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Research Paper

Microbiota and anthropic interference on antimicrobial resistance profile of bacteria isolated from Brazilian maned-wolf (*Chrysocyon brachyurus*)

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Abstract

Both the study of Brazilian wild mammal fauna and the conditions that foster the preservation of endangered species, such as Brazilian Maned-wolf (*Chrysocyon brachyurus*), in wild life are of extreme importance. In order to study the resistance profile of microbiota bacterial colonizing Brazilian Maned-wolf, this work investigated samples from eight male captive and free roaming animals originating from different Brazilian geographical regions. Samples for microbiological purposes were collected with swabs and kept in appropriate transport medium. Using routine microbiological techniques, the isolated bacteria were tested toward antimicrobial drugs by the agar disk diffusion method. Results showed that all samples from wild animals were sensitive toward all drugs tested. Conversely, the resistance profile of bacteria isolated from captive animals varied among strains and animal body site location. *Escherichia coli* samples from prepuce, anus and ear showed multi-resistance toward at least four drugs, especially against erythromycin and tetracycline, followed by *Proteus mirabilis* and *P. vulgaris* strains isolated from anus and ear. Among Gram-positive bacteria, strains of coagulase-negative staphylococci showed multi-resistance mainly toward erythromycin and amoxicillin. The work discusses these findings and suggests that profile of multi-resistance bacteria from captive subjects may be attributed to direct contact with human or through lifestyle factors such as feeding, predation or contact of animals with urban animals such as birds, rodents, and insects from surrounding environments.

Key words: Maned-wolf, *Chrysocyon brachyurus*, microbiota, antimicrobial resistance, anthropic pressure, environment.

Introduction

The Maned-wolf (*Chrysocyon brachyurus*) is the largest south American canid and despite of its wide distribution pattern covering open areas of South America, this species is listed as a Near Threatened (IUCN, 2007). Antropic pressure threatens endangered species mainly through deforestation of native forests, causing loss of native foraging areas, and forces animals to seek alternatives (Margarido and Braga, 2004). The Brazilian Maned-wolf *Chrysocyon brachyurus*, belongs to the Canidae family of the Carnivora order and developed in the Brazilian central highlands in the Pleistocene. In its natural environment this species feed on fruits, insects, and small birds, mammals and reptiles (Carvalho and Vasconcelos, 1995; Reis *et al.*, 2006). Studies have shown that human management interferes in the gut flora of captive wild animals, when they are fed commercially-prepared foods (Schwab *et al.*, 2011).

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Gastrointestinal tracts harboring *Salmonella* in captive *C. brachyurus* kept in Brazilian Zoos have been studied, with the authors suggesting a physiological adaptation of the bacterium to the animals' gut (Gilioli and Silva, 2000).

In dogs, the natural cavities such as nasal, oral and prepuce can house several bacteria and fungi which under special conditions may lead host infection (Birchard and Sherding, 1998; Sturgees, 2001). Domestic dogs with pathogenic resistant bacteria can also represent risks of infection to their owners (Guardabassi *et al.*, 2004). However, while in wild canidae no transmission of bacteria to human has been described to date, wild animals may indeed represent a zoonotic risk to humans (Chomel *et al.*, 2007). Based on the profile of antimicrobial resistance of bacteria towards drugs, this work discusses the possible anthropic pressure on microbiota of Brazilian Maned-wolf maintained in captivity when compared with their wildlife counterparts.

Material and Methods

Eight Brazilian Maned-wolf specimens were enrolled in the present work, with two wildlife specimens coming from Serra da Canastra, MG, and the other six captive animals coming from Zoos, one from Sorocaba, SP, two from São Bernardo, SP, one from Volta Redonda, RJ, and two from two private reserves one from Mogim Mirim-SP and one from Araxá-MG. Animals were chemically restrained with 5 mg/kg de PV of Zoletil® (hydrocloridrate of tiletamina + zolazepan cloridrate - VIRBAC®) via IM (Cunha *et al.*, 2007). Then samples from five previously selected body sites were collected: oral, nasal and ear cavities, and foreskin (prepuce) and perineal areas. Standard Stuart medium transport microbiological swabs (Copan®, Italy) were used and immediately conditioned to be analyzed in the laboratory. The following media (Acumedia®, USA) were used to isolate the microorganisms: Sabouraud agar, blood base agar supplemented with defibrinated sheep blood and McConkey agar. All inoculated mediuns were maintained at 37 °C incubation during 24-72 h, or as much as one week for the observation of fungi growth. After this, all colonies were characterized by morphotintorial Gram staining, and biochemical tests performed: catalase (Sigma, USA), coagulase (Laborclin, Brazil), oxidase (Dry slide test Difco, USA) and DNAse (DNAse agar, Merck, Germany). Colonies suspected as *Pseudomonas* spp. were also cultured on Cetremide agar (Merck, Germany). Colonies suspected to be *Staphylococcus aureus* were cultured on Vogel-Johnson agar (Acumedia®, USA). To differentiate micrococci from staphylococci, semi-solid O-F (Difco, USA) supplement with 1% glucose and 0.04 IU bacitracin (CEFAR, Brazil) tests were adopted. Standard strains *Micrococcus luteus* ATCC4698 and *Staphylococcus aureus* ATCC25923 were used as negative and positive controls, respectively, according to manufacturer instructions. Bacteria presenting clear zone around colonies above 10 mm were considered *Micrococcus*.

Bacteria were identified using Api ID32 Staph, Api ID32 Strep and Api ID32E, and interpretation was conducted by an automated system (miniApi, bioMèriéux, France). *Malassezia pachydermatis* yeast forms were classified by culture on Sabouraud agar in Petri dishes supplemented with and without sterile olive oil, following by incubation at 37 °C. All yeast colonies were checked by Gram staining and characteristic *Malassezia* forms were observed and *M. pachydermatis* growth in both medium guaranteed the species identification.

Antimicrobial assay

All bacteria identified were tested for sensibility towards 12 drugs for Gram-positive and 12 drugs for Gram-negative bacteria (Laborclin®, Brazil), by using the disk diffusion on agar method in Petri dishes containing 20 mL Muller Hinton agar (Acumedia®, USA) (CLSI, 2012). The drugs used for Gram-positive bacteria were amoxicillin (AMO, 10 μg), clindamycin (CLI, 2 μg), cephalotin (CFL, 30 μg), penicillin G (PEN, 10 U), oxacillin (OXA, 1 μg), tetracycline (TET 30 μg), ampicillin (AMP, 10 μg), erythromycin (ERI, 15 μg), sulphazotrim (SUL, 25 μg), gentamicin (GEN, 10 μg), cephoxitin (CFO, 30 μg) and vancomycin (VAN, 30 μg); and for Gram-negative bacteria the drugs were tetracyclin (TET, 30 μg), cloranphenicol (CLO, 30 μg), amoxicilin + clavulanic acid (AMC, 20/10 μg), gentamicin (GEN, 10 μg), cephoxitin (CFO, 30 μg), tobramicin (TOB, 10 μg), ampicillin (AMP, 10 μg), cephalotin (CFL, 30 μg), cotrimoxazole (SUT, 25 μg), enrofloxacin (ENO 5 μg), erythromycin (ERI, 15 μg) and ciprofloxacin (CIP, 5 μg).

All inoculums (0,5 McFarland scale) used for the above tests were prepared in sterile 0.95% saline solution and read ($DO_{550\ nm}$) in photometer (Densimat, bioMérrieux, France). All experiments were performed in triplicate.

Toxin production by staphylococci

All staphylococci were tested for enterotoxin (SEA-SEE) and TSST-1 toxin production by the Membrane Over Agar (MOA) followed by immunodifusion methods, as described by other researchers (Braga *et al.*, 2004). Positive controls and specific antibodies and antigen for toxin detection were kindly provided by Dr. Luis Simeão do Carmo, from Universidade Federal de Minas Gerais-UFMG (MG, Brazil). To identify toxin from bacteria the Ouchterlony immunodiffusion method in 1.2% Noble agar (Difco, USA) was employed using supernatant from MOA culture. A standard Food Research Institute model template was used to perforate wells for applying test samples. Each positive reaction was immersed for 72 hours in a 0.9% NaCl solution that was changed three times a day, followed by the staining step with Coomassie Blue R-250, for 30-

60 min at RT. A gentle unstaining step was performed with a methanol: ethanol solution, and samples were photodocumented (Su e Wong, 1995).

Results

The results reported in Tables 1-4 show the microorganisms isolated of samples from Brazilian Maned-wolf, according to body sites investigated.

With respect to enterotoxin investigation, from all coagulase-negative staphylococci (CNS) and one *S. intermedius* tested for SEA-SEE, only one strain of CNS from a captive animal received from the Sorocaba Zoo was positive for enterotoxin A.

The results reported in Tables 5 and 6 show the antimicrobial resistance profile of Gram-positive cocci isolated from captive and wild Brazilian Maned-wolf, respectively, and according to body sites investigated.

The only coagulase-positve *S. intermedius* strain was isolated from the oral cavity of a captive animal and showed full resistance towards amoxicillin, penicillin, and

Table 1 - Bacteria isolated from wild life Brazilian Maned-wolf according to body sites investigated.

Microorganisms	Body sites				
	Oral cav	Nasal cav	Auricular av	Prepuce	Perianal
Escherichia coli	X	X	X	X	X
Proteus vulgaris	X	X		X	X
Proteus mirabilis			X		
Pseudomonadaceas		X			
Serratia marcescens					X
CNS	X	X	X	X	X
Micrococcus sp.	X				

Abbreviation: Cav = cavity; CNS: Coagulase negative staphylococci.

Table 2 - Mycologic samples isolated from wild life Brazilian Maned-wolf, according to body sites investigated.

Microorganisms	Body sites				
	Oral cav	Nasal cav	Auricular av	Prepuce	Perianal
Candida albicans	X		X	X	
Aspergillus sp.		X	X	X	X
Penicillium sp.		X		X	X
Trichophyton metagrophyles			X		

Abbreviation: Cav = cavity.

Table 3 - Bacteria isolated from captive Brazilian Maned-wolf, from different geographic regions, according to body sites investigated.

Microorganisms	Body sites				
	Oral cav	Nasal cav	Auricular cav	Prepuce	Perianal
Escherichia coli	X	X	X	X	X
Proteus vulgaris	X	X	X	X	X
Proteus mirabilis		X	X	X	X
Pseudomonadaceas		X	X		
Serratia marcescens					X
Serratia rubidaea					X
CNS	X	X	X	X	X
Staphylococcus intermedius	X				
Micrococcus sp.	X	X			

Abbreviation: Cav = cavity.

Table 4 - Mycologic samples isolated from captive Brazilian Maned-wolf, from different geographic regions, according to body sites investigated.

Microorganisms	Body sites				
	Oral cav	Nasal cav	Auricular av	Prepuce	Perianal
Candida albicans	X		X	X	
Candida parapisilossis		X			
Aspergillus sp.		X	X	X	X
Penicillium sp.		X		X	X
Malassezia pachydermatis			X		
Trichoph metagrophytes			X		
Trichosporon asahii	X	X	X		
Cryptococus laurentii			X		

Abbreviation: Cav = cavity.

Table 5 - Resistance and susceptibility profiles of Gram-negative bacteria colonizing different sites of captive Brazilian Maned-wolf and tested drugs.

Cocci / site	Antibiotic											
	AMO	CLI	CFL	PEN	OXA	TET	AMP	ERI	SUT	GEN	CFO	VAN
	Number of isolates / Reading											
E. coli/ oral	3 / r 6 / i	2 / i										
E. coli / nasal	3 / i	3 / i							6 / i			
E. coli / auricular	3 / r5 / i	5 / i		3 / r1 / i					1 / i			1 / i
E. coli / preputial	3 / i	1 / r2 / i	5 / i	2 / i		1 / r3 / i						
E. coli / anal	1 / r5 / i	2 / r1 / i	3 / i			1 / i						
Proteus vulgaris / oral	1 / r		1 / r			1 / r			1 / r1 / i			
Proteus vulgaris/nasal	2 / i	1 / i							1 / i			
Proteus vulgaris/ auricular	1 / r3 / i	1 / r2 / i	1 / i	1 / i								
Proteus vulgaris/ prepucial	2 / i	2 / r	1 / r2 / i	1 / i		1 / i						
Proteus vulgaris/ anal	2 / r2 / i	1 / r1 / i	2 / r2 / i			1 / i						
Proteus mirabilis/ auricular	3 / i	4 / i		1 / r			1 / i		1 / i			
Proteus mirabilis/nasal	1 / i	1 / i							1 / i			
Proteus mirabilis/ preputial	3 / i	1 / r	1 / r1 / i									
Proteus mirabilis/anal	2 / i	2 / r	1 / i			2 / i						
Pseudomonadacea/ nasal	2 / i					1 / i			1 / i			
Pseudomonadacea/ auricular	2 / i	3 / i	1 / i				2 / i				2 / i	2 / r2 / i
Serratia marscencens/Anal	1 / r	1 / i										
Serratia rubidae/anal	2 / i	1 / i	2 / i									
E. coli/ oral	3 / r6 / i	2 / i										
E. coli / nasal	3 / i	3 / i							6 / i			
E. coli / auricular	3 / r5 / i	5 / i		3 / r1 / i					1 / i			1 / i

r = Resistant; i = intermediary; AMO = amoxicillin; CLI = clindamicin; CFL = cephalotin; PEN = penicillin; OXA = oxacillin; TET = tetracyclyn; AMP = ampicillin; ERI = erythromycin; SUT = cotrimoxazole; GEN = gentamicin; CFO = cephoxitin; VAN = vancomycin.

cotrimoxazole, while showing intermediary resistance profile towards oxacillin. (Tables 3 and 6).

The data showed multirresistant *E. coli* isolated from captive specimens, and bacteria antimicrobial profile from free roaming animals were all sensitive to all drugs tested.

Discussion

In order to contextualize the results found in the present work, one must consider the peculiar way of life led by this species, as described before, especially their natural diet and management choice adopted by each institution in order to offer conditions for the survival of individuals un-

Table 6 - Resistance and susceptibility profiles of Gram-positive cocci colonizing different sites of captive Brazilian Maned-wolf and tested drugs.

Cocci / body site	Antibiotic											
	AMO	CLI	CFL	PEN	OXA	TET	AMP	ERI	SUT	GEN	CFO	VAN
	Number of isolates / Reading											
CNS/*oral*	5 / r	1 / i			3 / r	2 / r1 / i	1 / i	5 /r3 / i	1 / r1 / i	2 / i		
CNS/nasal					2 / i	2 / i		1 / i				
CNS/*auricular*								1 / i				
CNS/*prepucial*						2 / i	1 / i					
CNS/*anal*	3 / r1 / i				1 / r		1 / i			3 / i		
S. intermedius/*oral*	1 / i			1 / r	1 / i				1 / r			
Micrococcus spp./ *oral*	2 / r			2 / r			1 / r2 / i	2 / i	1 / r1 / i	1 / i		
Micrococcus spp./ *nasal*					2 / i	1 / i		2 / i	1 / r1 / i	1 / i		

r = Resistant; i = intermediary; AMO = amoxicillin; CLI = clindamicin; CFL = cephalotin; PEN = penicillin; OXA = oxacillin; TET = tetracyclyn; AMP = ampicillin; ERI = erythromycin; SUT = cotrimoxazole; GEN = gentamicin; CFO = cephoxitin; VAN = vancomycin

der their care. Were proposed a comprehensive study of sources and movements of resistance genes among microorganisms, including physical forces, such as wind and water, and biological forces such as human activities and animals, insects and birds in general (Allen *et al.*, 2010). *C. brachyurus*, also known as "lobo-guará" is an endangered mammal species in the Brazilian wild. The data showed the diversity of microorganisms colonizing the cavities investigated in males specimens of the Brazilian Maned-wolf. The profile of resistance and susceptibility of bacteria to the drugs tested showed important differences between animals in the wild and in captivity. The role of humans or animals, including insects and birds, as carriers and transmitters of resistant bacteria to captive Brazilian Maned-wolves is proposed. *Salmonella*-causing diarrhea in one captive *C. brachyurus* in Brazilian Zoo was reported, but no other animal presented clinical signals of gut disturbance caused by this species (Gilioli and Silva, 2000). Others have highlighted wild animals diseases as a relevant public health issue, such as the case of wild birds carrying enterobacteriaceae *E. coli* and *Salmonella* Typhimurium (Tsiodras *et al.*, 2008). The detection of resistant bacteria in feces from wild Yellow-headed Blackbird was discussed, despite the absence of antimicrobial pressure in the environment (Gibbs *et al.*, 2007). Some of the same antibiotic used to treat human pathogens, such as amoxicillin and erythromycin, are among the drugs used to treat disease, promote growth and improve feed efficiency in animals. (Sarmah *et al.*, 2006). The *E. coli* resistance profile was shown to be affected by several drugs dispersed in an environmental myriad affecting animal wild fauna (Allen *et al.*, 2011). Free range feral swine from Brazilian wetlands presented an expressive colonization of body sites by resistant bacteria which raised the issue of the participation of water in the environment as a dispersing agent of these microorganisms and their resistance profile towards drugs (Lessa *et al.*, 2011). Here we have shown that captive and wild animals presented antibiotic resistant bacteria towards several drugs, including amoxicillin and erythromycin, but no confirmation as to what caused such a resistance, a question which needs additional investigation. Although some authors emphasize ongoing questions as to the influence of antimicrobial agricultural compounds on antimicrobial resistance and subtherapeutic exposure of bacteria, drugs of every important clinical class are utilized in agriculture, and human populations are exposed to antimicrobial-resistant pathogens via consumption of animal products as well as through widespread release into the environment (Silbergeld *et al.*, 2008). It is also known that the selection pressure applied by the antibiotics that are used in clinical and agricultural settings has promoted the evolution and spread of genes that confer resistance, regardless of their origins (Allen *et al.*, 2010). Finally, the study proposes that direct or indirect contact of humans and their subproducts with wild animals, such as the Brazilian Maned-wolf, could foster cross-contamination of captive animals since the resistance profile of bacteria isolated from animals in the wild clearly showed that antimicrobial pressure was insignificant on microorganisms isolated.

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