

Oral bacterial microbiota and traumatic injuries of free-ranging *Phrynops geoffroanus* (Testudines, Chelidae) in southeastern Brazil

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Abstract

Oral bacterial microbiota and traumatic injuries of free-ranging *Phrynops geoffroanus* (Testudines, Chelidae) in southeastern Brazil. During 2006 and 2007, we collected free-ranging *Phrynops geoffroanus*, from two anthropogenically altered rivers in southeastern Brazil. Oral microbiological samples were taken for isolation of aerobic and facultative anaerobic bacteria; a physical examination was performed; and we evaluated possible effects on the turtles' health. Twenty-nine species of bacteria were isolated in Piracicaba River turtles (n=10), and twenty-four species in Piracicamirim stream turtles (n=8), most of them gram-negative. In both sites, potential pathogens for reptiles were: *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter agglomerans*, *Citrobacter freundii*, and *Bacillus* sp. Although boat propeller lesions were common on the carapace of the turtles, we have not found turtles with signs of clinical diseases. The oral bacterial microbiota of *P. geoffroanus* inhabiting the Piracicaba River basin are composed of a diverse microbe spectrum, and long-term studies of the effects of pollution and traumatic injuries on this population and its microbial flora are warranted.

Keywords: Testudines, Chelidae, lesions, microbiology, pollution, southeastern Brazil.

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Resumo

Microbiota bacteriana oral e lesões traumáticas no cágado *Phrynops geoffroanus* (Testudines, Chelidae) no sudeste do Brasil. Entre 2006 e 2007, coletamos amostras microbiológicas da cavidade oral de cágados *Phrynops geoffroanus* de vida livre, em dois rios antropizados da região sudeste do Brasil, para o isolamento de bactérias aeróbicas e anaeróbicas facultativas, e realizamos um exame físico e avaliamos possíveis efeitos sobre o estado de saúde dos animais. Vinte e nove espécies de bactérias foram isoladas em cágados do rio Piracicaba (n=10), e vinte e quatro espécies em animais do ribeirão Piracicamirim (n=8), a maioria delas gram-negativas. Em ambos os sítios estudados foram isolados patógenos potenciais para répteis, como *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter agglomerans*, *Citrobacter freundii* e *Bacillus* sp. Embora lesões na carapaça dos cágados, causadas por hélices de barcos, foram um achado comum no exame físico, não encontramos animais com sinais clínicos de doenças. A microbiota bacteriana oral dos cágados que habitam a bacia do rio Piracicaba é composta por um amplo espectro de bactérias, e estudos de longa duração sobre os efeitos da poluição e de lesões traumáticas nesta população e de sua microbiota oral são recomendados.

Palavras-chave: Testudines, Chelidae, lesões, microbiologia, poluição, sudeste do Brasil.

Introduction

Bacterial infectious diseases have been associated with population declines in reptiles (Gibbons *et al.* 2000) and are common in captive and free-ranging turtles (Glazebrook and Campbell 1990a, Orós *et al.* 2005). A variety of bacteria can be present in healthy turtles, but these microbes can become pathogenic in susceptible animals living under stressful conditions (Santoro *et al.* 2006). The knowledge of bacterial microbiota can be a useful tool in understanding the role of bacteria as pathogenic agents in wild turtles (Santoro *et al.* 2006).

Anthropogenically-altered environments are characterized by changes such as chemical pollution (Saba and Spotila 2003), which, in turn, has led to host immunosuppression in several species (Saba and Spotila 2003, Keller *et al.* 2006). The expression of clinical disease, particularly bacterial infections, can reflect both the level of contamination and the physiologic stress that the contamination can cause to a host.

Phrynops geoffroanus is the South American freshwater turtle with the widest geographical distribution, ranging from the Colombian Amazon to southern Brazil and northern Argentina (Ernst and Barbour 1989). It inhabits pristine streams, rivers and lakes, as well as

disturbed urban aquatic environments (Souza and Abe 2001). Few studies report the ecological aspects of the species in such altered habitats (Souza and Abe 2000, 2001). The goal of this study was to describe the oral bacterial microbiota of *Phrynops geoffroanus* in two polluted rivers from the Piracicaba River basin, southeastern Brazil; to perform a physical examinations of these individuals; and to evaluate the possible effect on the turtles' health.

Materials and Methods

The study was conducted in two anthropogenically altered sites, the Piracicaba River and its tributary, the Piracicamirim stream. The Piracicaba River basin (12,400 km²) is mostly located in the state of São Paulo, southeastern Brazil. The main local industrial activities are paper and pulp, food crops, sugar and ethanol, textile, tanning, metallurgic, chemical and fuel refineries (CETESB 2001). Pasture, sugar cane and citrus plantations dominate land use in this region (Krusche *et al.* 1997). Although 92% of the human residences have sewage services (Krusche *et al.* 1997) only 16% of the total domestic sewage volume is treated. The Piracicamirim stream micro basin has an area of 133 km² (Ballester *et al.* 1999, Omoto *et al.* 2000). The region has intense

alterations due to non-planned urban settlement and the sugar cane industry. The main sources of pollution are domestic untreated sewage and fertilizers used in sugar cane crops (Ometo *et al.* 2004).

Turtles from Piracicaba River were captured monthly between August 2006 and June 2007 by active search (Lagueux *et al.* 1995), during the day or at night, using a motor boat and dip nets, in an area known as Monte Alegre (22°41'75" S, 47°33'58" W) located in the central part of the basin. The turtles were captured in the Piracicamirim between April 2006 and August 2007, on the campus of the Universidade de São Paulo (22°42'51" S, 47°37'36" W). We used four gill nets (nylon; mesh size 3-5 cm; 1.5-2 m deep; 15 m long) that were extended perpendicularly between the river banks (Souza and Abe 2000) during day time and checked every three hours.

The animals captured in both sites were transported to the laboratory in plastic containers. The microbiological samples were obtained by inserting a sterile cotton swab in the turtles' mouth. Swabs were then placed in Amies Agar Gel Transport Medium (CB Products, Ltd., Corumbataí, São Paulo 13540-000, Brazil), placed on ice and transported within 24 hours to the Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (FMVZ-USP). We cultured the samples on BHI Broth Medium (Oxoid, Ltd., Basingstoke, Hampshire RG24 8PW, England) (brain and heart infusion broth; incubation at 37° and 25°C for 24 hours) and on 5% Sheep Blood (Oxoid) and MacConkey Agar (Oxoid) (incubation aerobically at 37° and 25°C, examined in 24-96 hours). The BHI Broth Medium samples were subcultured on 5% Sheep Blood and MacConkey Agar, using the same procedure. Microbiological samples were incubated simultaneously at 37° C and room temperature because turtles are heterothermic animals and the temperature gradient can influence the bacterial growth. For isolation of *Staphylococcus* species, samples were placed in

Baird-Parker Agar (Oxoid) (incubation at 37°C for 24-48 hours). For *Salmonella* species detection we cultured on Tetrathionate Broth (Oxoid) (incubation at 37°C for 24 hours), Brilliant Green Agar (Oxoid) and Xylose-lysine-tergitol 4 (XLT4) Agar (Difco Laboratories, Detroit, Michigan 48201, USA) (incubation aerobically at 37°C, examined in 24-96 hours). After incubation on Tetrathionate Broth, samples were subcultured on Brilliant Green Agar and XLT4 Agar, using the same procedure. Aerobic and facultative anaerobic bacteria isolated in this investigation were identified and classified according to literature (Lennette *et al.* 1985, Krieg and Holt 1994, Murray *et al.* 1999).

After oral sample collection, gender was determined by external morphological characteristics (Ernst and Barbour 1989, Molina 1989, Souza and Abe 2000) and turtles were weighed, measured (midline straight carapace length, MSCL). In addition, a physical examination was performed on all animals, and their health status was evaluated, with particular attention to clinical signs such as lethargy, sunken eyes, cachexia, and emaciation.

Results

We captured ten animals for this study (two adult males and eight adult females) from the Piracicaba River and eight (five adult males, two adult females and one immature) from the Piracicamirim stream. Mean body mass was 2402 ± 946 g (range 1085-4200 g) and MSCL was 292 ± 40 mm (range 225-357 mm) in turtles from Piracicaba River. For Piracicamirim stream turtles, body mass and MSCL data were 2384 ± 1393 g (range 250-5200 g) and 289 ± 72 mm (range 136-365 mm), respectively. Eight out of ten turtles in Piracicaba River had traumatic carapace injuries (Figure 1). The injuries of two animals were not entirely healed, contrary to the others. Only two of eight turtles from the Piracicamirim stream had healed traumatic injuries. Despite the traumatic lesions,

all animals from both sites were active and apparently had no clinical signs of disease.

A total of 29 species of bacteria were identified from oral samples in Piracicaba River turtles and 24 species in Piracicamirim stream turtles (Table 1). There was a predominance of gram-negative microorganisms in both sites, nine gram-positive and 20 gram-negative in Piracicaba, and five gram-positive and 19 gram-negative in Piracicamirim. Fifteen species of bacteria were found in turtles from both sites. In animals from Piracicaba River, six species of bacteria grew only at 25°C, ten species only at 37°C, and 13 at both temperatures. In animals from Piracicamirim stream three species grew only at 25°C, 12 species only at 37°C, and nine at both temperatures.

At 25°C incubation temperature, the most commonly isolated species from Piracicaba River turtles were *Aeromonas sobria*, *Klebsiella oxytoca* and *Acinetobacter lwoffii* (40% of the animals). At 37°C, *Escherichia coli* (60%), *Micrococcus* sp and *Bacillus* sp. (50%), *Klebsiella oxytoca*, *Citrobacter amalonaticus*, *Edwardsiella tarda* and *Acinetobacter lwoffii* (40%), and *Klebsiella pneumoniae*, *Citrobacter freundii* and *Edwardsiella hoshinae* (30%) were the prevalent species.

In animals from Piracicamirim stream, *Acinetobacter lwoffii* (50% of the animals), *Bacillus sphaericus* and *Aeromonas hydrophila* (25%) were the most common species at 25°C. At 37°C, the most common were *Escherichia coli* (62.5%), *Klebsiella ozaenae* and *Bacillus* sp. (50%) and *Klebsiella oxytoca*, *Klebsiella rhinoscleromatis*, *Enterobacter agglomerans*, *Citrobacter amalonaticus*, *Edwardsiella tarda* and *Edwardsiella hoshinae* (25% each).

Discussion

The results of this investigation demonstrate that the oral bacterial microbiota of *P. geoffroanus* from Piracicaba River and Piracicamirim stream are composed of a diverse microbial spectrum, with several potential



Figure 1 - Traumatic injury in free-ranging *Phrynops geoffroanus* from Piracicaba River basin, southeastern Brazil.

pathogens for reptiles. Dickinson *et al.* (2001) isolated six species of nasal bacteria in free-ranging *Gopherus agassizii*. Fifteen species of bacteria were isolated from lesions of stranded *Caretta caretta* (Orós *et al.* 2005), and Santoro *et al.* (2006) found 27 species of nasal aerobic bacteria in nesting *Chelonia mydas*. The number of bacteria species isolated in the oral cavity of Piracicaba River animals is higher than reported for other turtle species.

Escherichia coli, the most common bacterium isolated in *P. geoffroanus* at 37°C, has been associated with several lesions in stranded *Caretta caretta* (Orós *et al.* 2005), and in cases of juvenile mortality in free-ranging *Chelonia mydas* (Raidal *et al.* 1998). *Citrobacter freundii* has been associated with cutaneous ulcerative disease in wild box turtle (Jackson and Fulton 1970) and in pulmonary and hepatic lesions in *Chelonia mydas* (Raidal *et al.* 1998). *Enterobacter agglomerans* was isolated in stomatitis and dermatitis in captive *Alligator mississippiensis* (Novak and Seigel 1986), and *Micrococcus* sp. in subcutaneous abscesses in iguanid lizards (Boam *et al.* 1970).

The isolation of opportunistic bacteria in the oral cavity of free-ranging turtles is not synonymous with disease (Santoro *et al.* 2006).

Table 1 - Bacteria isolated from oral samples of free-ranging *Phrynops geoffroanus* from Piracicaba river and Piracicamirim stream, southeastern Brazil, incubated at 25 and 37° C (number of turtles in cells).

Bacteria species	Piracicaba river (n=10)		Piracicamirim stream (n=8)	
	25°C	37°C	25°C	37°C
Gram-positive cocci				
<i>Enterococcus faecium</i>	—	1	—	—
<i>Micrococcus</i> sp.	2	5	—	1
<i>Staphylococcus capitis</i>	—	1	—	—
<i>Staphylococcus caseolyticus</i>	1	—	—	—
<i>Staphylococcus kloosii</i>	—	—	1	—
<i>Staphylococcus lentus</i>	1	—	—	—
<i>Staphylococcus saprophyticus</i>	—	1	—	—
<i>Staphylococcus sciuri</i>	1	—	—	—
<i>Staphylococcus warneri</i>	—	1	—	—
Gram-positive aerobic bacilli				
<i>Bacillus sphaericus</i>	—	—	—	1
<i>Bacillus</i> sp.	2	5	2	4
<i>Corynebacterium xerosis</i>	—	—	—	1
Gram-negative bacilli				
<i>Aeromonas caviae</i>	2	2	1	1
<i>Aeromonas hydrophila</i>	—	—	2	—
<i>Aeromonas sobria</i>	4	2	—	—
<i>Aeromonas veronii</i>	—	1	—	—
<i>Aeromonas</i> sp.	1	—	—	—
<i>Plesiomonas shigelloides</i>	—	1	—	—
Gram-negative enterobacteria				
<i>Buttiauxella brennerae</i>	—	—	—	1
<i>Citrobacter freundii</i>	—	3	—	1
<i>Citrobacter amalonaticus</i>	—	4	1	2
<i>Edwardsiella hoshinae</i>	—	3	—	2
<i>Edwardsiella tarda</i>	—	4	1	2
<i>Enterobacter aerogenes</i>	—	—	1	—
<i>Enterobacter agglomerans</i>	1	—	1	2
<i>Enterobacter cloacae</i>	—	1	—	1
<i>Escherichia blattae</i>	1	—	—	—
<i>Escherichia coli</i>	—	6	1	5
<i>Escherichia fergusonii</i>	—	—	—	1
<i>Escherichia hermannii</i>	—	—	—	1
<i>Klebsiella oxytoca</i>	4	4	1	2
<i>Klebsiella ozaenae</i>	—	2	1	4
<i>Klebsiella pneumoniae</i>	2	3	—	1
<i>Klebsiella rhinoscleromatis</i>	—	1	—	2
<i>Kluyvera cocheae</i>	—	—	—	1
<i>Serratia marcescens</i>	—	1	—	—
Non-fermenting gram-negative bacilli				
<i>Acinetobacter lwoffii</i>	4	4	4	1
<i>Shewanella</i> sp.	1	1	—	—

However we must consider their potential for pathogenicity in immunosuppressed states (e.g. secondary to pollution) (Dickinson *et al.* 2001, Santoro *et al.* 2006), stressful conditions (e.g. traumatic injuries) (Jackson *et al.* 1972, Glazebrook and Campbell 1990a, Orós *et al.* 2005), and in conjunction with parasitic infections (Glazebrook and Campbell 1990b, Raidal *et al.* 1998). It is unclear at this time whether the potentially pathogenic bacteria found in this study were introduced by contamination, or represent the normal oral flora for this species. Additional studies on the microbial flora of *P. geoffroanus* from cleaner locations would be useful for comparison to the present data.

Carapace injuries occurred in most of Piracicaba River turtles and some from Piracicamirim stream. Predation is not likely to be the cause in light of the shape of the lesions that usually include cracks and fractures (Figure 1). Most turtles had a single lesion on the carapace in the area medial to the anterior marginal scutes. The most likely cause of these traumatic lesions is boat propeller strikes, as the Piracicaba River has a considerable traffic of fishing boats. The wounded animals captured in Piracicamirim stream had probably moved from Piracicaba River, as their lesions were similar and Piracicamirim stream is too shallow for motorboat passage. Traumatic lesions in *P. geoffroanus* at Piracicaba River basin may provide a portal of contamination for industrial, agrochemicals and domestic discharges. However, the turtles had no signs of clinical disease, which could suggest that they do not seem to be immunocompromised, and the only dead animal found was hit by a car.

Although evidence suggests that *P. geoffroanus* can take advantage of polluted watercourses (Souza and Abe 2000, 2001), it is important to examine populations in this situation on a long-term basis (Balée and Erickson 2006). Turtles are long-lived animals, often living longer than 20 to 30 years (Gibbons 1987). Urban and industrial activities grew dramatically at Piracicaba River basin during

the 1970's (Negri 1992). Therefore, a small number of turtle generations have been exposed to such environmental change. Although no reduction in recruitment in the local population has been noted so far, it is impossible to predict how this species will be affected on a long-term basis after a number of generations have been exposed to such an unhealthy environment. Long-term studies of the effects of pollution and traumatic injuries on this population, and its microbial flora are warranted.

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