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Carbapenem resistant *Enterobacteriaceae* from port areas in São Paulo State (Brazil): Isolation and molecular characterization

Vanessa Costa Andrade[a,b,*], Tânia Caetano[c], Sónia Mendo[c], Ana Júlia Fernandes Cardoso de Oliveira[a,b]

[a] Departament of Biochemistry and Microbiology, São Paulo State University (UNESP), Rio Claro, Brazil
[b] Marine and Environmental Microbiology Laboratory, São Paulo State University (UNESP), Biosciences Institute, São Vicente, Brazil
[c] CESAM & Department of Biology, University of Aveiro, 3810-193 Aveiro, Portugal

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ABSTRACT

Coastal areas with important economic activities have high levels of contamination by metals, pathogenic bacteria, among other contaminants. The emergence of antibiotic-resistant bacteria is a global problem of public health. Carbapenem resistant Enterobacteriaceae (CRE) are a serious threat. The occurrence of carbapenem resistant bacteria was investigated in waters and sediments of a Brazilian coastal area, characterized by high levels of contamination. The samples of water and sediment were collected in two areas of the coast of São Paulo (Brazil). The study involved the characterization of the molecular mechanisms associated with the carbapenem resistance phenotype. No genes were detected for β-lactamases but the absence and/or presence of mutations in outer membrane proteins (OMPs) may justify the detected phenotype. The presented results show the need for further studies that allow a review of the current legislation and the importance of the reevaluation of monitoring policies of these environments.

1. Introduction

Natural environments, such as coastal areas, impacted by anthropogenic activities, tend to present higher levels of organic and inorganic contamination (Al Rashidi et al., 2015; Shibata et al., 2004). Coastal areas are also regions of important economic activities (*e.g.* tourism and harboring) and are, therefore, complex environments usually characterized by high population densities (Zampieri et al., 2017). The Brazilian coastline was disorderly occupied without the implementation of effluent treatment policies or strategies to mitigate the impacts of port activities. This has contributed to an increase of contaminants in adjacent areas, namely, metals and potentially pathogenic microorganisms such as antibiotic resistant bacteria (ARB) (Knapp et al., 2017; Gimiliani et al., 2016; Oliveira and Pinhata, 2008; Wright et al., 2006). Since 2014, WHO considers antimicrobial resistance (AMR) has one of the biggest threats to global health, food security, and development (WHO, 2014). The massive use of antibiotics, especially their inappropriate use, accelerated the spread of acquired bacterial resistance (Hocquet et al., 2016; Gullberg et al., 2011) not only in hospitals, but also in the natural environments (Taylor et al., 2011; Allen et al., 2010). In last few years, the emergence and rapid dissemination of Gram-negative bacteria resistant to β-lactams, especially carbapenems, are of great concern (Diene and Rolain, 2014). Carbapenems are a class of β-lactams usually used as a last resort for the treatment of multidrug-resistant bacteria (Janecko et al., 2016). Currently, carbapenem-resistant bacteria, especially *Enterobacteriaceae* (CRE) are a serious threat to global public health (Logan and Weinstein, 2017; Potter et al., 2016; Walsh et al., 2005). Resistance to carbapenems can be mediated by the inactivation of the antibiotic (enzymatic) or by target modification (*e.g.* mutations or lack of outer membrane proteins (OMPs)) (Bush, 2013; Walsh et al., 2005).

Due to physiological (cross-resistance) and genetic mechanisms (co-resistance), metal resistance also drives and selects for the spread of AMR (including carbapenems) (Seiler and Berendonk, 2012; Baker-Austin et al., 2006). This co-selection mechanism contributes to the increasing risk of occurrence of superbugs in the environment (Knapp et al., 2017; Zampieri et al., 2016; Leonard et al., 2015; Baker-Austin et al., 2006). Metals are more stable than antibiotics, lasting longer in the environment. They are, therefore, a long-drawn-out co-selection factor of ARB, possibly playing an important role in the proliferation and dissemination of these bacteria (Baker-Austin et al., 2006). This is particularly relevant in areas with a high metal content (*e.g.* cadmium, copper and zinc) such as polluted port and harbor areas.

The São Paulo state is considered the largest economic and

* Corresponding author at: Marine and Environmental Microbiology Laboratory, São Paulo State University (UNESP), Institute of Biosciences, São Vicente, Brazil.
E-mail address: costa.andrade@terra.com.br (V.C. Andrade).

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industrial center in the Southern Hemisphere. Its coast is highly impacted by anthropogenic activities such as harbors, fishery and tourism and it is a region with high population density. Specific examples include the largest port of Latin America (Port of Santos), petrochemical, metallurgy and fertilizer industries (Cortez et al., 2018; Torres et al., 2015). In addition, untreated effluents are discharged into the environment and sewage treatment is lacking, contributing to the increase of metals in water and sediment (Lino et al., 2016; Zampieri et al., 2016; Kim et al., 2016; Pinto et al., 2015; Abessa et al., 2005).

The present study aimed to investigate the occurrence of carbapenem resistant bacteria in water samples and sediment from polluted and preserved Brazilian coastal areas. The isolates were identified at the genus and/or species level and molecular analysis was employed to understand the resistance molecular mechanisms behind their phenotype. Finally, the susceptibility to metals and the correlation between antibiotics-metals resistance were evaluated in order to understand co-selection mechanism.

2. Material and methods

2.1. Study area and sampling

This study was carried out in two sites on the coast of the State of São Paulo State: Araça Bay (AB; 23°49′S, 45°24′W; Fig. 1A) and Santos (S; 24°00′S; 46°21′W; Fig. 1B). Sediment and water samples were collected between July of 2016 and January of 2018. Water samples were collected in sterile flasks and sediments were manually collected (shallow waters) or with the help of a dredge (deeper waters), and stored in sterile plastic bags. The samples were kept refrigerated (4 °C) until their analysis, which occurred within 12 h after of collection.

2.2. Bacterial isolation and identification

The microorganisms adhered to the sediments were recovered as described by Oliveira and Pinhata (2008). 100 μL of water or sediment suspension were plated on MacConkey agar plates with imipenem (1 μg mL^{-1}; IPM). IPM resistant colonies were selected after incubation at 37 °C for 24 h. The production of metallo-β-lactamase (MBL) was detected with the imipenem-EDTA double-disk synergy test as previously described (Lee et al., 2003). Biochemical identification of $IMP^{R}MBL^{+}$ strains was performed with the Enterokit (Probac® - São Paulo/SP, Brazil) according to the manufacturer's instructions. pDNA extraction was performed with NZYMiniprep (NZYTech, Portugal), as indicated by the manufacturer.

For bacterial identification at the molecular level, the 16S rDNA gene was amplified by colony PCR with the 27F and 1492R primers (Table 1), followed by nucleotide sequence determination. The PCR was performed in a final volume of 50 μL containing 2× Platinum SuperFi PCR Master Mix (Thermo Fisher Scientific), 0.3 pmol/μL of each primer and 1 μL of the DNA template. Amplification was carried out with an initial denaturation at 98 °C for 30s, 30 cycles of amplification which consisted of denaturation at 98 °C for 10 s, annealing at 56 °C for 10s and extension at 72 °C for 50s and a final extension at 72 °C during 5 min. The amplification products were purified and sequenced (Stabvida, Portugal). The resulting Sanger sequences were analyzed using FinchTV 1.5.0 (Geospiza Research Team, 2012) and CLC Sequence Viewer 8.0 (Qiagen). The obtained nucleotides sequences were compared to others deposited at NCBI GenBank using the Blastn (http://blast.ncbi.nlm.nih.gov).

BOX-PCR fingerprinting was performed with BOX A1R primer (Table 1). PCR reaction was made in a final volume of 25 μL and contained: 3 mM $MgCl_2$, 0.2mMdNTPs, 1× NZYTaq Buffer, 0.6 pmol/μL of primer, 1 μL of DNA template and NZYTaq II 1 U (NZYTech). Amplification was carried out with an initial denaturation at 95 °C for 5 min, followed by 30 cycles of amplification: denaturation 30 s at 95 °C, annealing 30s at 53 °C, extension 8 min at 72 °C and a final extension for 10 min at 72 °C. PCR products were separated in a 1% agarose gel for 60 min, in 1× TAE Buffer containing 50 μM thiourea.

2.3. Antibiotic susceptibility testing

Antimicrobial susceptibility tests were performed according to the guidelines of the Brazilian Committee for Antimicrobial Susceptibility Testing (BrCAST, 2019). Disk-diffusion susceptibility tests were performed with the following antibiotics (μg/disc): ampicillin (10), amoxicillin/clavulanic acid (30), aztreonam (30), cefotaxime (5), cefoxitin (30), ceftazidime (10), ceftriaxone (30), ertapenem (10), fosfomycin (200), gentamicin (10), imipenem (10), meropenem (10), norfloxacin (10) and tetracycline (30). The minimum inhibitory concentration (MIC) of ertapenem, imipenem and meropenem was determined, in three replicates, using the microdilution broth method and according to BrCAST guidelines (BrCAST, 2019) and Clinical and Laboratory Standards Institute (CLSI, 2019). *Escherichia coli* ATCC 25922 was used as quality control on both tests.

2.4. Metal susceptibility testing

The metals tested were selected based on previous studies performed in the sampling areas (Zampieri et al., 2016; Torres et al., 2015; Buruem et al., 2012; Cesar et al., 2007). The stock solutions of cadmium ($CdCl_2$), cobalt ($CoCl_2$ $6H_2O$), copper ($CuSO_4$ $5H_2O$), nickel ($NiCl_2$ $6H_2O$) and zinc ($ZnCl_2$) were prepared at a concentration of 256 mM using ultrapure water. The MIC was determined using the microdilution broth method, with CMP broth with a metal concentration ranging from 0 to 128 mM (Viana et al., 2018). Three replicates were performed. The plates were incubated at 37 °C, overnight and growth was visually inspected. *E. coli* ATCC 25922 was used as reference strain. The isolates were considered resistant if the MIC was higher than that of *E. coli* ATCC 25922 (Zampieri et al., 2016; Akinbowale et al., 2007; Ansari and Malik, 2007). Spearman's correlation was performed in PAST 9.23 software (Hammer et al., 2001) to evaluate the co-selection metals-antibiotics. In this analysis, Intermediate resistance was considered as resistance.

2.5. Screening of metallo β-lactamases

The genomic DNA from the positive strains tested for IPM-EDTA double disk synergy was purified with NZYGelpure (NZYTech, Portugal). The presence of the MBL genes bla_{NDM}, bla_{VIM}, bla_{IMP}, bla_{SIM}, bla_{DIM}, bla_{GIM}, bla_{SPM} and bla_{AIM} was screened by PCR with the primers previously described (Table 1). Colony PCR was carried out in a final volume of 25 μL containing 1× Platinum SuperFi PCR Master Mix (Thermo Fisher Scientific), 0.3 pmol/μL of each primer and 1 μL of DNA template. Amplification was carried out with an initial denaturation at 95 °C for 3 min, 30 cycles of amplification consisting of 30 s denaturation at 94 °C, annealing 30s at the appropriated temperature for each primer (Table 1) and 45 s extension at 72 °C, followed by a final extension at 72 °C, for 5 min.

2.6. Screening of other antibiotic resistance determinants

The presence of other β-lactamase genes bla_{KPC}, bla_{GES}, bla_{TEM}, bla_{CTX}, bla_{SHV}, bla_{OXA} and bla_{BIC} as well as class 1, 2 and 3 integrases were investigated using the primers described in Table 1. The detection of outer membrane proteins (OMPs) genes was also performed by PCR, using the following primers: OmpA, OmpC, OmpF, OmpK35, OmpK36 and OmpK37 (Table 1). PCR amplification setup and amplification parameters were performed as described in the previous section. After purification, the presence of mutations in the *omp* genes were investigated by Sanger sequencing (Stabvida, Portugal), followed by protein alignment with reference enterobacterial OMPs.

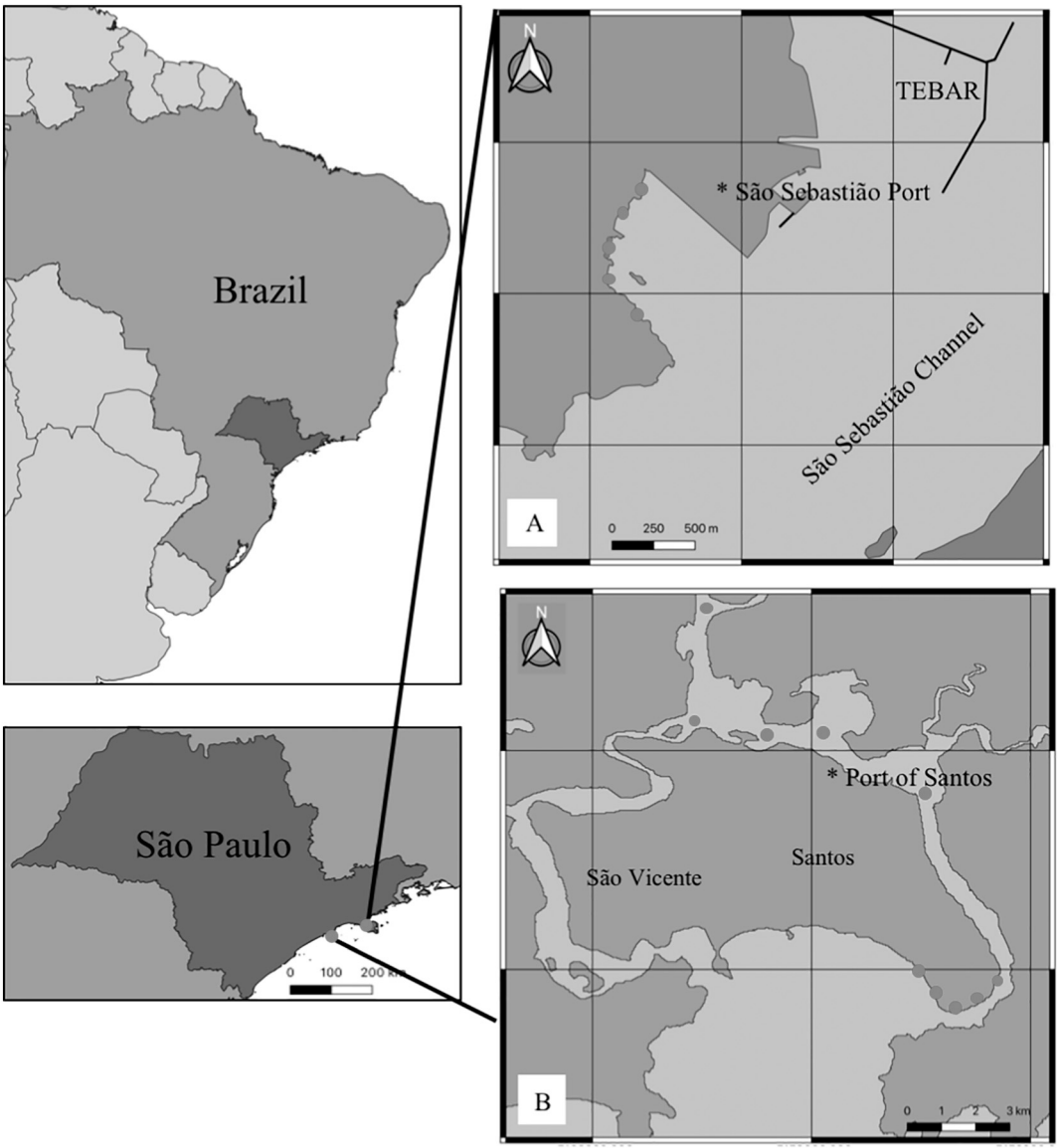

Fig. 1. Study areas and sampling points. A) Araça Bay, located in São Sebastião municipality. B) Santos, located in Santos Basin.

3. Results

3.1. Recovery of MBL-positive strains

A total of 331 strains were isolated. 48 of them were resistant to IPM and of these, 11 tested positive in the IPM-EDTA double-disk synergy test. Approximately 73% of the IPM-EDTA^{R} strains were recovered from samples collected in the Santos area and 64% from sediment samples from both study areas. The Santos area shelters the largest port of Latin America (Port of Santos) as well as a large industrial complex of petrochemical, metallurgy and fertilizer industries (Cortez et al., 2018; Torres et al., 2015). In this area, the collection and disposal of sewage is an important issue and a serious public health risk: most of the discharges are not adequately treated, being collected and released directly through Santos Submarine Emissary, resulting in the contamination of water and sediments (Gimiliani et al., 2016; Oliveira and Pinhata, 2008). Consequently, Santos has a high anthropogenic influence when compared to Araça Bay, which is considered one of the last preserved mangrove areas of the Municipality of São Sebastião (Lamas et al., 2016; Amaral et al., 2010). Araça Bay is part of two environmental protection areas: Marine Protected Area of the Northern Coast of São Paulo State (APAMLN) and Environmental Protected Area of the Alcatrazes Municipality (APAMA) (Kim et al., 2018). However, over the last years, the anthropic impacts in this area have been rising (Muniz et al., 2015). This is especially due to the sewage from a marine outfall, activities of São Sebastião harbor and the petrochemical Petrobras Waterway Terminal (TEBAR) that contribute to the increase of contamination, especially by metals (Zampieri et al., 2016).

The 11 IMP-EDTA^{R} strains were identified by biochemical and molecular methods and their results coincided. 36.4% of the strains belonging to *Escherichia coli*, 18.18% to *Lemiriorella* spp. and *Raoultella* spp. and 9.1% to *Budvicia aquatica*, *Enterobacter bugandensis* and *Klebsiella pneumoniae* (Table 2). BOX-PCR fingerprinting was performed and no closely-related strains (or even clones) could be identified, even among the *E. coli* isolates isolated from Santos area (S5, S8, S9 and S11; Fig. 2).

3.2. Antibiotic susceptibility of MBL-positive strains

Curiously, by disk diffusion antibiotic susceptibility testing none of

Table 1
Primers used in the present study, size of the expected amplicon and annealing temperature used in each reaction.

Primer	Nt. sequence (5′ – 3′)	Size (bp)	Annealing temp.	Reference
27F	AGAGTTTGATCCTGGCTCAG	1360	56 °C	Zampieri et al. (2016)
1492R	GGTTACCTTGTTACGACTT			
BOX A1R	CTACGGCAAGGCGACGCTGACG	ND	53 °C	Rademaker and de Bruijn (1997)
NDM-F	GGTTTGGCGATCTGGTTTTC	621	56 °C	Poirel et al. (2011)
NDM-R	CGGAATGGCTCATCACGATC			
VIM-F	GATGGTGTTTGGTCGCATATCG	390	58 °C	Poirel et al. (2011)
VIM-R	GCCACGTTCCCCGCAGACG			
IMP-F	GAATAGAGTGGATTAATTCTC	232	55 °C	Poirel et al. (2011)
IMP-R	CGTTTAACAAAACAACCACC			
SIM-F	TACAAGGGATTCGGCATCG	570	52 °C	Poirel et al. (2011)
SIM-R	TAATGGCCTGTTCCCATGTG			
DIM-F	GCTTGTCTTCGCTTGCTAACG	699	52 °C	Poirel et al. (2011)
DIM-R	CGTTCGGCTGGATTGATTTG			
GIM-F	TCGACACACCTTGGTCTGAA	477	52 °C	Poirel et al., 2011
GIM-R	AACTTCCAACTTTGCCATGC			
SPM-F	AAAATCTGGGTACGCAAACG	271	52 °C	Poirel et al. (2011)
SPM-R	ACATTATCCGCTGGAACAGC			
AIM-F	CTGAAGGTGTACGGAAACAC	322	52 °C	Poirel et al. (2011)
AIM-R	GTTCGGCCACCTCGAATT			
KPC-F	TGTCACTGTATCGCCGTC	798	52 °C	Poirel et al. (2011)
KPC-R	CGTTGACGCCCAATCC			
GES-F	ATGCGCTTCACGCAC	642	52 °C	Poirel et al. (2011)
GES-R	CTATTTGTCCCTCAGG			
TEM-F	AAAGATGCTGAAGATCA	425	44 °C	Poirel et al. (2011)
TEM-R	TTTGGTATGGCTTCATTC			
CTX-F	GTGCAGTACCAGTAAAGTTAAG	550	55 °C	Poirel et al. (2011)
CTX-R	CGCAATATCATTGGTGGTGCC			
SHV-F	GCGAAAGCCAGCTGTCGGGC	304	62 °C	Poirel et al. (2011)
SHV-R	GATTGGCGGCGCTGTTATCGC			
OXA-F	ACACAATACATATCAACTTCGC	438	52 °C	Poirel et al. (2011)
OXA-R	AGTGTGTTTAGAATGGTGATC			
BIC-F	TATGCAGCTCCTTTAAGGGC	537	52 °C	Poirel et al. (2011)
BIC-R	TCATTGGCGGTGCCGTACAC			
OmpA-F	ATTGCAGTCGCACTGGCTGG	1100	60 °C	Santos et al. (2017)
OmpA-R	GCCTGCGGCTGAGTTACAAC			
OmpC-F	GTTAAAGTACTGTCCCTCCTG	1200	54 °C	Santos et al. (2017)
OmpC-R	GAACTGGTAAACCAGACCCAG			
OmpF-F	AGCGCAATATTCTGGCAGTG	1000	54 °C	Santos et al. (2017)
OmpF-R	CTGGTAAACGATACCCACAG			
OmpK35-F	CAGACACCAAACTCTCATCAA	~1080	60 °C	Santos et al. (2017)
OmpK35-R	AGAATTGGTAAACGATACCCA			
OmpK36-F	CAGCACAATGAATATAGCCGA	~1110	60 °C	Santos et al. (2017)
OmpK36-R	GCTGTTGTCGTCCAGCAG GTT			
OmpK37-F	CATTCCGCAGAATGAGACGGC	~1115	60 °C	Santos et al., (2017)
OmpK37-R	CGACGATGTTATCGGTAGAGA			
IntI 1-F	ACATGCGTGTAAATCATCGTCG	500	65 °C	Santos et al. (2017)
IntI 1-R	CTGGATTTCGAATCACGGCACG			
IntI 2-F	GCAAACGCAAGCATTCATTA	400	62 °C	Santos et al. (2017)
IntI 2-R	ACGGATATGCGACAAAAAGG			
IntI 3-F	AGTGGGTGGCGAATGAGTG	600	55 °C	Santos et al. (2017)
IntI 3-R	TGTTCTTGTATCGGCAGGTG			

Table 2
Identification of isolates and respective sampling areas.

Strain	Species	Area (Sample)
AB1	*Budvicia aquatica*	Araça Bay (Water)
AB2	*Lemiriorella grimondii*	Araça Bay (Sediment)
AB3	*Lemiriorella richardii*	Araça Bay (Sediment)
S4	*Raoultella ornithinolytica*	Santos (Sediment)
S5	*Escherichia coli*	Santos (Water)
S6	*Raoultella plantiocola*	Santos (Sediment)
S7	*Klebsiella pneumoniae*	Santos (Water)
S8	*Escherichia coli*	Santos (Water)
S9	*Escherichia coli*	Santos (Sediment)
S10	*Enterobacter bugandensis*	Santos (Sediment)
S11	*Escherichia coli*	Santos (Sediment)

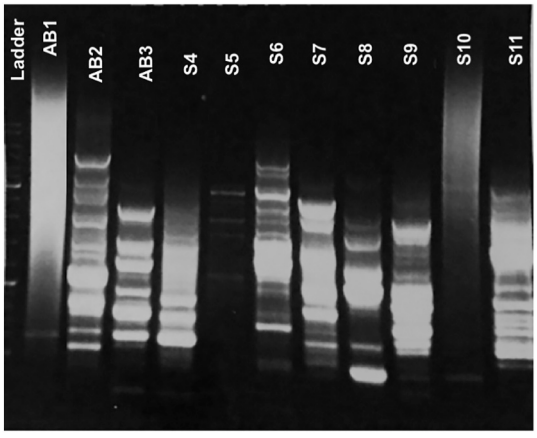

Fig. 2. Image of an agarose gel of the BOX-PCR performed with the 11 isolates under study.

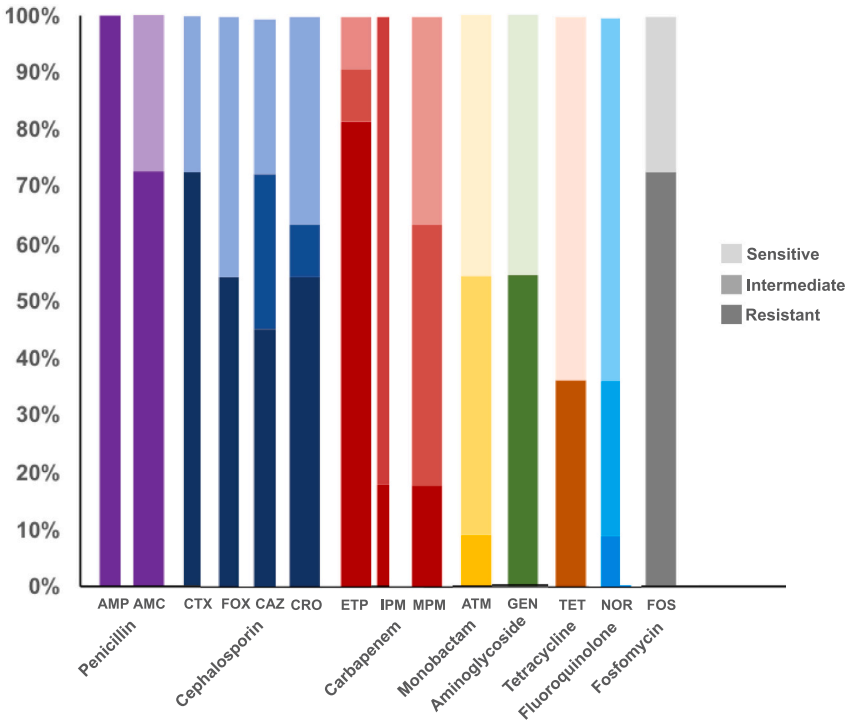

Fig. 3. Susceptibility results obtained by disk-diffusion method for the 11 isolates to different classes of antibiotics: i) Penicillin: ampicillin (AMP), amoxicillin + clavulanic acid (AMC); ii) Cephalosporins: cefotaxime (CTX), cefoxitin (FOX), ceftazidime (CAZ), ceftriaxone (CRO); iii) Carbapenems: ertapenem (ETP), imipenem (IPM), meropenem (MPM); iv) Monobactam: aztreonam (ATM); v) Aminoglycoside: gentamicin (GEN); vi) Tetracycline: tetracycline (TET); vii) Fluoroquinolone: norfloxacin (NOR); vii) Fosfomycin: fosfomycin (FOS). The resistance of each antibiotic is shown by the intensity of the colors, from dark (Resistant), medium (intermediate) and light (sensitive).

Table 3
Minimum Inhibitory Concentrations (MIC) of carbapenems and resistance phenotype according to BrCAST (2019) breakpoints. (R) resistant and (I) intermediate.

MIC (μg mL^{-1})											
Antibiotics	AB1	AB2	AB3	S4	S5	S6	S7	S8	S9	S10	S11
Ertapenem	8(R)	32(R)	32(R)	8(R)	32(R)	4(R)	16(R)	16(R)	32(R)	32(R)	32(R)
Imipenem	4(I)	16(R)	16(R)	16(R)	16(R)	16(R)	16(R)	8(R)	32(R)	16(R)	16(R)
Meropenem	8(I)	16(R)	32(R)	16(R)	16(R)	16(R)	8(I)	16(R)	16(R)	16(R)	64(R)

the isolates were susceptible to imipenem, and they are all resistant (18%) or intermediate resistant (82%) to this antibiotic (Fig. 3). Considering the other carbapenems, resistance to ertapenem was high (81%) and 18% of isolates were resistant to meropenem. Intermediate resistance to meropenem was approximately 45% (Fig. 3). The results of MIC presented that all the strains were resistant to ertapenem and the majority was also resistant to imipenem and meropenem (Table 3). Only *B. aquatica* AB1 strain presented intermediate susceptibility to imipenem and meropenem and *K. pneumoniae* S7 to meropenem (Table 3). Regarding other studied antibiotics (Fig. 3), all the isolates were resistant to ampicillin and 72.7% showed resistance to amoxicillin/clavulanic acid. In the cephalosporin susceptibility test, 54.5% of the isolates were resistant to cefotaxime, cefoxitin and ceftriaxone and 45% were resistant to ceftazidime. 36.3% were resistant to tetracycline. Low resistance levels were detected to the β-lactam aztreonam (9%), whereas intermediate resistance to this antibiotic was higher (45,5%). Resistance to other classes of antibiotics was also detected, namely to fosfomycin (72.7%), gentamicin (54.5%), tetracycline (36.4%) and norfloxacin (9%). It was found that 54.5% of the strains were MDR since they were resistant to three or more different classes of antibiotics (Table S1). Among these, *Budvicia aquatica* AB1 was identified as a MD4R strain, since it was resistant to four different classes of antibiotics.

3.3. Metal susceptibility of MBL-positive strains

There are no breakpoints established for metal resistance. Therefore, *E. coli* ATCC was used as reference of susceptibility (Table 4). All strains presented an equal or higher MIC than *E. coli* ATCC 25922 to all the metals tested. In general, the highest MICs were obtained for Ni, Cu and Zn. Spearman's correlation analysis was performed to evaluate the co-selection between metal and antibiotic resistance (Table 5) and co-selection was observed with a correlation higher than 0.7. The results show that resistance and/or decreased susceptibility to some

Table 4
MICs of metals for the 11 tested strains. *E. coli* ATCC 25922 (C) was included as reference strain.

MIC (mM mL^{-1})												
Metal	C	AB1	AB2	AB3	S4	S5	S6	S7	S8	S9	S10	S11
Cd	0.12	1	0.5	0.25	0.5	0.5	0.5	1	4	1	1	0.25
Co	0.5	1	0.5	0.5	2	1	1	2	1	1	1	0.5
Cu	0.5	1	2	2	1	1	8	4	1	2	2	1
Ni	1	4	32	16	16	8	16	2	16	8	4	1
Zn	0.25	1	2	0.5	1	2	1	4	4	4	1	0.5

Table 5
Spearman's correlation. Values obtained between metal MICs and antibiotic-resistant strains.

Antibiotics	Metal				
	Cd	Co	Cu	Ni	Zn
Ampicillin	1*	1*	1*	1*	1*
Amoxicillin + clavulanic acid	0.25	0.02	0.59	0.28	0.21
Aztreonam	0.74*	0.26	0.82*	0.79*	0.91*
Cefotaxime	0.50	0.66	0.39	0.56	0.92*
Cefoxitin	0.41	0.15	0.20	0.42	0.71*
Ceftazidime	0.90*	0.16	0.58	0.81*	0.36
Ceftriaxone	0.65	0.15	0.52	0.93*	0.26
Ertapenem	0.55	0.91*	0.49	0.94*	0.82*
Fosfomycin	0.41	0.44	0.19	0.10	0.75*
Gentamicin	0.21	0.17	0.48	0.67	0.54
Imipenem	0.97*	0.93*	0.55	0.44	0.53
Meropenem	0.97*	0.93*	0.55	0.44	0.53
Norfloxacin	0.91*	0.12	0.63	0.21	0.64
Tetracycline	0.11	0.72*	0.76*	0.55	0.15

* Show a strong positive correlation (> 0.7).

Table 6
Detection of presence (+)/absence (−) of outer membrane proteins (OMPs) genes. (*) Mutation detected. (N/A) Not applicable.

	OmpK35	OmpK36	OmpK37	OmpA	OmpC	OmpF
B. aquatica AB1	+	−	−	+	+	+
L. grimontii AB2	+	+	+	+	+	+
L. richardii AB3	−	−	−	−	−	−
R. ornithinolytica S4	−	−	−	+	+	+
E. coli S5	+	−	−	+	−	−
R. plantiocola S6	+	−	−	+	+	−
K. pneumoniae S7	+*	+	+*	+	−	+
E. coli S8	+	−	−	+	+	−
E. coli S9	+*	+*	+	+	−	−
E. bugandensis S10	N/A	N/A	N/A	−	−	−
E. coli S11	+	−	−	+	+	+

antibiotics is related with higher MICs to metals, including: i) ampicillin with all metals, ii) aztreonam with Cd, Cu, Ni and Zn, iii) cefotaxime, cefoxitin and fosfomycin with Zn, iv) ceftazidime with Cd and Ni, v) ceftriaxone with Ni, vi) norfloxacin with Co and vii) tetracycline with Co and Cu (Table 5). Additionally, higher MICs of Co correlated with resistance to all carbapenems, whereas Cd correlated only with imipenem and meropenem and Ni and Zn only with ertapenem.

3.4. Screening of resistance genes and characterization outer membrane proteins (OMPs)

The IMP-EDTA^R strains tested negative for all the β-lactamase and integrase genes screened. In addition, we further investigated the presence of 6 OMP encoding genes (Table 6). All the OMP genes screened were amplified in L. *grimondii*, whereas none of them detected in L. *richardii* AB3 and *E. bugadensis* S10. The OmpK 35 and OmpA genes were amplified in all the *E. coli* strains. However, OmpK 36 and OmpK 37 were only present in strain S9 and OmpF in the strain S11. OmpC was successfully amplified in *E. coli* S8 and S11 isolates. Considering the two *Raoultella* spp., strain S4 has OmpA, OmpC and OmpF genes, whereas strain S6 has OmpK 35, OmpA, OmpC and OmpK 35. After sequencing, mutations were detected in OmpK 35 and OmpK 37 of *K. pneumoniae* S7 and OmpK 35 and OmpK 36 of *E. coli* S9 (Table 6). When compared to the reference sequence, *K. pneumoniae* S7 OmpK35 and OmpK37 have the following amino acid substitutions: DVEAA145HLQTT, V311L, S325L, D328N, V332G and S171T, D277N, D279N, R322G, I340F, respectively. The predicted OmpK 35 protein of *E. coli* S9 differ from the reference sequence on D73N, I97T, T116P, A122G, G132R, D135H, G137R, G141R, S234G and I264V. Amino acid residues substitutions were also detected in its OmpK 36 protein which included: L225Q, L236V, Y241F, Y250 and G257D and the deletion of SP residues at position 216.

4. Discussion

Marine environments are important for economic and leisure development, especially in coastal areas. However, they are also complex environments. They receive inputs of different types of contaminants, like sewage and port discharges. Effluents may contain antibiotics, antibiotic metabolites, as well as pathogenic bacteria that may be related to both household and hospital effluents (Marti et al., 2014; Vignaroli et al., 2018). It is recognized that *E. coli*, *K. pneumoniae* and *Enterobacter* spp. are important human pathogens (Maravic et al., 2015). Accordingly, ARB, in particular of Gram-negative resistant bacteria, is a public health problem. For instance, in the last 20 years, only two new classes of antibiotics have been developed, but none of them target Gram-negative bacteria (Luepke et al., 2017). Water and sediments play a significant role in the dissemination and evolution of ARB, by receiving and concentrating discharges of contaminants, such as from public sewage and hospital wastes (Marti et al., 2014; Oliveira and Pinhata, 2008). No less important are nonpathogenic soil bacteria that have a large collection of antibiotic resistance genes, contributing to the soil resistome and can also act as a source for the evolution and dissemination of antibiotic resistance determinants (Marti et al., 2014).

In the last decades, the excessive and intensive consumption of antibiotics, especially those of last resort, such as carbapenems, led to a rapid dissemination of ARB (Durão et al., 2018; Kelly et al., 2017). In Brazil, most of studies of CRE are focused on clinical isolates (Sampaio and Gales, 2016; Gales et al., 2012; Furtado et al., 2007), and very few of them are related to environmental isolates (Paschoal et al., 2017; Araujo et al., 2016; Montezzi et al., 2015). According to Rossi (2001), resistance is more relevant in the southern and southeast states. When compared to Europe and to the United States, Brazil has higher levels of ARB, especially among Gram-negative bacilli (Rossi, 2001). Paschoal et al. (2017) and Montezzi et al. (2015) studied and observed the presence of *Enterobacteriaceae* strains in Rio de Janeiro coast, detecting the presence of bla_{KPC}, bla_{GES}, bla_{CTX}, bla_{SHV}, bla_{TEM} (Montezzi et al., 2015), bla_{IMP} and bla_{NDM} (Paschoal et al., 2017). As observed in the present study, Araujo et al. (2016) found that the majority of *Enterobacteriaceae* strains isolated from Rodrigo de Freitas Lagoon and Carioca River (Rio de Janeiro) were resistant to CAZ, ATM, FOX and CTX. The predominant carbapenemase encoding genes found in isolates from Rodrigo de Freitas Lagoon and Carioca River were bla_{KPC}, bla_{GES} and $bla_{OXA\text{-}48\text{-}like}$. Apart from these three genes, bla_{NDM} was also detected in the water samples (Araujo et al., 2016).

Despite being IMP-EDTA^R, most of them were intermediate resistant to IMP by the disk diffusion assay. According to the MIC results, all the isolates were resistant to ETP, 90.9% were resistant to IPM and 81.8% to MPM. On the other hand, results from the disk-diffusion assay revealed that 81.8% strains were resistant to ETP, and only 18.2% were resistant to IPM and MPM. Consequently, the results on carbapenem resistance can depend on the antimicrobial susceptibility test used, as previously reported (Maalej et al., 2011; Tenover et al., 2006). We identified a higher rate of ETP resistance compared to IPM and MPM. This was also observed in other studies and might be explained by the penetration capacity rates through porins, where IPM and MPM are moderately active (Woodford et al., 2007; Elliott et al., 2006; Rossi et al., 2006).

Although the positive results in the double-disk synergy test, no carbapenemase encoding genes were detected in the isolates of the present study. Likewise, intermediate resistance or resistance to carbapenems has also been described for *K. pneumoniae* and *E. coli* isolates without any detectable carbapenemase encoding gene. Instead, these phenotypes were associated with the complete loss of outer membrane proteins or with nonsense mutations on those proteins, namely,

OmpK35 and OmpK36 (Adler et al., 2015; Tsai et al., 2013; Shin et al., 2012). For instance, Tsai et al. (2013) showed that the loss of these two OmpK in *K. pneumoniae* led to an increase of ertapenem (32 mg/L) and meropenem (8 mg/L) MICs, although with borderline breakpoints. In *E. coli*, mutations on other outer membrane proteins, OmpC and OmpF, were associated with resistance to meropenem and ertapenem (Adler et al., 2016). The loss of OmpK 35 in *E. coli* and *K. pneumoniae* was also associated with cefoxitin resistance (Ananthan and Subha, 2005). Thus, the absence of one or more of Omp genes detected in the *E. coli* strains of this study (S5, S8, S9 and S11) can justify the MIC for the carbapenems. Also, it can be hypothesized that the mutations detected in OmpK genes of *K. pneumoniae* S7 could be involved in the resistance phenotype (according to the MIC) to ertapenem and imipenem and the decreased susceptibility to meropenem. Regarding the other genera (*Budvicia* spp., *Leminorella* spp. and *Raoultella* spp.) there are few studies on susceptibility and its associated mechanisms (Sękowska, 2017; Westerveld et al., 2017; Chun et al., 2015; Haruki et al., 2014; Lam and Salit, 2014; Tomczak and Smuszkiewicz, 2014; Castanheira et al., 2009; Dalamaga et al., 2006; Blekher et al., 2000). Multidrug-resistance was detected in *R. ornithinolytica* (Chun et al., 2015; Haruki et al., 2014), *R. planticola* (Lam and Salit, 2014), *B. aquatica* (Tomczak and Smuszkiewicz, 2014), *Leminorella* spp. (Dalamaga et al., 2006; Blekher et al., 2000), but no encoding genes were investigated. Castanheira et al. (2009) described multidrug-resistant *R. plantiocola* and *R. ornithinolytica* encoding genes TEM-1, SHV-7 and KPC.

The absence of standardized methods for metal susceptibility testing as well as the absence of established resistance cut-offs make it difficult to compare metal resistance phenotypes between different studies. Nonetheless, herein, the highest MIC was observed to Ni, Cu and Zn. For *E. coli*, high MICs were obtained to Ni and Zn (S5, S8 and S9 strains), suggesting that the three strains are resistant to these metals. This resistance is not likely to be intrinsic since considerably lower MICs were detected for *E. coli* ATCC 25922 and *E. coli* S11. Marine sediments can concentrate pollutants, such as antibiotics and metals, favoring the emergence of co-selection and the spread of ARB (Zampieri et al., 2017; Whitman et al., 2014; Oliveira and Pinhata, 2008). Metals play an important role in this context mainly because they are highly stable elements that are resistant to degradation, thus persisting longer in the environment, when compared to antibiotics. Consequently, it is believed that metals contribute to the co-selection of genetic elements encoding metals and antibiotic resistance (Seiler and Berendonk, 2012). Herein, we identified association between antibiotics and metal resistance, suggesting a co-selection of these genetic determinants. This was particularly evident for ampicillin and to all the metals. Among the carbapenems, the highest correlations identified were: i) ertapenem and Zn, Ni and Co and ii) imipenem and meropenem with Cd and Co. Similar to what was observed in the present study, Berg et al. (2005) reported co-selection resistance to ampicillin occurring in soils contaminated with Cu. Koc et al. (2013), detected multidrug resistant *Raoultella plantiocola*, that were also resistant to Cu and Ni.

Since there is no Brazilian legislation regarding the concentration of metals in estuarine or marine sediments, CETESB uses the Canadian Sediment Quality Guideline (SQG) (CCME, 2002) as standard. Santos has chronical high levels of metals, probably, due Port of Santos activities and Cubatão Industrial Complex, which contributes to the environmental degradation (Kim et al., 2016; Azevedo et al., 2009; Cesar et al., 2007; Tessler et al., 2006). Kim et al. (2016) detected the presence of Cd, Cu, Ni and Zn, and also the influence of non-treated sewage release in the area. Tessler et al. (2006) refer that an enrichment of Cu and Zn occurred from 1950, and Azevedo et al. (2009) reported a high concentration of metals, including Co and Zn, on *Cathorops spixii* liver collected from Santos Bay, reinforcing the impact of industrial and harboring activities in the Santos area. On the other hand, Araça Bay presented metal levels within the SQG Canadian limits for Cd, Cu, Ni and Zn (Kim et al., 2018; Zampieri et al., 2016). These results demonstrate the influence of port activities in the area, especially due to the influence of the dynamics of São Sebastião Channel (Kim et al., 2018; Zampieri et al., 2016; Pereira et al., 2007).

Brazilian legislation considers only water samples and only few bacteria (*e.g. E. coli* and *Enterococcus* spp.). Another worrying aspect is the lack of legislation or public policies regarding ARB, especially in areas of intense economic activity and high population density, as Santos. Most of studies focus on pathogenic bacteria, and only recently more studies regarding environmental bacteria, including environmentally isolated ARB bacteria, appeared (Wright, 2019; Crofts et al., 2017). These natural ARB may act as reservoirs of antibiotic resistance determinants, especially antibiotics of last resort, such as carbapenems, which are considered to be of top level of risk (Manaia, 2017).

Thus, considering the results presented here, it is extremely important to implement programs to monitor water and sediments quality standards of coastal environments and at the same time, to update existing information, since to date, many genera and species that are characteristic of these environments have been neglected despite their clinical relevance and potential carriers and disseminators of ARG.

5. Conclusion

Multidrug resistant and carbapenem-resistant *Enterobacteriaceae* strains were isolated from Araça Bay and Santos, both on São Paulo State coast, in Brazil, although the genes that encode the most commonly known beta-lactamases were not detected. This suggests that the carbapenem resistance is either non-enzymatic or encoded by novel enzymes that are able to hydrolyze carbapenems. The absence and/or the mutations found in some *Omp* genes can be one possible cause for the resistance phenotype observed. Metal-antibiotic co-resistance has been suggested by other authors as a possible cause for this phenotype. A high MIC was detected for Ni in most of the isolates and it was found to be strongly associated with ertapenem resistance. The same was also observed for Co and Zn. Resistance to imipenem and meropenem correlated with a higher MIC for Cd and Co. Although most of the strains have been isolated from sediment samples, the Brazilian legislation does not include the evaluation of the microbial quality of the sediments.

Thus, the present study contributes to the recognition that such policies should be re-evaluated and that further and more studies should be undertaken. Sediments are ecosystems with high bacterial densities, where the persistence and evolution of the resistance determinants occurs. In addition, this evolution is most likely selected and accelerated by external factors, such as pollutants resulting from anthropogenic activities.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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