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Detection of resistance genes and evaluation of water quality at zoo lakes in Brazil

Detecção de genes de resistência e qualidade da água dos lagos de zoológico, Brasil

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ABSTRACT

*The investigation of the presence of antibiotic-resistance genes in aquatic environments is important to identify possible reservoirs of resistant microorganisms that could be a threat to human and animal health. The aims of this study were to analyze the presence of genes conferring resistance to antimicrobials in the aquatic environment and to assess the quality of water in zoo lakes. Results showed a pattern of genes conferring resistance to multiple antibiotics and turbidity, which was expected to be due to the presence of contaminants. The most frequent genes were sul I and sul II (sulfonamides), which were present in all the lakes, followed by genes encoding β-lactamases such as blaPSE I (77.8%) and ampC (66.7%). However, tet(K), tet(M), and ermC genes were not detected. There was a positive correlation between the number of **Enterobacteriaceae** and resistance genes. In conclusion, the source of contamination of all lakes was probably the neighboring urban sewage or wastewater that increased the frequency of the total coliforms and resistance genes, which in turn posed a threat to the conservation of the animal life inhabiting the zoo.*

Key words: *multidrug resistance, zoo pollution, aquatic environment, **Enterobacteriaceae**.*

RESUMO

*A investigação da presença de genes de resistência a antibióticos (ARGs) em ambientes aquáticos é importante para identificação de possíveis reservatórios de microrganismos resistentes que podem ser uma ameaça para a saúde humana e animal. O objetivo deste estudo foi analisar a presença de genes de resistência a antimicrobianos e a qualidade da água de zoológico. Os resultados mostraram um padrão de genes de resistência a múltiplos antibióticos e turbidez da água, devido à presença de contaminantes. Os genes mais frequentes foram sul I e sul II (sulfonamidas) que estão presentes em todos os lagos, e β-lactamases como blaPSE I (77,8%) e ampC (66,7%). Os genes tet(K), tet(M) e ermC não foram detectados. Houve correlação entre o número de **Enterobacteriaceae** e aumento na detecção de genes de resistência. As fontes de contaminação dos lagos são, provavelmente, esgoto urbano vizinho ou de águas residuais que aumentam a presença de coliformes totais e a frequência dos genes de resistência, que podem ser um risco para vida de animais silvestres em cativeiro.*

Palavras-chave: *resistência múltipla, poluição em zoo, ambiente aquático, **Enterobacteriaceae**.*

INTRODUCTION

Water is one of the most important natural resource as it is indispensable for the survival of animal species (DUARTE, 2011). It is considered as a critical factor for the successful performance and production of livestock, affecting various processes including the health status of animals (BARROS et al., 2010). Urban runoff water often has a poor quality and consequently may serve as a vehicle for several antibiotic-resistant microorganisms (GÕNI-URRIZA et al., 2000; DUARTE, 2011).

Pollution in urban zoos usually comes from small point sources (ZHAO et al., 2007). These sources, such as wastewater or untreated sewage, can have a profound effect on the ecological health of streams and reservoirs (DELETIC, 1998; SCHREIBER et al., 2001). Several studies have reported pollution due to runoff from urban-residential

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and industry-dominated areas (GROMAIRE-MERTZ et al., 1999; LEE & BANG., 2000; CHOE et al., 2002). Considering the paucity of data regarding antimicrobial resistance in the aquatic environment and zoos in Brazil as well as throughout the world, this study was aimed to investigate the presence of resistance genes in order to identify the possible reservoirs of resistant microorganisms.

MATERIALS AND METHODS

The study was conducted at the Zoo of Universidade Federal do Mato Grosso (UFMT), located in the city of Cuiabá-MT, Brazil (near residential neighborhoods), which has an area of 11 hectares and is inhabited by approximately 500 animals of 80 different species including birds, reptiles, and mammals.

From November 2013 to April 2014, water samples from nine different points of the zoo were collected as follows: stream before Jaguar's enclosure (P1); stream after the Jaguar's enclosure (P2); Big Lake (P3); Nesting Alligator (P4); monkey's lake (P5); tortoise's enclosure (P6); Albino Alligator's Lake (P7); seasonal spring stream (P8) and Tapir's lake (P9) (Figure 1).

For the detection of resistance genes, approximately 1-L water samples were collected in sterile plastic bags. The samples were filtered in a vacuum pump system (Sigma-Aldrich^{TM}) through a polyethersulfone membrane of 0.2µm pore size (Pall^{TM}), with a maximum interval of 3 hours between sample collection and processing.

The filters were then washed in sterile buffered water, cut into several pieces, placed in polystyrene tubes and resuspended in 1.8mL of SET solution (20% sucrose, 50mM EDTA and 50mM Tris-HCl [pH 8.0]) and subsequently frozen (SOMERVILLE et al., 1989). The DNA was extracted using 360µL of lysozyme buffer (5mg mL^{-1}, 1mM EDTA, 10mM NaCl) and incubated at 37°C for 1h. Sodium dodecyl sulfate (SDS 1.0%) and 25µL of proteinase K (20mg mL^{-1}) were subsequently added and the samples were further incubated at 37°C for 2h with horizontal shaking at 150rpm. Finally, 280µL of CTAB (cetyltrimethylammonium bromide 10%) was added, followed by incubation at 65°C for 20min.

DNA was purified using phenol–chloroform–isoamyl alcohol and precipitated using isopropanol, washed with 70% ethanol and resuspended in ultrapure 200µL water (SOMERVILLE et al., 1989; RIVIERA et al., 2003).

Figure 1 - Stream before Jaguar's enclosure (P1); stream after the Jaguar's enclosure (P2); Big Lake (P3); Nesting Alligator (P4); monkey's lake (P5); tortoise's enclosure (P6); Albino Alligator's Lake (P7); seasonal spring stream (P8) and Tapir's lake (P9).

The detection of 16 resistance genes was performed using the Polymerase Chain Reaction (PCR) in a final volume of 25µL, along with a positive control, and these genes were confirmed by DNA sequencing. The primer sequences of the antimicrobial resistance genes are listed in table 1. The amplification products were analyzed by electrophoresis on 2% agarose gel (Invitrogen™) at 5 volts per cm, stained with Gel

Table 1 - Sequence of oligonucleotides used for amplification of resistance genes.

Target Genes	Sequences of Primers	Amplicon (bp)	Reference
sul I	5' – CGCACCGGAAACATCGCTGCAC – 3' 5' – TGAAGTTCCGCCGCAAGGCTCG – 3'	163	PEI et al. (2006)
sul II	5' – TCCGGTGGAGGCCGGTATCTGG – 3' 5' - CGGGAATGCCATCTGCCTTGAG – 3'	191	PEI et al. (2006)
*amp*C	5' – TCCTATCAAMACTGGCARCC – 3' 5' - CCYTTTTATGTACCCAYGA – 3'	500	SCHWARTZ et al. (2003)
*bla*PSE 1	5' – ACCGTATTGAGCCTGATTTA – 3' 5' – ATTGAAGCCTGTGTTTGAGC – 3	321	JACOBS & CHENIA (2007)
blaZ	5'- ACTTCAACACCTGCTGCTTTC -3' 5'- TGACCACTTTTATCAGCAACC -3'	173	MARTINEAU (2000)
*mec*A	5'- CTAGTAAAGCTCCGGAA -3' 5'- CTAGTCCATTCGGTCCA -3'	314	CHOI et al. (2003)
*fem*A	5'- AAAAAAGCACATAACAAGCG -3' 5'- GATAAAGAAGAAACCAGCAG -3'	132	DURAN et al. (2012)
*msr*A	5'- TCCAATCATTGCACAAAATC - 3' 5'- TCCAATCATTGCACAAAATC - 3'	163	MARTINEAU (2000)
*erm*A	5'- AAGCGGTAAACCCCTCTGA -3' 5'- TTCGCAAATCCCTTCTCAAC -3'	190	STROMMENGER et al. (2003)
*erm*B	5'- CTATCTGATTGTTGAAGAAGGATT -3' 5'- GTTTACTCTTGGTTTAGGATGAAA -3'	142	MARTINEAU (2000)
*erm*C	5'- AATCGTCAATTCCTGCATGT -3' 5'- TAATCGTGGAATACGGGTTTG -3'	299	STROMMENGER et al. (2003)
ant(4')-Ia	5'- AATCGGTAGAAGCCCAA -3' 5'- GCACCTGCCATTGCTA -3'	135	CHOI et al. (2003)
aac(6')-*aph*(2'')	5'- GAAGTACGCAGAAGAGA -3' 5'- ACATGGCAAGCTCTAGGA -3'	491	CHOI et al. (2003)
*aph*3'-IIIa	5'- AAATACCGCTGCGTA -3' 5'- CATACTCTTCCGAGCAA -3'	242	CHOI et al. (2003)
tet(K)	5'- GTAGCGACAATAGGTAATAGT -3' 5'- GTAGTGACAATAAACCTCCTA -3'	360	STROMMENGER et al. (2003)
tet(M)	5'- AGTGGAGCGATTACAGAA -3' 5'- CATATGTCCTGGCGTGTCTA - 3'	158	STROMMENGER et al. (2003)

Red™ (Biotium®) and observed under ChemiDoc XRS™ system using ImageLab™ software. We used a standard molecular weight marker consisting of 100 bases pairs (Fermentas®).

Approximately 2L of water from P3, P4, P5, P6, P7, P8, and P9 was collected in individual bottles and sent for microbiological and physicochemical analyses, following the methodology of the American Public Health Association (RICE et al., 2012). Local data to assess the type of environment, communication between the enclosures, presence, and type of contaminants, and occupation density were collected according to the instructions of IBAMA 169/2008 (BRASIL, 2008). Statistical analysis was performed using software R version 3.0.1. (FOX, 2005; R DEVELOPMENT CORE TEAM, 2011). Associations between the resistance and other variables were calculated using a binomial regression between genes, microbiological examination, and physical and chemical examination. Spearman correlation coefficient was used to assess the association between microbiological analysis (total and thermotolerant coliforms) and the presence of resistance genes. In all cases, p-value ≤ 0.05 was defined as significant.

RESULTS AND DISCUSSION

The results of the microbiological analysis of the total and thermotolerant coliforms revealed high counts of ***Enterobacteriaceae*** in all sampling sites (Table 2), with P5 showing the lowest and P6 showing the highest counts. There was a positive correlation between total microbial (r=0.763, P=0.0457) as well as thermotolerant (r=0.807, P=0.0281) counts and increased numbers of detected antibiotic-resistance genes. Previously it was shown that the discharge of urban waste resulted in an increase in resistant strains and antibiotic-resistance genes, and that these discharges were usually derived from domestic sewage (GÕNI-URRIZA et al., 2000; STOLL et al., 2012).

During the physicochemical analyses (Table 2), changes were observed in turbidity in P3 (90.1NTU), and in OD (Oxygen Demand) (0.2mg L^{-1}) and BOD (Biological Oxygen Demand) (6.4mg L^{-1}) in P6, based on CONAMA Resolution 357/05 (BRASIL, 2005). High turbidity may indicate an excessive amount of microorganisms or organic matter. Moreover, at point P3, the water surface was covered by aquatic vegetation and was associated with a slight change in OD. Although this site has not yet fully undergone eutrophication, care is needed so that

Table 2 - Microbiological, physicochemical and epidemiological characteristics in water samples from collection points of UFMT Zoo, November /2013 - April /2014.

	P3	P4	P5	P6	P7	P8	P9
Total Coliforms (nº $100mL^{-1}$)	$1.1x10^6$	$8.9x10^5$	$7x10^4$	$1x10^8$	$7.6x10^6$	$9.5x10^5$	$3x10^5$
Thermotolerant Coliforms (nº $100mL^{-1}$)	$6.4x10^4$	$7.5x10^4$	$5.5x10^3$	$3.6x10^6$	$2.2x10^5$	$6.4x10^4$	$2.3x10^4$
Turbidity(NUT)	90.1	30.3	24.8	2.9	38.7	11.7	20.2
Color (U.H.)	34.2	19.6	54.6	28.8	27	41.4	15.9
pH	6.8	7.38	8.41	6.96	7.45	7.86	7.14
OD (mg L^{-1} O_2)	5.5	7.1	6.40	0.2	6.8	7.3	7.1
BOD 20,5 (mg L^{-1} O_2)	2.4	1.6	4	6.4	1.2	0.9	3.6
Total alkalinity (mg $CaCO_3$ L^{-1})	104.3	93.8	62	116.8	93.2	63.6	110.6
Total Hardness (mg aCO_3 L^{-1})	141.3	114.8	76.2	124.7	99.4	79.8	120.6
Suspended Solids (mg L^{-1})	225	147	118	54	130	94	87
Total Nitrogen (mg L^{-1})	0.44	0.4	0.35	0.53	0.46	0.27	0.38
Nitrate (mg L^{-1})	0.12	0.12	0.10	0.18	0.15	0.09	0.16
Phosphorus (mg L^{-1})	0.04	0.05	0.03	0.07	0.05	0.02	0.04
Environment	Lk	Lk	Lk	Hs	Lk	Hs	Lk
Enclosure's communication	+	+	+	+	+	-	+
Animal density	Hi	Hi	Hi	Hi	Lo	Hi	Lo
External contaminats	Ww	-	Ww	Ww,Se	-	Ww	-

Legend: OD: Oxygen demand; BOD: Biological oxygen demand; Lk = lake, Hs = headspring, + =presence, - =absence, Hi = High, Lo = Low, Ww = wastewaters, Se = Sewage. P1 and P2 microbiological analysis were not performed because high cost and it had no contact with zoo animals.

the local situation does not get worse. In a similar previous study, changes in OD and BOD were reported at some sites, especially where there was a discharge of wastewater (GÕNI-URRIZA et al., 2000).

All the nine samples tested were positive for resistance to at least three of the antimicrobial classes. Moreover, 13 of the 16 antimicrobial-resistance genes tested were detected; only *ermC*, *tet(K)*, and *tet(M)* were not detected.

The highest gene frequency was observed in P6 (62.5%), followed by that in P1 (50%), P3 (50%), and P4 (50%), as depicted in table 3. A pattern of genes conferring resistance to multiple antibiotics was expected due to the presence of external contaminants (wastewater and sewage) discharged into the zoo lakes. The presence of resistance genes are linked to the presence of sewage and other sources of contamination such as residual water, which increases the amount of resistant bacteria (GÕNI-URRIZA et al., 2000).

Sulfonamide-resistance genes (*sul I* and *sul II*) were present in all samples. COLUMER-LLUCH et al. (2014) demonstrated that the *sul I* gene was more prevalent in water samples in Tunisia and DOBIASOVA et al. (2013) reported that the *sul I* and *sul II* genes were mostly detected in ***Escherichia coli*** isolates from stool samples from zoo animals. Although sulfonamides are widely used in veterinary medicine, their use is even more widespread in human medicine (SKÖLD, 2000). Since the genes encoding resistance to sulfonamides are present in all lakes, it is probable that contamination of these lakes occurred from human contaminants.

β-Lactam-resistance genes were detected in all samples, except P7. The frequency of resistance genes varied between 77.8% (*blaPSE I*) and 11.1% (*blaZ*). Studies have also demonstrated the presence of the β-lactamases (*ampC*) genes from the DNA samples of biofilms in wastewater treatment stations, drinking water, and hospital water (SCHWARTZ

Table 3 - Results of antimicrobial class's analyses and resistance genes detection in water samples from UFMT Zoo, November /2013 - April /2014.

Class	gene	Samples									Positive (%)
		P1	P2	P3	P4	P5	P6	P7	P8	P9	
Sul	sul I	x	x	x	x	x	x	x	x	X	9 (100)
	sul II	x	x	x	x	x	x	x	x	X	9 (100)
β-Lac	ampC	x	x	x	x		x		x		6 (66.7)
	blaPSE I	x		x	x	x	x		x	x	7 (77.8)
	blaZ								x		1 (11.1)
Meth	mecA				x	x	x	x		x	5 (55.6)
	femA	x	x	x	x					X	5 (55.6)
	msrA	x	x		x		x	x			5 (55.6)
Macro	ermA	x		x	x		x				4 (44.4)
	ermB	x					x	x	x		4 (44.4)
	ermC										0 (0)
Amin	ant(4')-Ia						x	x			2 (22.2)
	aac(6')-aph(2")			x			x	x	x		4 (44.4)
	aph3'-IIIa			x		x				x	3 (33.3)
Tetra	tet(M)										0 (0)
	tet(K)										0 (0)
nº genes		8	5	8	8	5	10	7	7	6	-
nº class		4	3	5	4	4	5	4	4	4	-

Legend stream before Jaguar's enclosure (P1); stream after the Jaguar's enclosure (P2); Big Lake (P3); Nesting Alligator (P4); monkeys lake (P5); tortoise's enclosure (P6); Albino Alligator Lake (P7); seasonal spring stream (P8) and Tapir's lake (P9). Sulf: Sulfonamides; β-Lac: β-Lactamics; Meth: Methicillin; Macro: Macrolides; Amin: Aminoglycosides; Tetra: Tetracyclines.

et al., 2003), probably because of the presence of ***Enterobacteriaceae*** from wastewater or sewage contamination (HENRIQUES et al., 2006).

Among the macrolide-resistance genes, the *ermC* gene was earlier reported to be isolated from the dominant ***Staphylococcus epidermidis*** from biofilms (HONG-JING et al., 2014) and *ermA* and *ermC* genes from samples of a wastewater treatment station (FARIA et al., 2009). Contrary to this, no samples were positive for the *ermC* gene in the present study.

The *aac(6')-aph(2")*, *aph3'-IIIa* and *ant(4')-Ia* genes have been previously detected in ***Staphylococcus aureus*** isolates (DURAN et al., 2012). However, in the present study, the low rate of detection of *aph3'-IIIa* and *ant(4')-Ia* genes and absence of *ermC*, *tet(K)* and *tet(M)* may have been observed since no bacterial isolation was performed. Another factor to be considered in this regard is the low concentration of bacteria hosting these resistance genes (SCHWARTZ et al., 2003).

The *tet(K)* and *tet(M)* genes that encode tetracycline resistance were not detected in this study, probably because of the geographical location, as has also been previously described for rivers in German and Australia (STOLL et al., 2012). This result may be associated with the fact that these genes are not expected in aquatic environments because of the precipitation and/or hydrolysis of tetracyclines and penicillins in such environments (KEMPER, 2008).

CONCLUSION

Zoo lakes serve as reservoir for multiple antibiotic-resistance genes, which could be transferred to animal and human pathogens. The source of contamination of all these lakes is probably the neighboring urban sewage or wastewater. Thus, the findings of this study may assist in developing strategies to avoid the spread of antibiotic-resistant bacteria.

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