

***The use of antimicrobials in selective coliform media
as a tool for quantifying resistant bacteria in aquatic ecosystems
in Rio de Janeiro, Brazil***

Utilização de Antimicrobianos em meios de detecção de coliformes
como forma de quantificação de bactérias resistentes em ecossistemas aquáticos
do Rio de Janeiro, Brasil

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Abstract

Introduction: Water samples were collected from two areas of the Guanabara Bay, which suffered minor and major environmental degradation. **Objective:** To isolate the total coliforms (TC) and faecal coliforms (FC) present in the water and to determine the similarity of their multidrug-resistance profiles with the ones found in nosocomial bacteria. **Material and Methods:** A conventional and a modified most-probable-number (MPN) tests were performed in parallel. The modified test comprised the use of 8mg/ml of gentamicin in each set of EC and Lactose tubes and another set with 32mg/ml cephalothin. The differences on the MPN between the three sets of tubes was then determined. **Results:** The NMP found in both TC and FC were lower in the set of tubes containing gentamicin when compared with the set of tubes containing cephalothin nevertheless with an exception from the samples belonging to Guaxindiba river (minor environmental degradation area) and Mangue channel (major environmental degradation area) where the MPN of both TC and FC were higher in the set of tubes containing gentamicin. **Conclusions:** The results obtained in this study suggest that the modified MPN may be a promising technique to assist in the management of hydric resources. This study also raises the issue of wastewater treatment and disposal in hospitals, which have a high level of bacterial selective pressure, being discarded in the vicinity of the area studied here enabling animals and humans to be exposed to treatment challenging pathogenic bacteria. **Keywords:** Water quality. Coliforms. Enterobacteriaceae., Environmental degradation. Multidrug-resistance.

Resumo

Introdução: Realizamos coletas de amostras de água em duas regiões da Baía de Guanabara com maior e menor impacto ocorrido por processos de degradação ambiental. **Objetivo:** buscar coliformes totais (CT) e coliformes fecais (CF) presentes na água, com características de multirresistência compatíveis com as de bactérias de origem hospitalar. **Materiais e Métodos:** Realizamos o teste de colimetria convencional e, paralelamente a este realizamos outro, no qual foram adicionados 8µg/ml de gentamicina e 32µg/ml de cefalotina aos tubos contendo caldo lactosado e caldo EC, para avaliação de diferenças nos índices de números mais prováveis (NMP) encontrados, nos tubos com e sem antimicrobianos. **Resultados:** Os índices de NMP encontrados, tanto de CT quanto de CF, foram menores naqueles contendo gentamicina, quando comparados com os mesmos índices para os que continham cefalotina, a exceção do rio Guaxindiba (localizado na área com menor degradação ambiental) e do Canal do Mangue (localizado na área maior degradação ambiental), onde os índices de NMP de CT e CF foram elevados nos meios contendo gentamicina. **Conclusão:** Os resultados obtidos indicam que o método proposto pode ser importante ferramenta para gestão sanitária de recursos hídricos, havendo forte indício de que esgotos oriundos de ambientes com alta pressão seletiva por uso de antimicrobianos, como hospitais, vêm sendo lançados sem o tratamento adequado nas proximidades dos locais estudados, expondo animais e humanos a agentes de infecções de difícil tratamento.

Palavras chave: Qualidade da água. Coliformes. Enterobacteriaceae. Degradação ambiental. Multirresistência.

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INTRODUCTION

Over the last decades, the quality of hydric resources used for human consumption and leisure activities has been of great concern to public health, and it has presented visible signs of degradation.^{1,2} Many pollutants have a direct biological action towards microorganisms in an aquatic environment and, when continuously emitted, they may contribute to both ecological imbalance and selection of resistant microbial populations, increasing the levels of degradation.³ The selection of resistant microorganisms in nature may be due not only to the production of these compounds by soil bacteria, but also to the elimination of human and animal faeces containing antimicrobial residues.^{4,5} The use of antibiotics in humans, husbandry and veterinary activities induces selective pressure, leading to the colonization and infection by resistant strains, that may interfere with therapeutics.^{6,7,8}

The human intestines, as well as for other animals, provide an important reservoir for Gram-negative bacilli, including Enterobacteriaceae species involved with infectious diseases both in community and nosocomial environments.^{9,10} Total coliforms are the standard microbiological quality indicators for treated and untreated water used for human consumption, while faecal coliforms are used as recreational water quality indicators.¹¹

Over the past years, we have been investigating the occurrence of Enterobacteriaceae harbouring antimicrobial resistance genes in different aquatic reservoirs, like rivers and lagoons, in Rio de Janeiro city.¹² These genes may be plasmid-encoded, transferable by bacterial conjugation and capable of interfering with therapeutics of human and animal infections. By using this semi-quantitative methodology it was possible to gather information regarding the prevalence of these resistant bacteria which are important in environmental surveillance.

MATERIAL AND METHODS

Study area – Samples were collected at Guapimirim, Caceribu and Guaxindiba rivers and at Magé channel (Guapimirim Environmental Protected Area –Magé-RJ) in April 2009. In July 2009 other samples were collected in rivers belonging to Fundão Island (in two different areas), Bica beach (Governador Island) and Mangue channel.

Colimetric tests – water samples were analyzed by the tube dilution method¹³, in order to determine the presence of total and faecal coliforms. In addition to the conventional colimetric test, gentamicin (8µg/mL) and cephalothin (32µg/mL) were added to both lactose and *Escherichia coli* broths. We also estimated the Most Probable Number (MPN) of total and faecal

resistant coliforms, with the aim of selecting those with multidrug-resistance characteristics, compatible with nosocomial strains. Water samples were also inoculated in selective solid media containing gentamicin and cephalothin, in order to increase the detection of resistant strains in these environments.

Antimicrobial susceptibility testing – the Antimicrobial Susceptibility Test (AST), was carried out through agar diffusion method, according to Clinical Laboratories Standards Institute.¹⁴ *Escherichia coli* strain ATCC 25922 was used as control. The following antimicrobial agents were used with their respective potencies: cotrimoxazol (Sut- 25 µg), cephalotin (Cph- 30 µg), ceftazidime (Caz- 30 µg), cefoxitin (Fox- 30µg), cefotaxime (Ctx- 30 µg), cefuroxime (Crx- 30 µg), ceftriaxone (Cro- 30 µg), cephalzoline (Cfz- 30 µg), aztreonam (Atm- 30 µg), ertapenem (Etp- 10 µg), cefepime (Cpm- 30 µg), piperacillin/tazobactam (Ppt- 100/10 µg), amoxicilin/clavulanic acid (Amc- 20/10 µg), Ampicilin/sulbactam (Asb- 10/10 µg), chloranphenicol (Chl- 30 µg), ciprofloxacin (Cip- 5 µg), norfloxacin (Nor- 10 µg), tetracycline (Tet- 30 µg), ampicillin (Amp- 10 µg), gentamicin (Gen- 10 µg), amikacin (Ak- 30 µg), kanamycin (Kan- 30 µg), tobramycin (Tob- 10 µg).

The strains resistant to second and third 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.¹⁴ *Klebsiella pneumoniae* strain ATCC 700603 was used as control.

RESULTS

The use of antimicrobial agents in colimetric tests enabled drug resistance detection in both total and faecal coliforms found in all analyzed water samples (Table 1).

From the eight waterways sampled, seven showed MPN values of TC \geq 1600/100mL in media without antimicrobials and these seven samples were then inoculated in media containing gentamicin and cephalothin.

The MPN obtained for both total and faecal coliforms was smaller in media containing gentamicin than those with cephalothin. Nevertheless, in Guaxindiba River (Guapimirim Environmental Protection Area) and in the Mangrove Channel, the MPN obtained for total gentamicin resistant coliforms was \geq 1600/100 mL – the same observed for cephalothin. In Fundão Island, the MPN obtained for both total and faecal coliforms in cephalothin containing media was also \geq 1600/100 mL. We identified at this collection point a *Klebsiella ozaenae* strain with resistance profiles compatible with those observed in nosocomial isolates, including carbapenems.

We could isolate multidrug-resistant bacterial strains, including Extended-Spectrum Beta-Lactamases (ESBLs) producers in five out of seven water samples (Table 2).

Table 1 – MPN index 100 mL in water samples collected from effluent rivers of Guanabara Bay.

Collection points	media without antibiotics		selective media with gentamicin		selective media with cephalotin	
	TC	FC	TC	FC	TC	FC
Guapimirim river	≥1600	130	<2	<2	900	34
Caceribus river	≥1600	26	<2	<2	500	14
Guaxindiba river	≥1600	≥1600	≥1600	170	≥1600	<2
Fundão Island I	≥1600	≥1600	<2	<2	26	9
Fundão Island II	≥1600	≥1600	350	17	≥1600	≥1600
Bica's Beach	140	140	<2	<2	12	<2
Mangrove channel	≥1600	≥1600	≥1600	280	≥1600	21

TC- total coliforms; FC- faecal coliforms

Table 2 – Geographical origin of enterobacteria strains, determination of antibiotic resistance profiles and confirmatory tests to ESBL

Strain	Isolation site	Resistance profile	Presence of ESBL
Ec 1Cfd	Guapimirim river	Cph/Cro/Cfz/Amp/Tet/Cip/Nor/Amc/Sut	(-)
Kp 2Ge	Caceribus river	Cph/Cro/Ctx/Cfz/Crx/Atm/Amp/Tet/Cip/Nor/Gen/Cpm/amc/Sut	(+)
Ec 3Cflc	Guaxindiba river	Cph/Cfz/Crx/Amp/Amc	(+)
Kc 4Gd	Fundão Island I	Amp/Tet/Cip/Gen/Kan	NT
Koz 5Cfl	Fundão Island II	Cph/Cro/Ctx/Cfz/Caz/Crx/Atm/Amp/Tet/Cip/Nor/Gen/Kan/Tob/Etp/Cpm/Ppt/Amc/Asb/Sut	(+)
Ec 6Ga	Bica's beach	Cph/Cro/Ctx/Cfz/Crx/Amp/Tet/Clo/Cip/Nor/Gen/Tob/Cpm/Amc/Sut	(+)
Kp 7Gb ₁	Mangrove Channel	Cph/Cro/Ctx/Cfz/Caz/Crx/Atm/Amp/Tet/Cip/Nor/Gen/Kan/Tob	(+)

Ec- *E. coli*; Kp- *K. pneumoniae*; Koz- *K. Ozaenae*; Amp- ampicillin; Cph- cephalotin; Fox- ceftiofexim; Cro- ceftriaxone; Crx- cefuroxime; Ctx- cefotaxime; Cfz- cephazoline; Chl- chloranphenicol; Gen- gentamicin; Cip- ciprofloxacin; Atm- aztreonam; Caz- ceftazidime; Kan- kanamycin; Nor- norfloxacin; Tet- tetracycline; Tob- tobramycin; Etp- ertapenem; Cpm- cefepime; Ppt- piperacillin/tazobactam; Amc- amoxicillin/clavulanic acid; Asb- ampicillin/sulbactam; Sut- cotrimoxazol; NT- strain no tested.

DISCUSSION

We could verify consistency in the total coliforms MPN results (resistant or not), since it was smaller or equal MPN results for faecal coliforms. The relationship between ESBL-producing strains and aminoglycoside resistance was also detected by phenotypic methods.¹⁴ The possibility of co-transmission may not only contribute to the rise in the occurrence of resistance markers but it also confers an evolutionary benefit to these strains, leading to selection environments with persistent antibiotic pressure, such as beta-lactams, aminoglycosides and quinolones.¹⁵ The frequency of antimicrobial-resistant isolates has increased dramatically in the environment, as a consequence of the wide use of these drugs in hospitals and community, as well as in veterinary activities.¹ Our approach focused on two antimicrobial groups whose bacterial resistances might indicate either nosocomial transmission (or other environments submitted to high antibiotic selective pressures, like sewage and hospitalized ICU patients) or the selection itself inside the community settings, can be able to indicate the event of multidrug resistance.¹⁶ ESBLs may represent important obstacles to the use of third generation cephalosporins, while aminoglycoside modifying enzymes (AMEs) can determine resistance profiles to different aminoglycosides.^{17,18} The occurrence of carbapenem-resistant enterobacteriaceae (CRE) is an emerging public health problem all over the world. We could identify in one collection point a Klebsi-

ella pneumoniae subspecies ozaenae strain resistant to imipenem and ertapenem, as well as to other antibiotics. Different studies have been associating the incidence of ESBLs (including carbapenemase) with plasmid-mediated quinolone resistance (PMQR) and AMEs.^{19,20}

The MPN of total and faecal coliforms determined by the conventional test was higher or equal to that found in media containing antimicrobials. The selective media allowed the detection of a "special" group of microorganisms with resistance characteristics inside a much bigger one with sensitivity profiles. It is possible to consider that the occurrence of these "special" isolates was due to faecal contamination.

We suggest this methodology as a useful additional tool for the colimetric test, especially in monitoring and preventing environmental contamination, mainly when it is associated with antibiotic-resistant microorganisms. It will be possible to carry out a simple and economic quantitative test in many different circumstances for assessment of multidrug-resistance in waterborne coliforms. This method can be highly reproducible and provides results to specific and justifiable situations.

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