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Identification and molecular analysis of antibiotic-resistant gram-negative bacteria isolated from mangrove sediment samples from the Anil River

Identificação e análise molecular de bactérias gram-negativas resistentes a antibióticos isoladas de amostras de sedimento de mangue do Rio Anil

Geovane Santos Muniz[1]; Edilson Santos Castro[2]; Fábio Henrique Ramos Braga[3]; José Maria do Amaral Filho[4]; Maria Raimunda Chagas Silva[5]; Andrea de Souza Monteiro[6]

[1]Master's student in Environment at CEUMA University, São Luís - MA, Brazil. E-mail: professorgilmarx@hotmail.com
[2]Master's student in Environment at CEUMA University, São Luís - MA, Brazil. E-mail: edilsonsantoscastroo@gmail.com
[3]Master's degree in Environment from CEUMA University, São Luís - MA, Brazil. E-mail: fabiosus@hotmail.com
[4]PhD candidate in Environment at CEUMA University, São Luís - MA, Brazil. E-mail: jmamaralf@gmail.com
[5]Professor of the Medicine Course at CEUMA University, São Luís - MA, Brazil. E-mail: marirah@gmail.com
[6]Professor of the Medicine Course at CEUMA University, São Luís - MA, Brazil. E-mail: andreasmont@gmail.com

Abstract

Mangrove sediments harbor diverse microbial communities that play crucial roles in biogeochemical processes and may serve as reservoirs for antimicrobial resistance genes (ARGs). This study aimed to isolate, identify, and determine the antimicrobial susceptibility profile of Gram-negative bacteria from mangrove sediments of the Anil River, São Luís, Maranhão, Brazil, and to detect β-lactamase-encoding genes. Sediment samples were collected at three sites over 10 months (June 2017–April 2018), covering seasonal variations. A total of 188 Gram-negative bacteria were isolated using MacConkey agar supplemented with antibiotics. Species identification was performed by MALDI-TOF mass spectrometry, and antimicrobial susceptibility was determined by disk diffusion. Molecular screening for β-lactamase genes (blaKPC, blaOXA-58, blaSHV, blaCTX-M, blaTEM) was conducted by multiplex PCR. The predominant genera were Ochrobactrum (44.68%) and Serratia (33.51%), with O. anthropi, O. intermedium, O. tritici, and S. marcescens being the most frequent species. High resistance rates were observed for cephalosporins, particularly cefuroxime (40.95%), cefepime (39.89%), ceftriaxone (36.70%), and cefotaxime (30.85%). The blaKPC gene was detected in 22 isolates, mainly O. anthropi, O. intermedium, O. tritici, and S. marcescens. The blaOXA-58 gene was found in 21 isolates, predominantly Ochrobactrum spp. (61.90%). No isolates carried blaSHV, blaCTX-M, or blaTEM genes. These findings indicate that Anil River mangrove sediments function as environmental reservoirs for opportunistic pathogens harboring clinically relevant resistance determinants, emphasizing the need for integrated surveillance strategies and proper wastewater management to mitigate the dissemination of ARGs between environmental and clinical settings.

Keywords: Antimicrobial resistance; Mangrove sediments; Ochrobactrum; Serratia; β-lactamases; BlaKPC; BlaOXA-58.

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Autor correspondente:
Andrea de Souza Monteiro
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Resumo

Sedimentos de manguezal abrigam comunidades microbianas diversificadas que desempenham papéis centrais em processos biogeoquímicos e podem atuar como reservatórios de genes de resistência a antimicrobianos (ARGs). Este estudo teve como objetivo isolar, identificar e determinar o perfil de suscetibilidade de bactérias Gram-negativas provenientes de sedimentos de manguezal do rio Anil, em São Luís, Maranhão, Brasil, bem como detectar genes codificadores de β-lactamases. Amostras de sedimento foram coletadas em três pontos ao longo de 10 meses (junho de 2017 a abril de 2018), contemplando variações sazonais. No total, 188 bactérias Gram-negativas foram isoladas em ágar MacConkey suplementado com antibióticos. A identificação foi realizada por espectrometria de massas MALDI-TOF e a suscetibilidade a antimicrobianos foi determinada pelo método de disco-difusão. A triagem molecular para genes de β-lactamases (blaKPC, blaOXA-58, blaSHV, blaCTX-M, blaTEM) foi conduzida por PCR multiplex. Predominaram os gêneros Ochrobactrum (44,68%) e Serratia (33,51%), com destaque para O. anthropi, O. intermedium, O. tritici e S. marcescens. Observaram-se elevadas taxas de resistência a cefalosporinas, sobretudo cefuroxima (40,95%), cefepima (39,89%), ceftriaxona (36,70%) e cefotaxima (30,85%). O gene blaKPC foi detectado em 22 isolados, principalmente de O. anthropi, O. intermedium, O. tritici e S. marcescens, enquanto o gene blaOXA-58 foi identificado em 21 isolados, majoritariamente pertencentes a Ochrobactrum spp. (61,90%). Nenhum isolado apresentou os genes blaSHV, blaCTX-M ou blaTEM. Esses achados indicam que os sedimentos de manguezal do rio Anil funcionam como reservatórios ambientais de patógenos oportunistas portadores de determinantes de resistência clinicamente relevantes, ressaltando a necessidade de vigilância integrada e manejo adequado de efluentes para mitigar a disseminação de ARGs entre os ambientes ambiental e clínico.

Palavras-chave: Resistência a antimicrobianos; Sedimentos de manguezal; Ochrobactrum; Serratia; β-lactamases; BlaKPC; BlaOXA-58.

INTRODUCTION

Microbial communities in mangrove sediments play a key role in the biogeochemical processes of this ecosystem, with their composition and structure modulated by biotic and abiotic factors[1]. Horizontal gene transfer (HGT) is the main mechanism for the spread of antimicrobial resistance, transferring antibiotic resistance genes (ARGs) from commensal and environmental species to clinically relevant opportunistic pathogens[2,3]. Although conjugation is recognized as the most influential mechanism in the spread of ARGs[4], recent evidence suggests that transformation and transduction are more relevant than previously established. Understanding the extent of the environmental resistome and the mechanisms of mobilization for pathogenic bacteria is essential for strategies to control the spread of ARGs[5].

Soils and sediments harbor an estimated bacterial density of up to 10101010 cells per gram, comprising 400-700 distinct species and biomass of 300-30,000 kg/hectare[6]. This bacterial diversity has a high capacity to convey resistance genes via HGT mechanisms[4]. Bacterial resistance spreads faster than the introduction of new antimicrobial compounds in clinical practice, posing a public health crisis. Historically, most antibiotics have been obtained by screening soil microorganisms[7]. Multidrug-resistant (MDR) bacteria develop or acquire resistance to two or more classes of antimicrobials[8].

β-lactam antibiotics, characterized by the presence of the β-lactam ring, constitute a class of significant therapeutic importance. Bacterial resistance to β-lactams primarily results from the expression of β-lactamases that hydrolyze the β-lactam ring, inactivating the drug[9]. Carbapenem-resistant Enterobacteriaceae (CRE) represent the most relevant species in the hospital environment, causing high mortality in critically ill patients in intensive care units, with conventional antibiotics generally inactive against CRE[10]. Carbapenemase-producing Enterobacteriaceae are particularly concerning due to their tendency to spread and therapeutic difficulty. The most prevalent

groups include Klebsiella pneumoniae carbapenemase (KPC), New Delhi Metallo-β-lactamase (NDM), and oxacillinases such as OXA-48 and OXA-58[10].

Among emerging pathogens, MDR Serratia marcescens is an important agent of hospital infections, widely distributed in soil and water[11]. S. marcescens causes a diverse spectrum of infections: wounds, urinary tract, respiratory tract, central nervous system, and bloodstream[12,13]. The genus Ochrobactrum comprises two clinically relevant species, O. anthropi and O. intermedium, with reduced susceptibility to β-lactams[14]. O. anthropi, an emerging opportunistic pathogen phylogenetically related to Brucellaceae, is associated with tissue infections in immunocompetent and immunocompromised patients, often related to medical devices, catheters, and probes[15]. Cases of bacteremia caused by Ochrobactrum spp. are predominantly associated with immunocompromised patients, including HIV carriers, diabetics, and dialysis patients[16-19].

The blaKPC and blaOXA-58 genes confer resistance to all β-lactams, including carbapenems, by encoding subgroup 2f enzymes. Gram-negative bacteria expressing KPC and OXA-58 β-lactamases are associated with hospital outbreaks, requiring efficient systematic screening[20]. Studies show that bacteria from aquatic environments can harbor genes such as blaOXA-58, including species of the genera Stenotrophomonas and Pseudomonas, with high resistance to carbapenems[21]. OXA-58, a carbapenem-hydrolyzing β-lactamase originating from MDR Acinetobacter baumannii, hydrolyzes penicillins, cephalosporins, and carbapenems[22,23]. KPC-2 has emerged as a carbapenemase that also hydrolyzes penicillins and cephalosporins, conferring multidrug resistance in several Gram-negative species[24].

Considering the discharge of anthropogenic pollutants (domestic sewage, pharmaceutical and hospital waste) into water bodies, microbiota organisms can harbor resistance genes. Rivers and sediments are reservoirs for the spread of infectious agents, and their monitoring is essential to assess the extent of resistance markers and the distribution of emerging pathogens. This study aimed to isolate Gram-negative bacteria resistant to antimicrobials from sediments in the mangroves of the Anil River, São Luís do Maranhão, determine susceptibility profiles, and identify species carrying resistance.

METHODOLOGY

Obtainment and Identification of Bacterial Isolates

Samples were collected at three sites along mangrove areas of the Anil River, São Luís Island, Maranhão State, Brazil. At each demarcated point (1 m²), three sediment samples (200 g) were obtained in seasonal periods over 10 months (June 2017 to April 2018), totaling three sampling events (rainy, transitional, and dry periods). The samples were homogenized, and 1 g of each

composite sample was weighed on an analytical balance, added to 9 mL of sterile saline solution (0.9%) or peptone water, and serially diluted up to 10^{-3}. Aliquots of 100 µL were inoculated onto MacConkey agar supplemented with increasing concentrations of the selected antibiotics (4, 8, 16, and 32 mg/mL) by surface spreading using a Drigalski loop. After incubation at 37°C for 72 h, colonies were counted, morphotypes were characterized, and isolates were purified by streaking on sterile MacConkey agar (Difco), followed by a new incubation at 37°C for 72 h. The resulting strains were preserved in BHI broth with 20% glycerol at -20°C. Samples were processed at the Applied Microbiology Laboratory of CEUMA University and identified at the Aquaculture Laboratory of the Federal University of Minas Gerais and at the Cedro Clinical Analysis Laboratory (São Luís, MA), between July 2017 and October 2018.

Determination of Antimicrobial Susceptibility Profile

The antimicrobial susceptibility profile was determined by the Kirby–Bauer disk diffusion method, using the following antibiotics: amikacin (30 µg), ampicillin+sulbactam (20 µg), cefepime (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg), cefuroxime (30 µg), cefotaxime (30 µg), ofloxacin (5 µg), ertapenem (30 µg), gentamicin (120 µg), and imipenem (10 µg). The analyses followed the manufacturer's recommendations and the guidelines of the Clinical and Laboratory Standards Institute CLSI (2017). Species identification was confirmed by MALDI-TOF mass spectrometry. Individual colonies grown on Tryptic Soy Agar (TSA) (Difco, USA) were applied as a thin film onto a 24-spot steel target plate (Bruker Daltonics, Bremen, Germany) and, after drying, co-crystallized with 1 µL of a saturated solution of α-cyano-4-hydroxycinnamic acid (HCCA; Bruker Daltonics) in 50% acetonitrile/2.5% trifluoroacetic acid (Sigma-Aldrich, St. Louis, MO, USA. Mass spectra were acquired in positive reflector mode using the MicroFlex LT system (Bruker Daltonics) under standard settings. Spectra were analyzed with MALDI Biotyper 2.0 software (Bruker Daltonics) using the following criteria: a score ≥2.000 indicated identification at the species level; 1.700–1.999 indicated identification at the genus level; and <1.700 was considered not identified. Escherichia coli ATCC 8739 was used as a positive control.

Extraction of Bacterial DNA

Bacterial DNA was extracted according to the protocol described by[25]. Isolates were grown on BHI agar (Brain Heart Infusion, Himédia, Difco, Detroit, MI, USA) for 24 h at 37°C. Colonies were resuspended in a 2.0 mL microtube containing 300 µL of TE-lysozyme buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, 50 mg/mL lysozyme, pH 8.0) and ground glass, vortexed for 2 min, and placed on ice. Then, 500 µL of a solution containing guanidine thiocyanate (5 mmol/L), EDTA (100 mmol/L),

and sarcosyl (0.5%) were added, followed by inversion mixing and incubation for 10 min at room temperature. Next, 300 µL of ammonium acetate were added, followed by inversion mixing and incubation for 10 min on ice. Subsequently, 500 µL of chloroform:isoamyl alcohol were added, followed by mixing and centrifugation at 12,000 rpm for 10 min. The aqueous phase was transferred to new tubes, an equal volume of isopropanol was added, and the mixture was incubated at -20°C for 12 h. After centrifugation at 12,000 rpm for 10 min, the supernatant was discarded and the pellet was washed three times with 70% ethanol. After drying, the DNA was resuspended in 50 µL of nuclease-free water. DNA concentration and purity were quantified using a NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) at 260 and 280 nm.

RESULTS AND DISCUSSION

Isolation and identification of Gram-negative bacteria

In this study, 188 Gram-negative bacteria were isolated and identified from mangrove sediment samples along the Anil River in São Luís, Maranhão. The most frequent species belonged to the families Brucellaceae and Enterobacteriaceae, with a predominance of the genera *Ochrobactrum* and *Serratia*.

Overall, *Ochrobactrum* represented 44.68% of the isolates (n=84) and *Serratia* 33.51% (n=63)[26,27]. Among the *Ochrobactrum* species, the most notable were *O. anthropic* (18.08%; n=34), *O. intermedium* (15.96%; n=30), and *O. tritici* (10.64%; n=20). Among the *Serratia* species, the most prevalent was *Serratia marcescens* (30.32%; n=57), followed by *Serratia aquatilis* (3.20%; n=6). Other identified bacteria included *Acinetobacter towneri* (6.91%; n=13), *Pseudomonas putida* (5.32%; n=10), *Stenotrophomonas maltophilia* (4.25%; n=8), *Rhizobium radiobacter* (3.20%; n=6), *Pseudomonas monteilii* (1.06%; n=2), *Acinetobacter johnsonii* (0.53%; n=1), and *Sphingomonas paucimobilis* (0.53%; n=1) (Table 1).

Table 1. Identification profile of the isolated bacterial species – collection 1 2 3.

Bacterial Species	Collection	Point 1	Point 2	Point 3
Ochrobactrum anthropi	1	03	02	02
	2	–	09	08
	3	06	–	04
Ochrobactrum intermedium	1	03	02	02
	2	01	08	02
	3	–	07	05
Ochrobactrum tritici	1	–	–	02
	2	–	03	04
	3	–	06	04
Serratia marcescens	1	04	04	05
	2	02	06	05
	3	08	09	13
Serratia aquatilis	1	–	01	01
	2	–	–	–

	3	–	03	01
Acinetobacter towneri	1	–	–	–
	2	02	03	04
	3	04	–	01
Acinetobacter baumannii	2	01	–	–
Acinetobacter johnsonii	2	–	–	01
Pseudomonas putida	1	–	02	01
	2	02	–	–
	3	02	01	–
Pseudomonas monteilii	2	–	–	02
Rhizobium radiobacter	2	01	04	01
Sphingomonas paucimobilis	1	02	–	–
	2	–	–	01
	3	–	–	–
Stenotrophomonas maltophilia	3	02	03	03

Source: Authors, 2025.

Most of the identified species are recognized as opportunistic human pathogens, with the potential to cause infections in the gastrointestinal and respiratory tracts, as well as in the bloodstream, both in the community and in hospital settings[28,29]. The detection of *S. marcescens* and *O. anthropi*, in particular, is relevant, as these species have been associated with decreased susceptibility to cephalosporins and other clinically used antimicrobials[26,27].

The presence of these bacteria in mangrove sediments indicates that the Anil River functions as an environmental reservoir for opportunistic microorganisms, likely influenced by discharges of domestic, hospital, and industrial effluents. This scenario favors both the persistence and dissemination of potentially resistant strains among environmental compartments and, subsequently, to human populations[30].

Antimicrobial susceptibility and resistance profile

The 188 Gram-negative isolates were subjected to susceptibility testing using the disk diffusion method against 11 antimicrobial drugs. Overall, a high percentage of resistance to cephalosporins was observed, especially among the most prevalent species. Considering all isolates, the highest resistance rates were observed for cefuroxime (40.95%; n=77), cefepime (39.89%; n=75), ceftriaxone (36.70%; n=69), and cefotaxime (30.85%; n=58) (Table 2).

Table 2 – Antimicrobial Susceptibility Profile (Collections 1, 2 and 3).

Drug	Collection	Point 1	Point 2	Point 3
Amicacina	1	0	0	10
	2	0	30	40
	3	77	0	0
Ampicilina + Sulbactam	1	30	30	25
	2	100	85	13
	3	60	10	10
Cefepima	1	40	10	60
	2	75	90	79

	3	65	80	65
Cefotaxima	1	70	60	70
	2	100	88	60
	3	60	70	79
Ceftazidima	1	28	20	50
	2	20	65	22
	3	62	100	55
Ceftriaxona	1	60	20	55
	2	80	100	82
	3	78	40	22
Cefuroxima	1	50	60	50
	2	100	95	80
	3	22	47	37
Ertapenem	1	40	15	15
	2	35	15	15
	3	–	–	–
Gentamicina	1	0	0	12
	2	20	35	27
	3	0	3	0
Imipenem	1	10	6	9
	2	–	–	–
	3	65	100	57
Ofloxacina	1	0	5	25
	2	–	–	–
	3	–	–	–

Source: Authors, 2025.

When specifically analyzing the four most frequent species (*S. marcescens, O. anthropi, O. intermedium*, and *O. tritici*), a resistance pattern consistent with that reported in the literature for clinical and environmental isolates was found. In *S. marcescens*, the highest frequencies of resistance were observed for cefepime (43.85%; n=25), followed by cefotaxime (29.82%; n=17), ceftriaxone (21.05%; n=12), and cefuroxime (21.05%; n=12). For *O. anthropi*, the most significant resistance profiles included cefotaxime (32.35%; n=11), ceftazidime (26.47%; n=9), cefepime (23.52%; n=8), and ampicillin + sulbactam (23.52%; n=8). In *O. intermedium*, resistance to cefotaxime (33.33%; n=10), cefepime (30%; n=9), and ceftriaxone (30%; n=9) was notable, while in *O. tritici*, higher percentages were observed for cefotaxime (25%; n=5), ceftriaxone (20%; n=4), and cefuroxime (15%; n=3).

These findings collectively demonstrate a significant reduction in the efficacy of third- and fourth-generation cephalosporins against the main isolated genera, especially *Ochrobactrum* and *Serratia*. This is highly worrisome, as cefotaxime (a third-generation cephalosporin) and cefepime (fourth-generation) are widely used in the treatment of infections caused by aerobic Gram-negative bacilli in hospital settings. Previous studies had already shown that isolates of *O. anthropic* and related species can exhibit resistance to first, second, and third-generation cephalosporins, corroborating the pattern observed in this work[27].

The literature describes that clinical strains of *S. marcescens* often exhibit resistance to ampicillin, amoxicillin, ampicillin/sulbactam, and narrow-spectrum cephalosporins like cefuroxime, in addition to high rates of resistance to ciprofloxacin, cefotaxime, aztreonam, and cefepime[31]. The detection, in this study, of environmental *S. marcescens* isolates with resistance profiles similar to those reported in hospitals reinforces the hypothesis of a flow of resistance genes between clinical and natural environments.

Carbapenems are last-line drugs in the treatment of infections caused by multidrug-resistant bacteria, including strains resistant to cephalosporins and quinolones. Environments impacted by sewage and hospital effluents are recognized as important reservoirs for carbapenem-resistant bacteria[32,33]. In this study, it was observed that isolates of the genus *Ochrobactru* exhibited resistance not only to cephalosporins but also to imipenem, with 9 isolates resistant to this drug, in addition to 10 resistant to cefotaxime and 9 to cefepime. In *S. marcescens*, strains resistant to cefepime (n=4) and imipenem (n=2) were detected.

CONCLUSION

This study demonstrated the dissemination of bacteria with antibiotic resistance profiles, particularly to cephalosporins such as ceftriaxone, cefotaxime, cefepime, and others, in mangrove sediments located in the Anil River in São Luís, Maranhão. In the analyzed mangrove sediment samples, it was possible to detect mainly *O. anthropi*, *O. intermedium*, and *S. marcescens* harboring the *bla*OXA-58 and *bla*KPC genes, indicating probable transmission via horizontal gene transfer mechanisms. The presence of the *bla*KPC gene in bacteria such as species of the *Ochrobactrum* and *Serratia* genera of environmental origin suggests significant disposal of wastewater derived from hospital settings without effective sanitary control. The environment acts as a reservoir for carbapenem-resistant bacteria. Further investigations into the evolution and dissemination of antibiotic resistance in environmental bacteria are necessary to understand and prevent the emergence of resistance in clinical settings.

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