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β-LACTAM RESISTANCE GENES IN GRAM NEGATIVE BACTERIA ISOLATED FROM A STREAM IN PORTO ALEGRE

Daniele V. de Oliveira, Tiele Carvalho, Aline W. Medeiros, Ana Paula Guedes Frazzon, Sueli T. Van Der Sand*

Laboratório de Microbiologia Ambiental, Departamento de Microbiologia, Imunologia e Parasitologia, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.

*Corresponding author: svands@ufrgs.br

ABSTRACT

The Dilúvio stream is part of an important watershed in the city of Porto Alegre, southern Brazil. It receives rain runoffs and domestic and hospital sewage, which carries a highly diverse microbial population. Some of these microorganisms may show resistance to different antimicrobials, acting as disseminators of resistance genes. This study characterizes the Gram-negative population present in the Dilúvio stream according to their antimicrobial resistance profiles, searching for the presence of β -lactams resistance genes. The resistance profile was determined according to the disk diffusion method using different classes of antimicrobials. The detection of the resistance genes bla_{TEM}, bla_{SHV} and bla_{CTX-M} was carried out by PCR. Approximately 67% of the isolates from sampling points 1 and 3, and about 59% of the isolates collected from point 2 and more than 95% from site 4 were resistant to two or more antimicrobials. These strains were submitted to the PCR reaction looking for the genes bla_{TEM}, bla_{SHV} and bla_{CTX-M}. The results showed a higher prevalence of bla_{TEM} gene in 21 of the isolates. Two isolates were positive for two genes, bla_{TEM} and bla_{SHV}, six for bla_{SHV} and one isolate was positive for bla_{CTX-M} gene, revealing the transfer of different resistance genes to the environment.

Keywords: Dilúvio stream - β-lactams resistance genes - Gram negative bacteria

1. INTRODUCTION

Water is one of the natural resources essential to the existence of living beings. However, intense human activities produce large amounts of polluting substances that, when discharged into waterways, cause serious environmental problems. The quality of the water available for different purposes, such as public and industrial supplies, irrigation, sport, and leisure can be a limiting factor in the economic and social development of cities, affecting population growth [1, 2]. Misconceptions in the use of water available for human consumption might lead to contamination with microorganisms from soil, human and animal waste, and other sources. Ultimately, this scenario represents a serious health risk since these sources may contain pathogenic microorganisms. The dumping of untreated wastewater in any drainage basin may affect natural water resources. Reducing quality of life for populations nearby these basins, exposing inhabitants to infectious, carcinogenic and/or teratogenic agents that may cause outbreaks and epidemics [3-5].

The Dilúvio stream crosses ten highly urbanized neighborhoods in the city of Porto Alegre and flows to Lake Guaíba, the main source of water for the of Porto Alegre. The stream receives domestic sewage and many effluents, raising concerns about water quality and the use of water resources for primary consumption. Bacterial resistance to antibiotics and other antimicrobial drugs is a natural phenomenon under selective pressure. However, due to the indiscriminate and recurrent use of these drug classes, resistance has become a major problem. Antimicrobials and other drugs are discharged into the environment without any prevention [6], causing the natural selection of the fittest organisms [7]. The indiscriminate use of antimicrobials stands as a potential risk for the success of therapeutic approaches to treat diseases caused by bacteria since these can carry the information of resistance to one or more classes of antimicrobials. Study revealed [8] that the higher the number of antimicrobials used during treatments and/or the longer treatment approaches are in place; the greater are chances for selection of more resistant strains.

Antimicrobial resistance is observed in the microbiota of different aquatic environments, such as rivers, springs, estuaries, coastal waters and the sea. It might be associated with resistance genes, which indicates that these may be present even in environments little exposed to selective pressure, if any [9, 10].

Environments exposed to the effects of anthropogenic action or agricultural activities show a higher concentration of bacteria carrying antimicrobial resistance genes, which can also be detected in wastewater [11].

Most research on aquatic environments has focused on antimicrobial resistance of bacteria from fecal origin, mainly because these are associated with infectious diseases and could cause outbreaks of water-borne diseases [12].

The antimicrobial class of β -lactams is the largest group of antibiotics available for human use. These antibiotics have a β -lactam ring in its central structure. It is the site of action of these drugs on the bacterial cell wall, inhibiting the activity of the enzymes that finalize the synthesis of the bacteria cell wall [13]. β -lactam antibiotics are highly specific and non-toxic to human cells.

Resistance to β -lactams may develop according to three different mechanisms: enzymatic inactivation by β -lactamases, alteration of the target receptors, and altered drug transport [13]. There is a wide variety of β -lactamases, and these are found in bacteria that inhabit various environments and are exposed to different selective pressures [14]. So, the production of these enzymes is the major cause of antimicrobial resistance in Gram-negative bacteria [15].

In this sense, this study characterizes the Gram negative bacterial population present in the waters of Dilúvio Stream, according to their resistance to different antimicrobial classes. Additionally, the presence of β -lactams resistance genes was assessed.

2. MATERIAL AND METHODS 2.1. Samples

The samples were isolated from the water of the Dilúvio stream collected during the year 2009. Sampling was carried out at five points: following the water course of the stream: Park Saint'Hilaire (point 1), Antonio de Carvalho Avenue (point 2), Guilherme Alves Street (point 3), Ramiro Barcelos Avenue (point 4), and Borges de Medeiros Avenue (point 5). Collections were carried out taking into consideration the four seasons of the year. Isolation and biochemical identification of isolates were performed as described in Oliveira [16]. The isolates used in this study are part of the Laboratory of Environmental Microbiology Collection, Department of Microbiology, Immunology and Parasitology, UFRGS.

2.2. Antimicrobial susceptibility assay

The susceptibility to antimicrobials was evaluated by the Kirby-Bauer method according to the Clinical and Laboratory Standards Institute [17]. The following 17 antimicrobial agents were tested: amoxicilin/ clavulanic acid (10 μ g), ampicilin (30 μ g), aztreonam (30 μ g), ceftazidime (30 μ g), cephalothin (30 μ g) cefoxitin (30 μ g), ciprofloxacin (5 μ g), chloramphenicol (30 μ g), ceftriaxone (30 μ g), cefotaxime (30 μ g), streptomycin (10 μ g), gentamicin (10 μ g), imipenem (10 μ g), nitrofurantoin (300 μ g), norfloxacin (10 μ g), trimethoprim/ sulfamethoxazole (25 μ g), tetracycline (30 μ g). After an incubation period of 16-18 h, the size of halos was measured.

All the assays were performed using the control strain *Escherichia coli* ATCC 25922 to validate the analysis.

The isolates that showed resistance to two or more antimicrobials were submitted to further analysis looking for β -lactam resistance genes. For this purpose, the primers for bla_{TEM} , bla_{SHV} and $bla_{\text{CTX-M}}$ genes were used as described in the literature [18]. Prof. Dr. Alfonso Barth kindly provided the *Klebsiella pneumoniae* strain as a positive control for the genes.

2.3. DNA extraction

The extraction of chromosomal DNA followed the protocol described by Sambrook et al. [19], with modifications. The samples were grown in 4 mL of TSB at 37°C for 18h after centrifugation at 13,000rpm for 5min. Supernatant was discarded, and the pellet resuspended in 1mL of TE buffer (10mM Tris, 1mM EDTA, pH 7.8) and centrifuged again. Cells were resuspended in 200 µL of buffer solution to which, 200μ L of TE³N (TE-5X + NaCl), 5μ L of SDS 10% and 5μ L of proteinase K (20mg/mL) were added. The sample was incubated in a water bath for 1 h at 37°C. Later, 30 µL of 5M NaCl and 400µL phenol-chloroform (1:1) was added to the suspension and homogenized. The mixture and then subjected to centrifugation for 15 min at 13,000rpm. The aqueous solution transferred to a new tube and 400μ L of chloroform-isoamyl alcohol (9:1) was added. The mixture was again homogenized and centrifuged for 15min at 13,000rpm. The aqueous phase was collected and transferred to another tube, supplemented with 1mL of ethanol 100% and incubated at -20°C for 1h. After this period, the sample was centrifuged; the supernatant was discarded and the pellet resuspended in 100µL of TE. The DNA was stored in a freezer.

2.4. Molecular analysis of the isolates

In Table 1 are described the primers for amplification of each of the genes bla_{TEM} , bla_{SHV} and $bla_{\text{CTX-M}}$ and the amplification conditions. The PCR reaction was carried out in 0.2mL tubes with a total volume of 25μ L. The mixture contained 1X reaction buffer, 1.5mM MgCl₂, 1U Taq polymerase, 10pmol of each primer, 0.2mM of deoxynucleotide, and 20ng of DNA. The reaction products were analyzed by electrophoresis for 1h 30min at 75V in 1.5% agarose gel.

2.5. Isolates identification

The positive isolates for the molecular analysis of gene resistance were identified using the VITEK2 Compact (BioMeriéux, France) automated system.

Genes	Primers	Amplification conditions				
		Denaturation	Annealing temperature	Extension		
bla _{TEM} ^a	F: 5'GTGCGCGGAACCCCTATT	94°C,	E 1º C	72°C		
	R: 5'TTACCAATGCTTAATCAGTGAGGC	1 min	52 C	1 min		
bla _{SHV} ª	F: 5'CTTTACTCGCCTTTATCGGC	94°C,		72°C		
	R: 5'TTACCGACCGGCATCTTTCC	1 min	50 C	1 min		
bla _{CTX-M} ^b	F: TTGCGATGTGCAGTACCAGTAA	95°С,	E 2ºC	72°C		
	R: CGATATCGTTGGTGGTGCCATA	20 s	52 C	30 s		

Table 1. Primers for the amplification of the genes

^a [19]; ^b [20].

3. RESULTS

3.1. Antimicrobial resistance profile of the isolates

The resistance profiles of 86 Gram negative isolates using the 17 antimicrobials were determined. The antimicrobials to which the isolates showed greater susceptibility were imipenem (98.83% of the isolates) followed by norfloxacin and ciprofloxacin, with 96.51% susceptible isolates (for each drug). Cephalothin showed the lowest efficiency, with 61.62% of resistant isolates. In the present study, we observed a high number of isolates, 72.09% (62/86), which were resistant to at least two antimicrobials, characterizing a multidrug resistance profile. A higher prevalence of multiresistant isolates was observed with the isolates collected at the fourth sampling period (spring) (Table 2). Only six (6.97%) isolates were resistant to one antimicrobial, and no isolate was susceptible to all antimicrobials tested.

Table 2. Resistance profile, listing the antimicrobial with the Dilúvio stream collections in the period from January to December 2009

			Number of resistant isolates			
Class		Antibiotic	1*	2*	3*	4*
	יווי. ת	Ampicillin	11	6	9	15
	Penicillin	Amoxicillin+ Clavulanic Acid	11	9	10	19
	Cephalosporins	Cephalothin	8	12	14	19
		Cefoxitin	9	6	6	15
β- lactams		Ceftriaxone	4	0	1	2
		Aztreonam	4	0	0	0
		Ceftezidima	4	0	0	0
		Cefotaxime	3	0	0	0
	Carbapenem	Imipenem	0	0	1	0
Tetracycline		Tetracycline	3	4	4	14
Aminaglugagidag		Gentamicin	0	0	2	12
Ammogrycosides		Streptomycin	0	2	1	9
Sulfonamide		Trimethoprim+sulphamethoxazole		4	3	14
Quinolone		Ciprofloxacin		0	1	0
Anfenicol		Chloramphenicol		4	1	15
Fluoroquinolone		Norfloxacin		0	1	0
Nitrofuran		Nitrofurantoin	7	7	7	1

*collection 1, total isolates= 12; collection 2, total isolates= 13; collection 3, total isolates= 16; collection 4, total isolates= 21

The isolates from the first collection (summer) showed a higher prevalence of resistance to amoxicillin/clavulanic acid and ampicillin, both with 11/18 (61.11%) isolates and cefoxitin, with 9/18 (50%) isolates (Table 2). In the same collection, all isolates were sensitive to streptomycin, gentamicin, and imipenem.

In the second collection (autumn), 12/22 (54.54%) isolates were resistant to cephalothin, 9/22 (40.90%) to amoxicillin/clavulanic acid, and 7/22 (31.81%) resistant to nitrofurantoin. Aztreonam, ceftazidime, cefotaxime, gentamicin, ceftriaxone, norfloxacin, ciprofloxacin and imipenem were the antimicrobials to which all isolates showed sensitivity (Table 2).

In the third collection (winter) the antimicrobials with the highest number of resistant isolates were: cephalothin 14/24 (58.33%), amoxicillin/clavulanic acid 10/24 (41.66%) and ampicillin 9/24 (37.5%) were. Aztreonam, cefotaxime, and ceftazidime were the antimicrobials with the lowest number of resistant isolates (Table 2).

In the spring collection (4th collection) 86.36% of the isolates were resistant to amoxicillin/clavulanic acid and cephalothin; 68.18% resistant to ampicillin, chloramphenicol, and cefoxitin. Also in this collection, it was observed the highest rate 63.63% of isolates resistant to tetracycline and trimethoprim / sulfamethoxazole.

The resistance profile of the isolates unidentified in this study shows that 29.06% of the isolates were resistant to tetracycline and, out of these, 56% were isolated from samples collected in spring.

3.2. Presence of resistance genes to β -lactams

After the susceptibility assay, the isolates that showed resistance to two or more antimicrobials were submitted to PCR assay, looking for the presence of β -lactam resistance genes.

The amplification product revealed the presence of the gene bla_{TEM} in 33.87% (21/62) of the isolates. While the bla_{SHV} gene was detected in 9.67% (6/62) and the gene $bla_{\text{CTX-M}}$ in 1.61% (1/62) of the microorganisms. Considering the results obtained, 43.54% (27/62) of the isolates were positive for at least one of the genes. Among the genes analyzed, two isolates showed both bla_{TEM} and bla_{SHV} (3.22%; Table 3). Considering the positive results for the *bla* genes the isolates were submitted for identification using the VITEK 2 automated system. From the 27 isolates only 26 we were able to recover and the classification of these isolates are in Table 3.

Table 3: Identification of the bacteria isolated from the water of the Dilúvio stream that showed positive results for the presence of genes bla_{TEM}, bla_{SHV} and bla_{CTX-M}

Species	Percentage (%)					
	bla _{TEM}	bla _{shv}	bla _{CTX-M}	Species		
E. coli*	73.07	15.38	-	84.61		
E. cloacae	3.84	-	3.84	7.69		
S. marcescens	-	3.84	-	3.84		
Unidentified	2 01	3.84	-	2 94		
isolates ^a *	5.04			5.04		

(-) absent for gene; (*) has two genes: blaTEM and blaSHV; (a*) isolate not recovered for identification by VITEK2, but has two genes: blaTEM and blaSHV.

4. DISCUSSION

Of the collections of water from the Dilúvio stream, samples collected in autumn showed the lowest rate of isolates resistant to the tested antimicrobials. This difference may be associated with the collection period, April 2009, when rainfall was very low, causing a reduction in bacteria concentration and, as a consequence, in levels of resistance. Canal [21], who analyzed water samples from Lagoa dos Patos, observed that most resistant isolates were observed in summer, different from the present study.

Tetracycline showed a resistance index that can be very disturbing, because its site of action is the inhibition of protein synthesis. This drug has long been used as a growth promoter in animal feed. However, today this use is banned in many countries by the World Health Organization (WHO). According to Peak *et al.* [22], there is a relationship between the use of antimicrobials and frequency of antimicrobial resistance genes in surface waters due to its release or its final destination being in the environment. Canal [21], reports that this resistance can be found side by side with the resistance to other antimicrobials due to the acquisition of genetic determinants.

A high frequency of ampicillin-resistant isolates was found in the water samples analyzed. Similar result was found by Schneider *et al.* [23] studying ground and surface water, where 36% of isolates were resistant to this antimicrobial agent. High rate of resistance to ampicillin was also observed by Batista *et al.* [6] in a study with enterobacteria of urban effluents.

Resistance to multiple antibiotics of different classes such as ampicillin, cephalosporins, aminoglycosides, and fluoroquinolones has increased in Gram-negative nosocomial isolates such as *K. pneumoniae* and *Enterobacter* sp. [8].

In our study, we observed a high prevalence of isolates resistant to penicillins and cephalosporins belonging to a large class of β -lactams. This class of antimicrobials acts interacting with the penicillin-binding proteins (PBP), inhibiting bacterial cell wall synthesis. Resistance may be associated with the fact that these antibiotics are the most widely used in human medicine. They have been available on the market much longer, and that they also have been used indiscriminately, which makes microorganisms more adapted to the presence of these drugs.

The increasing and indiscriminate use of antimicrobials in human and veterinary medicine, aquaculture and agriculture can lead to contamination of soil and waters. This may change the microbial ecosystems and spreading antimicrobial-resistant organisms in the environment [24]. This study warns about the need not only to consider the clinical environment in terms of resistant bacteria, but also the natural environment, where a high molecular diversity associated with resistance may be present. Although hospital or domestic effluents are diluted by the municipal sewage and rainfall, substances such as antimicrobials, household disinfectants other or pharmaceuticals may be present in the environment, because most of the components are persistent [25].

The resistance profile of the highest number of antimicrobials was observed in isolates from the winter collection. This high resistance index may be linked to the different seasons of the year, since rainy seasons may lead to increased concentrations of microorganisms in natural waters. Where higher levels of resistant strains might be included, since sunlight is acknowledged to reduce the presence of resistance genes [26, 22]. In the waters analyzed in the present study, the coexistence of a high number of microorganism species was observed. This condition may select for more resistant strains in the enterobacteria group through gene mechanism exchange such as conjugation or transformation.

When antibiotics are released into the environment, they may end up interfering with the population living in that area, and in high concentrations, can cause significant impacts [25; 27] , reaching humans directly or indirectly in this environment. The Dilúvio stream flows into the primary source of water supply for the city of Porto Alegre. For this reason the organization responsible for the water supply should take antimicrobial resistance into consideration, because it is a question of public health.

A considerable amount of waste from various sources is released daily in the stream. The presence of bacteria from the Enterobacteriaceae group is consistent with the fact that the Dilúvio stream receives a massive discharge of untreated domestic sewage. It is important to identify the microbial species present in this environment because this determination can produce valuable information about the sources of pollution of the surface water [28].

The microbiota in the environment is exposed to stressing survival conditions, which may determine the prevalence of more adapted species such E. coli. The characteristics of the Dilúvio stream may have influenced the survival of coliforms, which is extensively used as a water quality indicator. Among these, E. coli is the bacterium most widely used to assess fecal pollution [29], since it occurs naturally in the microbiota of the intestinal tract of humans and of most homothermic animals, being commonly detected in feces. The presence of E. coli in water or foods is an indication of contamination with human or, more rarely, animal feces. In this sense, the Dilúvio stream receives feces of several of these animals, mainly at the river head, located in a native wood where these animals move freely. Antimicrobial resistance of the β -lactam class, more specifically extended- spectrum β -lactamases (ESBL), has been studied for many years and in different environments, including water [23, 24, 30, 31]. ESBLs have been described in the literature, many of which were found in Gram-negative bacteria [32].

ESBL represents the first group in which the resistance mediated by β -lactamase, against the β -lactamic drugs, is the result of a modification in the enzyme substrate, and is the most common mechanism of resistance in Gram-negative bacteria [14, 33, 34]. The rate of ESBL-positive samples in the Dilúvio stream was almost twice as high as that identified by Dropa *et al.* [13], in a study were 17.30% of clinical samples from a hospital in Sao Paulo had the *bla*_{TEM} gene.

Jones *et al.* [18], studying 272 clinical specimens, observed that 88% of the samples were ESBL producers, but only six samples were TEM-type ESBL. However, in our study the most prevailing ESBL type was TEM, and the least prevailing was the CTX-M type (Table 3). For the gene bla_{SHV} only 10% of the samples were positive, a number much lower when compared with the index of 34% found for the gene bla_{TEM} (Table 3). Dropa *et al.* [13] detected the bla_{SHV} gene in 63% of the isolates from a clinical specimen.

Nogueira *et al.* [35] evaluated samples from patients from a hospital in Curitiba and detected a very high rate of Enterobacteriaceae ESBL-producing, where 78% of samples were positive. Santos *et al.* [36] found 25% and 67.7% of positive samples for ESBL in two hospitals in the city of Goiânia. These authors also reported that the incidence of ESBL-producing microorganisms in hospitals is increasing worldwide, but in Brazil this ratio is higher than elsewhere. Moreover, these microorganisms are emerging from the community.

Oliveira *et al.* [37], studying samples that came from patients in the University Hospital of Santa Maria (RS), reported that 43.3% of the samples had both TEM and SHV genes. TEM was identified in 82.2% of the samples and the SHV in 67.8% of the selected samples. According to Luzzaro *et al.* [38], the presence of both TEM and SHV genes is the association most commonly found in bacteria.

The TEM and SHV families are the largest. TEM was first described in Enterobacteriaceae [39]. The *bla* genes are the main genes responsible for the spreading of resistance across these bacteria [14]. These genes are dissiminated through horizontal transfer.

In South America, several reports on ESBLs have been published, mainly concerning *Klebsiella pneumonia*. In Brazil, Colombia and Venezuela, 30 to 60% of the confirmed cases of ESBLs were caused by this bacterium [33]. The most frequently reported bacterial species, in the Enterobacteriaceae family, host for ESBLs, is *K. pneumoniae*, followed by *E. coli* [32, 39].

The results obtained in this study reveal a higher prevelance of the bla_{TEM} gene in isolates, possibly due to the fact that it was the first of the Enterobacteriaceae family identified and that it was the most widely spread. Therefore, it was the first gene to present structural changes at amino acid level, triggering the evolution of new resistance genes [40]. For Braford [40], more that 90% of resistance to ampicillin in *E. coli* is due to the production of enzymes derived from the TEM enzyme.

The bla_{CTX-M} gene was detected in only 1.61% (1/62) of the water samples of Dilúvio stream. Dropa *et al.* [13] identified this gene in 33.9% of samples collected from patients admitted to a hospital in São Paulo, Brazil. A study that analyzed samples of a Tertiary Medical Center in Western Pennsylvania revealed that the bla_{CTX-M} gene was the most prevalent, being detected in 81% of isolates [41]. These prevalence indices of the bla_{CTX-M} gene confirm the significant increase in the spread of these resistance genes worldwide, even in Brazil. The present study, which analyzed water samples from the Dilúvio stream, Porto Alegre, southern Brazil, may be the first to report the presence of this gene in natural waters in the region. However, Lopes *et al.* [42] have published a study that accounts for the presence of this gene in clinical samples collected in northeastern Brazil.

5. CONCLUSION

The results obtained allowed identification and characterization of the resistance profile of bacteria from Dilúvio Stream water samples. The presence of resistance genes comes as no surprise since untreated hospital sewage is released into the stream. However, it should be stressed that we used bacterial cultures isolated in the laboratory from crude samples, that is, outside these microorganisms natural environment, which may underestimate resistance levels. However, the results obtained are useful since it is known that resistance genes are emerging in the environment worldwide.

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