Journal of Zoo and Wildlife Medicine 44(3): 765–768, 2013

SALMONELLA ENTERICA PREVALENCE IN LEATHERBACK SEA TURTLES (*DERMOCHELYS CORIACEA*) IN ST. KITTS, WEST INDIES

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Abstract: *Salmonella* spp. are gram-negative bacteria capable of causing diseases in a wide range of aquatic and terrestrial animals, including humans. Sea and terrestrial turtles have been recognized as carriers of this zoonotic pathogen. In this project, conventional and molecular diagnostic methods were combined to investigate the prevalence of *Salmonella enterica* in leatherback sea turtles (*Dermochelys coriacea*) that used the island of St. Kitts, West Indies as a nesting ground during 2011 ($n = 21$). Isolates obtained from selective media were screened and colonies suspected of being *Salmonella* spp. were confirmed by fluorescence resonance energy transfer polymerase chain reaction. The prevalence of *S. enterica* within this sample population during this period was found to be 14.2%. Moreover, due to the increasing risk of antibiotic resistance in enteric bacteria, antimicrobial susceptibility was investigated in all recovered *Salmonella* spp. isolates utilizing the broth microdilution method. All isolates were susceptible to the lowest concentration of kanamycin, gentamicin, ciprofloxacin, enrofloxacin, nalidixic acid, and trimethoprim/sulfamethoxazole tested. Further research should be pursued to understand the interaction of this bacterial pathogen with the environment, host, and other microbial communities, and to further develop faster, more sensitive, and more specific diagnostic methods.

Key words: Leatherback sea turtles, PCR, *Salmonella*, St. Kitts, zoonotic.

BRIEF COMMUNICATION

Leatherback sea turtles (*Dermochelys coriacea*) are listed as critically endangered species by the International Union for Conservation of Nature.[8] Having a worldwide distribution, they prefer to nest on isolated beaches in tropic areas adjacent to deep water, laying an average of six clutches per reproductive season.[5] The island of St. Kitts (17°15′N, 62°40′W) is a 168-km^2 island located in the eastern Caribbean used by leatherback sea turtles as nesting ground from March through July.[3]

Salmonella spp. are gram-negative facultative intracellular bacteria that belong to the family Enterobactericeae. Members of this genus may pose health risks to both aquatic and terrestrial animals, including humans.[1,14] The genus *Salmonella* is composed of two species, *Salmonella bongori* and *Salmonella Enterica*, but there are more than 2,000 serovars of *Salmonella*.[7] Most *Salmonella* infections come from ingestion of contaminated water and/or food (fecal-oral transmission);[10,13,14] however, domesticated animals are considered to be the primary reservoirs of *Salmonella* that may infect humans.[6,14] Several studies have reported the prevalence of *Salmonella* in captive and wild reptiles.[1,2,6,9,16–19,21] Nevertheless, the prevalence of *Salmonella* spp. in wild sea turtles and risk factors associated with *Salmonella* transmission from wild sea turtles to human is largely unknown, and only in a few cases has this been investigated.[11,15,17,18,21] This study was conducted to investigate the prevalence and antimicrobial susceptibility of these potential pathogenic bacteria in nesting leatherback sea turtles on the island of St. Kitts during the 2011 nesting season.

Cloacal swabs were taken from 21 leatherback sea turtles that nested on St. Kitts during the 2011 nesting season. Immediately following egg laying, a sterile BactiSwab® (Remel, Lenexa, Kansas 66215, USA) was introduced 5 to 6 cm into the cloaca and rotated five times. The swabs were stored on wet ice immediately following sample acquisition and then submitted to the Ross University School of Veterinary Medicine Marine Laboratory. These swabs were then used to

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inoculate MacConkey agar plates (Remel). The inoculated plates were incubated for 48 hr at 27°C. Colonies from primary isolation agar plates were replated for purity of culture on *Salmonella–Shigella* agar (Remel), used to select for *Salmonella* spp., and incubated overnight at 37°C. Once single colonies were observed and purity of the isolate was determined, the isolates were frozen at −80°C in 1× phosphate-buffered saline (PBS) containing 20% glycerol (Thermo Fisher Scientific, Waltham, Massachusetts 02451, USA) for later use. All recovered bacteria isolates were initially Gram-stained and tested for cytochrome oxidase and catalase activity (Thermo Fisher Scientific). Molecular identification of gram-negative, rod-shaped bacteria that did not produce cytochrome oxidase was achieved using fluorescence resonance energy transfer polymerase chain reaction (FRET PCR).[20] Briefly, a loop of the bacterium was suspended in 500 µl of sterile 1× PBS and subjected to DNA extraction using the DNeasy blood and tissue kKit (Qiagen Inc., Valencia, California 91355, USA) following the manufacturer's suggested protocol for gram-negative bacteria. Extracted DNA was stored at −20°C until further use.

A part of the *ttrRSBCA* locus encoding proteins for tetrathionate respiration was chosen as the target to design a FRET PCR for *Salmonella* spp.[12] The inclusivity and exclusivity of this technique was found to be 100% while applying 110 *Salmonella* strains and 87 non-*Salmonella* strains,[12] All oligonucleotides were designed by use of the Vector NTI software (Invitrogen™ Corporation, Carlsbad, California 92008, USA). The copy number of *Salmonella* spp. genomes was determined by FRET-PCR performed in a LightCycler® real-time platform with software version 4.0 (Roche Molecular Biochemicals, Indianapolis, Indiana 46250, USA) in a similar approach as previously described.[20]

The primers (upstream:5′-CAC TCA TCA TTG AAG AAG TGC TGG CTC-3′; downstream:5′-GGT AAT CGC ACA GGT AAT GGC AAT CAG-3′) and the probes (5′-TTT GCC TGT TAT CTT CAC TGG CGG AA-[6-FAM]-3′; 5′-[Bodipy 630/650]-AAA GAC GCC GCA ACA GAA GAA AAT CG-[phosphate]-3′) were designed to amplify and detect all reported *Salmonella* strains. The fluorescein probe was 3′-labeled with carboxyfluorescein (6-FAM) and used unpurified as a FRET energy donor probe excited by 488-nm light. The Bodipy 630/650 probe was 5′-labeled, 3′-phosphorylated, high performance liquid chromatography–purified, and used as acceptor probe (Integrated DNA Technologies, Coralville, Iowa 52241 USA). Nucleotide fragments representing partial *ttrRSBCA* were synthesized and inserted in the pIDTSMART cloning vector (Integrated DNA Technologies). The plasmid was linearized with *Hind* III (Promega, Madison, Wisconsin 53711, USA), followed by inactivation of the restriction enzyme at 65°C for 20 min. DNA was quantified by PicoGreen® DNA fluorescence assay (Molecular Probes, Inc., Eugene, Oregon 97402, USA) for preparation of quantitative standards. Specificity of the *Salmonella* FRET-PCR was confirmed by amplification of four isolates of *Salmonella* Typhimurium and the synthesized target gene of *Salmonella*, and a lack of amplification from extracted DNAs of *Escherichia coli*, *Pasteurella multocida* and *Pseudomonas aeruginosa*. PCR product was verified in 4% MetaPhor agarose gel electrophoresis, and isolated and purified for automated DNA sequencing with a QIAquick PCR purification kit (Qiagen). Nucleotide sequencing was performed using an ABI automatic DNA sequencer (Model 377; Perkin-Elmer, Waltham, Massachusetts 02451, USA) at the Genomic Sequencing Laboratory (Auburn University, Auburn, Alabama, USA) using the forward and antisense primers described above.

The minimal inhibitory concentrations of antimicrobial agents to *S. enterica* isolates from leatherback sea turtles and quality control (*E. coli* ATCC 25922) were tested using the Sensititre Urinary Plate Format, plate code CMV1BURF; Sensititre Staphylococci Plate, plate code EUST; and the Sensititre Custom Plate Format, plate code CMV2AGNF (Trek Diagnostic System, Inc., Cleveland, Ohio 44131, USA), using the manufacturer's suggested protocol and the Clinical and Laboratory Standards Institute published protocols.[4]

Initially, 171 bacterial isolates were isolated on MacConkey agar plates. After use of selective media screening (*Salmonella–Shigella* agar) and FRET PCR, seven isolates were identified as *S. enterica*. The seven isolates came from five of the 21 turtles that came to nest in St. Kitts during 2011, indicating a prevalence of *Salmonella* to be 14.2% of the nesting leatherback females sampled in 2011. The recovered *Salmonella* were susceptible to kanamycin, gentamicin, ciprofloxacin, trimethoprim/sulfamethoxazole, enrofloxacin, and nalidixic acid.[4]

In contrast to the findings in this project, previous studies reporting the frequency of *Salmonella* in wild turtles have found the prevalence to be very low.[16–18] Previously, researchers utilized

selective media and biochemical analysis to analyze cloacal swabs from 94 wild-caught turtles in central North Carolina, USA.[16] Interestingly, *Salmonella* was not recovered from any of the cloacal swabs.[16] In another report, researchers identified 189 bacterial isolates from 70 nesting leatherback sea turtle cloacal swabs in Pacuare Nature Reserve, Costa Rica.[18] Utilizing selective media and biochemical analysis, five of these isolates were identified as *Salmonella*. In a similar study on 70 nesting green sea turtles (*Chelonia mydas*) in Tortuguero National Park, Costa Rica, cloacal swabs were taken from each turtle, and using selective media and biochemical analysis, 352 bacterial isolates were found and none were identified as *Salmonella*.[17]

Compared to the frequency of *Salmonella* being shed in wild turtles, those turtles kept in captivity tend to have a higher prevalence of *Salmonella* spp. Using standard bacteriologic media and biochemical methods to isolate and identify enteric bacteria, *Salmonella* spp. were found in all red-eared sliders (*Trachemys scripta elegans*) analyzed from 18 pet shops in Puerto Rico.[19] In another study, a survey of nine major zoos throughout the United States (during which cloacal swabs were taken from 124 turtles, belonging to 14 species, 13 genera, and five families) revealed the presence of *Salmonella* in 15 turtles when utilizing selective media and biochemical screening tests.[9]

In this study, the prevalence of *Salmonella* was found to be significantly higher than previous work done in the same species in different geographic locations.[18] The higher sensitivity as well as the reduction in time and labor makes the molecular diagnosis assay an excellent alternative to biochemical methods for diagnostic purposes, surveillance, and research studies. A similar approach can be used to investigate and compare the prevalence of *Salmonella* spp. and other zoonotic pathogens in captive and wild populations of sea turtles from different geographic regions. The absence of any antibiotic resistance in the tested *Salmonella* isolates argues for colonization by genuine leatherback-adapted *Salmonella* spp. that have not been exposed to resistant populations of enteric bacteria. A side-to-side comparison of *Salmonella* isolates recovered from captive and wild turtles may help clarify resistance development of bacterial agents in animals with close contact with human populations.

Further research will continue on the St. Kitts leatherback population to further establish a baseline standard of health for this population. Since leatherback sea turtles typically do not nest annually but rather every 2 to 5 yr, sampling will be continued in an effort to find the most accurate prevalence of *Salmonella* in this nesting population. Moreover, the use of real-time PCR for the detection of *Salmonella* and other pathogens directly from fecal, cloacal, water, soil, and sand samples will be investigated in order to clarify the ecologic niches and risk factors associated with *Salmonella* transmission to human and animal populations.

Acknowledgments: The authors wish to thank the St. Kitts Department of Marine Resources for their permission to work on sea turtle nesting beaches and Ross University School of Veterinary Medicine, the Georgia Sea Turtle Center, and the St. Kitts Sea Turtle Monitoring Network for their assistance in sample acquisition, financial support, and technical support.

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Received for publication 10 September 2012