

Influence of Preoperative Oxytetracycline Administration on Community Composition and Antimicrobial Susceptibility of Cloacal Bacterial Flora of Loggerhead Sea Turtle, *Caretta caretta*, Post-Hatchlings

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ABSTRACT: Cloacal cultures from loggerhead sea turtle, *Caretta caretta*, post-hatchlings were obtained and evaluated for bacterial flora composition and antimicrobial susceptibility before and after laparoscopic surgery with or without preoperative oxytetracycline. Eight of 16 turtles received 25 mg/kg oxytetracycline IM. An equivalent volume of saline was administered to eight control turtles. Cultures were performed in all turtles immediately prior to treatment, at one week, and at one month following treatment. Minimum inhibitory concentration of tetracycline testing was performed. Cloacal bacterial diversity was also evaluated prior to and after administration of oxytetracycline in each group. There was an apparent shift in the community composition and diversity of cloacal bacterial flora in both groups between treatment times. In the saline treated hatchlings, the cloacal bacterial species diversity was unchanged or increased following treatment, whereas the bacterial flora diversity in the oxytetracycline treated hatchlings declined following treatment. Five percent (1/19) of the cloacal isolates in both groups of loggerhead hatchlings exhibited tetracycline resistance before treatment. After treatment, 24% (6/25) of the isolates from the oxytetracycline group were resistant to tetracycline, whereas 4% (1/27) in the saline group exhibited resistance to tetracycline. The shift in cloacal flora isolated from the hatchlings precluded comparisons between antimicrobial susceptibility of individual isolates before and after administration of oxytetracycline. However, the apparent shift in community composition of cloacal bacterial flora to a higher percentage of tetracycline resistant isolates in the oxytetracycline treated hatchlings compared to the saline treated hatchlings suggests that one dose of parenteral oxytetracycline may select for enteric bacteria with tetracycline resistance in loggerhead sea turtles.

KEY WORDS: loggerhead sea turtle, *Caretta caretta*, antimicrobial resistance, oxytetracycline, tetracycline.

INTRODUCTION

The rise in antimicrobial resistance from various human, veterinary, and agricultural sources during recent years and its possible implications for public health have led to an intensified surveillance of microbial susceptibility. Oxytetracycline is an antimicrobial that has been used to

treat or prevent bacterial infections for many years and has a wide application including human, veterinary, and agricultural uses throughout the world. Tetracyclines are broad-spectrum antimicrobials, exhibiting activity against a wide range of gram-positive and gram-negative bacteria (Chopra and Roberts, 2001).

Morbidity and mortality due to Gram-negative bacteria

such as *Vibrio* spp., *Pseudomonas* spp., and *Aeromonas* spp., have been documented in sea turtles (George, 1997). Isolates of all of these bacteria have been reported to contain more than one tetracycline resistance gene (Prescott, 2000). Recently, there has been widespread emergence of resistance to tetracyclines including numerous reports of bacterial resistance among other antimicrobials in aquaculture (Chopra and Roberts, 2001, Chelossi, *et al*, 2003). There is also evidence of transfer of tetracycline resistance encoding plasmids between aquaculture environments and humans (Rhodes, *et al*, 2000).

Tetracyclines are incorporated into mineralizing tissue at the time of administration, and therefore can be used as biological markers to label bone for aging studies. The oxytetracycline label can be used to validate sea turtle age estimation methodology by counting growth marks based on the position of the label and known time of administration. This procedure has been performed with biopsies or cross sections of sea turtle humeri using a dose of 25 mg/kg oxytetracycline (Klinger and Musick, 1992, Coles, *et al*, 2001).

The pharmacokinetics of oxytetracycline has recently been reported in loggerhead sea turtles. A dose of 25 mg/kg oxytetracycline administered IM to two year old loggerhead sea turtles resulted in a high volume of distribution, and means for bioavailability (91.8%), maximum plasma concentration (1.6 mg/ml), and elimination half life (61.9 hr). After an IM dose of 25 mg/kg, the plasma oxytetracycline concentration exceeded 1 µg/ml for less than one day (Harms, *et al*, 2004). Therefore, the oxytetracycline dose reported for bone marking in sea turtle skeletochronology studies would likely provide only limited extended protection against susceptible pathogens if administered as a one-time dose preoperatively, although it could have an initial protective effect in preventing infection due to contamination during surgery (Harms, *et al*, 2004). The efficacy of single dose prophylactic antibiotics in preventing infection due to surgical contamination in mammals is a matter of debate, with some reports of prophylaxis and others of no effect (Whittem, *et al*, 1999). The objective of this study was to evaluate the effect of a single preoperative oxytetracycline administration on community composition and tetracycline susceptibility of cloacal bacterial flora in loggerhead sea turtle hatchlings.

MATERIALS AND METHODS

This study was approved by Duke University's Institutional Animal Care and Use Committee. Loggerhead sea turtle hatchlings were collected September of 2003 after emergence from various nests on east coast beaches from Georgia to North Carolina and brought to Duke University Marine Laboratory (Beaufort, NC) as part of an on-going study to track natural sex ratios and post-hatchling gonadal development using laparoscopic examination, gross morphology, and histology (Wyneken, *et al*, 2003). The hatchlings were initially housed individually in 18 cm X 18 cm X 6 – 10 cm deep (depending on depth of water) plastic baskets that were placed in 123 – 357 L tanks containing turtles from the same nest. Baskets with dimensions of 18 cm X 26 cm X 6 – 10 cm were used

when the turtles outgrew the initial smaller baskets. The water source consisted of natural seawater (salinity 25 – 36 psu), maintained between 22 and 34°C (71 – 93°F) with a pH range of 7.4 – 8.5. Ammonia levels ranged from 0 – 6 mg/L and were measured after feeding and right before the daily water change. Full spectrum lighting was provided on a 12 hr light cycle.

The tanks were drained and disinfected daily. Disinfection consisted of scrubbing the surfaces of the tank with chlorhexidine (Nolvasan® Solution, Fort Dodge Animal Health, Fort Dodge, IA) then rinsing with fresh and salt water before refilling. The sea turtle clutches were separated by tank and tanks were disinfected separately. Hand washing occurred between handling turtles of different clutches.

Both treatment groups were fed a diet consisting of shrimp and a gel food composed of ground fish, Mazuri® turtle pellets (Purina Mills, St. Louis, MO), Miner-All I® Calcium powder (Sticky Tongue Farms, Romoland, CA), spinach, and carrots in Knox® gelatin (Kraft Foods, Tarrytown, NY). Turtles were initially fed at 20% of their body weight and gradually decreased over four months to 5% of the body weight.

Sixteen of the loggerhead sea turtles from two clutches (eight turtles per clutch) were chosen for this study. At the time of the study, the turtles were six months old and weighed between 190 and 270 g. None of these turtles had received previous antimicrobials, nor had they been housed within four meters or in the same tank with a turtle that had received antimicrobials. All of the turtles were washed in dilute povidone-iodine (10% solution diluted to 1% with water, Equate, Clay-Parks Labs, Inc., Bronx, NY) just prior to laparoscopic examination of the gonads with particular attention to the cleanliness of the incision site (right inguinal fossa) and the area surrounding the cloaca. In all of the turtles, a sterile rayon-tipped swab (BBL CultureSwab, Becton Dickinson and Company, Sparks, MD) was introduced into the cloaca, twisted 360°, removed, and immediately placed into sterile transport medium (BBL Port-A-Cul Tubes, Becton Dickinson and Company, Sparks, MD) and kept at 4°C (39°F) until shipment to the Clinical Microbiology Lab at North Carolina State University Veterinary Teaching Hospital (Raleigh, North Carolina). The cultures were plated within 12 – 24 hr of collection employing Columbia sheep blood agar (CBA; Remel Inc., Kansas City, KS), desoxycholate agar (DES; Becton Dickinson, Inc., Cockeysville, MD), and thioglycollate broth (Becton Dickinson, Inc., Cockeysville, MD) per standard methods. The CBA plates were incubated for 48 hr at 35°C (95°F) with 5 – 7% CO₂ added. The DES and thioglycollate broth were incubated for 48 hr at 35°C (95°F) in ambient air.

Sensitivity of the isolates to tetracycline was tested using the E-test (AB-Biodisk, North America, Inc., Piscataway, NJ). Bacterial isolates with minimum inhibitory concentration (MIC) values greater than 5 mg/ml were considered resistant. Cloacal bacterial diversity was evaluated in the two groups of turtles before and after treatment based on number of different bacterial isolates and total number of isolates.

Eight of 16 turtles randomly chosen out of the two

clutches received a preoperative dose of 25 mg/kg oxytetracycline (Liquamycin LA-200, Pfizer Inc., New York, NY, 200 mg/ml stock solution diluted to 25 mg/ml with sterile water) IM approximately 2 cm deep into the triceps muscle complex. The remaining eight control turtles received a 0.5 ml dose of sterile saline (0.9% sodium chloride, Baxter, Deerfield, IL) IM and did not receive any antimicrobials.

The 16 turtles were kept in a separate room (8.7 m X 7.6 m) for one week post-operatively. Each turtle was kept in a separate aquarium (38 – 76 L) filled 3/4 with water, which was placed into a 712 L, 1.2 m diameter tank (two aquaria per tank). Turtles of the same clutch and treatment group were housed within the 1.2 m diameter tank. The water consisted of seawater from the same source and was changed daily. Disinfection was performed under the same protocol as mentioned above. After one week and for the remainder of the study, the turtles were moved back to the original room due to facility space constraints, but were kept in an identical set-up with separate aquariums.

During this time, the cloacal cultures were repeated at one week following treatment for all sixteen turtles, and thirteen of the turtles were cultured again at one month following treatment under the same protocol as mentioned above (three of the turtles were removed from the study due to requirements of the larger on-going study under

which this research was being performed). The three most abundant isolates were selected for identification and antimicrobial susceptibility testing from the cultures obtained before treatment and at one week following treatment, unless fewer than three colony types were present. The single most abundant isolate was selected for identification from the culture obtained one month following treatment. A two-tailed Fisher's exact test was used to compare tetracycline resistance between groups after treatment with either saline or oxytetracycline (JMP, SAS Institute, Cary, NC) (Glantz, 1992). The Wilcoxon rank sum test was used for comparison of body weight fluctuations between treatment groups (JMP, SAS Institute, Cary, NC).

RESULTS

There was an apparent shift in the dominant cloacal bacterial flora in the oxytetracycline treated turtles from *Pseudomonas* spp. (26%) and *Shewanella putrefaciens* (21%) before treatment, to *Citrobacter freundii* (53%) and *S. putrefaciens* (26%) at one week after administration, and *Escherichia coli* (83%) at 1 month after administration of oxytetracycline (Table 1). There was also an apparent shift in the dominant cloacal bacterial flora isolated from the saline group from predominantly *Pseudomonas* spp. (37%) and *Aeromonas hydrophila* (26%) before treatment, to *C. freundii* (30%), *S. putrefaciens* (15%) and *A. hydrophila* (15%) at one week after administration, and *Pseudomonas* spp. (57%) and *E. coli* (29%) at one month after administration of saline (Table 1).

Table 1. Cloacal microbial flora, by number of isolates of each organism, in loggerhead sea turtle, *Caretta caretta*, hatchlings treated with oxytetracycline or saline.

Organism	Oxytetracycline group			Prior to treatment	Saline group	
	Prior to treatment	1 week post-treatment	1 month post-treatment		1 week post-treatment	1 month post-treatment
<i>Aeromonas hydrophila</i>	1			5	3	
Asaccharolytic gram negative rod	1				1	
<i>Citrobacter freundii</i>	1	10			6	
<i>Citrobacter</i> sp. CDC group 17	1				1	
<i>E. coli</i>	1	1	5		1	2
<i>Enterobacter cloacae</i>			1			1
<i>Flavobacterium</i> spp.	1					
<i>Flavobacterium oryzihabitans</i>	1					
<i>Pasteurella</i> -like organism	1			1		
<i>Pseudomonas</i> spp.	3			3		1
<i>Pseudomonas aeruginosa</i>	1				1	
<i>Pseudomonas fluorescens</i>	1	3		2		3
<i>Pseudomonas stutzeri</i>				2		
<i>Serratia marcescens</i>				1		
<i>Shewanella algae</i>					1	
<i>Shewanella putrefaciens</i>	4	5		2	3	
<i>Vibrio fluvialis</i>	1			1	2	
<i>Vibrio parahaemolyticus</i>	1			2		
<i>Vibrio vulnificus</i>					1	

In the saline treated hatchlings, the cloacal bacterial species diversity was unchanged or increased following treatment (nine different species out of 19 isolates before treatment, ten out of 20 isolates at one week post-treatment, and four different species out of seven total isolates one month after treatment whereas the bacterial flora diversity in the oxytetracycline treated hatchlings declined following treatment (14 different species out of 19 total isolates before treatment, four different species out of 19 total isolates one week after treatment, and two different species out of six total isolates one month after treatment).

Five different organisms exhibited tetracycline resistance

in this study including *C. freundii*, *Citrobacter* spp. CDC group 17, *E. coli*, *S. putrefaciens*, and *Serratia marcescens*. Five percent (1/19) of cloacal isolates in both groups of loggerhead hatchlings exhibited tetracycline resistance before treatment. After treatment, 24% (6/25) of the isolates from the oxytetracycline group were resistant to tetracycline, whereas 4% (1/27) in the saline group exhibited resistance to tetracycline. This difference between the two groups was statistically significant (Fisher's exact test, $p=0.046$). It should be noted, however, that one turtle contributed three of the tetracycline resistant isolates in the oxytetracycline treatment group.

Table 2. Minimum inhibitory concentrations of cloacal bacterial isolates from loggerhead sea turtle, *Caretta caretta*, hatchlings with one or more isolates exhibiting oxytetracycline resistance before and/or after treatment with oxytetracycline (Oxytet) or saline (Sal) (Tetracycline (Tet) resistance MIC > 5µg/ml).

Turtle	Oxytet or Sal	Isolate	Tet MIC (µg/ml) pre-treatment	Tet MIC (µg/ml) 1 week post	Tet MIC (µg/ml) 1 month post
NCON 0506	Oxytet	<i>A. hydrophila</i>	1		
		<i>Citrobacter</i> CDC group 17	1.5		
		<i>Pseudomonas</i> spp	1		
		<i>C. freundii</i>		>256	
		<i>S. putrefaciens</i>		2	
		<i>P. fluorescens</i>		3	
NCON 0507	Oxytet	<i>S. putrefaciens</i>	2		
		ASAGNR	2		
		<i>Pseudomonas</i> spp	2		
		<i>P. fluorescens</i>		4	
		<i>P. fluorescens</i> type 2		2	
		<i>E. coli</i>			>256
NCON 0508	Oxytet	<i>S. putrefaciens</i>	1	>256	
		<i>Pasteurella</i> - like org	3		
		<i>C. freundii</i>		3	
		<i>E. coli</i>		3	
NCON 0510	Oxytet	<i>C. freundii</i>	192	>256	
		<i>V. parahaemolyticus</i>	0.75		
		<i>F. oryzi</i>	3		
		<i>S. putrefaciens</i>		2	
		<i>C. freundii</i> type 2		>256	
		<i>E. coli</i>			>256
NCON 0504	Sal	<i>A. hydrophila</i>	1		
		<i>A. hydrophila</i> type 2	1		
		<i>V. fluvialis</i>		1	
		<i>Citrobacter</i> sp CDC group 17			256
		<i>S. putrefaciens</i>		3	
		<i>E. cloacae</i>			2

One week after administration of oxytetracycline, three of eight oxytetracycline treated sea turtles had a cloacal isolate exhibiting tetracycline resistance. Two of these organisms were also isolated prior to treatment with oxytetracycline. At this time, one exhibited tetracycline resistance (*C. freundii*) and the other (*S. putrefaciens*) was susceptible to tetracycline (Table 2). The third tetracycline resistant isolate at one week after administration of oxytetracycline was not isolated prior to treatment, so no comparisons within this isolate could be made between the two time periods.

One month after oxytetracycline administration, tetracycline resistant *E. coli* was isolated from two oxytetracycline treated hatchlings (40% (2/5) of *E. coli* isolates in this treatment group) (Table 2). *Escherichia coli* was not isolated from either of these turtles at previous sampling times for comparison. Tetracycline resistant *Citrobacter* sp. CDC group 17 was isolated from one saline treated hatchling at one week post-treatment. This organism was not isolated prior to treatment. No other tetracycline resistance was observed in the saline treated hatchlings.

Median body weight gain over the course of the study was 12.5 g (4 – 24.7 g) for the saline treatment group and 12.2 g (6.1 – 13.6 g) for the oxytetracycline treatment group. The difference in body weight gain between the treatment groups was not statistically significant (Wilcoxon rank sum, $p=0.53$).

DISCUSSION

There was an apparent shift in the cloacal microbial flora isolated from hatchlings of both treatment groups between the different sampling times. In the oxytetracycline treatment group, this was associated with a decrease in cloacal bacterial diversity and selection of three *C. freundii* isolates and one *S. putrefaciens* isolate exhibiting tetracycline resistance at one week post-treatment, and two *E. coli* isolates exhibiting tetracycline resistance at one month following treatment. Apparent shifts in the cloacal bacterial flora isolated from the saline treated hatchlings also occurred, but with only one turtle having a resistant isolate (*Citrobacter* sp. CDC group 17). The changes in microbial flora occurring with oxytetracycline treatment may reflect

the replacement of antibiotic susceptible isolates with isolates exhibiting pre-existing resistance. Antimicrobials often cause a shift in intestinal microbial populations in other species (Savage and Dubos, 1968), and therefore could also have the same effect in loggerhead sea turtles. Alternatively, stress and altered gastrointestinal motility have been reported to affect the community composition of intestinal microbial flora in laboratory rodents, and therefore could be a contributing factor to the apparent change in cloacal microbial flora in the turtles (Tannock, 1997).

Subtherapeutic antimicrobial treatment as a result of inadequate dosing or treatment time has been shown to select for antimicrobial resistance (Tannock, 1997). The prolonged elimination phase of oxytetracycline in loggerhead sea turtles (Harms, *et al*, 2004) may result in subtherapeutic concentrations of the drug leading to antimicrobial resistance. Therefore, acquired resistance as a result of oxytetracycline is also a possibility in this study. However, re-isolation of the same bacteria following treatment occurred at too low a rate to address this possibility. The changes in cloacal bacterial flora composition and diversity, with an increased percentage of resistant isolates in the oxytetracycline treated hatchlings, suggest that administration of a single dose of oxytetracycline has the potential to select for resistant bacteria. Further studies involving isolation and molecular characterization of tetracycline genes are necessary to investigate the development of acquired tetracycline resistance in the cloacal bacterial flora of immature loggerhead sea turtles.

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