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Identification and antibiotics resistance profile of gram-negative bacteria isolated from sediment Anil river in Maranhão, Brazil

Identificação e perfil de resistência à antibióticos de bactérias gram-negativas isoladas do sedimento do rio Anil no Maranhão, Brasil

Identificación y perfil de resistencia a antibióticos de bacterias gramnegativas aisladas de sedimentos del río Anil en Maranhão, Brasil

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ABSTRACT
The Manguezal ecosystem suffers impacts from human anthropic actions, which can cause transformations in the microbiota and selection of antibiotic-resistant variants. Thus, this study aimed to isolate and identify gram-negative bacteria to characterize the resistance genes to β-lactam antibiotics from sediment samples from the Anil River – MA (Manguezais area). The bacterial isolate was obtained from sediment samples plated on Macconkey Agar supplemented with antibiotics ceftazidime and meropenem. MALDI-QTOF identified the isolated bacterial species. The bacterial isolates were subjected to the Kirby-Bauer method to evaluate the antibiotic susceptibility profile. The PCR technique detected the genetic determinants. This study identified three and two bacteria; 20 isolates were classified as *Ochrobactrum intermedium*, 09 *Ochrobactrum tritici*, 02 *Stenotrophomonas maltophilia,* and 01 *Klebsiella pneumoniae*. In antibiotic susceptibility tests, it was observed that 100% (n=32) of the isolates were resistant to cefepime, 96.8% (n=31) to ofloxacin and ceftriaxone, and 90.6% (n=29) to imipenem. The molecular profile showed that 50% (n=16) presented the *bla*$_{GES}$ gene. In addition, genes belonging to the *bla*$_{OXA}$ profile were identified in 21 bacteria isolates from the genus *Ochrobactrum*, with the *bla*$_{OXA1}$ gene being the most prevalent among the isolates, identified in 11 bacterial lines. This study confirms that environments such as mangroves, which act as reservoirs of bacteria resistant to multiple antimicrobial drugs for clinical use, become a risk to human health.

Keywords: Antibiotics. Mangrove. Bacteria. Resistance Genes. Environmental.

RESUMO
Ecossistema manguezal sofre impactos com as ações antrópicas do homem, isso pode provocar transformações na microbiota e seleção de variantes

resistentes à antibióticos. Sendo assim, o objetivo deste estudo foi isolar e identificar bactérias gram-negativas, caracterizar os genes de resistência a antibióticos β-lactâmicos de amostras de sedimentos do Rio Anil – MA (área de Manguezais). O isolamento das bactérias foi obtido a partir de amostras de sedimento plaqueadas em meio Ágar Macconkey suplementado com antibióticos ceftadizima e meropenem. As espécies de bactérias isoladas foram identificadas pelo MALDI-QTOF. Os isolados bacterianos foram submetidos ao método de *Kirby-Bauer para avaliação do perfil de suscetibilidade à antibióticos.* Os determinantes genéticos foram detectados pela técnica de PCR. Neste estudo, um total de trinta e duas bactérias foram identificadas, dentre estas 20 isolados sendo classificados como *Ochrobactrum intermedium,* 09 *Ochrobactrum tritici,* 02 *Stenotrophomonas maltophilia e* 01 *Klebsiella pneumoniae*. Nos ensaios de suscetibilidade aos antibióticos foi observado que 100% (n=32) dos isolados eram resistentes à cefepima, 96,8% (n=31) para ofloxacina e ceftriaxona e de 90,6% (n=29) para imipenem. O perfil molecular mostrou que 50% (n=16) apresentaram o gene *bla*$_{GES}$. Em adição, genes pertencentes ao perfil *bla*$_{OXA}$, foram identificados em 21 isolados de bactérias do gênero *Ochrobactrum, sendo que o gene bla*$_{OXA1}$ foi o que prevaleceu entre os isolados, sendo identificado em 11 linhagens bacterianas. O presente estudo confirma que ambientes como manguezais, atuam como reservatórios de bactérias resistente a múltiplas drogas antimicrobianas de uso clínico, estas por sua vez se tornam um risco para a saúde humana.

Palavras-chave: Antibióticos. Mangue. Bactérias. Genes de Resistência. Ambiental.

RESUMEN

El ecosistema Manguezal sufre impactos por acciones antrópicas humanas, que pueden provocar transformaciones en la microbiota y selección de variantes resistentes a los antibióticos. Así, este estudio tuvo como objetivo aislar e identificar bacterias gramnegativas para caracterizar los genes de resistencia a los antibióticos β-lactámicos a partir de muestras de sedimentos del río Anil – MA (área de Manguezais). El aislado bacteriano se obtuvo de muestras de sedimento sembradas en Macconkey Agar suplementado con antibióticos ceftazidima y meropenem. MALDI-QTOF identificó las especies bacterianas aisladas. Los aislados bacterianos fueron sometidos al método de Kirby-Bauer para evaluar el perfil de susceptibilidad a los antibióticos. La técnica de PCR detectó los determinantes genéticos. Este estudio identificó tres y dos bacterias; Se clasificaron 20 aislamientos como Ochrobactrum intermedium, 09 *Ochrobactrum tritici*, 02 *Stenotrophomonas maltophilia* y 01 *Klebsiella pneumoniae*. En las pruebas de sensibilidad a los antibióticos se observó que el 100% (n=32) de los aislados fueron resistentes a cefepima, el 96,8% (n=31) a ofloxacino y ceftriaxona, y el 90,6% (n=29) a imipenem. El perfil molecular mostró que el 50% (n=16) presentó el gen *bla*$_{GES}$. Además, se identificaron genes pertenecientes al perfil *bla*$_{OXA}$ en 21 aislados de bacterias del género *Ochrobactrum*, siendo el gen *bla*$_{OXA1}$ el que predominó entre los aislados, identificándose en 11 linajes bacterianas. Este estudio confirma que ambientes como los manglares, que actúan como reservorios de bacterias resistentes a múltiples fármacos antimicrobianos de uso clínico, se convierten en un riesgo para la salud humana.

Palabras clave: Antibióticos. Mangle. Bacterias. Genes de Resistencia. Ambiental.

1 INTRODUCTION

Mangrove areas comprise about 60 to 70% of the coastline in Earth's tropical and subtropical regions. Brazilian mangroves account for 8.5% of total mangroves on the planet, the second country in extension of mangroves worldwide (Andreote; *et al.*, 2012; Spalding; Kainuma; Collins, 2010). Mangroves suffer impacts related to anthropic actions, such as sewage, garbage, and industrial waste dumping, port, fishing activities, and the extraction of minerals and oil (Freitas; Capeti; Sampaio, 2017). These activities impact the biogeochemical process of this ecosystem (Chakraborty *et al.*, 2015).

Microbial composition and structure are known to be influenced by biotic and abiotic factors in the mangrove sediments (Chakraborty *et al.*, 2015). The microorganisms suffer a constant adaptive pressure due to the characteristics found in the mangrove (Chakraborty *et al.*, 2015). Furthermore, there is more likely to be a greater combination and dissipation of resistance genes in aquatic environments, as these environments are constantly subject to human intervention (Von Wintersdorff *et al.*, 2016).

Genetic factors mediate horizontal gene transfer (HGT), facilitating evolution and adapting bacterial genomes to environmental changes (Von Wintersdorff; *et al.*, 2016). Through transfer processes such as conjugation, transformation, and transduction, mobile genetic elements (MGEs) (bacteriophages, plasmids, transposons, and integrons) mediate the transfer of DNA sequences between bacterial species (Blair *et al.*, 2015; Hu *et al.*, 2015). Genomic analysis has shown that antibiotic resistance genes (ARGs) appear abundantly in strains of environmental and clinical bacteria (Yang *et al.*, 2019). In recent decades, numerous studies have highlighted the abusive use of antibiotics in clinical treatment, aquaculture, and livestock farming, improving the rapid dissemination and transfer of ARGs in clinical and natural settings (Liu *et al.*, 2023). Coastal mangrove ecosystems always serve as a natural receptor of sewage that always

carries ARGs and high antibiotic concentrations. In that context, some evidence exists that some clinically relevant resistance genes originated in natural environmental microbes (Jiang *et al*., 2021; Zhao; *et al.*, 2019).

Bacteria found in the environment act as receptors for genes associated with antibiotic resistance and can subsequently infect humans (Bem *et al.*, 2019).Opportunistic pathogens found in the soil, such as *Ochrobactrum intermedium*, were reported as reservoirs for resistance to quinolones, sulfonamides, tetracyclines, macrolide,s and aminoglycoside streptomycin genes, as well as β-lactamase AmpC, which confers resistance to β-lactam antibiotics (Johnning *et al.*, 2013).

The members of the Enterobacteriaceae family are composed of gram-negative bacteria such as the genera *Escherichia*, *Enterobacter*, *Klebsiella*, *Citrobacter*, *Salmonella*, and *Shigella*, which are related to severe infections in hospitalized individuals or individuals in the general community (Tanner *et al.*, 2019). In turn, species of the *Ochrobactrum* are being considered emerging opportunistic pathogens, which have gained visibility by presenting a profile of resistance to multiple antimicrobial drugs (Caron; *et al.*, 2018; Yagel; *et al.*, 2020).

The present study aimed to isolate and identify gram-negative bacteria from mangrove sediments from Rio Anil-MA, characterize the molecular mechanisms involved in spreading resistance genes from Gram-negative bacteria, and evaluate the presence of integrons associated with the spread of gene resistance.

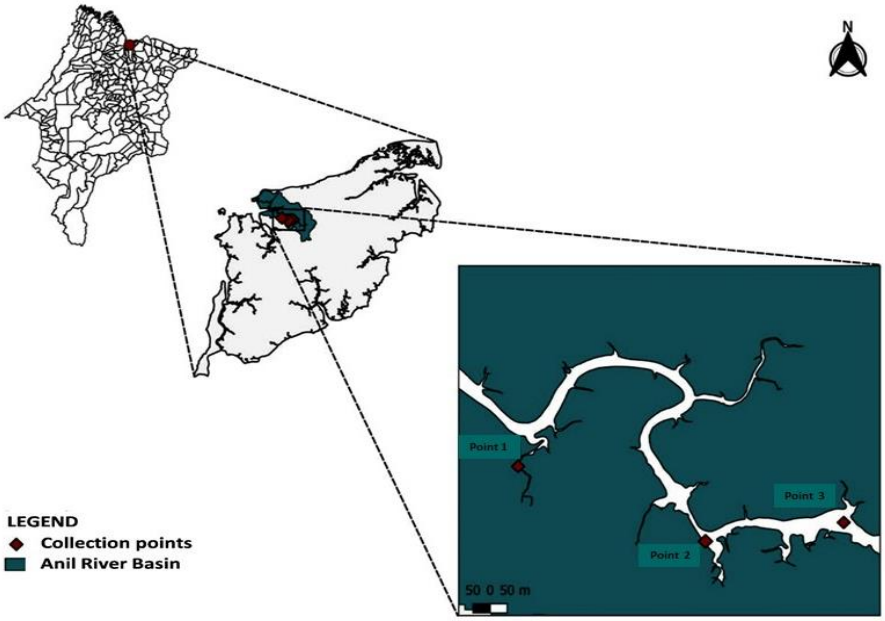
2 METHODOLOGY

2.1 STUDY AREA

The Anil River basin is located in the Northwest portion of São Luís between the latitudes 02º29'14 ”and 02º34'47” S and the longitudes 44º19'15 ”and 44º12'55” W, limited by the ocean basins to the North, with the Rio Paciência basin to the east, the Bacanga to the south and the São Marcos bay to the northwest. Its springs are located in Chapada do Tiririca (District Aurora), descending

at sea level approximately 9.5 km straight, with the directional axis oriented from SE to NW from the source (Alcântara; Mochel; Amorim; Thevand). The collection points this research occurred in strategic areas of the Ipase, Liberdade, and Vila Palmeira neighborhoods in three different points (Figure 1). These neighborhoods are fully urbanized, comprising a hospital, houses, colleges, stilts, and old medication companies along the route under the River Anil. After sample collection, the study followed the experimental design shown in Figure 1.

Figure 1. Map of the location of collection points along the river Anil São Luís - MA.



Source: Prepared by the authors

2.2 OBTAINING AND MAINTAINING BACTERIAL ISOLATES

The collection took place in 2022, and each point was dimensioned in n 1 m², where three samples of 200 g mangrove sediment were taken from three different collection points. Sediment samples were then taken to the CEUMA University Applied Microbiology Laboratory, where they were processed. The three samples from each point were homogenized, forming a unique sample for each point. After the homogenate, 1 g was added in 9 mL of sterile saline (NaCl, 0.9%),

diluted until $10^{-3,}$ and 100 µL was spread on of MacConkey Agar medium (Difco, Laboratories, Detroit, Mich.) previously prepared to contain 8, 16, 32 and 64 µg/mL of ceftazidime and meropenem antibiotics.The culture plates were incubated at 37 °C for 72 h. After growth, bacterial colonies and their characterized morphotypes were counted. The purified colonies were stored in Brain Heart Infusion broth (BHI, Difco) added with 20% glycerol and stored at -20 ºC.

2.3 IDENTIFICATION OF BACTERIAL ISOLATES

Bacterial species isolated from sediment samples were identified by *Matrix-assisted laser desorption/ionization-time of flight* (MALDI-TOF) mass spectrometry (MS) (Biotyper 2.0 software, Bruker Daltonics, Bremen, Germany). *Escherichia coli* ATCC 8739 was used as a positive control (Egli *et al.*, 2015).

2.4 DETERMINATION OF THE SUSCEPTIBILITY PROFILE TO ANTIMICROBIAL DRUGS

The Kirby-Bauer method determined the susceptibility profile to antimicrobial drugs for all isolates (Bauer; Kirby; Sherris; Turck, 1966). The antimicrobial drugs used in the tests were amikacin (30 µg), gentamicin (10 µg), ampicillin + sulbactam (20 µg), piperacycline + tazobactan (30/6µg), cefepime (30µg), ceftazidime (10µg), ceftriaxona (30µg), cefotaxime (5µg), ofloxacin (5 µg), norfloxacin (10 µg), cefuroxime (30 µg), ciprofloxacin (5µg), meropenem (10 µg) and imipenem (10 µg). Susceptibility analyses were carried out according to the manufacturer's recommendations by Brazilian Committee on Antimicrobial Susceptibility Testing (BrCAST 2022).

2.5 MOLECULAR ANALYSIS

2.5.1 Extraction of bacterial DNA

For molecular analyses, bacterial DNA was extracted according to the protocol described by Pitcher (Pitcher; D.G.; Sanders; Owen, 1989). The concentration and purity of the product obtained were quantified in a Nanodrop-TM 1000 spectrophotometer at 260 and 280 nm (Thermo Fisher Scientific, Waltham, MA, USA).

2.5.2 Polymerase Chain Reaction (PCR) for the detection of genes related to β-lactamase enzymes

The Polymerase Chain Reaction (PCR) assays for the genes encoding the β-lactamase enzymes were carried out in a 2720 Thermal Cycler (California, USA). In the PCR reactions, the GoTaq® Green Master Mix solution (Promega, Madison, WI, USA) was used, plus each specific pair of the initiator (10 ρmol/L), nuclease-free water, and the DNA template, corresponding to approximately 100 ηg of DNA. The amplification protocol was performed according to described in Table 1.

Table 1. Cycle conditions for amplifying the β-lactamases genes

	*bla*KPC	*bla*IMP	*bla*SPM1	*bla*GES	*bla*NDM	*bla*VIM2	*bla*TEM	*bla*SHV	*bla*CTX-M
Cycle	35	35	35	35	35	35	30	30	30
Initial denaturation	95 ºC - 5 min	94º C - 3 min	94º C - 3 min	94º C - 3 min	94º C - 3 min	94º C - 3 min	94º C - 3 min	94º C - 3 min	94º C - 3 min
Denaturation	95º C - 1 min	94º C - 1min	94º C - 1min	94º C - 1min	94º C - 1min	94º C - 1min	94º C - 1min	94º C - 1min	94º C - 1min
Annealing	55º C - 40 s	55º C - 1min	55º C - 1min	55º C - 1min	55º C - 1min	55º C - 1min	54º C - 1min	54º C - 1min	54º C - 1min
Extension	72º C - 90 s	72º C - 2 min	72º C - 2 min	72º C - 2 min	72º C - 2 min	72º C - 2 min	72º C - 2 min	72º C - 2 min	72º C - 2 min
Final extentsion	72º C - 10 min	72º C - 7min	72º C - 7min	72º C - 7min	72º C - 7min	72º C - 7min	72º C - 5 min	72º C - 5 min	72º C - 5 min

Source: Prepared by the authors

Then, the fragments were visualized on an agarose gel ranging from 1.5% to 2% according to the length base pairs and stained with UniSafe Dye (20,000x)

(Uniscience do Brasil, Brazil) according to the manufacturer's recommendations. After electrophoresis, the gel was photographed under ultraviolet lighting (Nordmann; Boulanger; Poirel, 2012). The Simplex-PCR technique was used to detect genes related to the enzymes *Klebsiella pneumoniae* carbapenemase (KPC), Verona imipenemase (VIM2), New Delhi metallo-β-lactamase (NDM). The multiplex-PCR technique was used for the target genes related to the β-lactamases enzymes Temoreira (TEM), sulphydryl variable (SHV), and cefotaxime enzyme (CTX-M), as well as for the amplification of the *bla*$_{IMP}$, *bla*$_{SPM1,}$ and *bla*$_{GES}$ (Dallenne *et al*., 2010; Mosca *et al*., 2013; Liu *et al*., 2012; Woodford *et al*., 2006).

2.5.3 Molecular identification by Polymerase Chain Reaction (PCR) of genes related to oxacillinase enzymes (bla$_{OXA1}$, bla$_{OXA23}$, bla$_{OXA24}$, bla$_{OXA51}$ and bla$_{OXA58}$)

The PCR reactions for the oxacillinase genes were performed in a 2720 Thermal Cycler (California, USA) in a final volume of 25 µL, containing 0.5 µL of each specific primer (10 pmol/L) described in Supplementary Material 1, plus 12.5 µL of PCR Master Mix - Promega® (Promega, Madison, WI, USA (Taq DNA polymerase, dNTPs, $MgCl_2$, PCR buffer [pH 8.5]), 4.5 µL of nuclease-free water and 3 µL of the DNA template, corresponding to approximately 100 ρg of DNA The amplification reaction occurred under the following conditions: 94°C for 5 minutes (initial denaturation) followed by 30 cycles of 94 °C for 1 minute, 52°C for 1 minute and 72 °C for 1 minute and final extension at 72°C for 7 minutes. The PCR products were applied on 2% agarose gel and stained in a Nucleic Acid staining solution - UniSafe Dye (20,000x) (Uniscience do Brasil, Brazil) according to manufacturer's standards. After electrophoresis, the gel was photographed under exposure to UV illumination.

3 RESULTS

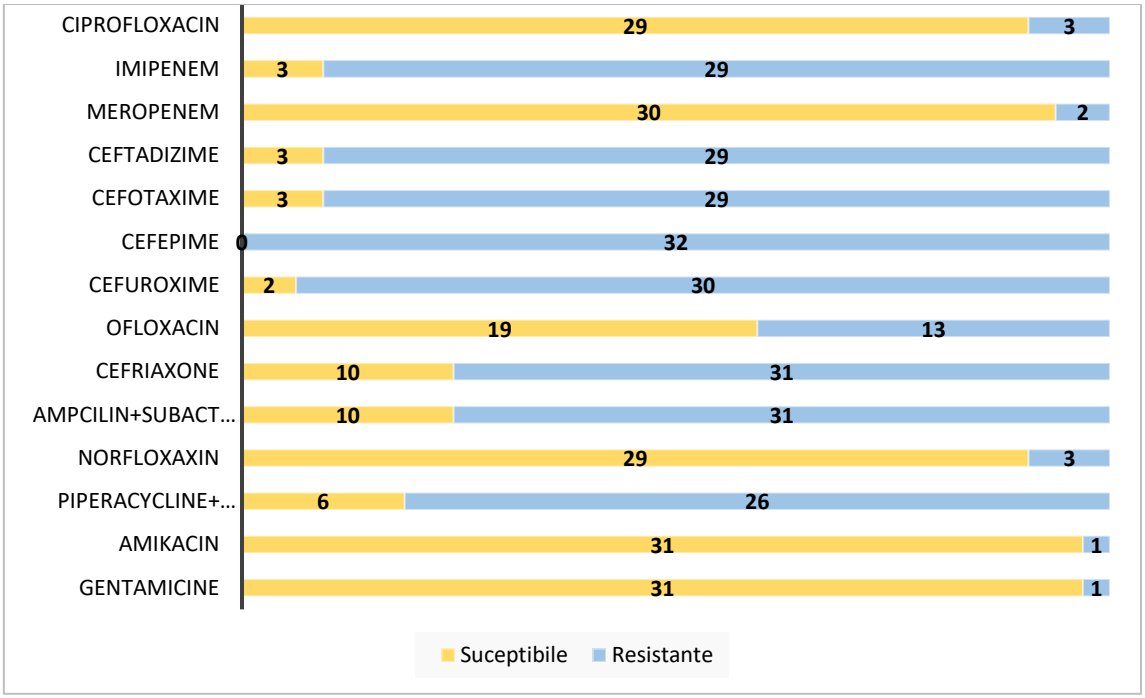
3.1 ISOLATION AND IDENTIFICATION OF GRAM-NEGATIVE BACTERIA OBTAINED FROM SAMPLES COLLECTED FROM RIO ANIL SEDIMENTS IN SÃO LUÍS-MA

In this study, 32 Gram-negative bacteria were isolated and identified, distributed in 4 different species: *O. intermedium* 62.5% (n = 20), *O. tritici* 27.27% (n = 9), *Stenotrophomonas maltophilia* 6.06% (n = 02) and *Klebsiella pneumoniae* 3.03% (n = 01).

3.2 DETERMINATION OF THE BACTERIAL SUSCEPTIBILITY PROFILE TO ANTIMICROBIAL DRUGS

In this study, susceptibility tests to antimicrobial drugs were performed using the disk diffusion method. Thirteen antimicrobial drugs were used for Gram-negative bacteria-selected isolates in the first and second collections. In this study, 32 isolates (100%) were resistant to cefepime, 96.8% (n = 31) to ofloxacin and ceftriaxone, and 90.6% (n = 29) to imipenem. While the sensitivity profile was 96.8% (n = 31) for amikacin, followed by 93.7% (n = 30) for meropenem (Figure 2).

Figure 2. Susceptibility profile of bacterial isolates to antimicrobial drugs isolated from Rio Anil-MA sediment.



Source: Prepared by the authors

3.3 DETECTION OF GENES RELATED TO B-LACTAMASES ENZYMES (bla^{shv}, $bla_{ctx\text{-}M}$, AND bla_{tem}) AND KLEBSIELLA PNEUMONIAE CARBAPENEMASE (KPC) ENZYME

Among the 32 strains of bacteria isolated and analyzed in the present study, 30.3% (n = 10) showed a positive result for the *bla*$_{SHV}$ gene. Four bacteria isolates (12.12%) had the *bla*$_{TEM}$ gene (Table 2).

3.4 DETECTION OF GENES RELATED TO METALLO B-LACTAMASES ENZYMES (bla_{ndm}, bla_{imp}, bla_{spm1}, bla_{ges}, AND bla_{vim2})

None of the bacterial isolates had the *bla*$_{IMP}$ and *bla*$_{VIM2}$ genes. However, 48.4% (n = 16) of the bacterial isolates tested positive for the *bla*$_{GES}$ gene. Three isolates (9.09%) had the *bla*$_{SPM1}$ gene. One isolate identified as *Ochrobactrum intermedium* tested positive for the *bla*$_{NDM\text{-}1}$ gene.

3.5 DETECTION OF THE GENES ENCODING SERINE CARBAPENEMASES (bla_{oxa1}, bla_{oxa23}, bla_{oxa24}, bla_{oxa51} AND bla_{oxa58})

The *bla*$_{OXA1}$ gene was the most detected and was found in 12 (36.36%) bacterial isolates analyzed (Table 2). In addition, the *bla*$_{OXA51}$ gene was detected in 5 bacterial isolates (15.15%). The *bla*$_{OXA58}$ gene was identified in two (6.06%) samples of the species *Ochrobactrum intermedium*. The *bla*$_{OXA23}$ and *bla*$_{OXA24}$ genes were detected in two bacterial isolates (3.03%) species *Ochrobactrum intermedium*.

Table 2: Markers of resistance and susceptibility to antimicrobial drugs of the 32 baceteria isolates collected.

Sample	species	Antimicrobial Profile (R)	Resistance genes	Integrons
P20M4	*Stenotrophomonas maltophilia*	CPM, CRO, IPM, ASB, AMI, CRX, MRP.	--	--
P20C16	*Ochrobactrum tritici*	CPM, CTX, OFX, CRO, CAZ, IPM, ASB, CRX, PTZ.	--	--
P21C16	*Ochrobactrum tritici*	CPM, CTX, CRO, CAZ, IPM, ASB, CRX, PTZ, AMP.	--	Int-1
P21M4	*Stenotrophomonas maltophilia*	CPM, CTX, CRO, IPM, ASB, CRX,MRP.	--	Int-1
P22C16	*Ochrobactrum intermedium*	CPM, CTX, OFX, CRO, CAZ, IPM, ASB, CRX, PTZ, NFX.	--	Int-1, Int-3
P31C2	*Ochrobactrum intermedium*	CPM, CTX, CRO, CAZ, IPM, ASB, PTZ, AMP.	--	--
P313M16	*Klebsiella pneumoniae*	CPM, CTX, OFX, CRO, CAZ, IPM, ASB, AMI, CRX, NFX, MRP, CIP.	*bla*$_{KPC}$, *bla*$_{OXA-1}$, *bla*$_{CTX-M}$, *bla*$_{SHV}$	--
P32C8	*Ochrobactrum intermedium*	CPM, CTX, CRO, CAZ, ASB, CRX, PTZ, AMP.	*bla*$_{OXA58}$	Int-1
P38C8	*Ochrobactrum intermedium*	CPM, CTX, CRO, CAZ, ASB, PTZ.	--	Int-1
P37C8	*Ochrobactrum intermedium*	CPM, CTX, CRO, CAZ, ASB, PTZ, IPM.	--	Int-1
P33C8	*Ochrobactrum intermedium*	CPM, CTX, CRO, CAZ, ASB, PTZ.	--	Int-1
P32M8	*Ochrobactrum intermedium*	CPM, CTX, OFX, CRO, CAZ, IPM, ASB, CRX,; NFX, CIP.	--	Int-1
P36C8	*Ochrobactrum intermedium*	CPM, CTX, CRO, IPM, ASB, PTZ, NFX, CIP.	*bla*$_{OXA24}$, *bla*$_{OXA58}$, *bla*$_{GES}$	Int-1
P110^{0}16C3	*Ochrobactrum tritici*	PTZ, ASB, CRO, CRX, AMP, CPM, CTX, CAZ, IPM		--

			bla$_{OXA1}$, *bla*$_{SPM}$, *bla*$_{GES}$, *bla*$_{TEM}$	
P110^{0}8C1	*Ochrobactrum tritici*	PTZ, ASB, CRO, CRX, AMP, CPM, CTX, CAZ, IPM	*bla*$_{GES}$, *bla*$_{SHV}$	Int-1
P110^{0}8C2	*Ochrobactrum intermedium*	PTZ, ASB, CRO, CRX, AMP, CPM, CTX, CAZ, IPM	*bla*$_{OXA1}$, *bla*$_{GES}$, *bla*$_{SHV}$	Int-1, Int-3
P110^{0}16C2	*Ochrobactrum tritici*	PTZ, ASB, CRO, CRX, AMP, CPM, CTX, CAZ, IPM	*bla*$_{GES}$	Int-1
P110^{0}16C4	*Ochrobactrum tritici*	ASB, CRX, CPM, CAZ, IPM	*bla*$_{OXA1}$	Int-1
4CP110^{0}2	*Ochrobactrum intermedium*	CRO, CRX, AMP, CPM, CAZ, IPM	--	Int-1
P210^{0}8C4	*Ochrobactrum intermedium*	PTZ, ASB, CRO, CRX, AMP, CPM, CTX, CAZ, IPM	*bla*$_{OXA51}$, *bla*$_{OXA1}$, *bla*$_{GES}$, *bla*$_{SHV}$	Int-1
P210^{-1}8C1	*Ochrobactrum tritici*	PTZ, ASB, CRO, CRX, AMP, CPM, CTX, CAZ, IPM	*bla*$_{OXA51}$, *bla*$_{OXA1}$, *bla*$_{GES}$, *bla*$_{TEM}$	Int-3
P210^{0}4C1	*Ochrobactrum intermedium*	PTZ, ASB, CRO, CRX, AMP, CPM, CTX, CAZ, IPM	*bla*$_{GES}$, *bla*$_{SHV}$	--
P210^{0}16C5	*Ochrobactrum tritici*	PTZ, ASB, CRO, CRX, AMP, CPM, CTX, CAZ, IPM	*bla*$_{OXA1}$, *bla*$_{OXA51}$, *bla*$_{GES}$, *bla*$_{TEM}$, *bla*$_{SHV}$	Int-1
P210^{0}16C2	*Ochrobactrum intermedium*	PTZ, ASB, CRO, CRX, AMP, CPM, CTX, CAZ, IPM	*bla*$_{OXA1}$, *bla*$_{OXA51}$, *bla*$_{SPM}$, *bla*$_{GES}$, *bla*$_{NDM}$, *bla*$_{SHV}$	Int-1
P210^{-1}8C3	*Ochrobactrum intermedium*	PTZ, ASB, CRO, CRX, AMP, CPM, CTX, CAZ, IPM	*bla*$_{GES}$, *bla*$_{SHV}$	Int-1
P210^{0}16C3	*Ochrobactrum intermedium*	PTZ, ASB, CRO, CRX, AMP, CPM, CTX, CAZ, IPM	*bla*$_{OXA1}$, *bla*$_{GES}$, *bla*$_{TEM}$	Int-1
P210^{0}8C2	*Ochrobactrum intermedium*	PTZ, ASB, CRO, CRX, AMP, CPM, CTX, CAZ, IPM, AMI	*bla*$_{SPM}$, *bla*$_{GES}$, *bla*$_{SHV}$	--
P210^{0}4C2	*Ochrobactrum intermedium*	PTZ, ASB, CRO, CRX, AMP, CPM, CTX, CAZ, IPM	*bla*$_{OXA1}$, *bla*$_{OXA23}$, *bla*$_{GES}$	Int-1, Int-3
P210^{0}4C5	*Ochrobactrum intermedium*	PTZ, ASB, CRO, CRX, AMP, CPM, CTX, CAZ, IPM	*bla*$_{OXA1}$, *bla*$_{GES}$	Int-1
P210^{0}16C1	*Ochrobactrum intermedium*	PTZ, ASB, CRO, CRX, AMP, CPM, CTX, CAZ, IPM	*bla*$_{OXA1}$, *bla*$_{OXA51}$, *bla*$_{GES}$, *bla*$_{SHV}$	Int-1
P210^{0}16C4	*Ochrobactrum intermedium*	PTZ, ASB, CRO, CRX, AMP, CPM, CTX, CAZ, IPM	--	--
P210^{-1}8C2	*Ochrobactrum tritici*	PTZ, ASB, CRO, CRX, AMP, CPM, CTX, CAZ, IPM	--	--

CPM, Cefepime; CTX, Cefotaxime; OFX, Ofloxacin; CRO, Ceftriaxone; CAZ, Ceftadizime; IPM, Imipenem; ASB, Ampicillin+Sulbactam, AMI Amicacin; CRX, Cefuroxime; GEN, Gentamicin; PTZ, Piperacycline+Tazobactan; NFX, Norfloxacin; AMP, Ampicillin; MRP, Meropenem; CIP, Ciprofloxacin: -, negative. Source: Prepared by the authors

4 DISCUSSIONS

Mangroves suffer from man's constant action (Zhao *et al.*, 2019). In these environments, microbial pathogens with antimicrobial-resistant profiles have already been described (Imchen; Vennapu; Ghosh; Kumavath, 2019). In this study, most isolates were resistant to more than one antimicrobial agent. A hundred percent of the isolates were resistant to cefepime, a 4th generation cephalosporin, and 96.8% exhibited resistance to ampicillin+sulbactam and ceftriaxone. Resistance to β-lactam antibiotics have already been described in isolates obtained from estuaries mangrove (Ghaderpour *et al.*, 2015), sediments in India (Imchen; Vennapu; Ghosh; Kumavath, 2019), and natural mangrove reserves (Liu *et al.*, 2020). These results are relevant since Gram-negative bacteria with MDR profiles are a major global health problem, and most are no longer sensitive to the antimicrobials commonly used in clinical practice. This problem has been attributed to the irrational use of these drugs (Banin; Hughes; Kuipers, 2017).

The species of the genera *Ochrobactrum* and *Klebsiella* detected in the sediment samples analyzed in the present study are considered opportunistic pathogens and identified as susceptible to several antimicrobials (Navon-Venezia; Kondratyeva; Carattoli, 2017; Yagel *et al.*, 2020). *Ochrobactrum* spp. has already been detected both in the environment and the clinic, with most infections related to using catheters in immunocompromised patients. Endocarditis, peritonitis, meningitis, osteomyelitis, endophthalmitis, septic arthritis, and bacteremia are infections also associated with this genus (Yagel *et al.*, 2020).

The genus *Ochrobactrum* has limitations because it does not have a cut-off point for the minimum inhibitory concentration and norms for the defined susceptibility profile. In this way, the results obtained are interpreted based on available studies. The species isolated showed resistance to cefepime and sensitivity to imipenem and gentamicin. These findings are similar to the data obtained in a study from five *Ochrobactrum* isolates of animal origin (Yagel *et al.*, 2020).

Studies indicate that resistance to beta-lactams is due to the presence of the *bla*$_{OCH}$ gene, which is equivalent to a class C beta-lactamase described by Ambler (Ryan; Pembroke, 2020; Yagel *et al.*, 2020).

The *K. pneumoniae* species is one of the most important genus from a clinical point of view. It is constantly associated with severe infections and isolates possessing an MDR profile (Caneiras *et al.*, 2019; Fasciana *et al.*, 2019). *K. pneumoniae* P313M16 presented resistance to most of the tested antimicrobials. *K. pneumoniae* is considered an opportunistic pathogen responsible for a range of nosocomial infections, thus becoming a public health concern, whose profile in most of these isolates is associated with infections by bacteria resistant to multiple antimicrobial drugs (Wyres; Lam; Holt, 2020). *K. pneumoniae* P313M16 was sensitive to gentamicin and resistant to cefepime and imipenem. Other studies corroborate these results (Soria *et al.*, 2020; Tekeli *et al.*, 2020). Still, the resistance profile to β-lactam antibiotics demonstrated by the *K. pneumoniae* P313M16 strain seems to be related to the presence of the *bla*$_{KPC}$ gene, evidenced by molecular tests.

Molecular detection assays for genes in *O. intermedium*, *O. tritici* related to β-lactamases showed that *bla*$_{OXA}$ and *bla*$_{GES}$ family genes predominate in 21 and 16 isolates, respectively. These findings may be linked to low-level expression of carbapenemases since many isolates were sensitive to imipenem and resistant to meropenem. However, a wide range of isolates resistant to the antibiotic cefepime was evidenced.

Bacteria that carry genes for the expression of β-lactamase enzymes are present in different environments, ranging from nosocomial to unpredictable locations. In the case of aquatic environment, bacteria have already been detected carrying the *bla*$_{CTX-M}$, *bla*$_{GES-2}$, *bla*$_{GES-4}$, *bla*$_{GES-13}$ genes in rivers (Marathe *et al.*, 2018; Yamashita; Katakawa; Tanaka, 2017); *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$ genes in drinking water channels (Adesoji; Ogunjobi, 2016); *bla*$_{IMP-1}$, *bla*$_{IMP-2}$, *bla*$_{OXA-23}$, *bla*$_{OXA-48}$, *bla*$_{CTX-M-8}$ and *bla*$_{SFC-1}$, *bla*$_{VIM-1}$ and *bla*$_{VIM-13}$ in domestic and hospital wastewater (Khan; Söderquist; Jass, 2019). The mangrove ecosystem is an essential carrier of microorganisms that carry genes responsible for antibiotic resistance (Liu *et al.*, 2020). Several factors corroborate the presence of AGRs in

aquatic environments since they are closely related to human anthropic actions. This environment serves as a reservoir for these microorganisms that, through genetic mechanisms, process the exchange of genetic, mobile, and immobile elements, disseminating them among the bacterial species present there (Hooban *et al.*, 2020).

Metallo-beta-lactamases are enzymes that hydrolyze carbapenem antibiotics. Their genes originated in soil bacteria, and their dissemination among gram-negative bacteria is observed in several environments (Fonseca; Andrade; Vicente, 2018). Among the isolates obtained, 16 of the *Ochrobactrum* genus had the *bla*$_{GES}$ gene, and 3 of the same genus were positive for *bla*$_{SPM-1}$. There are no reports in the literature on detecting these genes in *Ochrobactrum* species. Interestingly, the *bla*$_{NDM-1}$ gene was detected in *O. intermedium* strain in the present study. In a previous study, this gene was already detected in an isolate of *O. anthropi* in goat meat samples (Singh *et al.*, 2020).

In soil samples, the *bla*$_{OXA-1}$ gene was detected in isolates of *Ochrobactrum* spp (Furlan; Stehling, 2017). The *bla*$_{OXA-48}$ gene was amplified in *O. intermedium* isolate (Shanthini *et al.*, 2019). Bacteria carrying the *bla*$_{OXA-58}$ gene are considered responsible for several nosocomial outbreaks and environmental bacteria, such as *Pseudomonas*, *Rheinheimera*, *Shewanella*, *Raoultella*, *Stenotrophomonas*, *Vibrio*, *Pseudoalteromonas*, *Algoriphagus*, *Bowmanella,* and *Thalassospira* are identified as reservoirs of this gene (Xin *et al.*, 2019).

Previous studies have detected the presence of the *bla*$_{SHV}$ and *bla*$_{TEM}$ genes in *Ochrobactrum* spp and *O. anthropi* isolates, respectively. In urban river sediment samples (Lu *et al.*, 2010), in soil samples (Furlan; Stehling, 2017), and goat meat samples (Singh *et al.*, 2020).

In the *O. intermedium*, *O. tritici,* and *Stenotrophomona*s spp lineages, class 1 integrons and class 3 integrons were observed. These DNA sequences contribute significantly to the distribution and propagation of resistance to antimicrobials, as they can insert cassettes containing resistance genes in their structure, which are of significant importance in the adaptation and evolution of bacterial isolates (Akrami; Rajabnia; Pournajaf, 2019). These results are similar to those observed in other studies with Gram-negative bacteria from aquatic

environments. For example, these genetic elements have already been recorded in bacteria from urban aquatic systems (Chaturvedi *et al.*, 2021; Kaushik *et al*., 2019; Singh; *et al.*, 2020). Class 1 integron is the most commonly found in the environment and a nosocomial environment. The Class 1 integron is related to genes for resistance to disinfectants and heavy metals in pathogenic bacteria. It is often associated with the spread of resistance to antimicrobial drugs, as they are easily transferred horizontally to different bacterial isolates (Deng *et al.*, 2015; Huyan *et al.*, 2020). Thus, based on the high prevalence of the integron *int*1 gene, bacterial isolates can become vehicles for disseminating antibiotic-resistance genes among bacteria in the mangrove ecosystem.

5 CONCLUSION

With this study, it was possible to realize that Mangroves, as well as the rivers that makeup them, such as the Anil River – MA, are critical environments that serve as reservoirs of gram-negative bacteria resistant to multiple antibiotics, including variants that have genes for resistance, such as *bla*$_{GES}$ and *bla*$_{OXA}$. The identification of bacterial species such as *Ochrobactrum intermedium*, *Ochrobactrum tritici*, and *Klebsiella pneumoniae*, with high levels of resistance to antibiotics such as cefepime, ofloxacin, ceftriaxone, and imipenem, highlights the seriousness of the situation. By harboring microorganisms with significant resistance to clinical drugs, such environments represent a potential risk to human health, reinforcing the need to monitor and control human actions that affect these ecosystems. The results obtained in this research can assist society and the academic community by providing valuable insights into the prevalence and types of antibiotic-resistant bacteria in critical environments like mangroves. This information can guide public health policies, environmental protection measures, and strategies to mitigate the spread of resistant bacteria.The limitations of this study include the geographical scope restricted to the Anil River – MA, as well as the focus on a specific set of antibiotics and resistance genes. Future studies could expand the research area to include other mangroves and rivers, as well as investigate a broader range of resistance mechanisms to

antibiotics. Additionally, longitudinal studies would be beneficial to monitor changes in bacterial resistance over time and assess the impact of human interventions on the dynamics of these ecosystems.

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