

Original Article

## Tertiary hospital sewage as reservoir of bacteria expressing MDR phenotype in Brazil

Esgoto de hospital terciário como reservatório de bactérias expressando o fenótipo MDR no Brasil

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### Abstract

High doses of antibiotics used in hospitals can affect the microbial composition of sewers, selecting resistant bacteria. In this sense, we evaluated the antibiotic resistance profile and the multiresistant phenotype of bacteria isolated in sewage from a tertiary hospital in the interior São Paulo state, Brazil. For bacteria isolation, 10 µL of sewage samples were sown in selective culture media and the isolates were identified using VITEK-2 automatized system. The antibiotic sensitivity test was performed by disk diffusion. High percentages of resistance were found for amoxicillin, ampicillin, ceftazidime, clindamycin, vancomycin and the multidrug-resistant phenotype (MDR) was attributed to 60.7% of the isolates. Our results show bacteria classified as critical/high priority by WHO List of Priority Pathogens (*Enterococcus* and *Staphylococcus aureus* resistant to vancomycin and *Enterobacteriaceae* resistant to carbapenems) in hospital sewage. Therefore, the implementation of disinfection technologies for hospital sewage would reduce the bacterial load in the sewage that will reach urban wastewater treatment plants, minimizing superficial water contamination and bacterial resistance spread in the environment.

**Keywords:** drug resistance, sewage, phenotype, hospitals.

### Resumo

Altas doses de antibióticos utilizados em hospitais podem afetar a composição microbiana dos esgotos, selecionando bactérias resistentes. Nesse sentido, avaliamos o perfil de resistência a antibióticos e o fenótipo multirresistente de bactérias isoladas em esgoto de um hospital terciário no interior do estado de São Paulo, Brasil. Para o isolamento de bactérias, foram semeados 10 µL das amostras de esgoto em meios de cultura seletivos e os isolados foram identificados usando o sistema automatizado VITEK-2. O teste de sensibilidade aos antibióticos foi realizado por disco-difusão em ágar. Elevadas porcentagens de resistência foram encontradas para amoxicilina, ampicilina, ceftazidima, clindamicina, vancomicina e o fenótipo multirresistente (MDR) foi atribuído a 60,7% dos isolados. Nossos resultados mostram bactérias classificadas como prioridade crítica/alta pela Lista de Patógenos Prioritários da OMS (*Enterococcus* e *Staphylococcus aureus* resistentes à vancomicina e *Enterobacteriaceae* resistentes aos carbapenêmicos) no esgoto hospitalar. Sendo assim, implementação de tecnologias de desinfecção do esgoto hospitalar reduziriam a carga bacteriana no esgoto que chegará às estações de tratamento de esgoto urbanas, minimizando a contaminação dos ecossistemas hídricos receptores e a disseminação da resistência bacteriana no ambiente.

**Palavras-chave:** resistência microbiana a medicamentos, esgotos, fenótipo, hospitais.

## 1. Introduction

European Center for Disease Prevention and Control (ECDC), the Center for Disease Control and Prevention (CDC) and international experts' groups established a worldwide terminology for acquired resistance profiles

of several bacteria responsible for human infections (e.g., *Staphylococcus aureus*, *Enterococcus* spp., *Enterobacteriaceae* family, *Pseudomonas aeruginosa* and *Acinetobacter* spp.). The multidrug-resistant phenotype (MDR) has been defined

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as acquired resistance to at least one agent in three or more antimicrobial categories; extensively drug-resistant phenotype (XDR) as acquired resistance to at least one agent in all categories or susceptible to only one or two categories; and pandrug-resistant phenotype (PDR) defined as acquired resistance to all antibiotics in all categories (Magiorakos et al., 2012).

Hospital sewage is an environmental matrix that poses risks to human health, considering the presence of organic matter, chemical substances, medications and infectious agents (Carraro et al., 2016). High doses of antibiotics used in hospitals affect their sewage composition due to excretion of partially metabolized drugs in the patients' feces and urine (Amador et al., 2015). As a consequence, hospital sewage may become a highly selected reservoir for development of multiresistant bacteria (Zagui et al., 2020; Auguet et al., 2017). Nonetheless, environments with high concentrations of nutrients, such as sewage, favor horizontal gene transfer through plasmids and transposons containing antibiotic resistance genes (Korzeniewska and Harnisz, 2013). At the global level, there are several guidelines for maximum permissible limits for chemical substances and some microorganisms in treated effluents. However, there are no maximum limits established for antibiotics and resistant pathogens in most countries (Carraro et al., 2016). In Brazil, legislation allows hospitals to release sewage into the municipal sewage system without prior treatment, where urban wastewater treatment plants (UWWTP) exist (Brasil, 2002). However, evidence suggests that UWWTP are antibiotic resistance reservoirs and contribute to their dissemination in water resources (Rizzo et al., 2013; Auguet et al., 2017). In Brazil, few studies have addressed this issue and, considering the aspects mentioned above, evidence is needed to support new guidelines for hospital sewage treatment establishing. Thus, the present study aims to evaluate the prevalence of multiresistant phenotype (MDR, XDR and PDR) in bacteria from tertiary hospital sewage in Brazil.

## 2. Materials and Methods

### 2.1. Sampling site

Ribeirão Preto city is located in the northeast region of the São Paulo State, Brazil, with 711,825 inhabitants. Two UWWTP serve the city, treating 98% of the sewage (128,000m<sup>3</sup> of sewage/daily) using activated sludge technology (Zagui et al., 2020). The city comprises 13 tertiary hospitals, including one of the largest hospitals in Brazil, which is a reference center for various medical specialties, providing care for 4 million inhabitants from the region, in addition to patients from other Brazilian states and countries abroad. The hospital has approximately 750 beds and during 2019 there were 690,512 ambulatory care cases and 23,744 hospitalizations reported. According to Brazilian legislation, as mentioned previously, the hospital under study releases sewage without prior treatment into the municipal sewage system.

Samples were collected from three sewage inspection boxes that intercept effluents of the following hospital

sectors: i) ambulatory care; ii) patient's wards; and iii) confluent point of total hospital effluents. Four sampling campaigns were performed in each inspection box from April 2017 to June 2018. Triplicates samples were collected in previously sterilized glass vials and mixed to obtain a composite sample of 100 mL in each sampling point.

### 2.2. Microbiological analysis

Ten microliters of each sewage composite sample were seeded on Baird-Parker, Cetrimide and MacConkey (Kasvi) agar and incubated at 37 °C for 24 hours. Based on previous tests with this matrix, the volume of 10 µL was defined, as it presented better isolation results. Afterwards, isolated bacterial colonies were screening by Gram stain followed by identification using VITEK-2 automatized system (bioMérieux, Marcy l'Etoile, France) (Rave et al., 2019).

Antimicrobial susceptibility testing was performed using disk diffusion technique, according to the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2017) and antibiotics were selected according to Magiorakos et al. (2012), which establishes the criteria for the definition of acquired resistance phenotypes. The susceptibility of Gram-positive bacteria was tested for ampicillin (AMP), ciprofloxacin (CIP), clindamycin (CLI), erythromycin (ERI), gentamicin (GEN), linezolid (LNZ), nitrofurantoin (NIT), teicoplanin (TCP), trimethoprim-sulfamethoxazole (SXT), tetracycline (TET) and vancomycin (VAN). The susceptibility of the Gram-negative bacteria was tested for amikacin (AMI), amoxicillin (AMO), amoxicillin clavulanate (AMC), ampicillin (AMP), ampicillin/sulbactam (ASB), cefepime (CPM), cefotaxime (CTX), ceftazidime (CAZ), ceftriaxone (CRO), cefuroxime (CRX), ciprofloxacin (CIP), chloramphenicol (CLO), colistin (COL), ertapenem (ERT), gentamicin (GEN), imipenem (IPM), meropenem (MPM), piperacillin tazobactam (TZP), trimethoprim-sulfamethoxazole (SXT) and tetracycline (TET). The inhibition zone for each antibiotic was measured with a digital caliper and the results were compared to CLSI break-points (CLSI, 2017), in order to categorize bacteria as resistant, intermediate or susceptible.

### 2.3. Data analysis

The resistance phenotype (MDR, XDR and PDR) was attributed to bacteria according to Magiorakos et al. (2012). The antibiotic resistance percentages were assessed using graphs and Kruskal-Wallis test was used to evaluate differences of bacteria occurrence with resistance phenotypes between the sample points ( $p < 0.05$ ). The analyzes were performed using the GraphPad Prism 9 software.

## 3. Results

Twenty-eight bacterial colonies were isolated and 14 species identified, being *Escherichia coli* and *Klebsiella pneumoniae* the most prevalent bacteria, with 21.4% and 17.9%, respectively. As expected, Gram-negative bacteria were most frequently isolated, corresponding to 85.7% and other species of this group were identified, such as

*Citrobacter youngae*, *Enterobacter cloacae* Complex, *Hafnia alvei*, *Klebsiella ozaenae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Serratia liquefaciens* and *Yersinia enterocolitica*. Three species of the *Enterococcus* genus and *Staphylococcus aureus* were identified, representing Gram-positive bacteria.

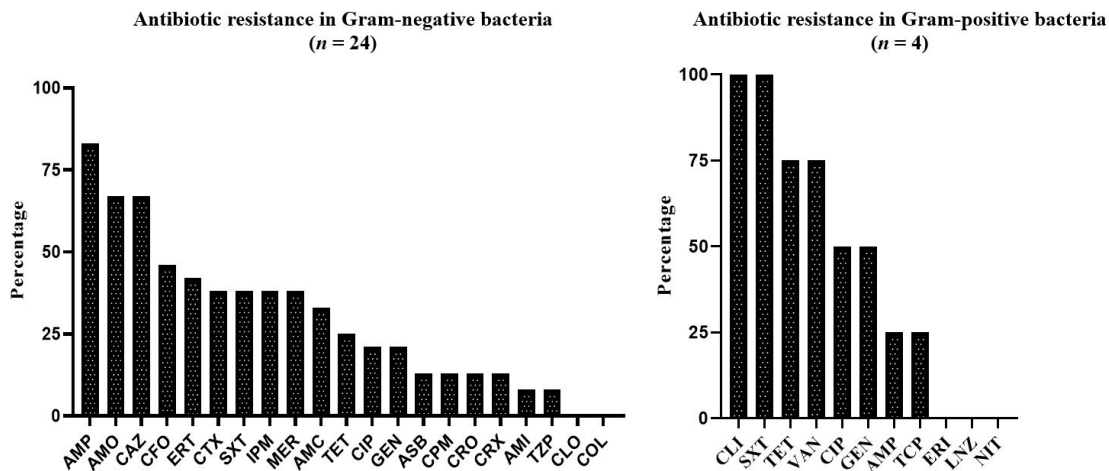
Table 1 shows the antibiotic resistance profile and multiresistant phenotype of bacteria isolated in different sample points (ambulatory care, patient wards, confluent point). All bacteria were resistant to at least one antimicrobial agent. A total of 60.7% were classified as MDR, most prevalent in ambulatory care sewage and patient's ward sewage. Among Gram-positive bacteria, *S. aureus*

presented resistance to a greater diversity of antibiotics in comparison to *Enterococcus* genus. Two isolates of the four species identified in this group were classified as MDR, with resistance to vancomycin. Among the Gram-negative bacteria, 62.5% were classified as MDR and six isolates presented resistance to at least ten antibiotics (*E. cloacae* Complex (1), *H. alvei* (2), *K. ozaenae* (2) and *K. pneumoniae* (1)). There was no statistically significant difference ( $p>0.05$ ) in occurrence of resistance phenotype among sampling points. According to antibiotic resistance profiles, there was no establishment of XDR and PDR phenotypes for any bacterial isolates.

**Table 1.** Antibiotic resistance profile and multiresistant phenotype of bacteria isolated in sewage from a tertiary hospital located in Ribeirão Preto, São Paulo, Brazil.

Sampling point	Species	Antibiotic resistance profile*	Resistance phenotype
Ambulatory care	<i>Proteus mirabilis</i>	AMO, AMC, AMP, CAZ, ERT, SXT, TET	MDR
	<i>Yersinia enterocolitica</i>	AMO, AMC, AMP, CTX, CFO, SXT	MDR
	<i>Enterococcus faecalis</i>	CIP, CLI, GEN, SXT, TET, VAN	MDR
	<i>Escherichia coli</i>	AMO, AMP, CAZ, SXT, TET	MDR
	<i>Escherichia coli</i>	AMO, AMP	-
	<i>Klebsiella pneumoniae</i>	AMO, AMP	-
Patient wards	<i>Enterobacter cloacae</i> Complex	AMI, AMP, ASB, CPM, CFO, CAZ, CRO, CRX, CIP, ERT, GEN, IPM, MER, TZP	MDR
	<i>Klebsiella ozaenae</i>	AMO, AMP, CTX, CFO, CAZ, CIP, ERT, GEN, IPM, MER, SXT, TET	MDR
	<i>Hafnia alvei</i>	AMO, AMC, AMP, CTX, CFO, CAZ, ERT, GEN, IPM, MER, SXT	MDR
	<i>Klebsiella ozaenae</i>	AMO, AMC, AMP, CTX, CFO, CAZ, CIP, ERT, IPM, MER, SXT	MDR
	<i>Escherichia coli</i>	AMO, AMC, AMP, CTX, CAZ, ERT, IPM, MER, TET	MDR
	<i>Staphylococcus aureus</i>	AMP, CIP, CLI, GEN, SXT, TCP, TET, VAN	MDR
	<i>Citrobacter youngae</i>	AMO, AMC, AMP, CFO	MDR
	<i>Klebsiella pneumoniae</i>	AMO, AMC, AMP, SXT	-
	<i>Enterococcus gallinarum</i>	CLI, SXT, TET, VAN	-
	<i>Pseudomonas aeruginosa</i>	CAZ, CIP, GEN	MDR
	<i>Escherichia coli</i>	AMO, AMP	-
	<i>Pseudomonas aeruginosa</i>	CAZ	-
Confluent point	<i>Klebsiella pneumoniae</i>	AMP, ASB, CPM, CFO, CAZ, CRO, CRX, CIP, ERT, GEN, IPM, MER, TZP	MDR
	<i>Hafnia alvei</i>	AMO, AMC, AMP, CTX, CFO, CAZ, ERT, IPM, MER, SXT	MDR
	<i>Serratia liquefaciens</i>	AMO, AMP, CTX, CFO, CAZ, ERT, IPM, MER	MDR
	<i>Pseudomonas aeruginosa</i>	AMI, AMP, ASB, CPM, CFO, CAZ, CRO, CRX	-
	<i>Klebsiella pneumoniae</i>	AMO, AMP, CTX, SXT, TET	MDR
	<i>Hafnia alvei</i>	CTX, CAZ, ERT, IPM, MER	-
	<i>Escherichia coli</i>	CFO, CAZ, TET	MDR
	<i>Klebsiella pneumoniae</i>	AMO, AMP, CAZ	-
	<i>Enterococcus faecium</i>	CLI, SXT	-
	<i>Escherichia coli</i>	AMP	-

\*AMI: Amikacin; AMO: amoxicillin; AMC: amoxicillin clavulanate; AMP: ampicillin; ASB: ampicillin sulbactam; CPM: cefepime; CTX: cefotaxime; CFO: cefoxitin; CAZ: ceftazidime; CRO: ceftriaxone; CRX: cefuroxime; CIP: ciprofloxacin; CLI: clindamycin; ERT: ertapenem; GEN: gentamycin; IPM: imipenem; MER: meropenem; TZP: piperacillin tazobactam; SXT: trimethoprim-sulfamethoxazole; TCP: teicoplanin; TET: tetracycline; VAN: vancomycin.



**Figure 1.** Distribution of antibiotic resistance (%) of bacteria isolated in sewage from a tertiary hospital in Ribeirão Preto, São Paulo, Brazil. AMI: Amikacin; AMO: amoxicillin; AMC: amoxicillin clavulanate; AMP: ampicillin; ASB: ampicillin sulbactam; CPM: cefepime; CTX: cefotaxime; CFO: cefoxitin; CAZ: ceftazidime; CRO: ceftriaxone; CLO: chloramphenicol; COL: colistin; CRX: cefuroxime; CIP: ciprofloxacin; CLI: clindamycin; ERI: erythromycin; ERT: ertapenem; GEN: gentamycin; IPM: imipenem; LNZ: linezolid; MER: meropenem; NIT: nitrofurantoin; TZP: piperacillin tazobactam; SXT: trimethoprim-sulfamethoxazole; TCP: teicoplanin; TET: tetracycline; VAN: vancomycin.

Figure 1 shows the antibiotic resistance distribution of bacteria isolated from the tertiary hospital sewage. For Gram-negative isolates, the most significant resistance percentages were found for ampicillin (83%), amoxicillin (67%) and ceftazidime (67%). Lower percentages of resistance were found for amikacin (8%), piperacillin tazobactam (8.3%) and no resistance to chloramphenicol and colistin. For Gram-positive isolates, the most significant resistance was found for clindamycin (100%), trimethoprim-sulfamethoxazole (100%), tetracycline (75%) and vancomycin (75%). It is important to note that the percentages may be underestimated due to the number of Gram-positive isolates ( $n = 4$ ). No resistance to erythromycin, linezolid or nitrofurantoin was found.

#### 4. Discussion

In this study, *E. coli* and *K. pneumoniae* presented higher frequency of isolation, corroborated with studies that verified the microbial profile in hospital effluents (Rabbani et al., 2017; Röderová et al., 2016). Wastewater composition varies according to local activities, however, most sewage microbial composition comes from gastrointestinal tract (Abdulhaq and Basode, 2015) and, from this perspective, most bacteria identified in this study can be found in human intestinal microbiota, as well as infections. In this sense, *E. coli* is the main etiological agent causing urinary infections (Chervet et al., 2018) and the existence of the same pathogenic bacteria found among inpatients and in sewage has been reported previously (Röderová et al., 2016). Röderová et al. (2016) found two genetically identical isolates of *K. pneumoniae*, one in the urinary catheter of a patient and another one isolated from urban sewer system of Olomouc city, Czech Republic. Such a finding supports the hypothesis that bacteria found in

hospital sewage addressed in this study may be related to inpatient infection.

The MDR phenotype percentage found (Table 1) were below the rate detected in a study conducted in Portugal, with 91.7% of MDR bacteria in hospital sewage resistant to penicillin, cephalosporins, sulfonamides and quinolones (Vaz-Moreira et al., 2016). There was a broad profile of antibiotic resistance in MDR isolates in the patient ward sewage, which may be justified by antibiotics administration in hospitalized patients, either for infections treatment or prophylactic measure to avoid infections related to health care. It is known that antibiotics use in such patients leads to occurrence bacteria carrying resistance genes in sewage, such as *bla* genes ( $\beta$ -lactam-resistant genes, the most widely used antibiotics class) (Korzeniewska and Harnisz, 2013). MDR isolates with resistance to broad spectrum antibiotics were also verified in the confluence point. Sewage discharge time is not reported, however, it is inferred that the bacteria exposure to products with antimicrobial potential, such as antibiotics, heavy metals and disinfectants, can contribute to resistant bacteria selection (Mania et al., 2018).

Due to the intrinsic resistance presented by different bacterial species such as *E. cloacae* Complex, *H. alvei*, *K. pneumoniae*, *P. aeruginosa* and *Y. enterocolitica* (CLSI, 2017), antimicrobial susceptibility test showed a high percentage of resistant bacteria to ampicillin and amoxicillin. High percentage was also found for ceftazidime, a broad-spectrum cephalosporin used for Gram-negative infection treatment. Other studies also showed broad bacterial resistance to several classes of antibiotics (Rabbani et al., 2017; Vaz-Moreira et al., 2016; Röderová et al., 2016) that may be associated with the epidemiological profile of infections and antibiotic therapy protocols adopted by different countries. Bacteria resistance to clindamycin and vancomycin showed high percentage.



Whereas susceptibility test with these antibiotics were performed only for Gram-positive bacteria ( $n = 4$ ), higher numbers of bacterial isolates could provide more real values for resistance to these antibiotics. Detection of vancomycin-resistant isolates in hospital sewage is relevant, given the dependence of this antibiotic for treatment of *S. aureus* and *Enterococcus* spp. infections. Increase of strains resistant to vancomycin is concern, which has led to approval some derivatives antibiotics such as dalbavancin, oritavancin and telavancin, as well as motivated the search for new semisynthetic drugs for clinical use (Pawlowski et al., 2016).

Antibiotics in hospital sewage and high concentrations of nutrients may favor the bacterial resistance development due selective pressure, as well as the horizontal transfer of mobile genetic elements containing resistance genes. In this sense, six isolates with an MDR phenotype (1 *E. cloacae* Complex, 2 *H. alvei*, 2 *K. ozaenae*, 1 *K. pneumoniae*) stand out because they presented a resistance profile to at least ten antibiotics of the following categories:  $\beta$ -lactams, quinolone, aminoglycosides, sulfonamides and tetracycline. The broad resistance profile of these bacteria may be a consequence of the highly selective medium that promotes the genetic diversity of chromosomes and/or mobile genetic elements in bacteria with the capacity to express various resistance mechanisms.

*Enterococcus* genus and *S. aureus* resistant to vancomycin and *Enterobacteriaceae* species resistant to carbapenems were identified. Such microorganisms are included in WHO Priority Pathogens List for research and development of new antibiotics (WHO, 2017). Cephalosporins and carbapenems are important antibiotics for serious infections treatments caused by Gram-negative pathogens; however, bacteria belonging to this group showed resistance to these antibiotics' categories ranging from 38 to 67%. None of the isolated bacteria were resistant to chloramphenicol, colistin, erythromycin, linezolid or nitrofurantoin. Colistin is a drug of last resort to treat infections caused by Gram-negative bacteria with MDR phenotype; however, currently a plasmid-borne colistin resistance gene (*mcr-1*) have been increasingly reported around the world, including in bacteria from hospital sewage (Jin et al., 2018; Sun et al., 2018). These findings show the need to monitor resistance patterns in hospital sewage to ensure the adequate antibiotics use, in order to avoid the spread of resistance determinants in the environment.

In conclusion, high incidence of MDR bacteria found in the tertiary hospital sewage is concern because resistance determinants spread rapidly in aquatic matrices. There is a need to evaluate effluents from tertiary hospitals due to frequent antibiotics use in care units. The findings reflect effluent bacteriological condition of a tertiary hospital that has more than 23,000 patients' internments annually, so it is necessary disinfection of sewage from large hospitals before the release into municipal sewage systems in Brazil, since the occurrence of resistant bacteria in municipal sewage is inherent to fecal pollution (Karkman et al., 2019). Hospital effluents treatment would reduce resistant bacteria into UWWTP and concomitantly resistance dissemination. In addition, new studies for establishment of maximum permissive limits for antibiotics concentrations and

resistant pathogens are necessary in order to avoid water resources pollution, since bacteria and/or resistance genes have already been detected in rivers impacted by UWWTP effluents (Amador et al., 2015; Giebułtowicz et al., 2018). Therefore, additional disinfection technologies for such effluents would reduce pollution of water resources and control the spread of resistant bacteria in the environment.

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