

Detection of bacterial samples on the aquatic ecosystems adjacent to Saquarema Lagoon – Rio de Janeiro

Detecção de cepas bacterianas no ecossistema aquático no entorno da Lagoa de Saquarema – Rio de Janeiro

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Abstract

Introduction: Saquarema Lagoon (RJ) has a high ecological and economic value owing to its multiple uses. The population's constant growth increases the amount of sewage containing bacteria and antimicrobial drugs that are discharged to the environment. **Objectives:** to detect Gram negative bacilli able to colonize or infect humans and animals and determine their antimicrobial resistance profiles. **Methodology:** samples were collected in the city centre in April 2010 and at Jaconé (Lagoon's most preserved site) in February 2011. The total and thermo tolerant coliforms were determined and the isolation of samples was made using agar media containing 32 µg/mL of cephalotin. All samples were tested for antimicrobial susceptibility (AST) and on 16 samples, plasmid DNA was extracted. **Results:** different Gram negative bacteria were detected, such as: *Enterobacter* spp, *Citrobacter freundii*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. The coliform results showed that the water quality is proper for Human recreation. AST results demonstrated the existence of bacteria resistant to antimicrobial drugs frequently used in the community. It was possible to detect high molecular weight plasmids and nine samples (56,25%) showed at least one plasmid DNA electrophoresis band. **Conclusions:** there were not detected resistant samples to antimicrobial drugs normally used in hospital settings, which may possibly refute the idea of a contamination by nosocomial and/or veterinary sewage discharge.

Keywords: Gram negative Bacilli. Enterobacteriaceae. Multidrug-resistance. Aquatic environment, Plasmid DNA.

Resumo

Introdução: a Lagoa de Saquarema (RJ) tem elevado significado econômico e ecológico devido aos seus múltiplos usos. O crescimento demográfico favorece o lançamento de efluentes contendo microrganismos e antimicrobianos, capazes de contaminar esse ambiente. **Objetivos:** procurou-se detectar a presença de bacilos Gram-negativos com potenciais de colonização/infecção humana e animal e analisar sua resistência a antimicrobianos. **Metodologia:** as coletas foram realizadas em Abril de 2010, no centro da cidade e, em Fevereiro de 2011, em Jaconé, parte mais preservada da Lagoa. Foram realizados testes de colimetria para detecção de coliformes. Para o isolamento das cepas, empregaram-se meios de cultura sem antimicrobianos e contendo 32 mg/mL de cefalotina. As cepas foram submetidas a testes de suscetibilidade a antimicrobianos (TSA) e em 16 cepas foram realizadas a extrações de DNA plasmidial. **Resultados:** foram identificadas diferentes espécies de bacilos Gram-negativos, em especial *Enterobacter* spp, *Citrobacter freundii*, *Klebsiella pneumoniae* e *Pseudomonas aeruginosa*. Os níveis de coliformes indicam que a qualidade da água na Lagoa é satisfatória para balneabilidade. O TSA exibiu perfil de resistência para antimicrobianos de uso frequente no ambiente comunitário. Foi detectada a presença de plasmídeos de alto peso molecular e nove cepas (56,25%) apresentaram pelo menos uma banda de DNA plasmidial. **Conclusões:** não foram detectadas cepas resistentes a antimicrobianos utilizados no meio hospitalar, podendo indicar ausência de contaminação por esgoto de origem nosocomial e/ou veterinária.

Palavras-chave: Bacilos Gram-negativos. Enterobacteriaceae. Multirresistência. Ecossistema aquático. DNA – plasmídeo.

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INTRODUCTION

The demographical growth and industry settlement around coastal lagoons increased the discharge of organic and chemical effluents in these lagoons with the outcome

being a modification and degradation of the autochthon microflora. This increase of discharged effluents can also increase the number of Gram-negative bacilli existent in the aquatic environment which eventually will cause eutrophication (IBEKWE; MURINDA; GRAVES, 2011; BAYRAM et al., 2012; DERRIEN et al., 2012).

The discharge of waste containing antimicrobial drugs may generate a selective pressure favoring the growth of an antimicrobial resistant population (GONÇALVES et al, 2014). According to Harnisz (2012) the antimicrobial resistance has become in the last two decades, a fast growing and out of control issue around the world. The acquired resistance to antimicrobial drugs on naturally resistant strains has began from natural selection and exposure to some antimicrobial drugs. Naturally occurring events, such as, mutations, gene transfer and genetic recombination can also contribute to the acquisition of antimicrobial resistant traits (KORZENIEWSKA; HARNISZ, 2013; ZHANG et al., 2012a; ZHANG et al., 2012b).

The main resistance mechanisms found in Gram negative bacilli include: modifying the antimicrobials target sites; production of inactivation enzymes, such as, Beta-lactams and aminoglycosides modifying enzymes; efflux pumps and modification of external membrane proteins associated to the antimicrobial permeability. Bacilli Gram negative can become resistant to several antimicrobial classes which may enable them to spread and colonize different hosts and environments in an easier manner (GIEDRAITIENE, 2011).

Studies by Gonçalves et al. (2014), it was possible to detect the presence of coliforms presenting antimicrobial resistance markers in water samples of Guanabara Bay, may be related to species from the hospital sewage.

The aquatic ecosystem of Saquarema may, in previous years, have untreated sewage discharges, currently there is a construction project taking place so that the sewage can be treated before discharging them into the ecosystem. The water in those ecosystems are used to supply the population commercialization uses, recreation, fishing and seafood aquaculture.

In the present study, the aim was to identify bacterial samples in water samples, collected at different sites around the Saquarema lagoon, to perceive a possible ecological degradation by the presence of multidrug resistant bacteria and thermo tolerant coliforms due to sewage discharge containing antimicrobial residues

METODOLOGIA

Study area – Samples were collected in April 2010 and in February 2011 at different sites around Saquarema lagoon (State of Rio de Janeiro, Brazil). In April 2010 the samples were collected from three sites: close to Saquarema city centre; at the Barra Franca waterway (connects the lagoon to the sea) and in front of the fishermen reside. In February the samples were collected at three sites (Jaconé I, II and III) on the Jaconé Borough.

Colimetric tests – Serial dilutions were used according to Standard... (2014), in order to determine the presence of total and thermo tolerant coliforms. Positive tubes, showing turbidity and gas production, were streaked onto Methylene Blue Agar (EMB) agar plates to isolate *Escherichia coli*. The growth on the different broths (Lactose, Brilliant Green and EC) was interpreted using the McGrady table.

Isolation and Identification of the strains – For each water samples from each site, it was inoculated two bottles of 100mL Brain Heart Infusion (BHI) Broth with double concentration. In only one bottle it was added 32 mg/mL of Cephalotin. The agar media Cystine Lactose Electrolyte Deficient – CLED was used, with and without antimicrobial drugs, to select the samples to be used for the study and the identification was performed according to Murray et al. (2007) where it was evaluated: motility, production of H_2S and indole, fermentation of glucose and lactose, urea production, use citrate as a carbon source, lysine and ornithine decarboxylation, arginine dehydrolysis, red methyl and voges-proskauer tests.

Antimicrobial Susceptibility Testing – The resistance profiles of the different strains was carried out through agar diffusion method, according to Clinical Laboratories Standards Institute – CLSI (PERFORMANCE..., 2012). It is important to emphasize that, although belonging to the same antibiotic class, they are from different generations once that the first and second generation of antimicrobials has been widely used both in community and hospital environment and can be found in large-scale on the environment. *Escherichia coli* sample ATCC 25922 was used as control. The following antimicrobial agents were used with their respective potencies: Cephalothin (CPH – 30µg), Cephazolin (CFZ – 30µg), Cefoxitin (FOX – 30µg), Cefuroxime (CRX – 30µg), Cefotaxime (CTX – 30µg), Ceftriaxone (CRO – 30µg), Cefazidime (CAZ – 30µg), Cefepime (CPM – 30µg), Gentamicin (GEN – 10µg), Amikacin (AK – 30µg), Kanamycin (KAN – 30µg), Tobramycin (TOB – 10µg), Ampicillin (AMP – 10µg), Piperacillin/Tazobactam (PPT – 100µg/10 µg), Amoxicillin/clavulanic acid (AMC – 20µg/10µg), Ampicillin/Sulbactam (ASB – 10µg/10µg), Ciprofloxacin (CIP – 5µg), Norfloxacin (NOR – 10µg), Imipenem (IPM – 10µg), Ertapenem (ETP – 10µg), Aztreonam (ATM – 30µg), Chloramphenicol (CHL – 30µg), Tetracycline (TCY – 30µg), and Cotrimoxazole (SUT – 25µg).

The samples resistant to 3rd generation cephalosporins in AST were submitted to confirmatory tests of extended-spectrum beta-lactamases (ESBLs) production by the double-disc synergy test and the approximation test, according to CLSI (2014). *Klebsiella pneumoniae* strain ATCC 700603 was used as control.

Plasmid DNA extraction – Sixteen samples (1.3, 1CFL2, 4.12, 4.13, 4.15, 4.16, 4.20, 4.39, 4CFL1, 4CFL2, 4CFL8, 5.2, 5CFL2, 5CFL3, 5CFL5, 6CFL3) showed resistance to three or more antimicrobial drugs and were

submitted to DNA extraction and 0.8% agarose gel electrophoresis for plasmid detection, according to Gonçalves et al. (2013). We used plasmid DNA of *E. coli* R861 sample as molecular weight markers and *E. coli* K12 R23 as negative control for the presence of plasmid DNA. The gels were dyed in an ethidium bromide solution (0,5 mg/mL), analyzed in ultraviolet transilluminator and photographed by a Kodak EDAS 120 system.

RESULTS

Colimetric tests – The MPN obtained in this study were considered unacceptable according to resolution CONAMA 274/00 (BRASIL. CONAMA, 2000) except samples from collection site II. The thermo tolerant coliforms results showed good water quality and according to the later resolution the water was satisfactory (Table 1).

Isolation of strains – On the six collection sites around Saquarema lagoon there were isolated 121 samples belonging to 13 different species, such as: *Enterobacter agglomerans* (23/121), *Pseudomonas aeruginosa* (19/121), *Enterobacter cloacae* (17/121), *Klebsiella pneumoniae* (16/121), *Klebsiella pneumoniae* subspecies *ozaenae* (12/121), *Citrobacter* spp. (7/121), *Enterobacter aerogenes* (7/121), *Enterobacter sakazakii* (7/121), *Citro-*

bacter freundii (4/121), *Escherichia coli* (5/121), *Serratia liquefaciens* (2/121) and *Serratia marcescens* (2/121).

Antimicrobial Susceptibility Testing – From the 121 samples tested, 41 (33, 9%) samples were resistant to three or more classes of antimicrobials, these classes, determined according to Magiorakos et al. (2012). The main classes that showed resistance were the 1st and 2nd generation of cephalosporins and penicillins, especially AMP (34 strains). Six samples were resistant to 3rd generation cephalosporins and 25 samples were resistant to CFZ. Seventeen samples showed resistance to AMI, 14 samples were resistant to GEN and 22 were TCY resistant (table 2).

Plasmid DNA extraction – From the 16 samples submitted to plasmid DNA extraction, 9 (56.25%) had consistent with plasmid DNA. Two samples (4.16 and 4.20) show a molecular weight of 147 kb while seven samples (4:16, 4:19, 4:20, 4CFL1, 5.2, 5CFL2 and 5CFL5) had molecular weight between 147 kb and 63 kb. Three samples (4.15, 4.16 and 4CFL2) had molecular weight below 63 kb. It is noteworthy the strain 4:16 showed three different size amplicons and strain 4:20 showed two different size amplicons.

Table 1 – MPN index 100 mL in water samples collected at sites around Saquarema lagoon

Parameters	Collection points					
	Itaúna	City Centre	Fishermen residence	Jaconé I	Jaconé II	Jaconé III
Total Coliforms	900	11	≥ 1.600	1.600	900	≥ 1.600
Thermo tolerant Coliforms	240	< 2	>240<350	<2	<2	<2

Table 2 – Antimicrobial resistance profile of the different samples isolated from the water samples collected at the Saquarema lagoon ecosystem.

Strains	Species	Resistance profile	Confirmatory test to ESBL
1.3	<i>E. sakazakii</i>	CFZ/CPH/CRX/AMP/AK	NT
1CFL2	<i>E. sakazakii</i>	CFZ/CPH/AMP/TCY	NT
2.10	<i>E. agglomerans</i>	CFZ/CPH/AMP/AK	NT
2.11	<i>K. ozaenae</i>	CFZ/CPH/AMP/TCY	NT
2CFL4	<i>P. aeruginosa</i>	CFZ/CFL/CRX/AMP/CHL	NT
4.2	<i>E. agglomerans</i>	CFZ/CPH/AMP/TCY	NT
4.5	<i>E. coli</i>	CFZ/CPH/AMP/TCY/CHL/GEN	NT
4.12	<i>K. pneumoniae</i>	CPH/CRO/AK/GEN/KAN	(-)
4.13	<i>E. agglomerans</i>	CPH/CRO/AK/GEN/KAN	(-)
4.14	<i>P. aeruginosa</i>	CFZ/CPH/CRX/AMP/TCY/KAN	NT
4.15	<i>C. freundii</i>	CPH/TCY/CHL	NT
4.16	<i>C. freundii</i>	CFZ/CPH/AMP/TCY	NT
4.17	<i>E. agglomerans</i>	CRX/AMP/TCY/CHL/AK/KAN	NT
4.19	<i>K. pneumoniae</i>	AMP/TCY/CHL	NT
4.20	<i>K. pneumoniae</i>	AMP/TCY/CHL/AK	NT
4.21	<i>E. aerogenes</i>	TCY/CHL/AK	NT
4.22	<i>K. pneumoniae</i>	CRX/AMP/TCY/CHL	NT
4.27	<i>K. pneumoniae</i>	CFZ/CPH/CRX/AMP/AK	NT
4.33	<i>E. agglomerans</i>	CFZ/CPH/AMP/AK	NT
4.39	<i>E. agglomerans</i>	CFZ/CPH/AMP/TCY/GEN	NT

Strains	Species	Resistance profile	Confirmatory test to ESBL
4CFL1	<i>P. aeruginosa</i>	CFZ/CPH/CRX/AMP/TCY/KAN	NT
4CFL2	<i>E. cloacae</i>	CRX/TCY/KAN	NT
4CFL5	<i>C. freundii</i>	CFZ/CPH/CRX/AMP/TCY	NT
4CFL8	<i>P. aeruginosa</i>	CFZ/CPH/CRX/CRO/AMP	(-)
4CFL9	<i>P. aeruginosa</i>	CFZ/CPH/AMP/CHL	NT
5.2	<i>E. agglomerans</i>	CRX/CRO/AMP/AK	(-)
5.7	<i>K. ozaenae</i>	CRX/AMP/GEN	NT
5.10	<i>E. agglomerans</i>	TCY/KAN/CIP	NT
5.13	<i>P. aeruginosa</i>	CPH/CRX/AMP/TCY/GEN/AK	NT
5.15	<i>K. pneumoniae</i>	AMP/TCY/GEN	NT
5CFL1	<i>E. agglomerans</i>	CPH/CRX/AMP/GEN	NT
5CFL2	<i>E. aerogenes</i>	CRX/CRO/GEN/AK/CIP	(-)
5CFL3	<i>E. agglomerans</i>	CPH/CRX/AMP/GEN/AK/KAN	NT
5CFL4	<i>P. aeruginosa</i>	CPH/CRX/AMP/GEN/AK	NT
5CFL7	<i>P. aeruginosa</i>	CPH/CRX/CRO/AMP/GEN/CIP	(-)
6.2	<i>P. aeruginosa</i>	CFZ/CPH/AMP/CHL	NT
6.4	<i>E. agglomerans</i>	CFZ/CPH/AMP/TCY	NT
6.5	<i>E. agglomerans</i>	CFZ/CPH/AMP/KAN	NT
6CFL1	<i>C. freundii</i>	CFZ/TCY/GEN	NT
6CFL2	<i>P. aeruginosa</i>	CFZ/CPH/CRX/AMP/TCY/GEN	NT
6CFL3	<i>P. aeruginosa</i>	CFZ/CPH/CRX/AMP/CHL/GEN	NT

CFZ – Cephazoline; CPH – Cephalothin; CRX – Cefuroxime; AMP – Ampicillin; AK – Amikacin; TCY – Tetracycline; CHL – Chloramphenicol; GEN: gentamicin; KAN – Kanamycin; CRO – Ceftriaxone; CIP – Ciprofloxacin; NT – not tested.

DISCUSSION

The increase in population around the Saquarema Lagoon leads to the discharge of untreated sewage causing the phenomenon of eutrophication in this lagoon. The water quality assessed in this study showed satisfactory results according to resolution CONAMA 274/00 (BRASIL. CONAMA, 2000). The study by Kolawole et al. (2011) suggests that thermo tolerant coliform levels can appear to be low in coastal lagoons due to the constant renewal of water, normally through a channel. These findings confirm the results of the present study, since in Saquarema lagoon this renewal is enabled by the Barra Franca channel.

Ash et al. (2002) studied the water from some rivers in United States of America finding mainly four bacterial genus, namely: *Citrobacter*, *Enterobacter*, *Pseudomonas* and *Serratia*. These findings corroborate with the results in the present study, although, the present study found that 23% of the isolated belonged to *Klebsiella* spp. while in the Ash et al (2002) study this genus was the least found. Brandt et al. (2012) described the genus, *Klebsiella*, *Escherichia*, *Enterobacter* e *Citrobacter* to be the most common found in coastal lagoons, which confirms the results of the present study. It was also found in the present study that 15% of the isolates were nonfermenters substantiating the results from Meirelles-Pereira et al. (2002).

According to Korzeniewska, Korzeniewska e Harnisz (2013), the high number of resistant microorganisms found in the wastewater may be associated with the presence of antibiotics in the environment. Resistance to antibiotics commonly used to treat humans was 44.1% by *Enterobacteriaceae* samples found in the environment. Magiorakos (2012) assert that the strains may be

considered multidrug-resistant once showing resistance to three or more antimicrobial groups, excluding the intrinsic resistances. Many authors state that the presence of antibiotics in an environment is the main force for the selection and spread of resistance. However, there is little evidence to confirm that antibiotics change and promote the selection of resistance in these environments (NOVO et al., 2013). The present study showed that besides resistant to different classes of antimicrobials the samples isolated did not show any resistance profile consistent to the ones found in nosocomial settings.

This study focussed on Gram negative bacilli, with some antimicrobial resistance, which may be found both in the community and in the hospital. Donskey (2006) found nosocomial Gram-negative bacilli resistant to cephalosporin, ciprofloxacin and aminoglycosides, which corroborates the results of the present study. Furthermore, according to the same author, Gram negative bacilli when resistant to different antimicrobial classes may enhance dissemination and colonization of pathogenic bacteria. Ribeiro et al. (2001) detected high numbers of *Enterobacteriaceae*, isolated from coastal sewage, that were resistant to ampicillin due to the widespread use of this antimicrobial drug in the community. In the present study, 82% (34/41) of all isolated strains were resistant to ampicillin.

In the present study there was no samples showing ESBL production on confirmation tests although, according to Vasques et al. (2011), *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter* spp. e *Serratia marcescens* are the most frequent reservoirs for ESBL enzymes.

Martinez e Baquero (2002) affirm that antimicrobial resistance in microorganisms is a genetic phenomenon

related to the existence of genes encoding different biochemical mechanisms that prevent the action of drugs. According to Donksey (2006), among Gram-negative bacilli, the production of ESBLs is the most common resistance mechanisms, and involves the participation of plasmids transferred to other microorganisms. It was possible to detect plasmids weighting from under 63kb to 147kb in strains showing resistance to different antimicrobial classes. Mayer (1988) showed that plasmids can be transferred between different bacterial genus and the most common transfers occur between Enterobacteriaceae and other Gram-negative bacilli. Carattoli (2009) demonstrated that resistance genes transfers are mostly associated with plasmid activity.

The dissemination of bacteria through aquatic ecosystems can lead to Human and Animal infections which can have a difficult treatment if these bacteria carry antimicrobial resistance genes in their plasmids. The free transit of bacteria between different ecosystems has occurred for many decades and it may have been aggravated by the lack of sanitation policies and poor soil management. Oliveira, França, e Pinto (2009) studied the water and sand on beaches in different areas of Sao Paulo State, Brazil, and isolated numerous multidrug-resistant samples in both water and sand in all areas. According to the authors all these areas have wastewater management and sewage treatment programmes, although, on holiday season these measurements do not suffice to maintain a good water quality in some areas. The existence of multidrug resistant bacteria in recreational aquatic environments represents an important public health concern because the continuous exposure of Humans and Animal to these bacteria may put them in a constant risk of colonization and infection.

The establishment of a sewage treatment programme can determines the preservation of Saquarema Lagoon, although we can notice many illegal activities that can compromise this preservation prospect. Our findings (useful towards environmental preservation) can be used as a comparative framework for periodic evaluations by colimetric tests and also as a trigger for further research of multidrug-resistant strains in a continuous environmental control program.

CONCLUSION

The presence of thermo tolerant coliforms above the legal values in the water of the Saquarema lagoon, may suggest the lack of commitment from the federal and state governments in regards to the public sewage and water treatments. The bacterial species isolated from the six collection sites are normally found in aquatic environments, and there was no bacteria found with an industrial or hospital source. This fact discards the possibility of hospitals and industries to have been the source of this bacterial presence. The dissemination of bacteria holding antimicrobial resistance genes in high weight plasmids may suggest that transference of those genes

can occur between bacterial species living in an aquatic ecosystem which may lead to human infection with a complex treatment. Saquarema Lagoon is in a preservation condition and it shows signs of good water quality, although, the constant presence of bacteria enclosing antimicrobial resistance genes in different sites of the lagoon may imply that, there are a natural resistance of the bacteria. A constant microbiological surveillance, including the assessment of resistance profiles, in ponds as Saquarema Lagoon, should allow detect the occurrence (or not) of progression in degradation process by population increasing and by the illegal discharge of sewage.

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