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Original Article

Oral microbiota in healthy *Bothrops atrox* (Serpentes: Viperidae) and in snakes with stomatitis

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The purpose of this study was to isolate bacteria found in the oral cavity of healthy *Bothrops atrox* and in snakes with stomatitis. The area around the snake fang sheaths were swabbed and the samples were placed in Stuart transport medium, and then seeded on blood agar and XLD agar. Gram staining and catalase and mannitol tests were performed to identify Gram positive bacteria, while biochemical screening with Rugai-lysine medium was used to identify Gram negative bacteria. *Proteus* spp. (37.5%), *Escherichia coli* (25%), *Citrobacter* spp. (18.76%), *Serratia* spp. (9.37%) and *Enterobacter* spp. (9.37%) were isolated from healthy snakes, while *Escherichia coli* (26.31%), *Citrobacter* spp. (21.05%), *Proteus* spp. (15.78%), *Salmonella* (10.52%), and *Staphylococcus* spp. (26.31 %) were isolated from snakes with stomatitis. *Staphylococcus* spp. in healthy snakes and in animals with stomatitis differed significantly, suggesting that this microorganism is associated with cases of stomatitis in *Bothrops atrox*.

INTRODUCTION

In Brazil, *Bothrops* and *Crotalus* are the genera of snakes most widely related with snake bites (BERNARDE, 2014). Jararaca from north of Brazil belongs to the family Viperidae, sub-family Crotalinae, genus *Bothrops* and species *Bothrops atrox* Linnaeus, 1758 (COSTA; BÉRNILS, 2015). Antivenom and medicines are researched and produced using a few fractions of the venom of snakes belonging to the genus *Bothrops*. The main toxic fractions in *Bothrops* venom are metalloproteinases and bothropsin. Thrombi may be formed, leading to renal ischemia due to decreased blood perfusion (CASTRO, 2006).

At most *Bothrops* breeding facilities, venom is extracted monthly by hand to prevent the formation of oral lesions, which could cause stomatitis. Giannotti et al. (2013), who studied morphological changes in the venom glands of snakes with low venom production, found lesions indicative of excessive pressure applied on the glands during the extraction procedure.

The usual signs of *Bothrops* sp. snakebite are tissue loss such as edema, abscess and necrosis caused by the action of proteolytic enzymes. In addition, formation of abscesses at the site of the bite is a commonly complication due to the large number of bacteria from the reptile's mouth (JORGE et al., 1994).

According to Jorge et al. (1990), the bacterial species found in the oral cavity of snakes in different regions of the world vary considerably. The main microorganisms found in the microbiota of snakes are Gram negative bacilli that can act opportunistically, causing disease in these animals (KOLENISKOVAS; GREGO; ALBUQUERQUE, 2006).

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The purpose of this study was to identify the bacteria in the oral cavity of healthy *Bothrops atrox* or of snakes with stomatitis bred in captivity, which are used for venom extraction, in order to determine whether the bacteria found in snakes with stomatitis are part of the normal microbiota of this snake species.

MATERIAL AND METHODS

Thirty healthy snakes of the species *Bothrops atrox*, 15 males and 15 females, and 12 *Bothrops atrox* snakes with stomatitis, eight females and four males were used in the present study (Figure 1). The samples were collected at a commercial snake breeding facility for venom extraction, Pentapharm of Brazil, located in Uberlândia, Minas Gerais, registered under no. 11904 at the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA).

To avoid adding a source of stress for the healthy snakes, the samples were collected during the routine procedures of the breeding facility, which follows all the international standards of animal welfare and biosecurity. The study was approved by SISBIO, Brazil's Biodiversity Authorization and Information System, under Permit no. 41060-1, and by the Animal Research Ethics Committee of the Federal University of Uberlândia, Protocol no. 142/13.

Figure 1. Oral cavity of *Bothrops atrox* with severe stomatitis.

Source: author's collection.

The snakes were physically restrained by placing a hook near the distal third of the head, and then grabbing the region of the animal's temporomandibular joints with the other hand to keep the animal's mouth open (WILKINSON, 2014). The secretion in the oral cavity of each snake was then collected by swabbing around each snake fang sheath with a sterile alginate cotton swab and submitted to microbiological examination.

These samples were stored in tubes containing Stuart transport medium and taken to the Laboratory of Infectious Diseases of the Federal University of Uberlândia. In the laboratory the samples were transferred to tubes containing thioglycollate broth, a highly nutritious medium that favors the growth of various microorganisms, and incubated in a bacteriological incubator at 37ºC for 24 hours (OPLUSTIL, 2004).

Using a platinum loop, the samples were seeded on Petri dishes containing blood agar and XLD agar (xylose-lysine deoxycholate), using the agar depletion technique, to isolate the bacterial colonies. The seeded dishes were placed in a bacteriological incubator at 37ºC for 24 hours and incubated again (QUINN et al., 2004).

Gram staining was performed on the blood agar colonies to identify Gram-positive and Gram-negative bacteria. Catalase and Mannitol tests were performed to identify the Gram-positive bacteria (OPLUSTIL, 2004).

The colonies grown on XLD agar were identified using commercial mini kits containing Rugai-lysine medium, to biochemically screen colonies growing on media selective for Gram negative bacteria belonging to the family Enterobacteriaceae. Rugai-lysine medium was used for each different XLD agar colony in order to identify each bacterial genus or species, as

recommended by the manufacturer (OPLUSTIL, 2004). A statistical analysis was performed using Fisher's exact test, considering 5% of significance.

RESULTS AND DISCUSSION

Bacterial growth occurred in the thirty samples from the oral cavity of healthy *Bothrops atrox* as well as in the twelve samples from *Bothrops atrox* with stomatitis, some of which showed the presence of more than one microorganism. The bacteria isolated from the thirty healthy snakes were: *Proteus* spp. (37.5%), *Escherichia coli* (25%), *Citrobacter* spp. (18.76%), *Serratia* spp. (9.37%) and *Enterobacter* spp. (9.37%), while those isolated from the twelve snakes with stomatitis were: *Escherichia coli* (26.31%), *Citrobacter* spp. (21.05%), *Proteus* spp. (15.78%), *Salmonella* spp. (10.52%) and *Staphylococcus* spp. (26.31%).

Fisher's exact test revealed a significant difference in *Staphylococcus* spp. between samples from healthy snakes and from snakes with stomatitis, suggesting that this microorganism is related with cases of stomatitis in *Bothrops atrox* (Table 1).

Table 1. Frequency of bacteria found in the oral cavity of healthy *Bothrops atrox* or in snakes with stomatitis.

Bacteria	Healthy snakes (30)	Frequency (%)	Snakes with stomatitis (12)	Frequency (%)	"P" value
Escherichia coli	8	25.00	5	26.32	0.4635
Staphylococcus spp.	-	-	5	26.32	**0.0009**
Citrobacter spp.	6	18.76	4	21.05	0.4331
Proteus spp.	12	37.50	3	15.78	0.4848
Serratia spp.	3	9.37	-	-	0.5453
Enterobacter spp.	3	9.37	-	-	0.5453
Salmonella spp.	-	-	2	10.53	0.0767
TOTAL	32	100	19	100	

In a microbiological study of the oral cavity of healthy snakes belonging to the families *Boidae, Colubridae, Elapidae* and *Viperidae*, Fonseca et al. (2009) identified the following bacteria: *Actinomyces* sp., *Burkholderia* sp., *Moraxella* sp., *Proteus* sp., *Sarcina* sp., *Bacillus subtilis*, *Staphylococcus aureus*, coagulase-negative *Staphylococcus* and *Yersinia enterocolitica*. Among them, the species *Proteus* spp. and *Staphylococcus* spp. were found in *B. atrox*, suggesting that these bacteria are part of the normal microflora of these reptiles. *Staphylococcus* spp. was isolated only in *Bothrops atrox* with stomatitis.

Gram negative bacilli, some of which zoonotic, are the mainly microorganisms found in oral and cloacal microbiota of reptiles, as evidenced in cases of complications after snakebite in humans, in which the site appears infected and sometimes necrotic (MADER, 1998). The *Salmonella* that was isolated from *Bothrops atrox* with stomatitis is an example of a zoonotic bacterium, and is widely described as part of the normal microbiota of snakes. Of course, *Salmonella* is not the only zoonotic bacterium present in snakes, and there are reports of other bacteria such as *Chlamydophila* spp. and *Mycobacterium* spp. (WILLIAMS, 2008).

The bacteria found in the oral cavity of healthy *Bothrops atrox* suggest that these are part of the normal microbiota of this snake species, which can cause diseases including stomatitis as a result of their opportunistic character when the animal is in a weakened state. It has been reported that the microorganisms commonly found as components of the microbiota in the digestive tract can act as etiological agents, but few researchers have defined these bacteria for Brazilian reptile species (DIAZ-FIGUEROA; MITCHELL, 2006).

In a study of microbiota of healthy *Bothrops jararac,a* Bastos et al. (2008) collected the samples directly from the colon. The authors succeeded in isolating several genera of the family *Enterobacteriaceae*, among which *Salmonella*, *Citrobacter* and *Escherichia* were the most frequent isolates. This indicates that these genera of bacteria are found in the intestinal microbiota of *Bothrops jararaca*, and these three genera were also isolated in our study on *Bothrops atrox*.

Like in *Bothrops atrox*, Jorge et al. (1990) isolated *Escherichia coli*, *Proteus* spp., *Staphylococcus aureus*, *Salmonella* Typhimurium and *Citrobacter* spp. in the microbiota from the fangs, fang sheaths and venom of *Bothrops jararaca*. The authors also described other microorganisms, such as group D *Streptococci*, *Providencia rettgeri*, *Providencia* spp., *Morganella morganii*, *Clostridium* spp. and *Pseudomonas* spp.

Careful prophylaxis and quarantine measures must be taken upon introducing free-living snakes in a stable breeding stock, because new animals can cause serious imbalances in the microbiota of captive specimens. The same care must be taken with the rodents that are fed to snakes, because they may carry pathogenic microorganisms that upset the balance of these microbiota (WILLIAMS, 2008).

In a recent study, Dehghani et al. (2016) analyzed oral cavity of venomous and non-venomous snakes and related that were similarly with the present study. The authors revealed the presence of *Staphylococcus* (34.5

%) being the highest rate of infection and the lowest rate was represented for *Pseudomonas* (3.1 %), *Proteus*, *Enterococcus*, and *Bacillus* (each 6.2 %), *Providencia* (each 12.5 %), *Salmonella* (18.8 %) and *Escherichia*. The authors stand out that the significant presence of bacterial pathogens in oral cavity of snakes demonstrates the need not only anti-venom treatment but also, the diagnosis and treatment of infections.

Several studies have focused on the pharmacological activity of the venom of snakes of the genus *Bothrops*; hence, it is important to know which microbiota inhabit the oral cavity of these animals (FREITAS-DE-SOUSA et al., 2015; HAYASHI; CAMARGO, 2005; MOSCA, 2008; PÁRAMO et al., 1998; STIVAL, 2011). This type of information also provides subsidies to improve the treatment of stomatitis and health care of snakes from captivity to ensure a high quality venom.

CONCLUSION

All the analyzed samples showed bacterial growth, underscoring the high relevance of this type of research on commercially bred and wildlife snake species. The presence of *Staphylococcus* spp. only in snakes with stomatitis suggests that this microorganism is correlated with the occurrence of the pathology. While *Proteus* spp., *Escherichia coli*, *Citrobacter* spp., *Serratia* spp., *Enterobacter* spp., and *Salmonella* spp. occur in normal microbiota of *Bothrops atrox*.

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