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Prevalence of enterobacteria in *Bothrops jararaca* in São Paulo State: microbiological survey and antimicrobial resistance standards

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ABSTRACT. There are few microbiological surveys on reptiles in Brazil. The study described here focuses on a species of snake of great medical interest and serves as basis for other studies and comparisons. The samples were collected directly from the colon of healthy adult Jararacas. The material was seeded in MacConkey Agar (Difco®) and XLT4 Agar (Difco®). The isolates were identified through the API 20E Identification System (BioMériux®). The isolates of *Salmonella* sp. were submitted to serotyping. Finally, the colonies were submitted to the antimicrobial sensitivity test. Several genera of the Enterobacteriaceae family were obtained (*Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Kluyvera*, *Morganella*, *Proteus*, *Providencia* and *Salmonella*), as well as a genus of morphologically similar Gram-negative bacteria (*Aeromonas*). *Salmonella, Citrobacter* and *Escherichia* were the most frequent isolates. Fourteen (14) serotypes of *Salmonella* were identified; 13 are of the subspecies IIIb and 1 of the subspecies IV. *Aeromonas*, *Enterobacter*, *Escherichia* and *Klebsiella* presented a higher resistance rate to antibiotics. *Kluyvera* and *Salmonella* were the genera most sensitive to the tested drugs. Aminoglycosides presented good antimicrobial action, but chloramphenicol was the only drug to which no isolate presented resistance.

Key words: *Bothrops jararaca*, snake, reptile, intestinal microbiota, Enterobacteriaceae, *Salmonella*.

RESUMO. Prevalência de enterobactérias em *Bothrops jararaca* no Estado de São Paulo: levantamento microbiológico e padrões de resistência antimicrobiana. Existem poucos levantamentos microbiológicos em répteis no Brasil. O estudo aqui descrito enfoca uma espécie de serpente de grande interesse médico e serve como base para outros estudos e comparações. As amostras foram colhidas diretamente do cólon de jararacas adultas e saudáveis. O material foi semeado em ágar MacConkey (Difco®) e ágar XLT4 (Difco®). Os isolados foram identificados por meio do Sistema API 20E de Identificação Bioquímica (Galerias API-BioMériux®). Os isolados de *Salmonella* sp. foram submetidos à sorotipagem. Por fim, as colônias foram submetidas ao teste de sensibilidade antimicrobiana. Foram obtidos vários gêneros da família Enterobacteriaceae (*Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Kluyvera*, *Morganella*, *Proteus*, *Providencia* e *Salmonella*) e um gênero de bactéria Gram-negativa morfologicamente semelhante (*Aeromonas*). *Salmonella*, *Citrobacter* e *Escherichia* foram os isolados mais frequentes. 14 sorotipos de *Salmonella* foram identificados, sendo 13 da subespécie IIIb e 1 da subespécie IV. *Aeromonas*, *Enterobacter*, *Escherichia* e *Klebsiella* apresentaram maior taxa de resistência aos antibióticos. *Kluyvera* e *Salmonella* foram os gêneros mais sensíveis às drogas testadas. Aminoglicosídeos apresentaram boa ação antimicrobiana, mas cloranfenicol foi a única droga para qual nenhum isolado apresentou resistência.

Palavras-chave: *Bothrops jararaca*, serpente, réptil, microbiota intestinal, Enterobacteriaceae, *Salmonella*.

Introduction

Bothrops jararaca is a species of venomous snake that occurs in Brazil, Paraguay and Argentina. Snakes of the genus *Bothrops* are responsible for higher human morbidity in the Americas than any other group of venomous snakes, and *Bothrops jararaca* is among the most important ones (Campbell and Lamar, 1989).

Infectious diseases are one of the largest causes of morbidity and mortality in reptiles. In reptiles, infectious diseases are almost always the result of immunosuppression, and this is often associated with

the stress of captivity (Rosenthal and Mader, 2006).

Gram-negative bacteria are the most common bacterial pathogens, although anaerobic bacteria and pathogenic fungi may be important components of reptilian disease (Rosenthal and Mader, 2006).

Most Gram-positive bacteria are not considered pathogenic in reptiles. They are common inhabitants. However, some Gram-positive bacteria can cause disease, especially in an immune-compromised animal (Rosenthal and Mader, 2006).

The gastrointestinal microbiota of reptiles is generally composed of aerobic and anaerobic Gram-positive and Gram-negative bacteria, yeast, and protozoa (Diaz-Figueroa and Mitchell, 2006).

Different authors report that the microorganisms normally found as microbiota components of the digestive system can act as etiological agents of gastrointestinal diseases in reptiles, but there are few researchers who have defined them for the Brazilian species of reptiles. The reports are scarce and many of them are outdated (Moreno *et al*., 1973; Belluomini *et al*., 1977; Iizuka *et al*., 1984; Calixto *et al*., 1986; Sá and Solari, 2001).

Therefore, it is of vital importance to know the microorganisms that form part of the microbiota of reptiles. This permits the construction of a solid basis for understanding of diseases and for improving the life condition of these animals in captivity.

The number of cultured isolates from the oral cavity and cloaca are limited. Due to this, some researches started to collect microbiological samples directly from the intestinal tract in order to be able to distinguish whether they differed from the samples collected from the oral cavity and cloaca (Diaz-Figueroa and Mitchell, 2006).

The aim of this work is to establish, through sample collection directly from the colon, the intestinal bacterial microbiota pertaining to the Enterobacteriaceae Family and Gram-negative bacteria closely related to the species of venomous snake *Bothrops jararaca*, and to evaluate the behavior of these isolates against different antibiotics.

Material and methods

Only adult and healthy animals of the species *Bothrops jararaca* were used (Table 1), originating from São Paulo State, Brazil, recently captured, and kindly provided by the Herpetology Laboratory of the Butantan Institute. Only one snake was found dead in the enclosure where it was housed.

An intestinal fragment containing the colon and the distal portion of the small intestine was removed immediately after euthanasia. In the Compared Pathology Laboratory for Wild Animals (Lapcom) of the Department of Pathology of the Faculty of Veterinary Medicine and Animal Science (FMVZ), University of São Paulo (USP), São Paulo State, Brazil, the organ was opened in a sterile manner and swabs of the mucosa were seeded in MacConkey Agar (Difco®) and XLT4 Agar (Difco®) and kept in microbiological oven at 37ºC under aerobiosis for 24 hours.

Table 1. Selected biological data of *Bothrops jararaca* submitted to colon bacterial culture.

Animal	Sex	SVL	TL	BW
01	F	84.2	95.0	310
02	F	88.6	100.0	320
03	F	124.0	140.0	890
04	F	106.3	120.0	580
05	F	89.5	101.0	330
06	F	101.0	114.0	410
07	F	92.1	104.0	215
08	F	115.2	130.0	410
09	F	106.5	121.5	300
10	F	100.0	113.0	310
11	F	87.7	98.7	210
12	F	88.6	100.0	260
13	F	109.5	124.2	305
14	M	67.5	78.0	95
15	M	73.0	84.0	121
16	F	118.0	131.5	570
17	F	94.7	108.7	170

F: female; M: male; SVL: snout-vent length (cm); TL: total length (cm); BW: body weight (g).

After isolation of the colonies, the material was submitted to the API 20E Identification System (BioMériux®).

Most of the *Salmonella* isolates were serotyped according to the Kaufmann-White Scheme (Popoff and LeMinor, 1997) at the Laboratory of Enteric Pathogens, Adolfo Lutz Institute, São Paulo, São Paulo State, Brazil, with a set of somatic and flagellar monovalent antisera against cell wall and flagellar antigens. The antisera are produced at the Adolfo Lutz Institute itself from standard *Salmonella* strains inoculated in rabbits.

Of 73 bacterial isolates, 68 were submitted to antimicrobial susceptibility test by the disk method. The tested antimicrobial agents were: amikacin (30 μg), ampicillin (10 μg), carbenicillin (100 μg), cefoperazone (75 μg), cefotaxime (30 μg), cefoxitin (30 μg), chloramphenicol (30 μg), trimethoprim-sulfamethoxazole (25 μg), gentamicin (10 μg), neomycin (30 μg), netilmicin (30 μg), polymyxin B (300 μg), tetracycline (30 μg) and tobramycin (10 μg).

Results and discussion

The incubation of the samples withdrawn from the colon was carried out only under aerobiosis at 37ºC, since under these conditions the bacteria grew well, foregoing the incubation at 25ºC, as Needham (1981) suggests.

Several attempts have been made to characterize the aerobic and anaerobic microbiota of the digestive system in different species of reptiles. However, these studies are based primarily on oral and cloacal swabs, and only represent the population of bacteria that are being eliminated (Diaz-Figueroa and Mitchell, 2006). In this study, the samples were collected directly from the intestine and are much more representative than cloacal or pericloacal swabs.

Enterobacteriaceae

For different bacterial isolates, only the genus was reached. For the majority, it was possible to identify the species, and for many *Salmonella* isolates, serotyping was possible to perform. In a study performed by Salb *et al.* (2002), of 47 isolates, only 20 could be characterized. Studies in birds and mammals also produce isolates of difficult characterization due to the limited number of biochemical tests.

Of 17 studied animals, several members of the Enterobacteriaceae Family can be isolated (Table 2), including representatives of the genus *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Kluyvera*, *Morganella*, *Proteus*, *Providencia*, *Salmonella* and a Gram-negative facultative anaerobic rod, which is morphologically similar to the members of the Enterobacteriaceae family, *Aeromonas*, confirming some recorded findings for reptiles in the literature (McCoy and Seidler, 1973; Moreno *et al*., 1973; Zwart, 1986; Johnson-Delaney, 2006).

Table 2. Enterobacteriaceae and *Aeromonas* sp. isolated from the colon of 17 *Bothrops jararaca.*

N	Genus	MC	XLT4	Animals	Species/Subspecies
20	*Salmonella*	7	13	01 (3)[a], 02 (2), 05 (2), 06, 07 (3), 09 (3), 11, 12, 15, 16 (2), 17	*S. enterica diarizonae* (13)[b], *S. enterica houtenae* (1), *Salmonella* ssp (3), *S. enterica* subspecies III (3)
19	*Citrobacter*	9	10	01, 02, 03 (2), 05, 06 (4), 09 (2), 10, 12, 13, 14 (2), 15, 16, 17	*C. braakii* (4), *C. freundii* (9), *Citrobacter* sp. (5), *C. youngae* (1)
9	*Escherichia*	8	1	04, 08 (6), 10, 14	*E. coli* (9)
6	*Klebsiella*	4	2	03 (2), 05, 10 (2), 12	*K. oxytoca* (6)
4	*Enterobacter*	2	2	04, 11 (2), 12	*E. cloacae* (4)
4	*Proteus*	4	0	12 (2), 14, 17	*P. mirabilis* (2), *Proteus* sp. (1), *P. vulgaris* (1)
3	*Aeromonas*	3	0	02, 03, 13	*A. hydrophila* alpha-hemolytic (1), *A. hydrophila* group 2 (1), *Aeromonas* sp. (1)
3	*Providencia*	3	0	10, 12, 16	*P. rettgeri* (2), *Providencia* sp. (1)
2	*Kluyvera*	2	0	11 (2)	*Kluyvera* sp. (2)
2	*Morganella*	2	0	06, 17	*M. morganii* (2)
1	*Serratia*	1	0	04	*S. marcescens* (1)
73	TOTAL	45	28		

a: the number in parenthesis represents the quantity of isolates obtained for each animal; b: the number in parenthesis represents how many species or subspecies were isolated for each genus of bacteria; N: number of bacterial isolates obtained; MC: MacConkey Agar; XLT4: XLT4 Agar.

The most frequent isolations were: *Salmonella* (27.3%), *Citrobacter* (26.0%) and *Escherichia* (12.3%). In one of the few studies with relation to bacteria in reptiles and amphibians in Brazil, Moreno *et al*. (1973) obtained similar data. The group isolated *Citrobacter* sp. and *Salmonella* sp. in, respectively, 35.3% and 20.55% of the cases.

In some cases, the same genus occurred 2 or more times in the same animal as different species or serotypes. For example: animal number 06 (4 *Citrobacter*), number 11 (2 *Kluyvera*), number 12 (2 *Proteus*) and number 07 (3 *Salmonella*). In fact, in a single reptile, more than 1 serotype of *Salmonella* can be found (Jephcott *et al*., 1969; Harvey and Price, 1983; Onderka and Finlayson, 1985; Van Der Walt *et al*., 1997; Madsen *et al*., 1998; Johnson-Delaney, 2006).

The high frequency of isolation of *Salmonella* (64.7% of the studied animals) is well documented (Johnson-Delaney, 2006) and several authors say that it may be higher than 90% (Iverson *et al*., 1969; Chiodini and Sundberg, 1981; Austin and Wilkins, 1998; Rosenthal and Mader, 2006).

From animal number 08, 6 colonies of *Escherichia coli* were exclusively isolated. These 6 colonies presented different biochemical behaviors. Some used lactose; others did not. The majority produced gas, and one did not. They were therefore considered different serotypes.

Salmonella

Salmonella colonizes the distal portion of the small intestine and colon of mammals, birds and reptiles, (Carvalho, 2006), and the most important intestinal pathological alterations in snakes, caused by salmonellosis, normally occur in the final half of the organ (Onderka and Finlayson, 1985). For these reasons, the colon was chosen for the harvest of samples destined to bacterial culture.

XLT4 Agar, selective for *Salmonella* sp., produced the growth of black colonies, which were biochemically identified as *Salmonella* sp. in their majority, or as *Citrobacter* sp. The growth of other types of colonies like white, yellowish or translucent color occurred eventually, and corresponded to bacteria of the genus *Enterobacter*, *Escherichia* and *Klebsiella.* This verified the high efficiency of XLT4 Agar, which even though permitting the eventual growth of other bacteria, presented better proportion of *Salmonella* sp. isolation in comparison to MacConkey Agar (Table 3). Some authors have also had excellent results with the medium for specific use in reptiles in the isolation of *Salmonella* (Mitchell, 2006a).

Table 3. Serotypes of *Salmonella* isolated from the colon of *Bothrops jararaca.*

N	Serotype	MC	XLT4
6	*Salmonella enterica diarizonae* 50:r:-	3	3
3	*Salmonella arizonae*	1	2
3	*Salmonella* sp.	1	2
1	*Salmonella enterica diarizonae* Rough	0	1
1	*Salmonella enterica diarizonae* 50:r:enz$_{15}$	0	1
1	*Salmonella enterica diarizonae* 60:r:z$_{15}$	0	1
1	*Salmonella enterica diarizonae* 65:	0	1
1	*Salmonella enterica diarizonae* 65:-:-	1	0
2	*Salmonella enterica diarizonae* 65:z$_{53}$:k	1	1
1	*Salmonella enterica houtenae* 65:-:-	0	1
20	TOTAL	7	13

N: number of serotypes obtained; MC: MacConkey Agar; XLT4: XLT4 Agar.

Of the 14 confirmed serotypes (Table 3), 13 pertained to the subspecies IIIb (diarizonae) and 1 to the subspecies IV (houtenae).

It is believed that ectotherms are the largest reservoir for subspecies III. In a study in South Africa, most of the serotypes isolated from snakes pertained to subspecies IIIa and IIIb (Van Der Walt *et al*., 1997). Subspecies II, IIIa, IIIb, IV and VI are found in ectotherms and in the environment (Popoff and LeMinor, 1997).

In a survey of *Salmonella* performed in Brazilian and imported pet reptiles, serotypes Hadar (I), Montevideo (I), 4,5:e,h:- (I), 17:z$_{10}$:e,n,x,z$_{15}$ (IIIb), 38:-:- (IIIb) and other 3 non-typable serotypes (IIIa, IIIb and IV) were found in snakes (Sá and Solari, 2001).

Some serotypes were not determined. There are reports where non-serotypable isolates of *Salmonella* were found, and it is possible that the reptiles still conceal unrecognized serotypes (Van Der Walt *et al*., 1997).

It was established, through serotyping, that particular *Salmonella* isolates that presented distinct physical characteristics in particular culture medium, pertained to different serotypes. It is the case of *S*. IIIb 60:r:z$_{15}$ and *S*. IIIb 50:r:- in XLT4 Agar for animal 01 and *S*. IIIb 65:-:- and *S*. IIIb 50:r:- in MacConkey Agar for animal 07.

Antibiogram

All isolates of *Aeromonas* presented resistance to ampicillin and to carbenicillin, and 33.3% of them to trimethoprim-sulfamethoxazole (Table 4).

Several strains of *Citrobacter* presented resistance to half of the tested antibiotics, including penicillins, cephalosporins and neomycin. *Enterobacter*, *Escherichia* and *Klebsiella* were the other genus that presented high antimicrobial resistance rates.

Kluyvera was the only genus sensitive to all tested antibiotics. Comparatively, *Salmonella* presented the second least degree of resistance. In a study in Nile Crocodiles (*Crocodylus niloticus*), the *Salmonella* isolates were sensitive to a number of commonly used antibiotics, except for 3 isolates that were streptomycin resistant. No multi-resistant strain was detected (Madsen *et al*., 1998).

The antibiotics that presented the highest resistance indexes were the semisynthetic penicillins, ampicillin (33.8%) and carbenicillin (23.5%). It is known that many natural penicillins, first-generation cephalosporins and macrolides have minimum effect against Gram-negative bacteria due to intrinsic resistance factors (Mitchell, 2006b).

The third-generation cephalosporins (cefotaxime and cefoperazone) presented a lower degree of resistance than that of the second generation (cefoxitin), as expected, given that those of the third generation present greater action against Gram-negatives than those of the second generation.

The tested aminoglycosides presented good action against the isolates (maximum of 5.88% resistance). Aminoglycosides are considered the best option for Gram-negative sepsis and are generally indicated for Gram-negative infections in reptiles including *E. coli*, *Klebsiella* sp., *Enterobacter* sp., *Pseudomonas* sp., *Proteus* sp., *Providencia* sp., *Salmonella* sp. and *Serratia* sp. (Mitchell, 2006b).

The potentiated sulfa (trimethoprim-sulfamethoxazole) had good action against the isolates (4.41% resistance).

Table 4. Antimicrobial resistance of Enterobacteriaceae and *Aeromonas* isolated from the colon of *Bothrops jararaca* (%).

Isolate	AMI	AMP	CAR	CPZ	CTX	CFO	CLO	SUT	GEN	NEO	NET	POL	TET	TOB
Aeromonas	0	100	100	0	0	0	0	33.3	0	0	0	0	0	0
Citrobacter	6.2	62.5	6.2	31.2	6.2	62.5	0	0	0	6.2	0	0	0	0
Enterobacter	0	25.0	25.0	25.0	0	100	0	0	0	25.0	0	0	0	25.0
Escherichia	0	11.1	22.2	11.1	11.1	0	0	22.2	11.1	0	11.1	0	33.3	11.1
Klebsiella	0	83.3	100	0	0	0	0	0	33.3	16.6	33.3	0	33.3	33.3
Kluyvera	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Morganella	0	0	0	0	0	0	0	0	0	0	0	100	0	0
Proteus	0	25.0	25.0	0	0	25.0	0	0	0	0	0	100	75.0	0
Providencia	0	66.6	66.6	0	0	0	0	0	0	0	0	100	66.6	0
Salmonella	0	0	0	5.0	0	0	0	0	0	5.0	0	0	0	0
TOTAL	1.4	33.8	23.5	11.7	2.9	22.0	0	4.4	4.4	5.8	4.4	11.7	14.7	5.88

AMI: amikacin (30 μg); AMP: ampicillin (10 μg); CAR: carbenicillin (100 μg); CPZ: cefoperazone (75 μg); CTX: cefotaxime (30 μg); CFO: cefoxitin (30 μg); CLO: chloramphenicol (30 μg); SUT: trimethoprim-sulfamethoxazole (25 μg); GEN: gentamicin (10 μg); NEO: neomycin (30 μg); NET: netilmicin (30 μg); POL: polymyxin B (300 μg); TET: tetracycline (30 μg); TOB: tobramycin (10 μg).

No isolate presented resistance against chloramphenicol. There are reports of use of chloramphenicol associated with ampicillin to eliminate *Salmonella* in lizards and chelonians, but it is known that this practice is inadequate, since these animals may become latent carriers and not actively shed *Salmonella* or the microorganisms may be eliminated in number lower than the sensitivity threshold of the assay (Mitchell, 2006b). Besides this, the use of antimicrobials may produce *Salmonella* resistant strains (Rosenthal and Mader, 2006).

Microbes can develop resistance either naturally through mutation (intrinsic) or as a result to exposure from the environment. The widespread use of antibiotics in humans and reptiles has led to an increased number of reports of antimicrobial resistant pathogens. In humans, thousands of deaths are associated with antimicrobial-resistant bacteria (Mitchell, 2006b).

The bacterial isolates of examined Jararacas presented low antimicrobial resistance rates against several tested drugs. This allows the use of these antibiotics with safety in the infectious process of these snakes. Additionally, the knowledge of antimicrobial resistance standards of reptile bacterial isolates is important for the treatment of human beings infected with reptile bacterial zoonoses.

Conclusion

It is expected that this work contributes to the basic knowledge of microbiology in reptiles and, in some way, to the treatment of these animals, allowing them to be kept in captivity in the best way possible, taking advantage of their potential of production of venom, leather and other sub-products in the likeness of production animals.

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