

Detection of antimicrobial-resistant gram-negative bacteria in hospital effluents and in the sewage treatment station of Goiânia, Brazil

Detección de bacterias gram negativas resistentes a antimicrobianos en efluentes hospitalarios y en la estación de tratamiento de aguas residuales de Goiânia, Brasil
 Detecção de bactérias gram-negativas antimicrobiano-resistentes em efluentes de hospitais e na estação tratamento de esgoto de Goiânia, Brasil

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ABSTRACT: The emergence of antimicrobial-resistant genes and the indiscriminate use of antibiotics contribute to the dissemination of resistant pathogens in the environment. The objective of the present study was to isolate *Pseudomonas aeruginosa*, *Acinetobacter* spp., *Klebsiella pneumoniae* and *Escherichia coli* from the sewage effluents of 10 hospitals located in Goiânia, Brazil, and from the sewage treatment station of the city, to determine their susceptibility profile and investigate their resistance mechanisms. The isolates from water samples were identified by biochemical tests and confirmed using API 20E (BioMerieux). Susceptibility profiling was performed by disc diffusion in accordance with the methodology established by the National Committee for Clinical Laboratory Standards. Extended-spectrum β -lactamase (ESBL) detection was carried out by the disk approximation method using phenotypic tests. Sixty-seven microorganisms were isolated and identified, including *E. coli* 10 (14,92%), *K. pneumoniae* 10 (14,92%), *P. aeruginosa* 3 (4,47%) and *A. baumannii* 1 (1,49%). Of the *E. coli* strains, 100% were resistant to aztreonam, 40% to ampicillin, 30% to piperacillin, 20% to ciprofloxacin and 10% to gentamicin. None of the bacterial strains produced ESBL or carbapenems. Of the *P. aeruginosa* strains, 100% were resistant to ampicillin-sulbactam, while 100% had intermediate resistance to gentamicin. Strains of *K. pneumoniae* were resistant to ampicillin (70%) and to piperacillin (20%); additionally, 50% showed intermediate resistance to piperacillin. Total resistance was not found in any of the isolates of *A. baumannii*, which showed intermediate resistance to aztreonam and ceftriaxone. Overall, resistance rates were low in the isolates of *E. coli*, *P. aeruginosa*, *K. pneumoniae* and *A. baumannii*.

KEYWORDS: Antimicrobial-resistant gram-negative bacteria. Dissemination of resistant pathogens – environment. Hospitals.

RESUMEN: La aparición de genes resistentes a antimicrobianos y la utilización indiscriminada de antibióticos contribuyen a la difusión de patógenos resistentes en el ambiente. El objetivo de este estudio fue aislar *Pseudomonas aeruginosa*, *Acinetobacter* spp., *Klebsiella pneumoniae* y *Escherichia coli* en efluentes de aguas residuales de 10 hospitales situados en Goiânia, Brasil, y de la estación de tratamiento de aguas residuales de la ciudad intentando determinar su perfil de susceptibilidad e investigar a sus mecanismos de resistencia. Los aislados de muestras de agua fueron identificados de promedio pruebas bioquímicas y confirmados utilizando API 20E (BioMerieux). El perfil de susceptibilidad fue realizado por difusión de disco de acuerdo con la metodología establecida por el National Committee for Clinical Laboratory Standards. La detección de beta-lactamasas de espectro extendido (ESBL) fue realizada de promedio el método de aproximación de discos utilizando pruebas fenotípicas. Sesenta y siete microorganismos fueron aislados e identificados, incluyendo *E. coli* 10 (14,92%), *K. pneumoniae* 10 (14,92%), *P. aeruginosa* 3 (4,47%) y *A. baumannii* 1 (1,49%). Las cepas de *Escherichia Coli* fueron 100% resistentes a aztreonam, 40% a ampicilina, 30% a piperacilina, 20% a ciprofloxacino y 10% a gentamicina. Ningunas de las cepas bacterianas produjeron ESBL o carbapenems. Las cepas de *P. aeruginosa* fueron 100% resistentes a ampicilina-sulbactam, mientras 100% presentaron una resistencia media a gentamicina. Las cepas de *K. pneumoniae* fueron resistentes a ampicilina (el 70%) y a piperacilina (el 20%); además, el 50% presentaron resistencia media a piperacilina. La resistencia total no fue encontrada en aislados de *A. baumannii*, que presentaron resistencia media a aztreonam y ceftriaxona. En términos globales, las tasas de resistencia fueron bajas en los aislados de *Escherichia Coli*, *P. aeruginosa*, *K. pneumoniae* y *A. baumannii*.

PALABRAS LLAVE: Bacterias gram negativas resistentes a antimicrobianos. Difusión de patógenos resistentes – ambiente. Hospitales.

RESUMO: A emergência de genes antimicrobiano-resistentes e o uso indiscriminado de antibióticos contribuem para a disseminação de patógenos resistentes no ambiente. O objetivo deste estudo foi isolar *Pseudomonas aeruginosa*, *Acinetobacter* spp., *Klebsiella pneumoniae* e *Escherichia coli* em efluentes de esgoto de 10 hospitais situados em Goiânia, Brasil, e da estação de tratamento de esgoto da cidade, para determinar seu perfil de susceptibilidade e investigar seus mecanismos de resistência. Os isolados das amostras de água foram identificados usando testes bioquímicos e confirmados com API 20E (BioMerieux). O perfil de susceptibilidade foi estabelecido pela difusão de disco de acordo com a metodologia estabelecida pelo National Committee for Clinical Laboratory Standards. A detecção de *Beta-Lactamase de Espectro Estendido* (ESBL) foi realizada pelo método de aproximação de discos usando testes fenotípicos. Sessenta e sete microorganismos foram isolados e identificados, incluindo *E. coli* 10 (14,92%), *K. pneumoniae* 10 (14,92%), *P. aeruginosa* 3 (4,47%) e *A. baumannii* 1 (1,49%). Dentre as cepas de *Escherichia Coli*, 100% foram resistentes a aztreonam, 40% a ampicilina, 30% a piperacilina, 20% a ciprofloxacina e 10% a gentamicina. Nenhuma das cepas bacterianas produziu ESBL ou carbapenems. Dentre as cepas de *P. aeruginosa*, 100% foram resistentes a ampicilina-sulbactam, enquanto 100% mostraram resistência média a gentamicina. As cepas de *K. pneumoniae* foram resistentes a ampicilina (70%) e piperacilina (20%); adicionalmente, 50% mostraram resistência média a piperacilina. Não houve casos de resistência total em alguns dos isolados de *A. baumannii*, que tiveram resistência média a aztreonam e ceftriaxona. De modo geral, as taxas de resistência foram baixas nos isolados de *P. aeruginosa*, *Escherichia Coli*, *K. pneumoniae* e *A. baumannii*.

PALAVRAS-CHAVE: Bactérias gram-negativas antimicrobiano-resistentes. Disseminação de patógenos resistentes – ambiente. Hospitais.

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Introduction

The indiscriminate use of antimicrobial drugs has caused a huge impact on public health by selecting bacterial strains resistant to conventional antibiotics, leading to an increase in the rates of hospital infection and high rates of morbidity and mortality. Some microorganisms that are important causes of infection in humans, such as gram-negative bacilli (GNB) that include *Enterobacter* spp. and *Pseudomonas aeruginosa*, are able to survive for long periods of time in the environment, thus contributing to the selection of resistant pathogens disseminated in the environment, as well as in hospitals, industry and veterinary facilities. These natural reservoirs of resistant genes may contribute to the appearance of resistant bacteria due to gene transfer mechanisms^{1,2,33}.

Gram-negative bacilli such as *Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter* spp. and *Pseudomonas aeruginosa*, among others, have been detected in three lakes in Rio de Janeiro, Brazil, and in rivers in the United States. The various microorganisms found in those water environments have shown different profiles of susceptibility and different antibiotic resistance mechanisms^{2,26,36}. Antimicrobial resistance, particularly multiple resistance, is a public health concern, and the presence of resistant microorganisms in the water is an worldwide emerging problem³⁵.

Materials and methods

Collection of samples of water from hospital sewage and culture conditions

Samples were collected during the months of May and June 2008, from 10 selected hospitals,

and from the sewage treatment station in the city of Goiânia, GO, Brazil. The collected samples were transported in sterile containers kept refrigerated until their microbiological analysis.

Using a sterile pipette, 8 mL from each sample of sewage water was transferred to a Falcon tube, shaken in a vortex and centrifuged for 10 minutes at 3000 rpm. For microbiological analysis, the sediment was suspended in 5 mL of brain heart infusion broth (BHI). The suspensions were incubated at 35°C for 4 hours. Next, each broth culture was homogenized and 200 µL was uniformly spread onto a blood agar plate (BA) which was incubated at 35°C for 24 hours.

Bacterial identification

Isolated colonies grown on BA were Gram stained and inoculated in bile-esculin agar, MacConkey agar, mannitol agar and blood agar for further identification.

The following biochemical tests were used to identify gram-negative bacilli: **motility-indole-ornithine (MIO) medium**, urea, catalase and triple sugar iron (TSI) agar. Isolated colonies recovered from TSI were biotyped using the API 20E system (BioMerieux). The isolated microorganisms were then stored in trypticase soy broth (TSB) supplemented with glycerol (15%) at -86°C in order to perform the microbiological tests described next.

Evaluation of in vitro sensitivity to antimicrobials

Antimicrobial sensitivity was performed on Mueller-Hinton agar (Hi-Media, India) by the standard disk diffusion method recommended by the National Committee for Clinical Laboratory Standards. (NCCLS)²⁰. Standard strains *Escherichia coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *K. pneumoniae* ATCC 700603 were used as con-

trols. Organisms were tested for antibiotic susceptibility. The diameter of the zone of growth inhibition was recorded and the isolates were classified as sensitive, intermediate resistant or resistant according to the criteria of NCCLS²⁰.

Phenotypic identification of extended-spectrum β -lactamase (ESBL) production

The bacterial strains that were possible producers of ESBL were identified according to the criteria established by the NCCLS (2002)²⁰, and the confirmatory analysis was carried out using the double-disk diffusion test. *E. coli* ATCC 35218 was used as control. An enhanced zone of inhibition with a difference of 5 mm around the amoxicillin/clavulanic acid disk as compared to the ceftazidime disk alone was interpreted as positive for ESBL.

Results

The following bacteria were found in the hospital sewage effluents: *E. coli*, 8 isolates (36,4%); *K. pneumoniae*, 10 (45,5%); *P. aeruginosa*, 3 (13,6%), one *A. baumannii*, (4,5%) and *E. coli*, 2 isolates (100,0%) from effluent sewage treatment station. Differences were found in the sensitivity profile of the *E. coli* isolates to some classes of drugs. All samples were sensitive to ceftazidime, cefotaxime, ceftriaxone, imipenem, amikacin, cefepime and cefpodoxime. Of the 10 isolates tested, six (60%) were sensitive to ampicillin, five (50%) to piperacillin, eight (80%) to ciprofloxacin and nine (90%) to gentamicin. Ampicillin, piperacillin, ciprofloxacin and gentamicin were ineffective against four (40%), three (30%), two (20%), and one (10%) *E. coli* strains, respectively. Two (20%) samples showed intermediate resistance to piperacillin

and all *E. coli* isolates were resistant to aztreonam (100%) (Table 1).

All *P. aeruginosa* samples were sensitive to piperacillin, aztreonam, piperacillin-tazobactam, ceftazidime, imipenem, ciprofloxacin and cefepime. Three (100%) were resistant to ampicillin-sulbactam. Intermediate resistance to cefotaxime was found in 100% of the isolates. The same pattern of sensitivity was found to ceftriaxone in 1 isolate (33.3%) and to gentamicin in another (33.3%) (Table 1).

The sensitivity profile of the *K. pneumoniae* isolates showed that seven (70%) were resistant to ampicillin and two (20%) to piperacillin. Intermediate resistance was observed for ampicillin (two strains – 20%), piperacillin (five strains – 50%), and for imipenem and ciprofloxacin (one strain- 10%). Ceftazidime, cefotaxime, ceftriaxone, gentamicin, amikacin and cefepime completely

inhibited growth of all the *K. pneumoniae* strains (Table 1).

No cases of total resistance were found in the unique strains of *A. baumannii* isolated in this study. This strain showed only intermediate resistance to aztreonam and ceftriaxone, being sensitive to all the other antimicrobials tested.

Finally, the search for ESBL producers showed that all the *E. coli* isolates that were resistant to aztreonam were not producers of extended-spectrum β -lactamase.

Discussion

The majority of therapeutic substances are partially metabolized by patients and discarded into the hospital's sewage disposal system and later into the public sewage system. In conditions of poor sanitation this effluent may be released into the environment, principally into riv-

ers or water reservoirs. Discharging organic effluents into reservoirs results in contamination by various pathogens, including bacteria bearing genes that are resistant to several antimicrobials. Genetic elements such as plasmids and transposons may further augment the problem, contributing towards increasing bacterial multiresistance since these mobile genetic elements may be transferred to other non-correlated genera and species of bacteria^{8,19,26,30}.

Studies conducted in various countries have detected the presence of antibiotics in different environmental compartments such as hospital effluents, municipal effluents and sewage treatment stations. The presence of these drugs in hospital effluents is known to contribute to the selection of bacteria containing genes resistant to antibiotics in the environment^{5,7,17,18,19,28}.

Table 1. Susceptibility profile of the microorganisms isolated from the sewage systems of 10 hospitals, and the municipal sewage treatment station of Goiânia city, GO, Brazil

Antimicrobials	E. coli			P. aeruginosa			K. pneumoniae			A. baumannii		
	N (%)			N (%)			N (%)			N (%)		
	S	I	R	S	I	R	S	I	R	S	I	R
AMP	6(60)	-	4(40)	-	-	-	1(10)	2(20)	7(70)	-	-	-
PIP	5(50)	2(20)	3(30)	3(100)	-	-	3(30)	5(50)	2(20)	1(100)	-	-
ATM	-	-	10(100)	3(100)	-	-	10(100)	-	-	-	1(100)	-
PTZ	-	-	-	3(100)	-	-	-	-	-	1(100)	-	-
CAZ	10(100)	-	-	3(100)	-	-	10(100)	-	-	1(100)	-	-
CTX	10(100)	-	-	-	3(100)	-	10(100)	-	-	1(100)	-	-
CRO	10(100)	-	-	2(66,6)	1(33,3)	-	10(100)	-	-	-	1(100)	-
IMP	10(100)	-	-	3(100)	-	-	9(90)	1(10)	-	1(100)	-	-
CIP	8(80)	-	2(20)	3(100)	-	-	9(90)	1(10)	-	1(100)	-	-
GEN	9(90)	-	1(10)	2(66,6)	1(33,3)	-	10(100)	-	-	1(100)	-	-
AMI	10(100)	-	-	-	-	-	10(100)	-	-	-	-	-
COM	10(100)	-	-	3(100)	-	-	10(100)	-	-	1(100)	-	-
AST	-	-	-	-	-	3(100)	-	-	-	1(100)	-	-
CPD	10(100)	-	-	-	-	-	10(100)	-	-	-	-	-
TIC	-	-	-	3(100)	-	-	-	-	-	1(100)	-	-

Abbreviations: AMP - ampicillin, PIP - piperacillin, ATM - aztreonam, PTZ - piperacillin-tazobactam, CAZ - ceftazidime, CTX - cefotaxime, CRO - ceftriaxone, IMP - imipenem, CIP - ciprofloxacin, GEN - gentamicin, MI - amikacin, CPM - cefepime, AST - ampicillin /sulbactam, CPD - cefpodoxime, TIC - ticarcillin.

S = sensibility; R = resistance; I = intermediate resistance.

In the present study, the microbial analysis of sewage effluents from 10 hospitals and the sewage station treatment of Goiânia, GO, Brazil, recovered 24 gram-negative microorganisms which could be identified as: 10 strains of *E. coli*, three of *P. aeruginosa*, 10 *K. pneumoniae* and one *A. baumannii*. Resistance of these isolates was expected to be higher, considering the presence of gram-negative bacteria resistant to multiple antibiotics present in the hospital environment^{2,24,26,27}. A study carried out in rivers in the United States identified the presence of *Acinetobacter*, *Alcaligenes*, *Citrobacter*, *Enterobacter*, *Pseudomonas*, *Serratia*, *Klebsiella* and *Proteus*, the latter two microorganisms having been isolated less frequently compared to the others². In another study, *K. pneumoniae*, *E. coli*, *E. cloacae*, *C. freundii*, *Aeromonas* spp., *S. marcescens*, *Citrobacter* spp., *K. oxytoca* and *A. calcoaceticus* were detected in samples taken from three lakes and a university teaching hospital in Rio de Janeiro, Brazil²⁶. Other microorganisms such as *Moraxella*, *Acinetobacter*, *Flavobacterium*, *Pseudomonas*, *Aeromonas*, *Bacillus*, *Proteus*, *Arthrobacter*, *Lactobacillus*, *Klebsiella*, *Plesiomonas*, *Pectobacterium*, *Chromobacterium*, *Serratia*, *Enterobacter*, *Staphylococcus* and *Micrococcus* were also found in rivers and in the Bay of Tillamook, Oregon, USA, in 1976^{2,16,24,26,37}.

In the present study, *E. coli* was found to be resistant to ampicillin, piperacillin, ciprofloxacin and gentamicin, and all the isolates were resistant to aztreonam. A study carried out in three sewage treatment stations in Australia in 2004 detected the presence of *E. coli* with a sensitivity profile similar to that described in the present study^{19,37}. Resistance to the antimicrobials tested may be associated with different resistance mechanisms such as extended-spectrum β -lactamase

production, efflux pump, mutation in the genes that codify DNA gyrase and topoisomerase, as well as porin loss and altered penicillin-binding protein (PBP)¹⁹.

It has been shown that *E. coli* is not a producer of ESBL and that its most likely resistance mechanism may be an alteration in the permeability of the membrane²². A study carried out in a Spanish hospital isolated 34 strains of *E. coli*, 10 of which were negative for ESBL production. Nevertheless, mutations in the attenuation and promoter regions of the AmpC gene were identified in eight isolates that were not ESBL-producers⁶. In our study, intermediate resistance to piperacillin suggests that a moderate selection has occurred, possibly originated from hospital-related activities. One of the hypotheses that may explain this resistance is that it is related to the mean of 13.3 hospitalizations per 100 inhabitants annually in Brazil, pointing to a close connection amongst the hospital, the community and the environment³⁴. Another hypothesis is that the increasing use of antibiotics in the domestic context may exert pressure that is sufficiently selective as to permit the appearance of strains with moderate resistance^{4,34}.

The present study has shown different antibiotic sensitivity profile among the samples of *P. aeruginosa*. All the isolates had intermediate resistance to cefotaxime a finding that is similar to the results of the study carried out in Goiânia in 2007 in which the isolates showed a high level of intermediate resistance (59.2%) or total resistance (40.8%) to this drug²³. A study carried out in rivers in the United States found that many gram-negative organisms, among them *P. aeruginosa*, were resistant to at least one antibiotic other than ampicillin, and a substantial frac-

tion were able to survive various antimicrobials².

Other authors have shown that dissemination of the use of biocides such as triclosan and quaternary ammonium salts in hospitals and home cares may encourage the selection of resistant bacteria. To a lesser extent triclosan has encouraged the selection of resistance of *E. coli*, and to a greater extent resistance of *P. aeruginosa*, to ciprofloxacin. Bacteria resistant to tetracycline, including *P. aeruginosa*, have also been identified in sewage systems^{12,13,14,30}.

The study carried out in United States' rivers found that more than 80% of the microorganisms resistant to cefotaxime and ceftazidime consisted of *Pseudomonas*. As a result of the dissemination of this pathogen in the environment, infections acquired in hospitals and in the community are usually associated with high mortality rates^{2,38,39}.

Despite the fact that this study did not find a high rate of resistance against quinolones, imipenem and cephalosporins, the National Nosocomial Infections Surveillance (NNIS) showed that the rates of resistance of *P. aeruginosa* isolated in 2003 to all these antipseudomonal antibiotics increased 9%, 15% and 20%, respectively, compared to those isolated between 1998 and 2002²¹.

Aztreonam was highly active against *P. aeruginosa*; however, other studies carried out in Brazil in samples of *Pseudomonas* from different states found high rates of resistance to this antimicrobial^{9,25}.

Intermediate resistance rates detected in the present study may be explained by the expression of one or the association of two resistance mechanisms such as alterations in the permeability of the membrane, increased action of the efflux pumps, or the expression of Ambler class A, B or D enzymes²³.

With respect to the sensitivity profile of *K. pneumoniae*, this microorganism had the highest rates of intermediate and total resistance to some of the antibiotics tested. In the present study, ESBL-producing *K. pneumoniae* was not detected; however, the emergence of the ESBL-producing pathogen has already been reported worldwide as an important cause of hospital infection. In USA and Canadian hospitals, the rate of ESBL-producing *K. pneumoniae* infection is less than 4%, while in Europe this rate varies from 15 to 20%. However, in Brazil rates are even higher, as shown in several regional studies^{10,11,29,31,32}.

In a study carried out in Goiânia, the mean prevalence of lineages of ESBL-producing *K. pneumoniae* was 38.2%. This finding is similar to the rate of 42.1% found in Brazilian hospitals and reinforces the fact that the prevalence of this microorganism is a major concern in Brazil^{31,32}. For this motive, measures are required to control the emergence of multiresistant bacteria, and barrier measures should be implemented to avoid the dissemination of pathogens with this resistance mechanism in hospitals.

A strain of *K. pneumoniae* with intermediate resistance to imipenem was found in the present study, contrary to the findings of the study carried out in hospitals in Goiânia, where 100% activity was found in the samples evaluated irrespective of ESBL production³⁴. Similar results have also been found in other studies carried out in Brazil and in other Latin American countries in which more than

90% of the samples were sensitive to carbapenems^{11,32}.

Studies carried out in 2002 and 2005 described high levels of antimicrobial-resistant bacteria isolated in rivers in the United States. Gram-negative bacteria resistant to imipenem were identified, recovered, and the molecular mechanisms involved in the resistance to these antibiotics were analyzed. Another study successfully isolated and identified 30 bacteria (*Aeromonas hydrophila*, *Stenotrophomonas maltophilia*, *Enterobacter spp*, *E. asburiae*), of which 29 were resistant to imipenem³. This finding draws attention to the possibility that many rivers in the United States may be reserves of broad spectrum carbapenemases^{2,3}.

In a study carried out in 1999 (15), over 80% of the isolates of *E. coli*, *K. pneumoniae*, *E. cloacae* and *P. aeruginosa* were susceptible to ciprofloxacin, in agreement with the results found in the present study in which few isolates of *E. coli* and *K. pneumoniae* were resistant to this antimicrobial. On the contrary, in that study both ceftazidime and ciprofloxacin were weakly active against *Acinetobacter baumannii*¹⁵, contradicting the results presented herein where all the strains analyzed showed susceptibility to these antibiotics.

Organisms resistant to ceftazidime, cefotaxime and imipenem were detected in many rivers in the United States in 2002². In this study, 20-30% gram-positive bacteria belonging to the cefotaxime-resistant *Bacillus* genus were identified. All the isolates resistant

to this antibiotic were capable of hydrolyzing nitrocefim, indicating the presence of β -lactamase. Many cefotaxime-resistant bacteria were also resistant to ceftazidime and these consisted principally of *Pseudomonas*^{2,38,39}.

Despite the resolution published by the National Council for the Environment n° 357/2005 (28) providing instructions on how to dispose of any pollutant including hospital effluents, none of the hospitals participating in the present study was complying with the instructions detailed in this resolution²⁸.

The susceptibility profile of pathogens isolated from the effluents of the hospital sewage system reveals possible limitations to this study such as the number of samples obtained and the time at which they were collected. Even so, we were able to demonstrate the presence of some resistant or intermediate sensitive pathogenic strains in the effluents studied, showing that it is a fact probably of high impact, so that is detected even when small number of samples are analyzed. Since the emergence of multi-resistant bacteria is a public health issue, our data support the need of monitoring the effluents of hospital sewage systems and to adopt sanitary measures to prevent the dissemination of resistant genes into the environment.

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