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Detection of Gram-Negative Bacteria Carrying the *bla*$_{KPC-2}$ Gene from Mangrove Sediments

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Abstract: Mangroves are environments that have fast cycles associated with high concentration of bacterial decomposers. These are impacted by anthropogenic pollution due to contamination of bacterial species carrying resistance genes. This study aims to evaluate the metabolic profile of the microbiota in mangrove sediments, and verify the presence of Gram-negative bacteria resistant to meropenem. The samples were obtained from location along the Anil river and were seeded in medium supplemented with the antibiotic meropenem in increasing concentrations. The DNA was analyzed by multiplex PCR for detecting resistance genes for β-lactam antibiotics. The bacteria were identified by Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). The genes were sequenced by ABI PRISM 3100, analyzed by MEGA 6.0 program, and the sequence identified was assessed by GenBank using the BLAST algorithm. Ecoplate® the kit was used to determine the metabolic profile of the microbiota. The results of the six bacterial isolates showed the *bla*$_{KPC-2}$ gene and were identified as *Stenotrophomonas maltophilia* and *Pseudomonas putida.* The samples showed a greater diversity Shannon index, a rich substrate consumption and high equity. There was a metabolic richness such as carbon consumption profiles, being a factor of adaptation of pathogenic bacteria carrying resistance genes to β-lactamics antibiotics.

Key words: Antibiotic resistant, meropenem, bacterial isolates.

1. Introduction

The Gulf Maranhense is located at the north end of the state of Maranhão, comprising the humid tropics, extending between latitude 15 °N and 15 °S which is of crucial importance for the environment, since it contains half the fresh water, solute particles and discharged into oceans [1]. The humid tropics are characterized by constant high rainfall (> 1,500 mm/year), high temperatures (> 20 °C) and low thermal drift. Considering a variety of economically important resources exploited by human coastal population, they are also important for the growth of several species of fish and shellfish [2].

The provision of domestic, hospital and industrial waste in the soil causes changes in physical and chemical properties by changing the catabolic processes of habitat microorganisms, making the search for strategies necessary to protect these areas [3]. Since the spread of antibiotic resistance genes presents a difficult challenge for health professionals on how to deal with infected patients [4]. Many resistance genes have contributed significantly to multidrug resistance (MDR) in bacteria, which are easily deployed from Gram-negative organisms because of their location in genetic mobile elements (MLE), such as transposons, integrons or plasmids conjugative [4, 5].

Once the population has direct contact with areas that may contain microorganisms carriers of antibiotic

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resistance genes, contamination is made possible by leveraging a public health problem. The bacterial resistance to β-lactam antibiotics evidenced in Gram-negative bacteria MDR is mainly conditioned by the action of β-lactamase enzymes [6]. The bacterial resistance to β-lactam antibiotics evidenced in Gram-negative bacteria MDR is primarily conditioned by the action of β-lactamase enzymes. The β-lactamase may inactivate penicillins, cephalosporins, carbapenems and monobactams [7]. They act in the hydrolysis of β-lactam ring hydroxylation by irreversible amide bond with antibiotic inactivation [7, 8]. The carbapenemases are hydrolytic enzymes which act on carbapenems, such as meropenem, imipenem and are classified into three classes according to Amber: Oxacillinases (OXA), Serine (for example *Klebsiella pneumoniae* carbapenemase (KPC) and metallo β-lactamase for example New Delphi Metallo-β-lactamase (NDM) [8, 9].

Taking into account the soil metabolic diversity, based on the comparison of the catabolic profiles of different microhabitats, the use of different carbon substrates (C) shows that a greater chemical diversity due to the degradation of the organic matter produced in rivers and sediments can favor throughout the time the microorganisms economic metabolism [2]. Thus, less of an organic substrate C is piped to the more energy metabolism and C is set in the microbial cells [2]. Being the metabolic diversity defined by the number, type and rate of use of the suborganism community, leads to genetic, environmental effects [10, 11]. The study of the functional diversity of soil microbes uses the Biolog system, and it can serve as an indicator of changes in soil quality or changes in response to stress [10, 12]. Despite all the context of the importance of mangroves to biodiversity and conservation, little is known about the identification and characterization of pathogens resistant to carbapenems in these environments. The mangroves on the island of São Luis, in this way, understanding the risk factors linked to the genes of bacterial resistance have become the factor that led to the realization of this research.

2. Method and Materials

2.1 Sample Collection and Isolation of Strains

Samples were collected in a region covered with mangrove vegetation along the Anil River in São Luís Island-MA. The points of the area were selected for coexistence feature with sewage release, along with the operation of the former MERCK chemical industry which was located in the region over a period of far use, now disabled. For sediment, collections were done at 10 cm below the surface layer thereof. For each point three (03) samples were harvested with a plot of 1 m^2 in area. Each sample consisted of 200 g each [13]. The sampling points were primary point located on latitude -2.538721, -44.263011 longitude and the secondary point downstream of such entries, latitude -2.537355, -44.263977 longitude (Fig. 1).

The samples were mixed, and each solid sample was withdrawn 1 g, weighed on an analytical balance. A 1 g sample was put up into tubes containing 9 mL of sterile NaCl solution (0.9%) and serially diluted (decimal dilutions).

The quantification of the concentration of Gram-negative bacteria CFU/g soil sample of about 100 µL was inoculated onto the surface of MacConkey agar (Difco, Inc., Detroit, MI. USA) which was added with 4 µg/mL, 8 µg/mL, 16 µg/mL, 32 µg/mL of meropenem antibiotic (Sigma-Aldrich Corporation, St. Louis, MO. USA). Samples were dispersed with the aid of a handle Drigalski for 5 min. After inoculation, the plates were incubated at 37 °C for up to 72 h for growth and quantification of colonies (CFU sediment/g). After staining and characterization by determination of the Gram colonies they were purified on MacConkey agar (by depletion method). Bacterial isolates were stored on brain heart infusion (BHI) (Difco, Inc., Detroit, MI. USA) with 20% glycerol at -80 °C.

The bacterial species were identified using mass spectrometry desorption and ionization by matrix assisted

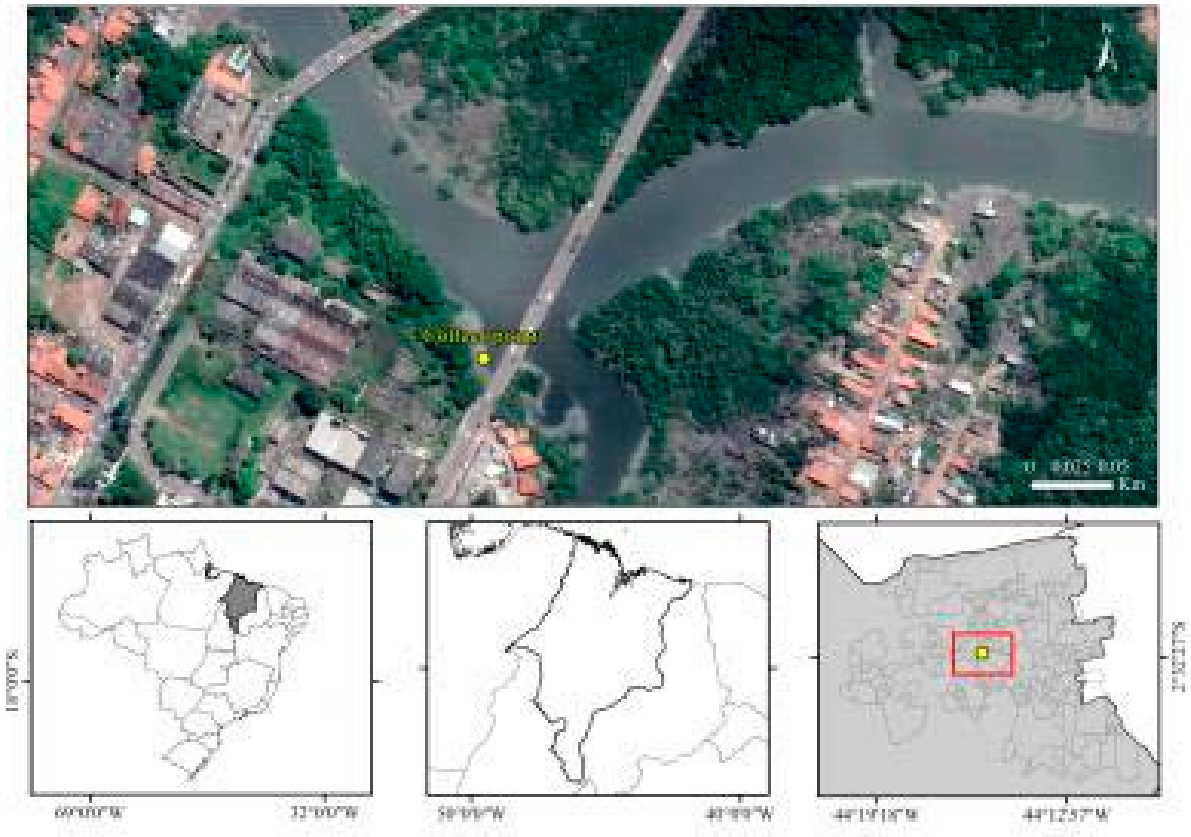

Fig. 1 Collection point map.

laser: time of flight (MALDI-TOF MS). Bacteria were grown for 24 h at 35 °C on brain heart infusion agar (BHI) (Difco, Inc., Detroit, MI. USA) in plates. Samples of the colonies were scraped off using a sterile plastic loop and applied as a thin film on a steel plate of 24 points (Bruker Daltonics, Bremen, Germany). The air dried sample was treated with 1 μL of a saturated solution of acid matrix-cyano-4-hydroxycinnamic acid (Sigma-Aldrich, St. Louis, MO, USA) in 50% acetonitrile/trifluoroacetic acid 25% (Sigma-Aldrich, St. Louis, MO, USA). Mass spectra were acquired in reflector positive mode in MicroFlex table LT instrument (Bruker Daltonics) using the default manufacturer settings. The captured spectra were analyzed using MALDI Biotyper software automation 2.0 (Bruker Daltonics). The identification criteria used in our analysis were the following: a score ≥ 2,000 indicated identification of the species level, 1,700-1,999 score indicated the genus level, and a score < 1,700 was interpreted as unidentified. Escherichia coli ATCC 8739 was used as a positive control for the analysis. And 700 was interpreted as unidentified.

2.2 DNA Extraction from Bacterial Isolates

Bacterial cultures were grown for up to 18 hours in trypticase soy broth (Difco, Inc., Detroit, MI. USA) to amplify the target gene fragments related to β-lactamases. After incubation, cells were centrifuged at 500 μL Tris EDTA buffer (TE, pH 8), 10,000 rpm for 20 minutes to obtain the cells. The DNA from the samples was obtained using the DNA Wizard® Genomic Purification Kit (Promega Corp., Madison, WI, USA), following the original manufacturer's protocol. Genomic DNA was quantified with NanoDrop™ 1000 Spectrophotometer (Thermo

Scientific, Pittsburgh, PA, USA) at 260 and 280 nm.

2.3 A Multiplex PCR Reactions and Sequencing Procedures

In order to analyze which genes related to β-lactamase enzymes present in the isolated Gram-negative bacteria were amplified by multiplex polymerase chain reaction (multiplex PCR) genes *bla*$_{KPC}$, *bla*$_{NDM}$, *bla*$_{OXA23}$, *bla*$_{OXA24}$, and *bla*$_{OXA58}$ [14, 15], Multiplex PCR reactions were performed in Mastercycler (Eppendorf AG, Hamburg, Germany) thermal cycler in a final volume of 25 µL. All PCR reactions were performed with GoTaq GreenMaster Mix (Promega, Madison, USA). The reaction contained 20 ρmol of each specific primer and were run using 15 µL of Master Mix, 2 µL 50 ng DNA and 1 µL of each primer (Exxtend Biotechnology, California, Br). The amplification reactions for the genes were performed under the following conditions: 94 °C for 5 minutes (initial denaturation) followed by 30 cycles of 94 °C for 1 minute, 54 °C for 1 minute and 72 ° C for 1 minute and final extension step at 72 °C for 5 minutes. After amplification, the fragments were separated by electrophoresis on 1.5% agarose gel with 0.5× TBE buffer (pH 8.0) at 100 V for 120 min.

The obtained gene fragments *bla*$_{KPC}$ were subjected to sequencing reactions using sequence DYEnamic ET Terminator cycles (GE Healthcare Life Sciences, Buckinghamshire, UK) according to the manufacturer's instructions. The fragments were analyzed in the ABI automatic sequencing system PRISM® 3100 Genetic Analyze (Applied Biosystems, USA). The quality of the eletropherogramas sequences obtained during the sequencing process was analyzed with the software ChromasPro (http://www.technelysium.com.au/chromas.html). For alignment, at least three fragment sequences of each *bla*$_{KPC}$ gene sequenced were selected from the database for alignment using MEGA 6.0 [16]. The similarity between the obtained nucleotide sequences was verified using BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.4 Metabolic Diversity Present in the Mangrove Using the Biolog System EcoPlates^{TM}

The Biolog EcoPlateTM (Biolog, Inc., Hayward, CA, USA) was used to determine the physiological profile of the Community (CLPPs) of mangrove sediments to assess the metabolic potential of the various microbial communities. Each plate contained 31 carbon sources, totaling 96 wells per plate, and a control with no carbon source, one sequence wells to the standard. A fresh mass equivalent of 1 g pellet was suspended in 10 mL of sterile NaCl 0.1% solution (w/v) and then stirred for 2 minutes at 1,000 rpm to pellet the bacteria extract. Subsequently, the suspension was diluted 100 times (v/v) with sterile NaCl solution 0.85% (w/v) inoculated and 120 µL of this suspension to each well. The microplates were incubated at 30 °C for 72 h in the dark. The plates were evaluated every 4 hours for the color content of the wells. After incubation calculated the average development of color per well (AWCD). The time to reach a AWCD 0.5 (AWCD 0.5) was determined by extrapolation to repeat each microplate. From this moment, the total number of substrates metabolized (metabolic wealth S) was determined using OD 0.1 as threshold for a positive response and Shannon diversity index (H).

2.5 Shannon Diversity Index, Equity and Wealth Substances

The Medium Heterotrophic metabolism (MHM) for each carbon source was calculated according to Garland [6]. The Shannon diversity index (H) which comprises both the richness of the substrates and the intensity with which they are used by the microorganisms was calculated according to Zak [17], using the

$$\Sigma pi\ H = (pi \ln (pi)). \quad (1)$$

where Pi is the ratio of the activity using a given substrate and usage activity of the entire substrate.

The richness of substrates (*S*) was determined by the total number of used substrates (MHM > 0) by microbial community sediment (ground). The substrate equality (*E*), which measures the uniformity of use of a substrate compared to the number of substrates used by the microorganisms, where *S* is the number of substrates, and *H* is the Shannon diversity index calculated according to Eq. (2).

$$E = H / LNS \quad (2)$$

3. Result and Discussions

The mangrove areas of the island of Saint Louis in São Luís Island region, located in the state of Maranhão, Brazil, currently correspond to an area of 120 square kilometers of extension. A special feature of these mangrove areas is the growing environmental impact, mainly caused by sewage discharges due to various human activities around. Particularly in this context, pollution Anil River, covering an area of 12.63 km^2, bordered in places by mangrove vegetation, receives various sewer outfalls, some connected to the hospital network of the city of São Luis. In the study, density analysis of Gram-negative bacteria resistant to meropenem observed in mangrove sediment ranged from 5.5 × 10^2 CFU/g (4 µg/mL) at 1 × 10^2 CFU/g (16 µg/mL) to the point 01 and 12.7 × 10^3 CFU/g (4 µg/mL) to 1.5 × 10^2 CFU/g (16 µg/mL) to point 02. No bacterial growth was observed at concentration of 32 µg/mL.

Considering the increasing movement of bacteria resistant to antibiotic drugs, trace and detect the first time of the presence of Gram-negative bacteria resistant to meropenem antibiotic that harbors the *bla*$_{KPC}$ gene in mangrove sediment of Anil river in Island Upaon-Acu (Brazil). In this study, we identified six (06) bacteria strains by MALDI Q-TOF MS/MS housing the *bla*$_{KPC-2}$ gene. The positive bacterial strains for *bla*$_{KPC-2}$ gene were C3 *Stenotrophomonas maltophilia* (resistant to 8 mg/mL—point 1), *Pseudomonas putida* (resistant to 8 µg/mL—point 1), *S. maltophilia* C1 (resistant to 16 µg/mL—point 2), *P. putida* C2 (resistant to 16 µg/mL—point 2), *S. maltophilia* C5 (resistant to 8 µg/mL—point 2), *S. maltophilia C6* (resistant to 8 µg/mL—point 2). Molecular approaches for the detection of genes for carbapenemases, such as *bla*$_{KPC}$ directed bacterial isolates in environmental samples are highly sensitive and efficient [18]. On the other hand, few studies have been developed to become a routine in the screening of bacteria carrying carbapenemases from environmental samples as part of surveillance systems [18, 19].

The amplified genes fragments of the isolates of *S. maltophilia* and *P. putida* were identified as *bla*$_{KPC-2}$ gene after sequencing and analysis of sequences, all sequences for *bla*$_{KPC-2}$ were deposited in GenBank with accession numbers KU695917 to KU696016. The similarity indices ranged from 99 to 100%, indicating that the sequenced region was highly conserved, *Stenotrophomonas maltophilia*-C1MK940321, *Stenotrophomonas maltophilia*-C3 MK940322, *Stenotrophomonas maltophilia*-C5MK940323, *Stenotrophomonas maltophilia*-C6 MK940324, *Pseudomonas putida*-C2 MK940325, *Pseudomonas putida*-C4 MK940326. The KPC-2 enzyme is a variant dominant and a factor that makes mediating factor carbapenems resistant in Enterobacteriaceae. On the other hand, *bla*$_{KPC-2}$ gene is emerging in other bacterial families, as well as other genes as *bla*$_{KPC-3}$ and *bla*$_{KPC-5}$ [19, 20]. Gram-negative bacteria that produce carbapenemases were isolated from river sediments, such as species *Escherichia coli* and *Enterobacter cloacae*, harboring *bla*$_{KPC-2}$ gene, and carrying IncN group plasmid, which is supposed to be an important dissemination of reservoir resistant to carbapenems [21]. *S. maltophilia* is a Gram-negative bacillus considered an opportunistic pathogen and is not highly virulent, however has been a major hospital pathogen associated with high mortality rates ranging 14% to 69% in patients with bacteremia [21, 22].

Aquatic environments such as rivers and lakes favor their survival in the environment, as in hospital areas they survive because of the characteristic having

favored in relation to its survival in moist environments, colonizing biological fluids such as urine, respiratory mucus, bladder and intravenous fluids [22, 23]. In the mangrove region from which samples were obtained from the Anil River a considerable impact is suffering due to the release of untreated domestic effluent. In addition, the area is directly influenced by emissaries of hospitals located in the Anil River, which may have favored the presence and dissemination of microorganisms of different or the same species carrying the *bla*$_{KPC-2}$ gene detected in *S. maltophilia* isolates [24].

In this study, there is a tolerance of up to 16 μg/mL meropenem for the isolates of *S. maltophilia*. That resistance profile is consistent with that observed for isolates obtained from samples of the lower respiratory tract, where it was observed that 100% of the microorganisms were resistant to imipenem and meropenem [25]. This study focused on bacteria obtained from sediment samples where this localized mangrove vegetation indicated the relevance of non-fermenting Gram-negative bacilli (NFGNB) as agents likely to disseminate the *bla*$_{KPC}$ gene in the aquatic environment.

The results in diversity of the Biolog System™ EcoPlates substrate consumption indicated a high diversity in point 02. For the Shannon diversity index it obtained a value of 2.448 and 3.216 for point 1 and point 2 respectively. For the evolution of microbial communities in environments of selective pressures and the heterogeneity of availability of nutrients such as carbon, nitrogen, sulfur and phosphorus occur in stabilizing metabolism favoring existing micro-organisms [26, 27]. Best environmental conditions favor the selective pressure and greater amount of biogeochemical compounds tend to increase in total biomass and reduced diversity due to the high competition for resources, prevailing with some groups over others, thus contributing to these environmental adaptations through genetic mechanisms elevating the spread of genes that favor the microorganisms resistant to the different classes of antimicrobial agents [28]. This shows that biological diversity is present at the point after the sewage releases, which was expected due to the presence of high load organic from the effluent, since the organic discharge promotes biodiversity and increases the diversity of metabolic profiles [29]. For the richness of the substrate (S) for sample 1 (one) equal to 44 (forty-four), and sample 2 of 65 (sixty-five), where S reflects the number of microorganisms found in the environment, there is a greater amount of microorganisms present in two points where there is a greater insertion effluent [28]. The corresponding equity of the points 1 and 2 was 0.646934461 and 0.77048394, respectively. These data support an increase in the microbial load in the region with greater distribution of substrate consumption in point two, leading to an increased diversity index (H) [28, 30].

4. Conclusions

Thus, we observed that increasing the organic matter deposited along the river in its Anil mangrove system causes a variation in the biodiversity considered, as the Shannon index and equity. It contributes to the increasing number of meetings among different microorganisms, thus facilitating the exchange of MLEs, which may be related to genes conferring resistance to antibiotics, such as *bla*$_{KPC-2}$ gene. The evidence of the presence of genes to inactivate carbapenem antibiotics in potentially pathogenic bacteria in the mangrove environment indicates that a rapid spread of *bla*$_{KPC-2}$ gene has its source likely sewage from untreated hospitals and households. The decontamination of hospital and domestic wastewater and stricter disposal are necessary actions for this problem to be alleviated. Moreover, there is an imminent risk of contamination of the local population with resistant bacteria, since these individuals use the fishing area and withdrawal crab feeding addition leisure, since the resistant bacterial counts were high for samples.

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