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Predictors of carbapenemase-producing bacteria occurrence in polluted coastal waters☆

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The spread of carbapenemase-producing bacteria is a worldwide concern as it challenges healthcare, especially considering the insufficient development of antimicrobials. These microorganisms have been described not only in hospitals, but also in several environmental settings including recreational waters. Community exposure to antimicrobial-resistant bacteria through recreation might be relevant for human health, but risk assessment studies are lacking. Absence of effective and feasible monitoring in recreational aquatic matrices contributes to such a knowledge gap. Here, we aimed at assessing predictors of occurrence of medically relevant carbapenemase-producing bacteria in coastal waters. We quantitatively assessed recovery of carbapenemase-producing Enterobacteriaceae, *Pseudomonas* spp., *Acinetobacter* spp. and *Aeromonas* spp. in superficial coastal waters showing distinct pollution history across one year, and registered data regarding tide regimen, 7-days pluviosity, salinity, pH, water temperature. We analyzed data using General Estimating Equation (GEE) to assess predictors of such occurrence. Our results suggest that the sampling site had the strongest effect over concentration of these antimicrobial-resistant microorganisms, followed by pollution indexes and tide regimen. Increased salinity, advanced sampling time, water temperature, rainfall and decrease of pH were related to decrease concentrations. We provide a list of factors that could be easily monitored and further included in models aiming at predicting occurrence of carbapenemase producers in coastal waters. Our study may encourage researchers to further improve this list and validate the model proposed, so that monitoring and future public policies can be developed to control the spread of antimicrobial resistance in the environment.

1. Introduction

Antimicrobial resistance is one of the main challenges of humankind in the 21st Century as it transcends the health sphere and threatens global health, economy and security (The Review on Antimicrobial Resistance, 2016; Laurence et al., 2019). Carbapenemase-producing gram-negative rods represent one of the greatest concerns regarding antimicrobial resistance in nosocomial-acquired infections (Rello et al., 2019). However, their dissemination is no longer restricted to hospital environments (Mills and Lee, 2019). These microorganisms are increasingly found in polluted aquatic environments, including those used for recreational purposes (Falgenhauer et al., 2019; Mahon et al., 2017; Mahon et al., 2019; Montezzi et al., 2015; Paschoal et al., 2017). Although wildlife in contact with polluted waters were found to carry antimicrobial-resistant bacteria (Dolejska & Papagiannitsis, 2018), consequences of such contact for humans remain unknown (Leonard et al., 2018).

The study of antimicrobial resistance in health institutions enables identifying risks related to healthcare associated infections, the development of efficient therapeutic choices, and the establishment of measures to limit its spread (Fournier et al., 2018). Antimicrobial resistance in the environment, on the other hand, lacks such understanding. Although lack of sanitation is considered the main factor favoring occurrence of carbapenemase producers in water sources, other factors in this scenario are unknown. This gap

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of knowledge hampers the development of statistical models aimed at predicting such occurrence. Such models would be valuable for monitoring as it is unfeasible to systematically investigate antimicrobial resistance based on culture. On the other hand, monitoring viable bacteria is imperative for assessing consequences and evaluating measures devoted to overcoming environmental dissemination of antimicrobial resistance.

Here we used General Estimating Equation to assess factors possibly related to the occurrence of medically relevant carbapenemase-producing bacteria in polluted coastal waters from Rio de Janeiro, Brazil (Paschoal et al., 2017).

2. Material and methods

2.1. Study site

Ten water samples were collected in five beaches from the south zone of Rio de Janeiro across one year, as described by Paschoal et al. (2017). Sampling sites included beaches located either inside (Flamengo and Botafogo) or in the vicinity of Guanabara Bay (Copacabana, Ipanema, and Leblon), an environment notorious for its pollution. This bay receives waters from 17 rivers crossing 15 municipalities, many of which do not collect and/or treat their sewage (SNIS, 2017). Botafogo beach harbors floodgates that prevent two channelized polluted rivers from reaching the coast in dry weather, favoring their course until an undersea emissary. Flamengo beach harbors Carioca river's mouth. This river crosses slum areas where it drains sewage from these communities, then follows channelized by the city until it reaches a wastewater treatment plant located close to its mouth, where it receives primary treatment (coagulation/flocculation). Copacabana, Ipanema and Leblon are open sea beaches. Copacabana beach is not under direct influence of rivers or channels. Ipanema hosts a submarine emissary discharging wastewater 27m deep and 3.3 km away from the coast. It also harbors Jardim de Alah's channel connecting Rodrigo de Freitas' lagoon and the beach, where floodgates control water flow. Leblon is where Visconde de Albuquerque's channel reaches the beach. Uncollected wastewater flows into rivers and storm water runoffs, most of which are channelized and directed to submarine emissaries. Floodgates prevent contaminated water from reaching the coast in dry weather, however, when it rains intensely, the system does not support the increased volume and contaminated waters ultimately flow into beaches (Fig. 1).

2.2. Measurements of physical–chemical and biological parameters

Water temperature was measured at the time of sampling. Salinity was assessed using a refractometer and results were expressed in parts per thousand (ppt) by weight. Rainfall and tide data were obtained at the National Institute of Meteorology and the Directorate of Hydrography and Navigation of the Brazilian Navy websites, respectively (INMET, 2015; Brazilian Navy, 2015). Thermotolerant coliforms counts were assessed using the multiple-tube fermentation technique (APHA, 2012), and total enterococci using samples diluted 1:10 and Enterolert® (IDEXX, Westbrook, Maine, USA), following manufacturer's instructions (APHA, 2012). Waters were considered suitable for primary contact if levels of enterococci and thermotolerant coliforms were $\leq$100 and $\leq$ 1000MPN/100 ml, respectively (CONAMA 274, 2000).

2.3. Search for carbapenemase-producing isolates

Culture procedures and methods to detect carbapenemase producers were described previously (Paschoal et al., 2017). Briefly, distinct volumes of samples were cultured in chromogenic agar supplemented with imipenem (1 μg/ml). All colonies grown in cultures yielding 20–50 colony forming unities (CFU) were subcultivated and the respective volume of sample inoculated was registered. Microorganisms were identified at the genus level using MALDI-TOF and screened for carbapenemase production using modified Carba NP test (Campana et al., 2017). The concentration of carbapenemase producers in samples were calculated by dividing the number of isolates showing phenotype consistent with carbapenemase production by the volume (ml) of coastal water inoculated in plate selected for the analysis and multiplying the result by 100.

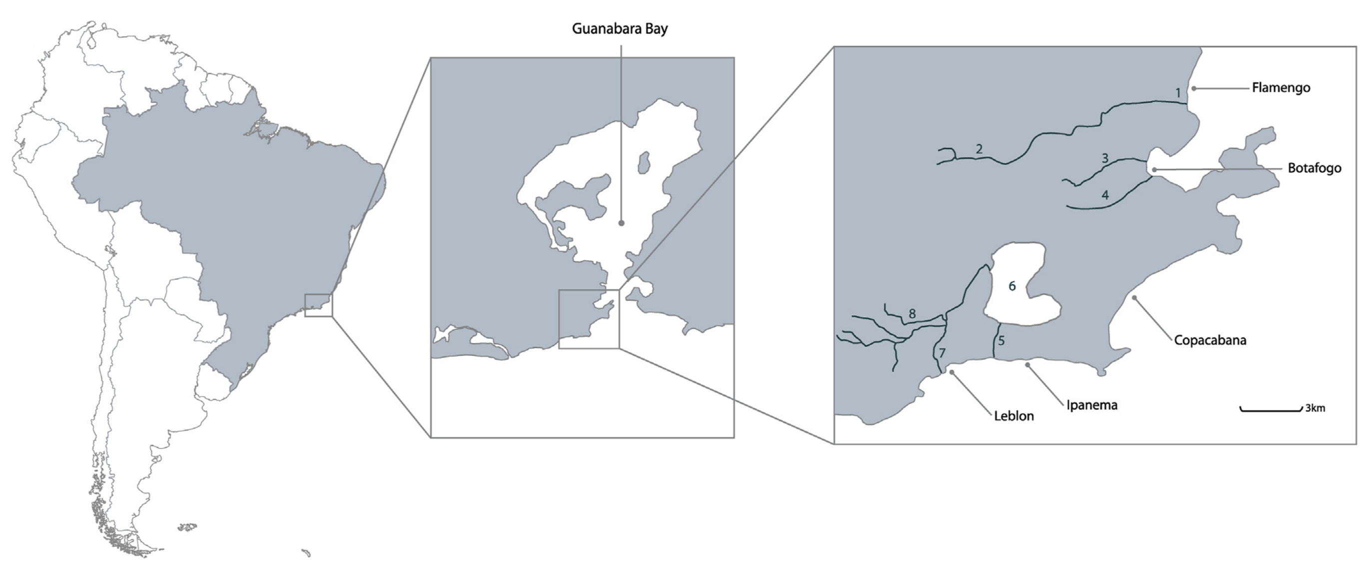

Fig. 1. Sampling sites. Flamengo and Botafogo are located inside Guanabara Bay; Copacabana, Ipanema and Leblon are open sea beaches; Carioca river's (1) mouth in Flamengo beach (2); Rivers that flow into Botafogo beach (3)(4); Jardim de Alah's channel (5) connecting Rodrigo de Freitas' lagoon (6) and the beach, where floodgates (5) control water flow. Visconde de Albuquerque's channel flow in Leblon beach (7) Rivers extensively polluted that flow in Visconde de Albuquerque's channel (8). Adobe Illustrator CS6, Adobe InDesign CS6, were used to figure designed and ©2017 Google maps as reference, https://www.google.com.br/maps.

2.4. Statistical analyses

Exploratory analyses of frequencies and variables distributions included Fisher's and Chi-square tests, and analysis of variance or bivariate correlations, when appropriate. We sought to apply a model that would test how environmental conditions could influence the occurrence and the variation in concentrations of carbapenemase producers across the study period, controlling for possible moderators and interacting effects, such as the amount of microorganisms and of medically relevant bacteria recovered from each sample, or suitability for primary contact. We hypothesized that the concentration of carbapenemase producers could behave as a Poisson-like distribution, since it represents a score of its occurrence in each beach (number of events of interest) for a fixed period. Also, that could present the characteristic of gamma distributions of having repeated values equal to 0, that is, a high probability of being a *mixed* distribution (Tweedie). Therefore, we chose to perform a General Estimating Equation having number of carbapenemase producers per 100 ml as dependent variable with a Tweedie distribution in a log link function. All five beaches selected as sampling sites were treated as subjects, with 10 measures each. Variables included in the model as predictors were both categorical (beach sites, water suitability for primary contact, and tide), and continuous (number of microorganisms, number of medically relevant bacteria, rainfall, pH, salinity, water temperature). The statistical package used in data analysis was SPSS version 18.0.

3. Results and discussion

Enterobacteriaceae; *Acinetobacter* spp.; *Aeromonas* spp.; and *Pseudomonas* spp. producing KPC, GES-like, NDM, IMP, VIM, SPM, and/or OXA carbapenemase were recovered from coastal waters evaluated across the whole study period, with the highest average concentration in Botafogo (1643.0 CFU/100 ml), followed by Leblon (6.4 CFU/100 ml), Flamengo (5.4 CFU/100 ml), Ipanema (4.2 CFU/100 ml), and Copacabana (0.04 CFU/100 ml) (Paschoal et al., 2017). The summary of variables registered for samples studied are listed in Supplementary Table 1. The model applied to assess the influence of environmental conditions on the variation of carbapenemase producers concentration in coastal waters across time suggested that beach's location had the strongest effect. Using Copacabana as reference, Botafogo was the main predictor, followed by Flamengo, Leblon, and Ipanema. Being suitable for recreation was related to decreased means of carbapenemase producers concentrations. Ebb tide was mainly related to increased concentrations of carbapenemase producers, while flood tide was related to decreased ones. Among covariates, increased salinity was related to decreased concentrations of carbapenemase producers, as was later sampling time (up to 11 a.m.), water temperature and rainfall, though with smaller magnitudes. Increased pH of samples was found to correlate with increased numbers of carbapenemase producing bacteria (Table 1).

Our findings suggest that the sampling site, microbiological quality of waters and tidal regimen had important effects on the concentration of carbapenemase producers of medical relevance. The constant flow of polluted rivers may explain the influence of the sampling site. Indeed, the only beach analyzed that did not receive water from contaminated rivers, Copacabana, showed a single carbapenemase producer of medical relevance during the whole study. However, we should not exclude the possibility that Copacabana waters may harbor other bacterial genera carrying carbapenemase-encoding genes of medical relevance whose growth was not prioritized by our study design. Water quality in Guanabara Bay increases at high tide and it is hypothesized that ebb and low tide may worsen the water quality of beaches nearby (Fistarol et al., 2015). Therefore, income of polluted waters from Guanabara Bay may explain the tide effect on concentrations of carbapenemase producers. Likewise, decreased salinity and higher pH values, both predicted higher concentrations of these bacteria, and are possible consequences of sewer contamination from freshwater runoffs; whereas later sampling time and higher water temperature probably predicted lower concentrations due to the incidence of ultraviolet radiation, which has both bactericidal and thermal effect. Rainfall was expected to relate to increased concentrations, as floodgates may allow contaminated water flow to the coast after rainy periods. In contrast, it exerted a slight effect

Table 1
Impact of categorical and continuous variables on the average of carbapenemase-producing bacteria in recreational coastal waters by GEE analysis.

	Parameter	Mean (SD)	Beta	95% Wald Confidence Interval		Hypothesis Test	
				Lower	Upper	Wald Chi-Square	p value
Categorical variables	Botafogo	1643 (2322.5)	**10.311**	8.40	12.23	111.30	0.000
	Flamengo	5.38 (4.0)	**9.195**	7.46	10.93	108.15	0.000
	Ipanema	4.2 (12.6)	**3.505**	3.21	3.80	541.18	0.000
	Leblon	6.42 (8.5)	**3.973**	3.43	4.52	204.37	0.000
	Copacabana	0.04 (0.1)	**0**[a]				
	Suitable	1.36 (2.9)	**−2.349**	−3.04	−1.66	44.29	0.000
	Unsuitable	636.83 (1612.5)	**0**[a]				
	Low tide	614.38 (1952.3)	**−3.503**	−5.05	−1.96	19.73	0.000
	Flood tide	27.0 (54.7)	**−4.278**	−7.11	−1.45	8.79	0.003
	High tide	647.42 (1698.9)	**−3.619**	−5.22	−2.02	19.55	0.000
	Ebb tide	204.45 (705.9)	**0**[a]				
Continuous variables	N microorganisms[b]	24.1 (7.5)	**−0.004**	−0.04	0.04	0.05	0.829
	N relevant bacteria[c]	5.7 (5.9)	**−0.007**	−0.04	0.03	0.18	0.672
	Rainfall (mm)[d]	32.2 (22.3)	**−0.090**	−0.12	−0.06	36.06	0.000
	Time of the day (h)	8.4 (1.3)	**−2.551**	−3.34	−1.76	40.30	0.000
	Water temperature (°C)	21.1 (2.3)	**−0.932**	−1.05	−0.82	254.28	0.000
	Air temperature (°C)	23.1 (2.9)	**0.950**	0.71	1.19	60.24	0.000
	pH	7.3 (0.3)	**1.705**	1.14	2.37	25.51	0.000
	Salinity	4.1 (0.4)	**−5.012**	−5.72	−4.30	191.45	0.000

[a] Set to zero because this parameter is redundant.
[b] Total number of CFU obtained in culture.
[c] Number of bacteria belonging to *Aeromonas* spp., *Acinetobacter* spp., Enterobacteriaceae, or *Pseudomonas* spp.
[d] Total pluviosity registered for seven days prior to sampling.

decreasing concentration of carbapenemase producers. Sewer dilution by pluvial water may explain this unforeseen finding, as the amount of rain registered was not clearly associated with floodgates position (Supplementary Table 1). This lack of association likely resulted from the evaluation of seven days pluviosity rather than rain intensity hours before sampling.

It is important to consider that the culture-based approach applied here was directed to recover microorganisms that may cause infections in humans against which beta-lactams would be therapeutic options. However, we may have neglected bacterial genera that could contribute to the spread of carbapenemases, but that grow in conditions different from the ones used. In addition, our study was not designed to assess the effect of sand, sediment, or wave size.

Given that carbapenemase producers are found in water matrices contaminated by sewer around the globe, this scenario is probably not restricted to Rio de Janeiro. For instance, there are reports of carbapenemase-producing bacteria in recreational water in different continents (Falgenhauer et al., 2019; Mahon et al., 2017; Mahon et al., 2019). Thus, the spread of carbapenemase producers in recreational waters is an actual concern that needs to be controlled and deserves attention from the scientific community and policy makers worldwide. Efforts to retard the evolution or control the spread of antimicrobial resistance in the environment will necessarily rely on the elucidation of factors influencing its intricacies.

4. Conclusion

Even though the statistical approach employed in this study is not intended to provide likelihood of events, it allowed identifying factors associated with variability in the occurrence of carbapenemase producers in coastal waters. Our findings may encourage future studies in modelling such factors, so that monitoring and future public policies can address the environmental dissemination of antimicrobial resistance in a broader perspective.

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CRediT authorship contribution statement

Raphael P. Paschoal: Investigation, Visualization, Writing - original draft. **Eloiza H. Campana:** Supervision, Investigation, Writing - original draft. **Laura de S. Castro:** Formal analysis, Writing - review & editing. **Renata C. Picão:** Conceptualization, Funding acquisition, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.114776.

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