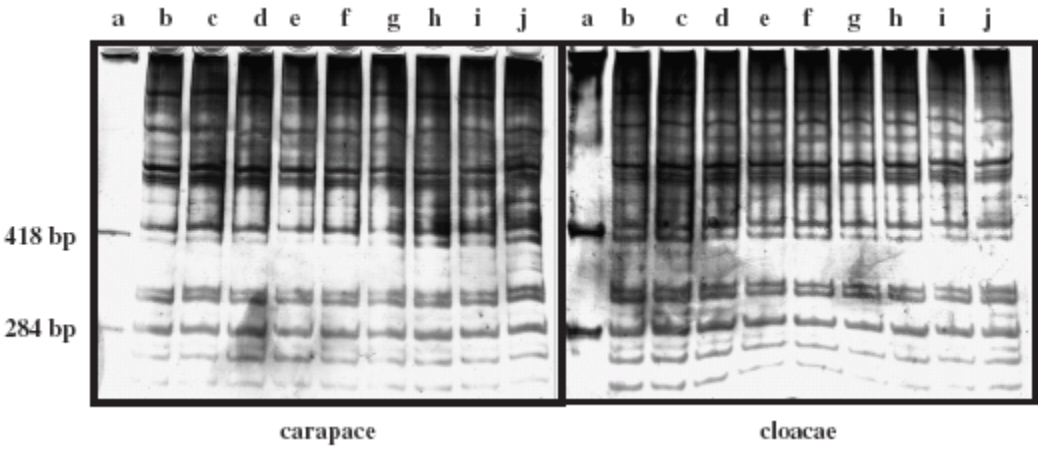

Fig. 6: Rep-PCR profiles of salmonellae (b-j) isolated from enriched samples taken from the carapace (left panel) or the cloacae (right panel) of the same musk turtle (*S. odoratus*) (F IX). Lane a represents a size marker with 284-bp and 418-bp fragments used for alignment.

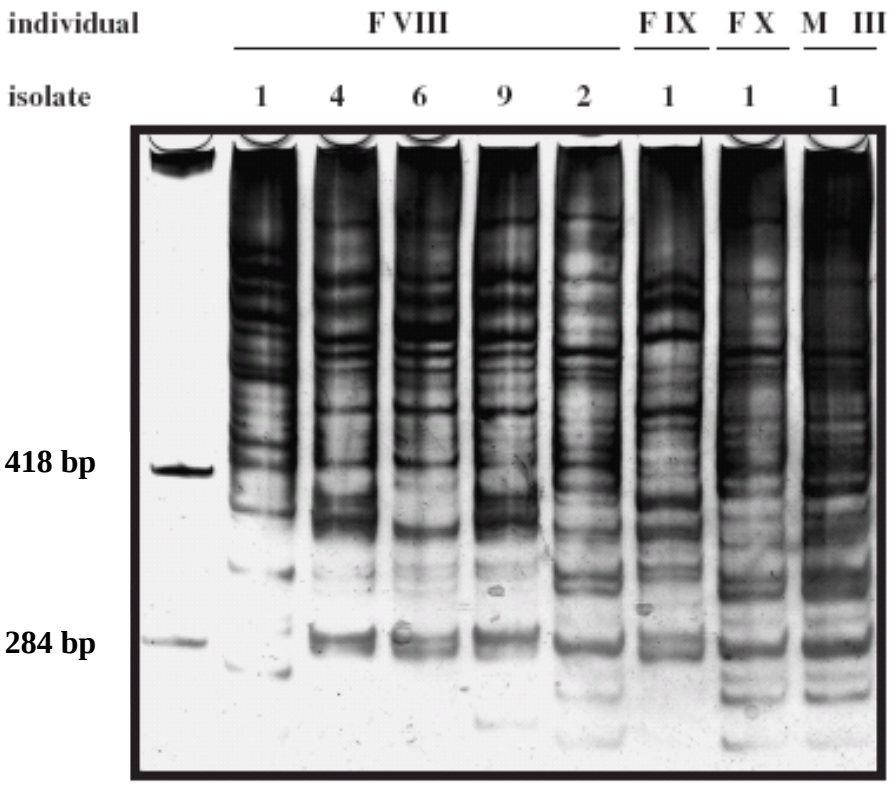

Fig. 7: Rep-PCR profiles of representative isolates from individual musk turtles (*S. odoratus*) F VIII, F IX, F X and M III. All isolates from F X and M III were identical, but different from those of F IX. In these three individuals isolates from the carapace and cloacae were identical. From the cloacae of F VIII, no isolates could be obtained and those from the carapace displayed higher diversity than those from the other individuals.

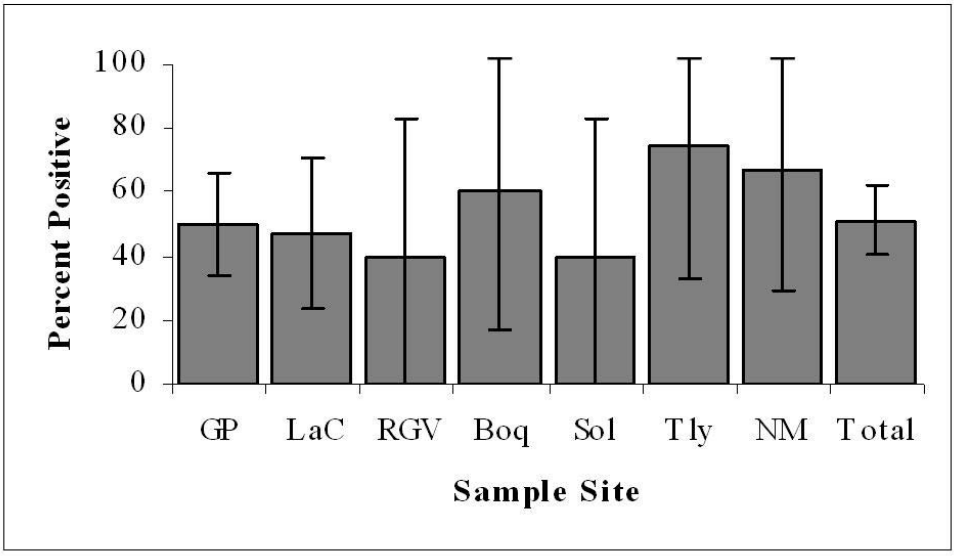

Fig. 8: Percentages and 95% confidence intervals of turtles captured from seven sites in the Rio Grande testing positive for the *inv*A gene present in *S. enterica* in at least one sample taken. Sample sites are Gravel Pit (GP), La Clocha (LaC), Rio Grande Village (RGV), Boquillas (Boq), Solis (Sol), Tally (Tly), and New Mexico (NM).

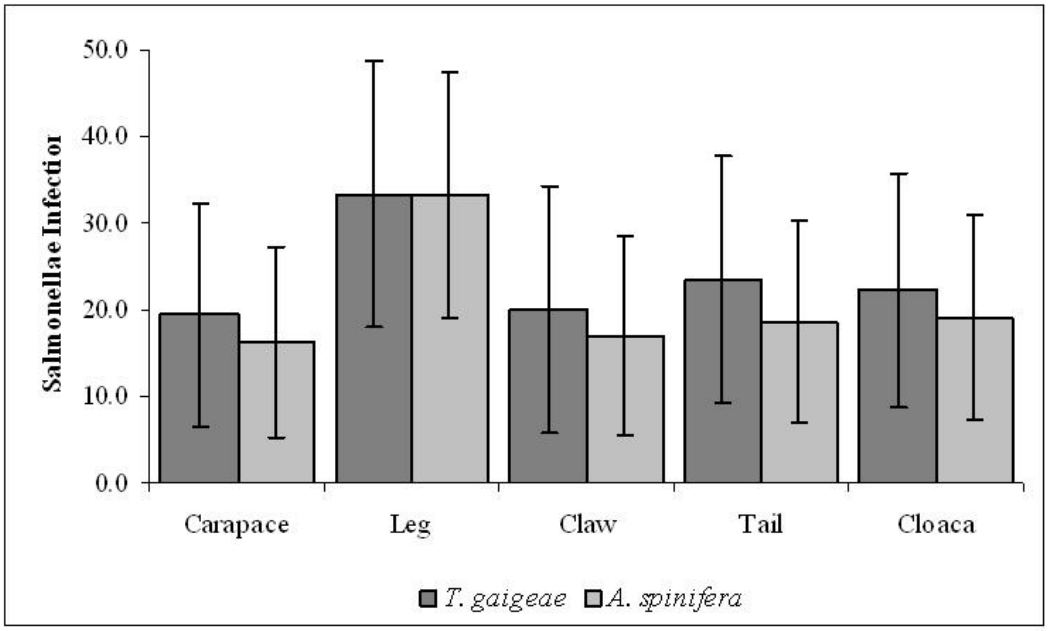

Fig. 9: Percentages and 95% confidence intervals of samples testing positive for the *inv*A gene present in *S. enterica* by PCR. Data given for *T. gageae* and *A. spinifera*. The only *C. picta* tested positive on the tail and in the cloacae (data not shown).

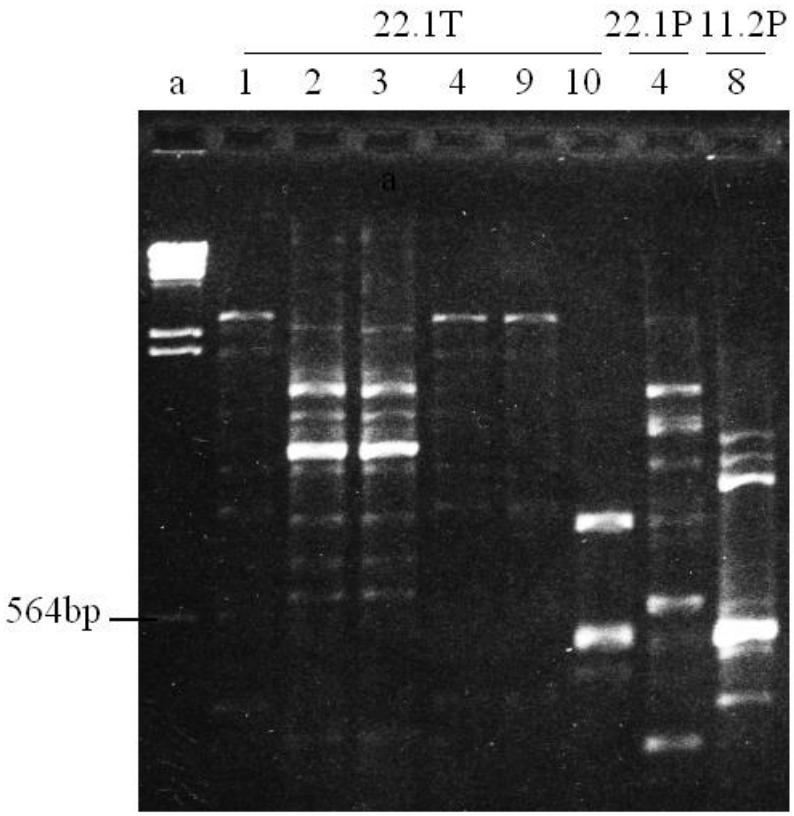

Fig. 10: Rep-PCR profiles of salmonellae isolated from enrichment samples taken from the carapace (P) and tail (T) of turtles (22.1 – *A. spinifera* and 11.2 – *T. gaigeae* captured in the Rio Grande. Lane a represents the Lamba HindIII size marker.

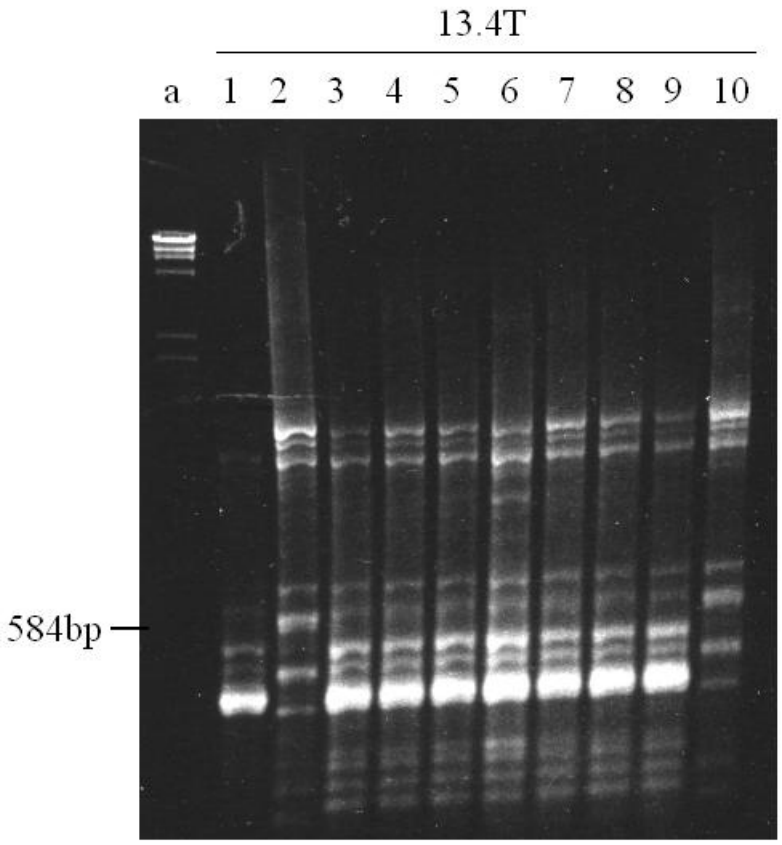

Fig. 11: Rep-PCR profiles of salmonellae isolated from an enrichment sample taken from the tail (T) of a turtle (13.4 – *A. spinifera*) captured in the Rio Grande. Lane a represents the Lamba HindIII size marker.

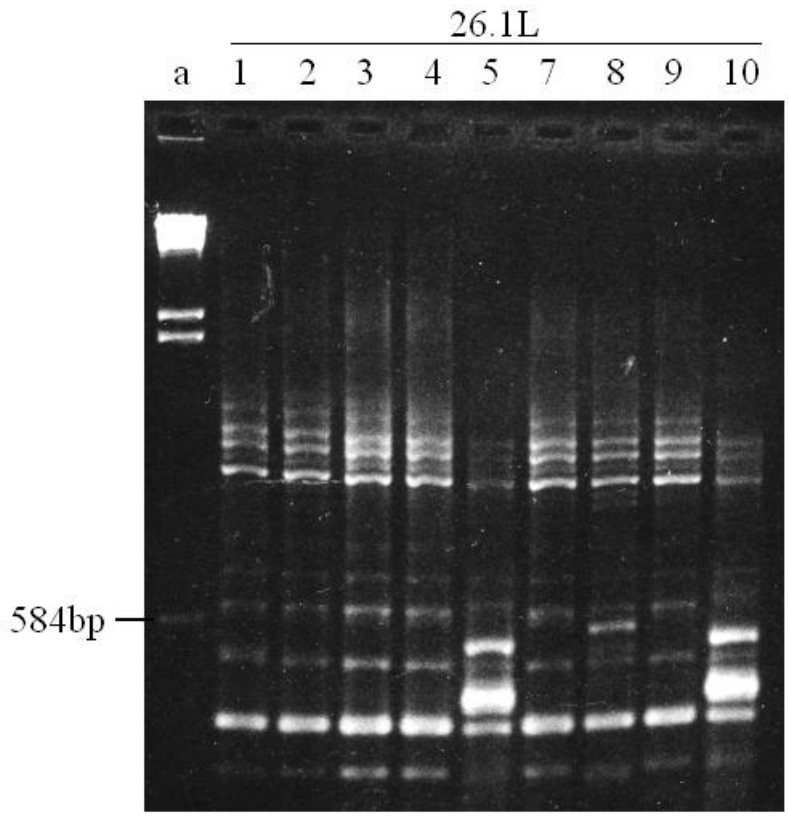

Fig. 12: Rep-PCR profile of salmonellae isolated from an enrichment sample taken from the leg (L) of a turtle (26.1 – *A. spinifera*) captured in the Rio Grande. Lane a represents the Lamba HindIII size marker.

V. DISCUSSION

Conventional pre-enrichment and enrichment culture techniques in combination with molecular identification procedures provided a fast and accurate tool for the detection of salmonellae in samples from captive, as well as, from wild turtles belonging to different species (*Callagur borneoensis, Siebenrockiella crassicollis, Graptemys nigrinoda, Trachemys gaigeae, Pseudemys gorzugi, Sternotherus odoratus, Chelydra serpentina, Apalone spinifera* and *Chrysemys picta*). Overlapping confidence intervals indicated that no significant difference was detected in the percentage of positive samples taken from the five sample spots chosen in and on the turtles sampled. These tools failed to detect salmonellae in water and sediment samples originating from Spring Lake, San Marcos, however, the *inv*A gene was detected by PCR amplification in 4/26 enrichment cultures originating from sediments from the Rio Grande. While the failure to detect salmonellae might reflect the accurate situation in Spring Lake, it could also have methodological causes since relatively small amounts of inoculum (e.g., cells from 1 ml of water) were used for pre-enrichment cultures. The inoculum amounts were not evaluated for potential detection limits, but used to provide comparable cell numbers from all samples as inoculum into pre-enrichment medium. Pre-enrichment and subsequent enrichment was necessary for detection of salmonellae by both PCR and *in situ* hybridization, although both techniques were supposed to require between 1 and 10 cells for detection (Fang et al. 2003, Malorny et al. 2003, Klerks et al. 2004). Differences

in signal intensities of amplicons most likely reflected cell densities of target organisms since faint signals of amplicons correlated with small numbers of salmonellae detected by *in situ* hybridization. Since enrichment conditions were only semi-selective, both techniques provided necessary and reliable tools to confirm the presence of salmonellae in the enrichment cultures, which allowed us to focus our isolation efforts on enrichments providing the most likely success.

Although isolation attempts focused on enrichment cultures in which salmonellae were detected, not all attempts were successful. This demonstrates the power of the molecular assays, but also the selectivity of our isolation approach. Since only 10 colonies were selected at random for subculture and subsequent identification, any enrichment of salmonellae to less than 10% would not have been sufficient to detect them by isolation. In our approach, this problem is not an issue because the major goal of isolation in this study was to validate the detection of salmonellae by molecular methods. Subsequent work on diversity of isolates, differences in salmonellae populations depending on the sampling site (locations on the turtle sampled as well as geographic location), or for epidemiological assessments, however, need to address this problem. Enhanced recovery of isolates could involve a much larger sampling size (i.e., retrieval of more than 10 isolates per site), subcultures in additional enrichment media and/or the isolation on different selective media.

Molecular techniques were valuable tools to monitor enrichment cultures and thus to direct isolation attempts, but also useful to rapidly identify isolates which could be used to increase the number of colonies screened and thus further increase chances for the detection of salmonellae. Recent studies (Saelinger 2006) that in contrast to this

investigation did not find any salmonellae in cloacal, fecal or gastrointestinal mucosal samples of wild North American turtles, including common musk turtles, might therefore be limited by a less sensitive methodology. The use of molecular tools provided an advantage with respect to time and sensitivity over detection techniques entirely based on isolation and subsequent morphological and physiological characterization.

Salmonellae have been detected in the gastrointestinal tract of many animals throughout the world (Gray 1995), including many amphibians and reptiles (Geue & Loschner 2002, Briones et al. 2004, Mermin et al. 2004). Lizards, snakes and turtles have been identified as significant reservoirs for human-associated salmonellosis (Cieslak et al. 1994, Ackman et al. 1995, Anonymous 1995, 1999), even though they are mostly asymptomatic carriers of salmonellae (Chiodini & Sundberg 1981, Anonymous 1995, Pasmans et al. 2002a). Although most of these studies focused on pet animals, studies on wild reptiles confirmed the potential of reptiles as reservoirs for salmonellae. In Spain, cloacal swabs retrieved salmonellae in 41% of lizards, 54% of snakes and 32% of turtles analyzed (Briones et al. 2004). The detection of salmonellae on the body and in the cloacae of 50% (9/18) of turtles captured in Spring Lake and 51% (41/80) of turtles captured in the Rio Grande supported the idea that wild turtles could serve as reservoir for salmonellae.

Detection of salmonellae was obtained in several turtle species with different life history traits. The three major types of turtles sampled in the wild, Big Bend slider (*Trachemys gaigeae*), common musk turtle (*Sternotherus odoratus)* and spiny soft-shelled turtle (*Apalone spinifera*), have very different diets that could potentially affect their exposure to salmonellae. The spiny soft-shelled turtle is carnivorous and typically

feeds on aquatic crustaceans, mollusks, carrion, fishes and amphibians. The musk turtle and Big Bend slider are both omnivorous when young, feeding on vegetation and slower moving prey such as earthworms, aquatic insects and mollusks and change with age to an almost entirely vegetarian diet as adults. It was originally hypothesized that because salmonellae are traditionally associated with animals, the carnivorous diet of the spiny soft-shelled turtle could potentially expose it to greater amounts of the pathogen. The data did not support this supposition when comparing cloacal samples from soft-shelled turtles to those from the omnivorous Big Bend sliders found in the same habitat. The detection rate of salmonellae in cloacal samples from *T. gaigeae* samples showed no significant difference (p value = 0.089) from that of *A. spinifera* (22% vs. 19% respectively, Fig. 9). Furthermore, the percentages of cloacal enrichments testing positive for salmonellae from turtles with similar, omnivorous, diets from different habitats were considerably more divergent, although not significant, i.e. 22% for *T. gaigeae* from the Rio Grande vs. 50% for *S. odoratus* from Spring Lake. A more exhaustive study including a multitude of turtle species from different habitats should be included in future studies to increase understanding of the interaction between turtles and salmonellae.

In contrast to the general assumption that salmonellae live primarily in the intestinal tracts of animals, our data on salmonellae on the carapace and other parts of the body of turtles demonstrated their ability to at least survive outside of the animals. At least 9 out of 18 tested samples from the carapace of turtles captured in Spring Lake were positive and 39 of the 80 turtles from the Rio Grande River also had at least one positive sample from outside the cloacae. Although salmonellae can replicate in contaminated food that

is rich in nutrients and stored improperly, it is not clear whether the bacteria were replicating or simply persisting on the bodies of turtles sampled.

Our results showed that both the intestinal tract of turtles as well as biofilms on their body were reservoirs for salmonellae. This potential source of contamination did not necessarily reflect the environmental conditions, i.e., the potential presence of salmonellae in water and sediment samples, since salmonellae could not be detected in some of the tested environments. This suggested that salmonellae were accumulating or persisting longer on the body and carapace of turtles than in water or sediment. Since major populations of salmonellae isolated from turtles captured in Spring Lake were generally identical in cloacal samples and on the carapace, contamination of the body through feces containing salmonellae was likely to be the source. This speculation was supported by the observation that turtles from Spring Lake without salmonellae in cloacal samples usually also did not have salmonellae on the body. On the other hand, one individual from Spring Lake was analyzed where no isolates could be obtained from the cloacae, but a high diversity of strains retrieved from the carapace. In addition to this individual, 23 of the turtles sampled from the Rio Grande possessed salmonellae on body while lacking any detectable salmonellae in the cloacae. These findings provided evidence that salmonellae could persist for some period of time on biofilms associated with the body of the turtles. In order to gain insight into this issue, additional studies on diversity and distribution of individual strains in and on individual turtles are needed.

This study was also designed to examine variation among turtle taxa as well as among individuals within a species in order to evaluate the biofilms supporting salmonellae. Musk turtles, Big Bend sliders and painted turtles tend to have significant algal mats

resident on the carapace, but the outermost shell components (scutes) are shed periodically, presumably along with the entire algal mat/biofilm community. This was not the case with the common snapping turtle, which was negative for salmonellae in our evaluations. Furthermore, the spiny soft shell turtle have a shell composed of bone and cartilage with a fleshy covering lacking horny scutes or any significant algal build-up. When taking into account differences in characteristics of the two turtles, a comparison between samples taken from the carapace of Big Bend sliders and spiny soft shell turtles from within the Rio Grande River showed overlapping confidence intervals and therefore no significant differences in the percentage of samples from the carapace testing positive for salmonellae (19% vs. 16%, respectively). Even though Big Bend sliders share similar diets and shell characteristics with musk turtles it is interesting to note that, although not significant, we detected salmonellae much more frequently (50% vs. 19%) in the enrichment cultures of samples from the carapace of musk turtles. These findings suggested that salmonellae on turtle biofilms have a life cycle, independent of environmental conditions and perhaps more complex than previously theorized. A greater number of samples including those from different species in future studies would help to increase our understanding of biofilms on the carapace of turtles as habitat for salmonellae. The ecology of turtle biofilms and the underlying ecology of salmonellae as a public health concern will be easier to pursue using the high fidelity molecular tools described in this study.

Infections in mammals and birds are usually caused by *S. enterica* subspecies *enterica* (Bäumler et al. 1998). From reptiles, all of the subspecies of *S. enterica* with subspecies *enterica* accounting for up to 50% of all isolates (Briones et al. 2004) as well

as *S. bongori* have been isolated (Bäumler et al. 1998). The major serotypes observed in turtles were Pomona, Java, Stanley, Poona, Muenchen, and Newport (Anonymous 1995, Woodward et al. 1997). Although many other serotypes have been detected in turtles and other reptiles (Johnson-Delaney 1996), serotype Rubislaw seems to be less common. However, detection of this serotype from turtles captured in Spring Lake was significant because the serotype Rubislaw had been identified as causal agent of salmonellosis in a case where a pet iguana was involved as potential reservoir (Anonymous 1995). Since samples from Spring Lake originated from a small area that covered only approximately 400 m^2, and only 4 turtles from one species were analyzed to serotype, the exclusive isolation of serotype Rubislaw might be result of our selectivity in this proof-of-concept approach. Select isolates from turtle samples representing a large stretch of the Rio Grande were also serotyped at the Texas Department of State Health Services. Two of the isolates serotyped were from the same sample but with unique genetic profiles as determined from rep-PCR. Both of these isolates were identified to be *Salmonella* spp. of an unknown serotype in the group -:y:1,7. This shows the high level of resolution possible using rep-PCR. Two other isolated were identified as belonging to serovars Newport and Assen, respectively. The serotype Newport is among the 20 most commonly isolated serotypes from human sources (CDC 2002) as well as one of the most common among reptiles (Anonymous 1995, Woodward et al. 1997). Serotype Assen is also associated with human sources, however, it is very uncommon and only accounted for three reported cases of salmonellosis in the United States from 1992-2002 (CDC 2002).

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