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Characterization of the Bacterial Microflora of the Tympanic Cavity of Eastern Box Turtles With and Without Aural Abscesses

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ABSTRACT: Aerobic bacterial cultures of the tympanic cavity of the middle ear were performed in eight eastern box turtles (*Terrapene carolina carolina*) with aural abscesses and 15 eastern box turtles without aural abscesses (controls) that were admitted to The Wildlife Center of Virginia, Virginia, USA during 2003. Twenty-two bacterial isolates were identified from 17 turtles including 10 gram-negative and 12 gram-positive bacteria. Ten of 15 control animals had bacterial growth, resulting in identification of 13 bacteria, including six gram-negative and seven gram-positive agents. Seven of eight turtles with aural abscesses had bacterial growth, and 10 isolates were identified, including four gram-negative and six gram-positive organisms. The most frequently isolated bacteria from control animals were *Micrococcus luteus* ($n=3$) and *Pantoea agglomerans* ($n=2$). *Morganella morganii* ($n=2$) was the only species isolated from the tympanic cavity of more than one turtle with aural abscesses. *Staphylococcus epidermidis* ($n=2$) was the only species isolated from both groups. A trend toward greater bacterial growth in tympanic cavities of affected turtles compared with turtles without aural abscesses was noted. No single bacterial agent was responsible for aural abscesses in free-ranging eastern box turtles in this study, an observation consistent with the hypothesis that aerobic bacteria are not primary pathogens, but secondary opportunistic invaders of environmental origin.

Key words: Aural abscess, bacterial flora, eastern box turtle, *Terrapene carolina carolina*, tympanic cavity, Virginia.

Aural abscesses are a common finding in free-ranging eastern box turtles (*Terrapene carolina carolina*) (Murray, 1996); they can be unilateral or bilateral, and vary dramatically in size. Clinical diagnosis is based on the observation of a swelling medial to the tympanic membrane. Histologically, lesions are characterized by squamous metaplasia, hyperplasia, and keratinization of the tympanic epithelium and a caseous plug consisting of central necrosis and an outer necrotic layer with mixed inflammatory cell infiltration (Brown et al., 2004).

The etiology of aural abscesses in free-living eastern box turtles is unknown. Hypovitaminosis A secondary to poor nutrition has been suggested as a predisposing factor for aural abscess formation in captive turtles, which, followed by bacterial pathogens ascending from the auditory tube, has been implicated as a likely cause of abscess formation (Murray, 1996). A recent study demonstrated that wild-caught box turtles with aural abscesses had significantly higher body burdens of organochlorine compounds (OC) (Holladay et al., 2001), a known vitamin A disruptor (Poon et al., 1995; Grasman et al., 1996), as compared to turtles without aural abscesses. Turtles with aural abscesses also had a trend toward lower serum and hepatic vitamin A levels (Holladay et al., 2001). In one study, multiple aerobic, gram-negative bacteria believed to be opportunistic pathogens were isolated from aural abscesses of free-living eastern box turtles; however, no control animals were cultured (Willer et al., 2003). Few studies have investigated the role of infectious agents associated with aural abscesses. Brown et al. (2004) found increased burdens of bacteria on histopathologic evaluation of eastern box turtles with abscesses compared to controls.

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Another study (Feldman et al., 2004) found no association between eastern box turtles positive for *Mycoplasma* sp. (via polymerase chain reaction [PCR]) and the presence of aural abscesses.

Little information is available regarding the bacterial microflora of the tympanic cavity of free-living eastern box turtles. Furthermore, to the authors' knowledge, there is no published comparison of the bacterial microflora from the tympanic cavity of free-living eastern box turtles with and without aural abscesses. The objective of this study was to describe the bacterial microflora associated with aural abscesses in free-living eastern box turtles and compare this to the tympanic cavity microflora of box turtles without aural abscesses. The intent was to expand upon current information regarding the etiopathogenesis of this condition.

Twenty-three eastern box turtles admitted to The Wildlife Center of Virginia (WCV), Virginia, USA (38°02′N, 78°55′W) between May and October 2003 and deemed nonreleasable as a result of aural abscess formation or severe traumatic injury were included in the study. Turtles were euthanized upon admission with an intravenous injection of 0.5 ml pentobarbital sodium (Beuthanasia-D, Schering-Plough Animal Health, Union, New Jersey, USA) in the subcarapacial sinus followed by an overdose of inhaled halothane (Halothane U.S.P., Halocarbon Laboratories, River Edge, New Jersey, USA). Animals were placed in an induction chamber with cotton impregnated halothane and euthanasia was confirmed by Doppler (Ultrasonic Doppler Flow Detector, Parks Medical Electronics, Inc., Aloha, Oregon, USA) when a heart beat was no longer detected.

Cases of aural abscesses were identified by observation of a unilateral or bilateral solid mass medial to the tympanum (Brown et al., 2004). Lesions were confirmed by surgical incision of the tympanic membrane and exposure of the inner caseous material. Control turtles consisted of animals presented for acute traumatic injuries not involving the skull (to avoid trauma-associated risk of aural bacterial contamination), were considered non-releasable, and had no evidence of swelling medial to the tympanic membrane. Non-abscess status was confirmed following surgical incision of the tympanic membrane and observation of visibly normal tympanic cavity epithelium of the middle ear.

Twenty-three free-living eastern box turtles, eight with aural abscesses and 15 controls, were included in the study. Random tables were used to either select the left or right tympanic membrane, which was aseptically prepared with betadine solution (Betadine solution 10%; Purdue Frederick Co., Norwalk, Connecticut, USA) and incised with a sterile #15 scalpel blade. A dry sterile swab was placed in the tympanic cavity of the middle ear and rolled over the caseous material or aural epithelium several times. Swabs were placed in sterile transport media (Starswab II™, Starplex Scientific Inc., Etobicoke, Ontario, Canada) and mailed within 24 hr on ice overnight to Virginia–Maryland Regional College of Veterinary Medicine (VMRCVM, Blacksburg, Virginia, USA) for routine aerobic bacterial culture.

Swabs received for aerobic bacterial isolation were immediately plated on Columbia agar with 5% sheep blood and MacConkey agar, and then incubated in 7.2% CO_2 and room air, respectively. Plates were incubated at 35 C and evaluated for growth and purity between 18 and 24 hr. Cultures with mixed populations were subcultured until a pure culture was achieved to decrease potential for competition of growth. Negative cultures were held for a minimum of three days before finalized as no growth. Standard procedures were used for bacterial isolate identification, including a Gram stain (Koneman et al., 1997). When necessary, automated biochemical testing (Sensititre®, Trek Diagnostic Systems,

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TABLE 1. Bacterial isolates, classification of Gram type and quantification of growth from aerobic cultures of the tympanic cavity of free-ranging eastern box turtles without aural abscesses admitted to The Wildlife Center of Virginia during 2003.

Bacterial isolate	Gram type	Number isolated	Quantity of growth[a]
Aeromonas sp.	Gram-negative	1	1+
Chryseobacterium meningosepticum	Gram-negative	1	2+
Citrobacter freundii	Gram-negative	1	1+
Klebsiella oxytoca	Gram-negative	1	2+
Pantoea agglomerans	Gram-negative	2	1+, 2+
Serratia marcescens	Gram-negative	1	2+
Bacillus sp.	Gram-positive	1	2+
Carnobacterium piscicola	Gram-positive	1	1+
Enterococcus avium	Gram-positive	1	2+
Enterococcus faecalis	Gram-positive	1	1+
Kocuria rosea	Gram-positive	1	1+
Micrococcus luteus	Gram-positive	3	1+, 1+, 2+
Staphylococcus epidermidis	Gram-positive	1	1+
No growth	Not applicable	5	Not applicable

[a] 1+ to 4+ grading system: 1+ = limited bacterial growth, typically only in the first quadrant to 4+ = heavy growth over the majority of the plate.

Inc., Cleveland, Ohio, USA) was utilized for isolate identification. Isolates were identified to species when possible; otherwise they were described to the generic level. The quantity of each isolate was estimated using a 1+ to 4+ grading system; 1+ corresponded to limited bacterial growth, typically only in the first quadrant, whereas 4+ indicated much heavier growth over the majority of the plate.

Twenty-two bacterial isolates were identified from the 23 turtles. Ten of 15 (67%; 95% CI 43%–91%) control animals had bacterial growth on culture resulting in the identification of 13 isolates (Table 1). Classification of bacteria type revealed six gram-negative and seven gram-positive agents, of which 11 isolates were identified to species and two were identified to genus. Seven of eight turtles with aural abscesses (88%; 95% CI 65-100%) had bacterial growth on aerobic culture (Table 2). Ten isolates were identified; four to the generic level and six to the species level. There were four different gram-negative and six gram-positive isolates. Six turtles, five controls (5/15; 33%) and one with an aural abscess (1/8; 13%), had no growth on aerobic culture.

The estimated quantity of growth for each bacterial isolate varied, particularly for turtles with aural abscesses (Table 2). All bacterial agents isolated from the tympanic cavity of control turtles had a 1+ (9/16; 56%) or 2+ (7/16; 44%) growth (Table 2). Tympanic cavity culture of turtles with aural abscesses resulted in a wider variety of growth per isolate ranging from 1+ to 4+ growth (Table 2). Aerobic culture from one affected turtle, which resulted in isolation of three bacterial agents, was not quantified.

Several animals from each group had more than one bacterial species isolated. Two turtles, one with an aural abscess and one without, had three different bacterial isolates identified. Three turtles with aural abscesses and four control turtles had two isolates identified. Eight turtles (three with aural abscesses and five controls) had one isolate identified. Bacterial species isolated from the tympanic cavity of more than one control turtle included *Micrococcus luteus* ($n=3$) and *Pantoea agglomerans* ($n=2$). *Morganella morganii* was the only bacteria species identified from the tympanic cavity of more than one turtle with aural abscesses ($n=2$). *Staphylococcus epidermidis* was the only species isolated from both control and affected

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TABLE 2. Bacterial isolates, classification of Gram type and quantification of growth from aerobic cultures of the tympanic cavity of free-ranging eastern box turtles with aural abscesses admitted to The Wildlife Center of Virginia during 2003.

Bacterial isolate	Gram type	Number isolated	Quantity of growth[a]
Citrobacter braakii	Gram-negative	1	2+
Morganella morganii	Gram-negative	2	2+, 2+
Pasteurella sp.	Gram-negative	1	4+
Proteus mirabilis	Gram-negative	1	Not available
Corynebacterium sp.	Gram-positive	1	Not available
Listeria monocytogenes	Gram-positive	1	Not available
Staphylococcus sp.	Gram-positive	1	1+
Staphylococcus epidermidis	Gram-positive	1	4+
Streptococcus group G	Gram-positive	2	2+, 3+
Streptococcus viridans	Gram-positive	1	1+
No growth	Not applicable	1	Not applicable

[a] 1+ to 4+ grading system: 1+ = limited bacterial growth, typically only in the first quadrant to 4+ = heavy growth over the majority of the plate.

turtles ($n=2$). In addition, *Citrobacter* spp. were isolated from each study group ($n=2$); however, different species of *Citrobacter* were cultured.

A variety of bacteria, including gram-positive and gram-negative agents, were isolated from the tympanic cavity of the middle ear of box turtles without gross evidence of aural pathology. Some of the organisms have been reported as reptile pathogens; however, estimation of growth of all isolates from the control group was quantified at a low growth level (1+ or 2+) demonstrating a probable lack of pathogenicity (Rosenthal and Mader, 1996). These results are consistent with previous studies that isolated low levels of mixed bacteria from the oral cavity of snakes (Soveri and Seuna, 1986; Blaylock, 2001) and the nasal cavity of desert tortoises (*Gopherus agassizii*) (Dickinson et al., 2001). In addition, other studies have isolated either predominantly gram-positive oral flora in snakes (Draper et al., 1981), or predominantly gram-negative flora (Johnson and Benson, 1996). To the authors' knowledge, this is the first description of the aerobic bacterial flora from the grossly normal tympanic cavity of eastern box turtles. The presence of negative samples, the variation in bacteria isolated, and low bacterial growth in control animals suggest that box turtles do not have permanent aural bacterial flora, but the flora is transient and probably of environmental origin.

No single bacterial agent was identified in association with the aural abscesses. Comparison of bacteria isolated from turtles with aural abscesses in this study and turtles described in Willer et al. (2003) show that only two genera and one species were identical out of a combined total of 41 bacterial isolates. These results are consistent with the hypothesis that aerobic bacteria are not primary pathogens, but secondary opportunistic invaders probably of environmental origin. Potential routes of bacterial invasion include hematogenous, direct penetration, and ascending infection via the auditory tube, with the latter being the most probable route. Gram-negative bacteria are commonly reported pathogens in reptiles, whereas disease associated with gram-positive bacteria is sporadic (Rosenthal and Mader, 1996). This study indicates that gram-positive aerobic bacteria should be considered as potential secondary pathogens in the pathogenesis of aural abscesses of eastern box turtles.

Culture of the tympanic cavity flora of eastern box turtles with aural abscesses

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was more likely to yield bacterial growth compared with controls, as suggested by a narrower and higher percentage confidence interval. In addition, bacterial culture of the tympanic cavity of box turtles with aural abscesses showed a trend towards greater amounts of bacterial growth than nonabscessed turtles, which is consistent with the findings of Brown et al. (2004). Bacterial flora isolated from the tympanic cavities of affected turtles differed markedly when compared to the control group. Whereas handling and transportation of swabs could affect the variety and level of bacterial growth, handling was identical for all samples and all were plated within 36 hr of sampling. This study demonstrates a trend towards an increased likelihood of bacterial isolation and increased amount of bacterial growth from the tympanic cavity of eastern box turtles with abscesses compared to those without.

Additional diagnostic testing of study animals might have been useful but was not available for this study. Brown et al. (2004) found occasional aural pathology associated with the absence of visible aural abscesses in eastern box turtles. Although subclinical aural abscesses cannot be ruled out without histologic examination of the tympanic cavity, the likelihood of this is small based on the previous report. Additionally, PCR detection of *Mycoplasma* sp. was not performed in this study; however, one previous study found no association between *Mycoplasma* sp. and the presence of aural abscesses in eastern box turtles (Feldman et al., 2006). Furthermore, infection of desert and gopher tortoises (*Gopherus polyphemus*) with *Mycoplasma agassizii* has been associated with subclinical and chronic upper respiratory tract disease without presence of aural abscesses (Brown et al., 1994; McLaughlin et al., 2000). Investigation of organochlorine compounds and vitamin A levels in affected turtles is ongoing and will be reported separately.

In conclusion, this study supports the role of aerobic bacteria in tympanic cavity abscesses as secondary opportunistic pathogens, and is consistent with the hypothesis that formation of aural abscesses requires predisposing factors such as epithelial squamous metaplasia. Furthermore, gram-negative and gram-positive bacteria should be considered as potential secondary pathogens involved in aural abscess formation in eastern box turtles. The health implications of aural abscesses in free-ranging populations of eastern box turtles is unknown and lack of understanding of the complete etiopathogenesis of this disease warrants further research.

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