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Diversity of bacteria carrying antibiotic resistance genes in hospital raw sewage in Southeastern Brazil

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ABSTRACT

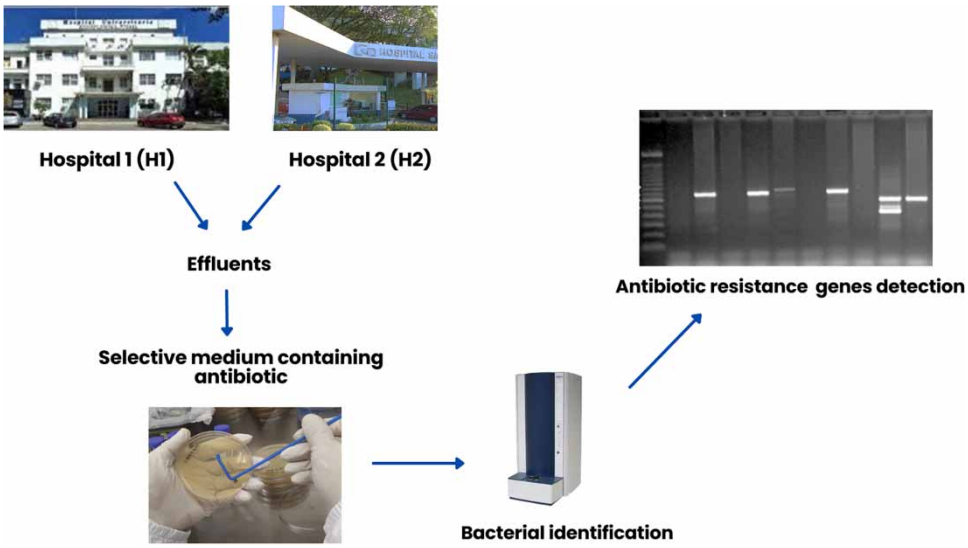
In recent decades, antibiotic-resistant bacteria (ARB) emerged and spread among humans and animals worldwide. In this study, we evaluated the presence of ARB and antibiotic resistance genes (ARGs) in the raw sewage of two hospitals in Brazil. Sewage aliquots were inoculated in a selective medium with antibiotics. Bacterial identification was performed by MALDI-TOF and ARGs were assessed by polymerase chain reaction (PCR). A total of 208 strains from both hospitals were isolated (H1 = 117; H2 = 91). A wide variety of *Enterobacterales* and non-*Enterobacterales* species were isolated and most of them were *Enterobacter* spp. (13.0%), *Proteus mirabilis* (10.1%), and *Klebsiella pneumoniae* (9.6%). *blaTEM* and *blaKPC* were the most frequent β-lactamase-encoding genes and the predominant macrolide resistance genes were *mph(A)* and *mel*. Many species had the three tetracycline resistance genes (*tetD*, *tetM*, *tetA*) and *strB* was the prevalent aminoglycoside resistance gene. Two *Staphylococcus haemolyticus* strains had the *mecA* gene. Quinolone, colistin, and vancomycin resistance genes were not found. This study showed that hospital raw sewage is a great ARB and ARG disseminator. Strict monitoring of hospital sewage treatment is needed to avoid the spread of these genes among bacteria in the environment.

Key words: antibiotic resistance genes, aquatic environment, bacterial resistance, Brazil, hospital raw sewage

HIGHLIGHTS

- Diverse *Enterobacterales* and non-*Enterobacterales* species have antibiotic resistance genes, including traditionally environmental bacteria.
- ESBL-encoding (bla_{TEM}) and carbapenemase genes (bla_{KPC}) were found in hospital raw sewage.
- Multiple macrolide and tetracycline resistance genes were reported.
- Hospitals contribute to ARB and ARG dissemination to the municipal sewage system.

GRAPHICAL ABSTRACT



INTRODUCTION

Antimicrobial resistance is recognized as a global challenge (WHO 2014). In recent decades, antibiotic-resistant bacteria (ARB) emerged and spread among humans and animals worldwide due to the selective pressure caused by the intensive and inappropriate use of antibiotics. In this context, cephalosporin, fluoroquinolone, and carbapenem resistance in *Enterobacterales*, carbapenem resistance in *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, methicillin resistance in *Staphylococcus* spp., and vancomycin resistance in *Enterococcus* spp. stand out (CDC 2019). This growing threat requires a global response and the development of strategies to inhibit its spread in hospitals and the environment. Besides these hospital pathogens, several traditionally environmental bacterial species have shown resistance to antibiotics, such as *Alcaligenes faecalis*, *Ochrobactrum* spp., and *Achromobacter* spp. (Furlan & Stehling 2017; Ngbede *et al.* 2020). By the presence of antibiotic resistance genes (ARGs), these bacteria are able to adapt to new environments and it shows the high gene transmission rate between hospital and environmental bacteria (Picão *et al.* 2013; Haller *et al.* 2018). ARG dissemination to environmental and zoonotic bacteria can be a potential risk to human health (Cacace *et al.* 2019; Ngbede *et al.* 2020). Ngbede *et al.* (2020) found mobile colistin resistance genes in *Enterobacterales* and *A. faecalis* of animal and human origin in Nigeria. Huang (2020) observed emergent extensively drug-resistant *A. faecalis* causing human infections. In Brazil, Furlan & Stehling (2017) reported a high level of resistance to β-lactam antibiotics and different β-lactamase-encoding genes in *Ochrobactrum* and *Achromobacter* bacteria isolated from soil.

Enterobacterales is the main group that links the hospital and the aquatic and soil environments (Picão *et al.* 2013; Haller *et al.* 2018). β-lactamase production is responsible for the most important resistance mechanisms among them and they are traditionally present in health care strains. Extended-spectrum β-lactamases (ESBLs) and carbapenemase-producing bacteria are global problems and strains with β-lactamases in environmental matrices are increasing (Woodford *et al.* 2014; White *et al.* 2016). In Brazil, *Enterobacterales* carrying ESBL genes, such as bla_{SHV}, bla_{TEM}, and $bla_{\text{CTX-M}}$, and carbapenemases, mainly bla_{KPC}, emerged from the soil and different aquatic environments (Picão *et al.* 2013; Furlan & Stehling 2017).

In hospitals, the use of antibiotics is more intensive and hospital effluents can receive antimicrobial residues and ARB (Harris *et al.* 2013; Varela & Manaia 2013). These effluents are discharged to municipal wastewater treatment plants (WWTP), which may also serve as important reservoirs for the transfer of ARGs by mobile genetic elements (MGEs) (Rizzo *et al.* 2013; Varela *et al.* 2014). The presence of resistance genes in the final WWTP effluents shows the inefficiency of the water treatment process and can contribute to ARB and ARG dissemination (Schwartz *et al.* 2003; Li *et al.* 2016).

This study aimed to monitor ARB and ARG in two hospital raw sewage systems from Espírito Santo, Southeastern Brazil, to subsidize strict legislation for environmental control of antibiotic resistance dissemination.

METHODS

Sample collection and hospital characteristics

This study was performed in two hospital raw sewage systems (H1 and H2) in the city of Vitória, Southeastern Brazil. Five samples were collected monthly from October 2020 to February 2021 in each hospital. H1 is a reference teaching institution for the treatment of tuberculosis and HIV/AIDS, which provides from low-complexity to highly specialized health services and has 295 beds and about 2,108 employees. H2 is a reference philanthropic institution for the treatment of various types of cancer and has 265 beds, around 1,500 employees, and over 400 doctors in the clinical staff. The distance between both hospitals is 100 m. At the end of the hospital raw sewage system, before its content was discharged into the municipal sewage system, the samples were collected at a depth of about 0.5 m from the surface. Then, 500 mL sterile plastic bottles were submerged in the effluent, immediately transported in a portable icebox to the laboratory, and processed during the following 6 h.

Sample processing and screening in selective medium

For primary growth, 100 μL of the raw sewage aliquot and its serial dilutions (10^{-1} and 10^{-2}) were inoculated in triplicate in plates with Mueller-Hinton agar (Oxoid, England) medium supplemented with antibiotics. Amikacin (16 μg/mL), cephalexin (32 μg/mL), erythromycin (2 μg/mL), colistin (2 μg/mL), levofloxacin (2 μg/mL), and tetracycline (4 μg/mL) were the antibiotics and concentrations used. Mannitol salt agar (Oxoid, England) with oxacillin (4 μg/mL) and bile-esculin agar (Himedia, India) with vancomycin (8 μg/mL) were also used to screen for methicillin-resistant *Staphylococcus* spp. and vancomycin-resistant *Enterococcus* spp., respectively.

After primary growth, a secondary isolation of colonies was performed on nutrient agar medium (Oxoid, England). For this isolation, the size, pigmentation, opacity, and edges of the colonies on the primary plate were analyzed. One colony of each profile and only one among all colonies with identical profiles were selected for isolation. All strains were stored in tryptone soy broth (TSB) (Himedia, India) with 20% glycerol at −20 °C.

Bacterial identification

Bacterial identification was performed by the matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) technique, using a Bruker Microflex™ MALDI-TOF/MS instrument (Billerica, MA, USA).

DNA extraction and ARG detection in ARB

For DNA extraction, the Wizard™ Genomic DNA kit (Promega, USA) was used, according to the manufacturer's technical specifications.

The presence of resistance genes in ARB was assessed by single or multiplex PCR, according to the antibiotic present in the culture medium for primary isolation (Table 1).

All PCRs conditions used in this study were validated in laboratory (according to conditions established in the references) and PCR amplicons were sequenced and DNA sequences analysis was performed using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Statistical analysis

Chi-square or Fisher's exact test was used to evaluate the difference in ARB and ARGs from the hospitals (H1 and H2). Statistical analyses were performed using GraphPad Prism (version 7.04) and $p < 0.05$ was considered significant.

RESULTS

Bacterial isolated from selective screening medium

A total of 208 strains were isolated from the two hospital raw sewage (H1 = 117; H2 = 91) and we identified 172 strains (82.7%) by MALDI-TOF. We did not identify the other 36 (17.3%) (Figure 1). We observed a wide variety of *Enterobacterales* species and the most isolated were *Enterobacter* spp. ($n = 27$; 12.9%), *Proteus mirabilis* ($n = 21$; 10.1%), *Klebsiella pneumoniae* ($n = 20$; 9.6%), *Escherichia coli* ($n = 18$; 8.6%), and *Pseudomonas aeruginosa* ($n = 16$; 7.7%) (Figure 1).

The prevalent species in H1 and H2 were significantly different. We found *P. mirabilis* only in H1 ($p < 0.0001$) and *Enterobacter* spp. mainly in H2 (25×2; $p < 0.0001$). *P. aeruginosa*, *K. pneumoniae,* and *E. coli* presented no significant differences between them ($p > 0.05$). In general, various species existed in only one hospital (Figure 1).

Table 1 | Genes used in PCR to detect antibiotic resistance genes

Antibiotic used in the screening medium (class)	Resistance genes[a]	References
Amikacin (Aminoglycosides)	*aacA, aacA1, aac(6')-Im, aacC1, aadA4, aph, aphA, aph2, strA, strB*	Szczepanowski *et al.* (2009)
Erythromycin (Macrolides)	*mph(A), mph(B), ermA, ermB, mefA, mel*	
Levofloxacin (Quinolones)	*qnrA3, qnrB1, qnr*	
Tetracycline (Tetracyclines)	*tetA, tetD, tetM*	
Cephalexin (β-lactams)	$bla_{\text{KPC1-5}}$, bla_{IMP}, bla_{VIM} (M-PCR 1) bla_{SHV}, bla_{TEM}, $bla_{\text{OXA1-like}}$ (M-PCR 2) $bla_{\text{OXA48-like}}$ $bla_{\text{CTX-Mgp1}}$, $bla_{\text{CTX-Mgp2,}}$ $bla_{\text{CTX-Mgp9}}$ (M-PCR 3) bla_{OXA23}, $bla_{\text{OXA24-like}}$, bla_{OXA51}, bla_{OXA58} (M-PCR 4) bla_{NDM}	Dallenne *et al.* (2010) Woodford *et al.* (2006) Poirel *et al.* (2011)
Colistin (Polimixins)	*mcr1, mcr2, mcr3, mcr4, mcr5* (M-PCR 5)	Rebelo *et al.* (2018)
Oxacillin[b] (β-lactams)	*mecA*	Milheiriço *et al.* (2007)
Vancomycin[b] (Glycopeptides)	*vanA, vanB* (M-PCR 6)	Depardieu *et al.* (2004)

[a]M-PCR (multiplex PCR). All other genes were detected by single PCR.
[b]used specifically to detect methicillin-resistant *Staphylococcus* spp. and vancomycin-resistant *Enterococcus* spp., respectively.

ARG detection in ARB

A total of 36 widely varied strains, including traditionally environmental bacteria (*Alcaligenes faecalis* and *Kluyvera* spp.), were isolated from the selective medium with cephalexin. Among them, 31 strains (86.1%) had at least one β-lactam resistance gene. bla_{TEM} ($n = 22$), bla_{KPC} ($n = 17$), and $bla_{\text{OXA-1-like}}$ ($n = 9$) were the most frequent genes (Table 2). We did not find bla_{IMP}, bla_{VIM}, bla_{NDM}, $bla_{\text{OXA-24-like}}$, $bla_{\text{OXA-23}}$, $bla_{\text{OXA-48}}$, $bla_{\text{OXA-51}}$, and $bla_{\text{OXA-58}}$. We found bla_{TEM} in 10 different species, mainly *Klebsiella pneumoniae* and *Enterobacter* spp., and bla_{KPC} in seven, mainly *Enterobacter* spp. (Table 2).

Only bla_{KPC} showed a significant difference ($p = 0.0054$) in gene detection between H1 and H2.

Among the 28 strains isolated from the medium with erythromycin, *mph(A)* ($n = 23$) was the prevalent gene. We found *mel* and *ermB* in 18 and 16 strains, respectively, and did not find *mph(B), ermA*, and *mefA*. Different species present the coexistence of distinct macrolide resistance genes. We found *mph(A)* in all identified species, except for *Klebsiella oxytoca,* which had no genes (Table 3).

The genes found in H1 and H2 presented no significant difference between them ($p > 0.05$).

All 31 strains isolated from the selective medium with tetracycline had at least one of the three genes: we found *tetA*, *tetD*, and *tetM* in 23 (74.2%), 27 (87.1%), and 26 (83.9%) strains, respectively. We found these genes in 10 different species, mainly *E. coli* ($n = 8$) and *K. pneumoniae* ($n = 6$) (Table 4).

We observed a significant difference between H1 and H2 regarding *tetA* ($p = 0.0109$) and *tetM* ($p = 0.0118$).

A total of 39 strains were isolated from the selective medium with amikacin and 29 (74.4%) of them had at least one resistance gene – *strB* ($n = 18$) and *strA* ($n = 11$) were prevalent. We also found *aacA* ($n = 10$), *aadA4* ($n = 7$), and *aph* ($n = 5$), but did not find *aph2, aphA, aacC1, aac(6)Im*, and *aacA1* (Table 5).

We observed that *strB* was more significantly detected in H1 ($p < 0.0001$). Other genes presented no significant difference.

A total of 33 strains were isolated from the medium with levofloxacin and we did not find *qnr, qnrA3*, and *qnrB1* in any strain. Considering $n = $ H1/H2, the following species were isolated: *Acinetobacter baumannii* ($n = 1/0$), *Alcaligenes faecalis* ($n = 0/2$), *Citrobacter freundii* ($n = 0/5$), *Corynebacterium* spp. ($n = 1/0$), *Enterobacter* spp. ($n = 0/1$), *Enterobacter cloacae* ($n = 0/1$), *Enterococcus faecalis* ($n = 1/0$), *Escherichia coli* ($n = 2/2$), *Klebsiella oxytoca* ($n = 1/0$), *Klebsiella pneumoniae* ($n = 2/3$), *Klebsiella* spp. ($n = 1/0$), *Morganella morganii* ($n = 1/0$), *Proteus mirabilis* ($n = 2/0$), *Pseudomonas aeruginosa* ($n = 2/1$), *Pseudomonas* spp. ($n = 1/0$), and unidentified ($n = 3/0$).

Moreover, 36 strains were isolated from the medium with colistin: *Aeromonas hydrophila* ($n = 1/0$), *Aeromonas* spp. ($n = 0/1$), *Chromobacterium violaceum* ($n = 0/1$), *Enterobacter* spp. ($n = 0/7$), *Escherichia coli* ($n = 1/0$), *Klebsiella pneumoniae* ($n = 1/1$), *Kluyvera* spp. ($n = 0/1$), *Ochrobactrum* spp. ($n = 0/1$), *Proteus mirabilis* ($n = 11/0$), *Providencia* spp. ($n = 0/1$), and unidentified ($n = 4/5$). We did not find *mcr1, mcr2, mcr3, mcr4*, and *mcr5* in any strain.

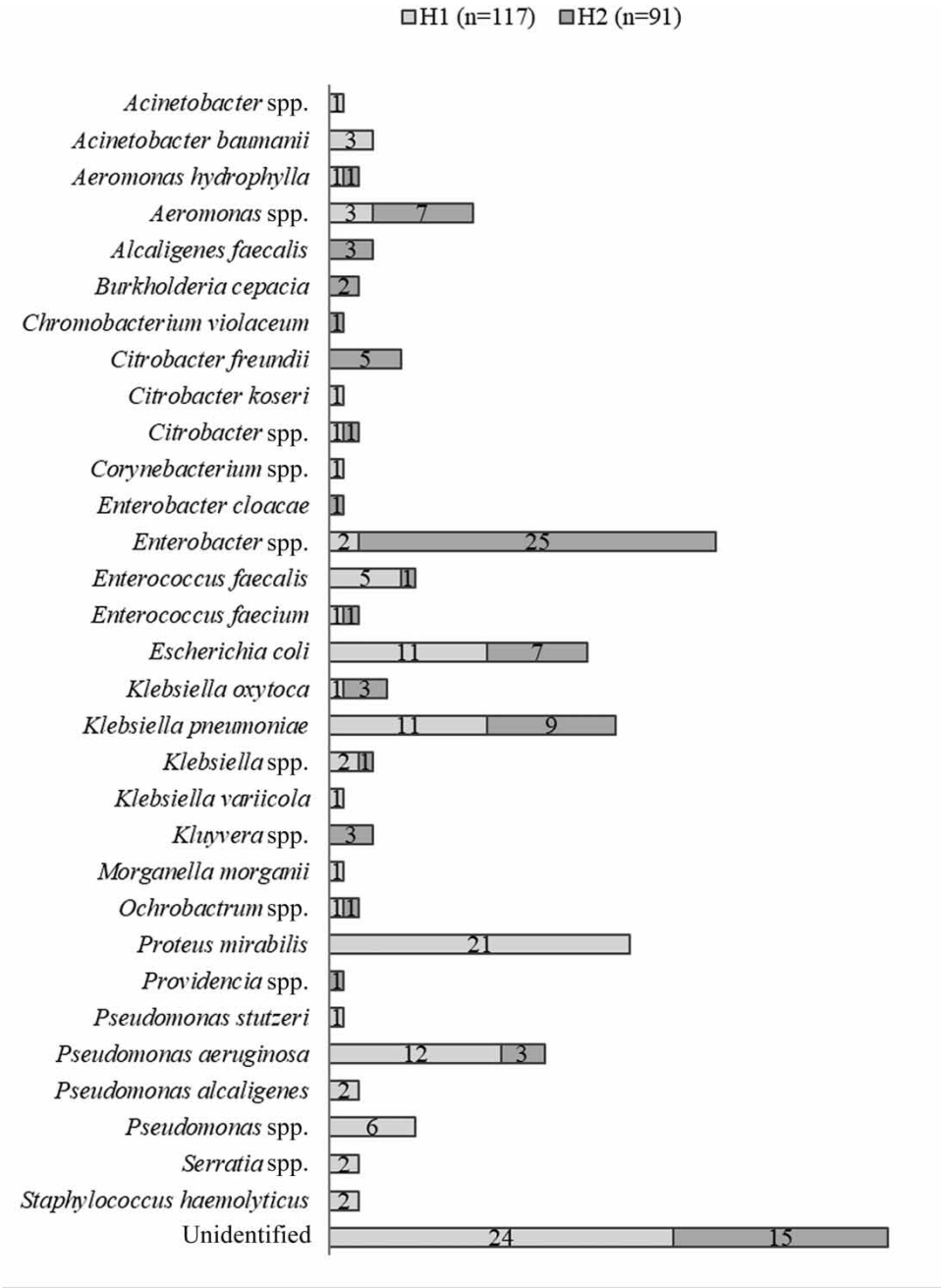


Figure 1 | Bacterial species isolated from two hospital raw sewage systems (H1 and H2) ($n = 208$).

Five gram-positive strains were isolated from mannitol salt agar supplemented with oxacillin: *Staphylococcus haemolyticus* ($n = 2/0$), *Enterococcus faecalis* ($n = 2/0$), and *Enterococcus faecium* ($n = 0/1$). The two *S. haemolyticus* strains had *mecA gene*.

We found no *Enterococcus* in the selective bile-esculin agar with vancomycin. Thus, we did not study *vanA* and *vanB* genes.

DISCUSSION

The flow of effluents and solid waste with chemicals, pharmacological pollutants, and persistent organic compounds has been changing aquatic ecosystems. In this study, we assessed a wide variety of species with different ARGs. *Enterobacterales* were

Table 2 | Presence of β-lactamase resistance genes in 36 strains isolated from the selective medium with cephalexin

	Resistance genes[a] (number of strains)													
	bla^{b}_{KPC1-5}		$bla_{CTX-M\ 1}$ group		$bla_{CTX-M\ 2}$ group		$bla_{CTX-M\ 9}$ group		bla_{TEM}		bla_{SHV}		$bla_{OXA1like}$	
Species (number of strains H1/H2)	H1	H2	H1	H2	H1	H2	H1	H2	H1	H2	H1	H2	H1	H2
Aeromonas spp. (1/1)	–	1	–	–	–	–	–	–	1	–	–	–	–	–
Alcaligenes faecalis (0/1)	–	–	–	–	–	–	–	–	–	1	–	–	–	–
Burkholderia cepacia (0/1)	–	1	–	–	–	–	–	–	–	–	–	–	–	1
Enterobacter spp. (2/4)	2	3	–	–	–	–	–	–	2	2	1	1	1	1
Escherichia coli (2/0)	–	–	2	–	–	–	–	–	–	–	–	–	2	–
Klebsiella pneumoniae (3/2)	–	2	1	–	–	–	–	–	2	1	–	–	1	–
Klebsiella oxytoca (0/1)	–	1	–	–	–	1	–	1	–	1	–	–	–	–
Kluyvera spp. (0/2)	–	2	–	–	–	–	–	2	–	1	–	1	–	1
Ochrobactrum spp (1/0)	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Proteus mirabilis (2/0)	1	–	–	–	–	–	–	–	1	–	–	–	–	–
Pseudomonas stutzeri (1/0)	–	–	–	–	–	–	–	–	1	–	–	–	–	–
Pseudomonas aeruginosa (3/0)	–	–	–	–	–	–	–	–	3	–	1	–	2	–
Pseudomonas spp. (1/0)	–	–	–	–	–	–	–	–	1	–	–	–	–	–
Serratia spp. (1/0)	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Unidentified (5/2)	3	1	–	–	–	–	–	–	4	1	1	1	–	–
Total (22/14)	6	11	3	–	–	1	–	3	15	7	3	3	6	3

[a]'–': absence. We did not find bla_{IMP}, bla_{VIM}, $bla_{OXA-24\ like}$, bla_{OXA23}, bla_{OXA-51}, bla_{OXA-58}, bla_{NDM1}, or bla_{OXA48} in any strain.
[b]: statistical difference in gene detection between H1 and H2.

prevalent (>65%) in a selective medium supplemented with antibiotics. Other studies found higher rates for this group in hospital wastewater (Picão *et al.* 2013; Haller *et al.* 2018). In our study, *Enterobacter* spp., *K. pneumoniae*, *P. mirabilis*, *E. coli*, and *P. aeruginosa* were the most isolated species. This group of bacteria is very relevant due to their association with serious infections and antibiotic resistance in health care (Bush & Bradford 2020). We observed significant differences between some prevalent species isolated in the two hospitals. We found most *Pseudomonas* species and *P. mirabilis* only in H1. On the other hand, we found most *Enterobacter* spp. in H2. The reasons for this difference are unknown; however, it may reflect the environmental characteristics and circulating microbiota in each hospital. *E. coli* and *Klebsiella* species presented no significant difference between hospitals.

β-lactams are among the most frequently prescribed antibiotics and are important in the treatment of numerous types of infections; however, the dissemination of β-lactamase-encoding genes, mainly the TEM, SHV, and CTX-M extended-spectrum β-lactamases and carbapenemases, such as KPC and NDM, are serious concerns for public health (Lee *et al.* 2016; Wang *et al.* 2018; CDC 2019). In Brazil, Picão *et al.* (2013) found KPC-2-producing *Aeromonas* spp. and *Enterobacterales*, including *Kluyvera* spp., in hospital effluents and different WWTP sites. The authors showed that ARB are continually discharged in urban rivers, even after a secondary sewage treatment. Similarly, in our study, bla_{KPC} was predominant in several *Enterobacterales*, including *Kluyvera* spp. and *Aeromonas* spp. A high concentration of bla_{KPC} in multispecies was found in the hospital wastewater and forepart stages of the WWTP in the United States (Loudermilk *et al.* 2022).

The *Burkholderia cepacia* complex is a group of gram-negative and glucose nonfermentative bacteria found in the environment that was isolated from opportunistic infection in immunocompromised patients (Uehlinger *et al.* 2009). Furlan *et al.* (2018) were the first to assess bla_{KPC} in the *B. cepacia* complex isolated from Brazilian soil. The authors also found $bla_{OXA-1-like}$ in these strains. We found bla_{KPC} and $bla_{OXA-1\ like}$ in only one *B. cepacia* strain. To our knowledge, this is the first study showing *B. cepacia* from hospital raw sewage systems with these genes isolated. These results are worrying and show the possibility of transfer to other glucose-nonfermentative bacteria, such as *Pseudomonas* spp. and *Acinetobacter* spp.

Conte *et al.* (2017) described ESBL and quinolone-resistant *Enterobacterales* from a hospital effluent, a sanitary effluent, an inflow sewage, an aeration tank, and an outflow sewage within a WWTP. Results show the high presence of ESBLs,

Table 3 | Presence of macrolide resistance genes in 28 strains isolated from the selective medium with erythromycin

	Resistance genes[a] (number of strains)					
	mph(A)		*ermB*		*mel*	
Species (number of strains H1/H2)	H1	H2	H1	H2	H1	H2
Citrobacter koseri (1/0)	1	–	1	–	1	–
Citrobacter spp. (0/1)	–	1	–	–	–	–
Enterobacter spp. (0/4)	–	3	–	1	–	2
Escherichia coli (3/0)	2	–	2	–	1	–
Klebsiella oxytoca (0/1)	–	–	–	–	–	–
Klebsiella pneumoniae (1/1)	1	1	–	1	–	1
Klebsiella spp. (1/1)	–	1	–	–	–	–
Klebsiella variicola (1/0)	1	–	1	–	1	–
Proteus mirabilis (3/0)	3	–	2	–	3	–
Pseudomonas aeruginosa (3/0)	3	–	2	–	3	–
Pseudomonas spp. (1/0)	1	–	1	–	1	–
Unidentified (3/3)	3	2	3	2	3	2
Total (17/11)	15	8	12	4	13	5

[a]'–' absence. We did not find *mph(B)*, *ermA,* and *mefA*.

Table 4 | Presence of tetracycline resistance genes in 31 strains isolated from the selective medium with tetracycline

	Resistance genes (number of strains)					
	tetA		*tetD*		*tetM*	
Species (number of strains H1/H2)	H1	H2	H1	H2	H1	H2
Acinetobacter spp. (1/0)	1	–	1	–	1	–
Aeromonas spp. (1/2)	1	1	1	2	1	2
Enterobacter spp. (0/3)	–	1	–	3	–	1
Escherichia coli (3/5)	3	3	3	5	3	3
Klebsiella oxytoca (0/1)	–	–	–	1	–	1
Klebsiella pneumoniae (4/2)	4	2	4	2	4	1
Proteus mirabilis (1/0)	1	–	1	–	1	–
Pseudomonas aeruginosa (1/0)	1	–	1	–	1	–
Pseudomonas spp. (1/0)	1	–	–	–	1	–
Serratia spp. (1/0)	1	–	–	–	1	–
Unidentified (4/1)	3	–	2	1	4	1
Total (17/14)	16	7	13	14	17	9

'–' absence.

mainly $bla_{\text{CTX-M}}$. Moreover, they found quinolone resistance genes at all sites, except in the inflow sewage and aeration tank. In our study, the rates of $bla_{\text{CTX-M}}$ were low and bla_{TEM} was the most isolated gene. We did not find any quinolone resistance gene (*qnr, qnrA3*, or qnrB1). Haller *et al.* (2018) evaluated effluents from two large hospitals in Singapore and found several β-lactamase-encoding genes: bla_{SHV} (41.1%), bla_{NDM1} (35.6%), $bla_{\text{CTX-M}}$ (35.6%), and bla_{KPC} (28.8%) were prevalent. In our study, we found no strains with bla_{NDM1}. A pan-European survey analyzing ARGs in treated wastewater and in the receiving water bodies showed that the absolute abundance (ARGs/100 mL) of the analyzed genes could be ranked according to the following order: $intI1 > sul1 > tetM > bla_{\text{OXA-58}} > bla_{\text{TEM}} > bla_{\text{OXA-48}} > bla_{\text{CTX-M-32}} > mcr\text{-}1 >$

Table 5 | Presence of aminoglycoside resistance genes in 39 strains isolated from the selective medium with amikacin

	Resistance genes[a] (number of strains)									
	aacA		*aadA4*		*aph*		*strA*		*strB*[b]	
Species (number of strains H1/H2)	H1	H2	H1	H2	H1	H2	H1	H2	H1	H2
Acinetobacter baumannii (2/0)	1	–	2	–	1	–	1	–	2	–
Aeromonas hydrophila (0/1)	–	–	–	–	–	–	–	–	–	–
Aeromonas spp. (1/3)	1	1	–	–	–	–	–	1	1	1
Burkholderia cepacia (0/1)	–	–	–	–	–	–	–	–	–	–
Citrobacter spp. (1/0)	1	–	–	–	1	–	1	–	1	–
Enterobacter spp. (0/6)	–	2	–	2	–	–	–	2	–	–
Enterococcus faecalis (2/1)	1	–	–	1	1	–	1	–	2	–
Enterococcus faecium (1/0)	–	–	–	–	–	–	–	–	1	–
Proteus mirabilis (2/0)	–	–	–	–	–	–	–	–	1	–
Pseudomonas aeruginosa (3/2)	–	1	–	–	–	–	1	–	3	–
Pseudomonas alcaligenes (2/0)	–	–	1	–	–	–	1	–	1	–
Pseudomonas spp. (2/0)	1	–	1	–	1	–	1	–	2	–
Unidentified (5/4)	–	1	–	–	1	–	2	–	2	1
Total (21/18)	5	5	4	3	5	0	8	3	16	2

[a]'–' absence. We did not find *aacA1*, *aac(6')Im*, *aacC1*, *aphA*, and *aph2*.
[b]: statistical difference in gene detection between H1 and H2.

$bla_{\text{CTX-M-15}} > bla_{\text{KPC}}$ (Cacace *et al.* 2019). We did not find $bla_{\text{OXA-58}}$, $bla_{\text{OXA-48}}$, and *mcr-1* in our study, however, we found *tetM* in most strains.

We described ARGs in various species that were predominantly isolated from aquatic environments and soil or have been involved in opportunistic human infections. *Alcaligenes faecalis* is a sporadic and opportunistic cause of infection in immunocompromised patients and extensively drug-resistant *A. faecalis* infections emerged (Huang 2020). Ngbede *et al.* (2020) found mobile colistin resistance genes (*mcr-1.1*) in *A. faecalis* of animal origin in Nigeria and Al Laham *et al.* (2017) found strains with different bla_{VIM} in Gaza, Palestine. In our study, *A. faecalis* strains had only bla_{TEM}. *Ochrobactrum* spp., another traditionally environmental pathogen, can also cause human infection (Dharne *et al.* 2008). In Brazil, Furlan & Stehling (2017) showed a high level of resistance to β-lactam antibiotics and found different β-lactamase-encoding genes ($bla_{\text{CTX-M-gp1}}$, bla_{SHV}, $bla_{\text{OXA-1-like}}$, and bla_{KPC}) in *Ochrobactrum* spp. and *Achromobacter* spp. In our study, the *Ochrobactrum* spp. strain, when isolated from a selective medium with β-lactam antibiotics, had bla_{TEM}, which confirms the emergence of resistance genes in these species.

Tetracyclines are used in agriculture, fish farming, and in the treatment of infectious diseases in humans and animals. Tetracycline resistance genes were found in different bacterial species isolated from aquatic environments in different areas of the world (Szczepanowski *et al.* 2009; Xu *et al.* 2015; Tuo *et al.* 2018). In our study, we found *tetA*, *tetD*, and *tetM* in most strains. Tuo *et al.* (2018) showed the prevalence of *tetM* and *tetA* in strains isolated from the Funan River, China. Szczepanowski *et al.* (2009) also found these genes in WWTP in Germany. Xu *et al.* (2015) evaluated the distribution of antibiotics and ARGs in WWTP in Beijing, China, and of the nine tetracycline genes studied, three were related to the efflux pump (*tetA*, *tetB*, and *tetE*) and three to ribosomal protection (*tetM*, *tetZ*, and *tetW*). Their rates were high, which is in accordance with our findings for *tetA*, *tetD*, and *tetM*.

Various authors demonstrated high rates of macrolide genes in WWTP and hospital effluents (Szczepanowski *et al.* 2009; Rodriguez-Mozaz *et al.* 2015; Milaković *et al.* 2020). We also found three macrolide resistance genes (*mphA*, *ermB*, and *mel*) among the six studied. In Germany, Szczepanowski *et al.* (2009) found 15 macrolide resistance genes out of 20 – *mphA*, *mphB*, *mph*, *ermA*, *ermB*, *mefA*, and *mel* were the most prevalent genes in activated sludge from WWTP and *mphA*, *mphB*, *mph*, *ermB*, and *mel* in its final effluents. In Spain, the presence of *ermB* in hospital effluents and WWTP effluents and influents was associated with the presence of antibiotics, such as clarithromycin and azithromycin (Rodriguez-Mozaz *et al.* 2015). Similarly to our study, two different Chinese studies found high *ermB* concentrations in hospital wastewater

from three tertiary public hospitals (Wang *et al.* 2018) and various sites throughout the Funan River (Tuo *et al.* 2018). Macrolides are used to treat gram-positive human infections and their activity against *Enterobacterales* is poor (Gomes *et al.* 2017). However, the activity of azithromycin against most common diarrhoeagenic pathogens, such as *E. coli*, *Shigella* spp., and *Salmonella* spp., was excellent (Lübbert 2016; Gomes *et al.* 2017). Similarly to our study, Gomes *et al.* (2019) showed that *mph(A)* was the most frequent macrolide resistance gene in *E. coli* isolated from stool samples of children under five years of age in periurban areas in Lima, Peru. Moreover, in this study, *erm(A)*, *erm(B)*, and *mef(A)* were less frequent. In our study, we did not find *ermA* and *mefA*, but found *erm(B)* in more than 50% of strains (16/28).

Regarding aminoglycoside resistance genes, Tuo *et al.* (2018) showed that *aac(6′)-Ib-cr* and *aph(3′)-IIIa* were prevalent *among* 24 *mcr*-positive strains. Aminoglycosides were the most abundant genes in all ARG groups (24.57%) from an urban general hospital sewage in Shantou, China – *aph(6)-Id*, *aph(3″)-Ib*, and *aac(6′)-Ib7* were the prevalent genes (Cai *et al.* 2021). In our study, we found only five out of the 10 genes analyzed and *strA, strb,* and *aacA* were the most isolated. These genes circulate in aquatic and animal strains. Lu *et al.* (2022) showed that, among the streptomycin-resistant *E. coli* isolated from chickens, 99.76% had the *strA* aminoglycoside resistance gene and 63.27% had *strB*. Graves *et al.* (2011) compared the presence of ARGs in *E. coli* isolated from swine manure, lagoon effluent, and soil collected from lagoon waste and showed that the most frequent aminoglycoside resistance genes were ARGs – *aadA, strA,* and *strB* were prevalent.

Methicillin resistance in staphylococci occurs by the acquisition of staphylococcal cassette chromosome *mec* (SCC*mec*), which has *mecA* gene and its homologues, such as *mecC* (Lakhundi & Zhang 2018). Several studies have already found *mecA* or *mecC* in hospital effluents and aquatic environments. Basode *et al.* (2018) found *mecA* in *S. aureus* in three out of four samples collected from a municipal sewage, including those of an animal slaughterhouse and a fish market in Saudi Arabia. Silva *et al.* (2022) found *mecA* in 45 methicillin-resistant staphylococci (28 *S. aureus* and 17 coagulase-negative staphylococci from the Hospital Center Trás-os-Montes e Alto Douro, Portugal). Silva *et al.* (2021) found *mec*C in methicillin-resistant *Staphylococcus aureus* (MRSA) from surface water in Portugal. MRSA is a serious global threat and is related to hospital and community infections (Lakhundi & Zhang 2018). In our study, we did not study *mecC* and only two strains had *mecA* (both were *Staphylococcus haemolyticus*). Another studied already showed that this species can serve as a reservoir of resistance genes for other staphylococcci species (Rossi *et al.* 2016). The presence of this gene in the environment may favor its dissemination to other strains of the genus, especially *S. aureus*, considering that the amount of community-associated methicillin-resistant strains is increasing (Fritz *et al.* 2020).

Although some Brazilian laws and regulations about waste and wastewater management in hospital and health care units exist, no detailed study links the discharge of hospital raw sewage with the main urban sewage collection line. This could represent an open risk for the environmental dissemination of antibiotic resistance among the urban population and highlights the need for specific legislation about proper hospital sewage treatment and waste disposal.

CONCLUSION

This study showed that a diversity of traditionally environmental bacterial species and *Enterobacterales* has relevant ARGs. *Enterobacter* spp., *Proteus mirabilis, Klebsiella pneumoniae*, and *Escherichia coli* had various ARGs, including *β*-lactams (bla_{TEM} and bla_{KPC}), macrolides (*mphA, mel,* and *ermB*), tetracycline (*tetA*, *tetD*, and *tetM*), and aminoglycosides (*strB* and *strA*). Studies on the presence of antibiotic-resistant bacteria in hospital raw sewage systems are essential to assess and establish a specific legislation for their treatment.

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AUTHORS' CONTRIBUTIONS

M.P.B.B.: draft of the article, data collection and interpretation, and methodology; F.S.C.: data interpretation and methodology; S.T.A.C. and R.P.S.: supervision, data analysis and interpretation, project administration, revision and editing of the article. All authors contributed to the study conception and design.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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