Antimicrobial resistance genes and bacterial diversity in sediments of Guandu River, Brazil

Rivers provide essential water resources for humans. However, appear to be reservoirs of antimicrobial-resistant genes and dispersal routes of resistant pathogens because human activities promptly influence them. Sediments are the most propitious substrates for bacteria survival and can act as an antimicrobial reservoir. Three sediment samples were collected upstream of the water catchment point of the Guandu Water Treatment Plant (ETA Guandu) to evaluate microbial diversity and antimicrobial resistance genes to sulfonamide (sul1, sul2), tetracycline (tetA, tetB) and beta-lactam (blaCTX-M, blaoxa24 and blaoxa58) by PCR. The most abundant phylum in all samples was Proteobacteria, which was also the most dominant in sediments presenting a pattern already reported in the literature for diversity in tropical and temperate climate rivers. Sulfonamide resistance genes were detected in all samples. The blaCTX-M gene was detected only in one sample, and tetA, tetB, blaoxa24 and blaoxa58 genes were not found in any sample. The presence of antimicrobial resistance genes sul1, sul2, and blaCTX-M indicate that sediments can act as a reservoir of resistance. These findings will provide new data about the bacterial community and antimicrobial resistance genes in Brazilians river sediments.

Keywords: Beta-lactam; Microbial diversity; Next generation sequencing; Sulfonamide; Tetracycline.

Genes de resistência a antimicrobianos e diversidade bacteriana em sedimentos do Rio Guandu, Brasil

Os rios fornecem recursos hídricos essenciais para os seres humanos. No entanto, eles podem ser reservatórios de genes de resistentes a antimicrobianos e rotas de dispersão de patógenos resistentes, uma vez que as atividades humanas os influenciam prontamente. Os sedimentos são os substratos mais propícios para a sobrevivência das bactérias e podem atuar como reservatórios de antimicrobianos. Três amostras de sedimentos foram coletadas a montante da captação de água da Estação de Tratamento de Água do Guandu (ETA Guandu) para avaliar a diversidade microbiana e a presença de genes de resistência à sulfonamida (sul1 e sul2), tetraciclina (tetA e tetB) e beta-lactâmicos (blaCTX-M, blaoxa24 e blaoxa58) por meio de PCR. O filo mais abundante em todas as amostras foram Proteobacteria, que também foi o mais dominante em sedimentos apresentando um padrão já relatado na literatura para diversidade em rios de clima tropical e temperado. Genes de resistência às sulfonamidas foram detectados em todas as amostras. O gene blaCTX-M foi detectado apenas em uma amostra, e os genes tetA, tetB, blaoxa24 e blaoxa58 não foram encontrados em nenhuma amostra. A presença dos genes sul1, sul2 e blaCTX-M indicam que os sedimentos podem atuar como reservatórios de resistência. Essas descobertas fornecerão novos dados sobre a comunidade bacteriana e genes de resistência a antimicrobianos presentes nos sedimentos fluviais brasileiros.

Palavras-chave: Beta-lactâmicos; Diversidade microbiana; Sequenciamento de nova geração; Sulfonamida; Tetraciclina.

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INTRODUCTION

The emergence of antimicrobial resistance genes represents a significant global health problem (TAYLOR et al., 2011). The magnitude of the problem worldwide affects environmental, animal and human health (WHO, 2014). Antimicrobial resistance has recently been widely examined from an environmental perspective, projecting new light on a problem that has traditionally been confined to a subset of clinically relevant resistant bacterial pathogens. Studies have revealed a crucial role of the environment in the spread and evolution of antimicrobial resistance in bacteria (BONDARCZUK et al., 2019).

Among the various environments, river systems have been received effluents from industrial and human activities and represent a unique scenario for the acquisition and dissemination of antimicrobial resistance genes (ARGs) and the proliferation of resistant bacteria (CHEN et al., 2019). Sediments are fundamental to understand the phenomenon of resistance in water systems. According to Chandran et al. (2011, cited by OHORE et al., 2019), "bacteria survive longer in sediments than in the water column, as they provide beneficial conditions for their nutrition and have a higher desorbing capacity of some antimicrobials". The presence of antimicrobials, heavy metals, and other residues might select different bacterial groups and subsequently change the microbial diversity and the antimicrobial resistance profile (OHORE et al., 2019). Rivers not only act as the pools of ARGs but also become the dispersal routes of resistant pathogens, increasing the risks for transmission back to humans (MARATHE et al., 2017; LARSSON et al., 2018).

The Guandu River and its hydrographic basin occupy 3,600 km, covering 15 municipalities in the Metropolitan and South-Central Regions of the State of Rio de Janeiro, Brazil. This basin is a peculiar characteristic, with no other example in the country, because much of the volume of water that composes it originates from the transposition of, on average, 120 m³ s^{-1} of the Paraíba do Sul River, in the municipality of Barra do Piraí (GUANDU COMMITTEE, 2012). The Guandu drinking water treatment plant supplies water to 10 million inhabitants of Rio de Janeiro's metropolitan region. However, the Paraíba do Sul System – Guandu is inserted in a region of high population and industrial occupation, also with significant agricultural production, with many environmental problems occurring: the natural release of domestic sewage in their water bodies, abrupt and irregular expansion of urban areas, pollution by industrial waste, mineral extraction (mainly sand), contamination by agricultural pesticides, deforestation of riparian forests (GUANDU COMMITTEE, 2012).

Independent cultivation techniques based on nucleic acids have been increasingly used in Environmental Microbiology studies. They allow reaching a larger portion of the bacterial community in their natural habitat since cultivable microorganisms represent less than 1% of the soil's microbial population (AMANN et al., 1995). We can extrapolate this advantage to studies of antimicrobial resistance where we can directly access antimicrobial resistance genes from the DNA of a broader range of soil bacteria and not only those grown in the culture medium. Therefore, this study aimed to evaluate antimicrobial resistance genes and microbial diversity in the Guandu River's sediments in Nova Iguaçu, Brazil, using the independent cultivation technique based on the nucleic acid.

MATERIALS AND METHODS

Sample collection

Three sediment samples were collected upstream from the water catchment point of ETA Guandu (Water Treatment Plant), on the border between Nova Iguaçu and Seropédica, using a sterile collector. After the collection, the samples were stored in sterile plastic bags. The samples were transported in a refrigerated container and subsequently stored at -20ºC.

Total soil DNA extraction

Total DNA extraction from soil samples was performed using the DNA PowerSoil kit (MO BIO, 2016), according to the protocol provided by the manufacturer. After extraction, the samples were stored at -20°C, and the quantity and quality of the DNA obtained were evaluated by spectrophotometer (THERMO SCIENTIFIC, 2010).

Amplification of the 16S rRNA and antimicrobial resistance genes by PCR (Polymerase Chain Reaction)

PCR reactions were optimized using initial parameters: Buffer 1X, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 0.4 µM of each primer, 1 U Taq DNA Polymerase, about 20 ng of total DNA, and ultra-pure water to complete the total volume of 25 µl. The primers selected and used are described in Table 1, as well as their sequences and fragment sizes.

Table 1: Sequence of primers and size of fragments generated in the amplification of the *16S rRNA* gene and resistance genes *sul*1, *sul*2, *tet*A, *tet*B, bla_{CTX-M}, bla_{oxa24} and bla_{oxa58}.

Gene	Primer	Sequence	Fragment	Reference
16S rRNA	27F	AGAGTTTGATCCTGGCTCAG	1500 pb	Lane (1991)
	1512R	ACGGCTACCTTGTTACGACT		
*sul*1	Sul 1 F	CGCACCGGAAACATCGCTGCA	162 pb	Pei et al. (2006)
	Sul 1 R	TGAAGTTCCGCCGCAAGGCTC		
*sul*2	Sul 2 F	TCCGGTGGAGGCCGGTATCTGG	190 pb	Pei et al. (2006)
	Sul 2 R	CGGGAATGCCATCTGCCTTGAG		
tetA	tetA F	GCGCGATCTGGTTCACTCG	164 pb	Aminov et al. (2004)
	tetA R	AGTCGACAGYRGCGCCGGC		
tetB	tetB F	TACGTGAATTTATTGCTTCGG	206 pb	Aminov et al. (2004)
	tetB R	ATACAGCATCCAAAGCGCAC		
bla_{CTX-M}	bla_{CTX-M} F	AAAAATCACTGCGCCAGTTC	862 pb	Geser et al. (2012)
	bla_{CTX-M} R	CCGTCGGTGACGATTTTAGCC		
oxa-24	oxa-24 F oxa-24 R	CAGTGCATGTTCATCTATT TCTAAGTTGAGCGAAAAG	702 pb	Chen et al. (2018)
oxa-51	oxa-51 F oxa-51 R	TCCAAATCACAGCGCTTCAAAA TGAGGCTGAACAACCCATCCA	639 pb	Chen et al. (2018)
oxa-58	oxa-58 F oxa-58 R	AAGTATTGGGGCTTGTGCTG CCCCTCTGCGCTCTACATAC	599 pb	Chen et al. (2018)

The protocol followed in the thermocycler for amplification of the 16S rRNA gene was: 5 minutes of initial denaturation at 95 ºC, 40 cycles of 95 ºC for 30 seconds, 60 ºC for 30 seconds and 72 ºC for 30 seconds, followed by a final elongation at 72 ºC for 7 minutes. The following parameters were used to amplify the sulfonamide resistance genes: 5 minutes of initial denaturation at 95 ºC, 40 cycles of 95 ºC for 30 seconds, 60 ºC for 30 seconds and 72 ºC for 30 seconds, followed by a final elongation at 72 ºC for 7 minutes. For the

*tet*A and *tet*B genes, the amplification condition followed from 5 minutes of initial denaturation to 94°C, 40 cycles of 94°C for 5 seconds and 61°C for 30 seconds, followed by a final elongation of 61° for 7 minutes. The *bla*$_{CTX-M}$ gene the amplification condition followed from 5 minutes of initial denaturation to 94 ºC, 40 cycles of 94 ºC for 1 minute, 55 ºC for 1 minute and 72 ºC for 1 minute, followed by a final elongation at 72 ºC for 5 minutes. The *bla*$_{oxa24}$ and *bla*$_{oxa58}$ genes the amplification condition followed from 5 minutes of initial denaturation to 94 ºC, 40 cycles of 94 ºC for 1 minute, 56 ºC for 30 seconds and 72 ºC for 1 minute, followed by a final elongation at 72 ºC for 10 minutes. The positive control of PCR reactions was obtained by extracting DNA from antimicrobial-resistant strains of *Escherichia coli* isolated from chickens from poultry farms located in Rio de Janeiro.

PCR products were separated by electrophoresis in 1.5% agarose gel containing SYBR Green dye (Life Technologies). The images were captured under UV light using the L-PIX EX (Loccus Biotecnologia) photo documentation system.

Library preparation, sequencing of the 16s rRNA gene, and bioinformatics analysis

The preparation of 16S rRNA library with amplicons of the variable region V3-V4 of the gene encodes 16S rRNA generated with amplification with primers Bakt_341F (CCTACGGGNGGCWGCA) and Bakt_805R (GACTACHVGGGTATCTAATCC) (PARADA et al., 2016) and sequencing in a 2x250 paired-end system on the MiSeq platform (Illumina, USA) were performed by Macrogen, Inc. (dna.macrogen.com).

The raw sequencing data were processed using the Mothur v.1.41.3 software (SCHLOSS et al., 2009). The forward and reverse sequences were paired in contigs. The sequences smaller than 245 base pairs (bp) or greater than 275 bp were removed, which contained ambiguity or more than eight homopolymers. Then, the sequences were aligned using a Silva database as a reference (QUAST et al., 2012). The resulting alignment was submitted to the screen.seqs and filter.seqs commands to remove misaligned sequences and non-informational columns in alignment. Then, the sequences were pre-grouped using the pre.cluster command using the parameter diffs=2. Chimeric sequences were detected and removed using the chimera.vsearch and remove.seqs commands, respectively. The resulting sequences were classified according to version 16 of the Ribosomal Database Project (COLE et al., 2009) using the classify.seqs command with a confidence threshold of 80. Sequences identified as chloroplasts, mitochondria, eukaryotes or Archaea and those identified as unknown realms were also removed. After normalizing the samples for the same number of sequences, these were grouped into operational taxonomic units (OTUs) using the cluster.split command, with a dissimilarity threshold of 3%. The rare OTUs were removed using the split.abund command and finally, the distribution of OTUs, the richness indexes of Chao (1984), of Shannon (2001) and Simpson (1949) and the rarefaction curves were calculated.

RESULTS AND DISCUSSION

The 16S rRNA gene was detected in all soil samples (Table 2), evidencing that there is DNA in quantity

and quality for PCR amplification of genes encoding antimicrobial resistance.

Table 2: Detection of 16S rRNA gene and antimicrobial resistance genes bla_{CTX-M}, bla_{oxa24}, bla_{oxa58}, $sul1$, $sul2$ tetA, and tetB in Guandu river sediments.

Samples	16S rDNA	bla_{CTX-M}	bla_{OXA24}	bla_{OXA58}	$sul1$	$sul2$	tetA	tetB
			Resistance genes					
A	+	-	-	-	+	+	-	-
B	+	+	-	-	+	+	-	-
C	+	-	-	-	+	+	-	-

Presence (+) and absent (-) of gene.

The genes encoding resistance to sulfonamides $sul1$ and $sul2$ were detected in all three samples. The genes encoding resistance to tetracyclines tetA and tetB and the bla_{oxa24} and bla_{oxa58} genes were not detected in any samples. The bla_{CTX-M} gene (responsible for CTX-M beta-lactamase production) was detected only in one sample (sample B).

Studies indicate that the most abundant and diverse antimicrobial resistance genes in the river system are associated with drugs widely used in this area, suggesting dissemination due to selective pressure resulting from antimicrobials (CHEN et al., 2019). Furthermore, various contaminants such as metals, biocides, and other pollutants may accumulate in the sediments that act as a reservoir of pollutants and inadvertently promote resistant bacteria's persistence (CHENG et al., 2014). It indicates that river sediments may be one of the crucial reservoirs of antimicrobial resistance due to the selective pressure resulting from discarding the waste from industries, from the urban and agricultural in the river.

OTU rarefaction curves tended to reach the plateau with the total number of sequences analyzed (Figure 1), which indicates that the sampling effort was sufficient to cover the existing diversity. That is, the increase in value would have a minimal impact on the number of OTUs (HUGHES et al., 2005).
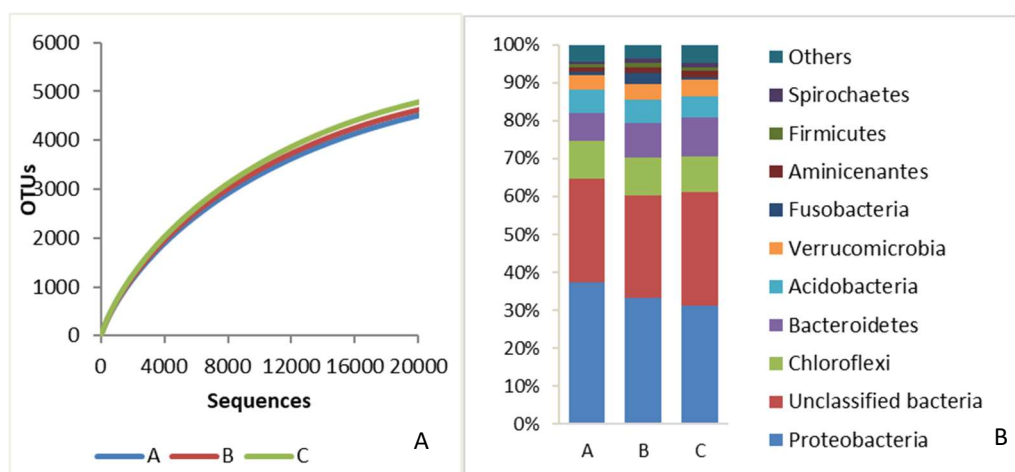

Figure 1: (A) OTU rarefaction curves with 97% similarity per sample of Guandu River sediments. (B) Bacterial phyla with an average relative abundance higher than 1% found in Guandu river sediments.

The dominant bacterial phylum in sediments was Proteobacteria, followed by Chloroflexi, Bacteroidetes, and Acidobacteria. The results of diversity in temperate and tropical climate rivers present a pattern already reported in the literature, and the phylogenetic groups are the most abundant (KÖCHLING et al., 2017).

Proteobacteria are the most common, being the dominant phylum in the riverine system. According to Köchling et al. (2017) Chloroflexi and Acidobacteria phyla can be almost exclusively found in sediments. Bacteria of the class Gammaproteobacteria have colonized several different environments because they present wide metabolic diversity (WANG et al., 2012). They can be found on the surface of sediments and are associated with environments rich in organic matter and being involved with sulphur reduction (HALLER et al., 2011; WANG et al., 2012). It is common in more polluted environments to find bacteria of the Beta and Deltaproteobacteria classes, where the latter class is typical to find sulphate and iron reducers (FeIII); betaprotobacteria are degrading and recalcitrant, but both are found in contaminated sediments (HALLER et al., 2011).

Chloroflexi phylum's presence abundantly in three sediment samples is suggested for its essential role in the degradation of organic material in the environment (KÖCHLING et al., 2017). Acidobacteria has its abundance related to specific pollutants, and in other reports, it was a predominant phylum in the analysis of microbial diversity of sediments with the Chloroflexi (KÖCHLING et al., 2017).

Regarding the phylum Bacteriodetes, they are saprophyte organisms, specialized in the mineralization of organic material, directly involved in its degradation contributing to the flow of carbon within the ecosystem (THOMAS et al., 2011). They are found as the river's depth increases, concluding that they are efficient in degrading macromolecules (LAPÉBIE et al., 2019). The presence of this phylum, Bacterioidales and Bacteroides are associated with high contamination with organic matter, especially fecal contamination (KÖCHLING et al., 2017).

Fusobacteria is more found in the oral cavity and present in intestinal tracts (OLSEN, 2014). The discharge of raw sewage at the sampling point suggests that the family's presence is mainly associated with domestic effluent. In the section of The Fusobacterias Family, Olsen (2014) shows that some fusobacterias "as *F. nucleatum* exhibited a significant portion of beta-lactamase producers, which may deteriorate treatment with beta-lactam antimicrobials". A gene that confers resistance to beta-lactams was found in one of our samples.

Studies indicate that beta-lactamase production increases in some Bacteroides and Fusobacteria, showing a great one health concern about which antimicrobial would still be suitable for future infections caused by these bacteria (OLSEN, 2014). These results express a significant concern that riverine systems could act as a reservoir of antimicrobial resistance.

CONCLUSIONS

Proteobacteria was the most abundant phylum, presenting a pattern reported in the literature for diversity in tropical and temperate climate river sediments. The presence of antimicrobial resistance genes *sul*1, *sul*2, and *bla*$_{CTX-M}$ indicate sediments can act as a reservoir of antimicrobial resistance, and the waste from industries, from the urban and agricultural environment, can increase the persistence of resistance in bacterial communities through the selection pressure of antimicrobials, biocides and heavy metals. These

findings provide new data about the bacterial community and antimicrobial resistance genes in Brazilians river sediments.

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