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ORIGINAL ARTICLE

Multiresistance, beta-lactamase-encoding genes and bacterial diversity in hospital wastewater in Rio de Janeiro, Brazil

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Abstract

Aims: To investigate the bacterial diversity, antimicrobial resistance patterns and types of beta-lactamase genes in Gram-negative bacteria isolated from a hospital sewage treatment plant.
Methods and Results: Between July and December 2008, we collected samples from influent, clarifier tank effluent and chlorine contact tank effluent from a sewage treatment plant service of a hospital located in the city of Rio de Janeiro, Brazil. Of the 221 isolates identified, 40% were characterized as extended-spectrum beta-lactamase (ESBL) producers. Nonpathogenic micro-organisms and some pathogenic genera were quantified. The most common ESBL-producing isolates were *Klebsiella pneumoniae*, *Enterobacter cloacae* and *Escherichia coli*. The bla_{TEM}, bla_{SHV} and $bla_{CTX\text{-}M}$ genes were detected in 82, 48 and 67% of bacterial isolates, respectively.
Conclusions: Results showed that hospital wastewater treatment plant is not suitable systems for the removal of all antibiotic-resistant micro-organisms present in hospital wastewaters.
Significance and Impact of the Study: This study provides evidence that bacteria resistant to multiple antibiotics and their resistance genes that are usually present in the hospital can reach the environment, even after the use of hospital wastewater treatment plants.

Introduction

Antimicrobial drugs and antimicrobial-resistant bacteria are discharged in large quantities in the environment (Halling-Sorensen *et al.* 1998). Antimicrobial-resistant bacteria and antimicrobial-resistant genes have been detected in different environments, such as domestic sewage, hospital sewage and sewage-contaminated river waters (Tennstedt *et al.* 2003; Reinthaler *et al.* 2003; Costanzo *et al.* 2005; Arikan and Aygan 2009; Reinthaler *et al.* 2010; Galvin *et al.* 2010). However, it is important to note that despite the levels of human activity or water pollution, antimicrobial-resistant genes may also be found in non-native bacteria selected by low levels of antibiotics present in soil and water (Pruden *et al.* 2006; Storteboom *et al.* 2010).

Hospital waste and domestic sewage constitute a special category of waste that is highly hazardous because of its infectious and toxic characteristics (Tsakona *et al.* 2007). Healthcare centres, including hospitals, constantly generate wastewater and a consequent discharge of effluents that require appropriate treatment and destination. Hospital sewage releases a variety of multiresistant bacteria and substances such as antimicrobials, pharmaceuticals, disinfectants, anaesthetics, radioisotopes, heavy metals and drugs not metabolized by patients (Meirelles-Pereira *et al.* 2002; Emmanuel *et al.* 2005; Baquero *et al.* 2008).

Beta-lactam antibiotics are commonly used to treat hospitalized patients infected by Gram-negative bacteria. The production of beta-lactamases is the principal mechanism involved in the inactivation of these antibiotics. The extended-spectrum beta-lactamases (ESBLs) confer resistance to the penicillins, third-generation cephalosporins and aztreonam. The most abundant ESBL types are represented by SHV, TEM and CTX-M. (Paterson and Bonomo 2005).

In Brazil, few studies have compared the antimicrobial resistance patterns of bacteria in hospital sewage plants. In addition, the bacterial diversity in wastewater is largely unknown. In this study, we sought to determine the bacterial diversity and to determine the antimicrobial resistance profile and types of beta-lactamases (TEM, SHV and CTX-M) in Gram-negative bacteria isolated from a hospital sewage treatment plant in the city of Rio de Janeiro, Brazil.

Materials and methods

Wastewater sampling and characterization of the sewage treatment plant

We collect wastewater samples from a sewage treatment plant serving a hospital located in the metropolitan area of the city of Rio de Janeiro (RJ), Brazil. The wastewater comes from all units of the hospital, including laboratories, rehabilitation, dialysis, hospitalization and surgery units, clinics, maternity, laundry and the cafeteria.

The hospital sewage treatment plant uses an extended aeration-activated sludge process with a hydraulic retention time of 18 h, sludge age of 20 days, operating temperature of 18–25°C and mean influent flow of 5·0 l s^{-1}, followed by post-treatment (disinfection of final effluent by chlorination). An extended aeration-activated sludge plant uses a biological process by which nonsettleable substances occurring in dissolved and colloidal forms are converted into settleable sludge. The plant is divided into three parts: an aeration tank (continuous stirred tank reactor with sludge recycle), a clarifier tank and a chlorine contact tank. In the pretreatment stage, the plant has a screen for the removal of gross solids.

Sampling was performed eight times between July and December 2008. Three samples (1000 ml) were collected on each day from influent (raw sewage), a clarifier tank and a chlorine contact tank by submerging sterile bottles in the tanks. After collection, all samples were refrigerated and transported to the laboratory for immediate analysis.

Determination of environmental parameters

The physicochemical parameters pH, BOD_5 (biochemical oxygen demand over 5 days), chemical oxygen demand (COD) and nitrogen as nitrate ($N\text{-}NO_3$) and as ammonium ($N\text{-}NH_3$) were determined.

The pH was determined by pH meter (Digimed DM20; Digimed Instrumentação Analítica, Brazil). The COD test was performed by the closed reflux method followed by photometric determination, using a COD reactor (Hach Company, Loveland, CO, USA) and visible spectrophotometer (model DR-2500; Hach Company). BOD_5, nitrate and ammonium were determined using the potentiometric method with selective electrodes Orion 081010MD, Orion 9707BNWP and Orion 9512HPBNWP, respectively (Hach Company).

Total and faecal coliforms were analysed using the Colilert P/A Quanti tray 2000 kit (IDEXX Laboratories, Westbrook, ME, USA) according to the manufacturer's instructions; results are expressed as most probable number (MPN) in 100 ml^{-1}.

The methodologies used to assess the physicochemical parameters and total and faecal coliforms were consistent with the methods described in the *Standard Methods for Examination of Water and Wastewater* (APHA 1998).

Bacterial diversity determination by partial sequencing of 16S rDNA

Three samples (1000 ml) were collected on the same day from influent (raw sewage), a clarifier tank and a chlorine contact tank. Effluent samples were filtered onto a 47-mm-diameter, 0·22-μm nitrocellulose filter (Millipore, MA, USA) and stored at −80°C for later DNA extraction. DNA was extracted using the UltraClean Soil DNA kit (Mo Bio Laboratories, Carlsbad, CA, USA) according to the manufacturer's protocol. The metagenomic 16S rDNA was amplified by PCR using the primer pair PRBA63f (Marchesi *et al.* 1998) and UN518r (Øvreås *et al.* 1997) for the domain bacteria. Agarose gel electrophoresis of 150 μl of PCR product was performed prior to purification with the QIAquick gel extraction kit (Qiagen, Hilden, DE) according to the manufacturer's instructions. Purified amplicons were cloned using the pGEM-T Easy Vector (Promega, WI, USA) according to the manufacturer's protocol and then transformed into competent DH5-alpha *Escherichia coli* cells. Ampicillin- and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)-amended LB agar was used for the blue-white screening of transformants, which were subject to direct whole-cell PCR to amplify the plasmid insert. Each insert was sequenced using the BigDye terminator system and an ABI-3730 automatic capillary sequencer (Applied Biosystems, CA, USA).

The electropherogram sequencing files were processed using the Phred program (Ewing and Green 1988) for base calling and for trimming of vector and low-quality

(<20) sequences. The high-quality sequences located between the rRNA primers were used for further analysis.

The sequences were chimera-checked using the Mallard program (Ashelford *et al.* 2006), and putative chimeras were excluded from further analysis. Valid sequences were then aligned using ClustalX 1.81 (Thompson *et al.* 1997). The PHYLIP format output alignments were used to construct distance matrices within each library using DNADIST provided in the PHYLIP 3.6 package (Felsenstein 1993), with default parameters and using the Jukes–Cantor model option. The generated matrices were used as input files for DOTUR (Schloss and Handerlsman 2005) to calculate the species richness using Chao1 (Chao 1987) and ACE (Chao and Lee 1992) estimators, the rarefaction curves and the Shannon–Weaver diversity index (Shannon and Weaver 1949). The Good's coverage estimator was used to calculate the sample coverage using the formula $C = 1 - (n_i/N) \times 100$, where N = total number of sequences analysed and n_i = number of reads that occurred only once among the total number of reads analysed using $DOTUR_{0.03}$ (Good 1953; Good and Toulmin 1956).

The bacteria phyla composition was determined by taxonomic assignment using the RDP Classifier (Wang *et al.* 2007) with default parameters through the web service provided by RDP II (Cole *et al.* 2008). For tree construction, the nearest neighbours of the 16S rRNA were obtained using the ALIGN tool (DeSantis *et al.* 2006). All sequences were aligned with the ClustalW aligner (Thompson *et al.* 1994) of the mega 4.0 program (Tamura *et al.* 2007), using the neighbour-joining method, the Jukes–Cantor model (Jukes and Cantor 1969) option and a bootstrap value of 1000.

Identification of wastewater isolates

A volume of 100 μl from each sample was inoculated onto the culture media. To optimize the bacterial count, a decimal dilution series (10^{-1}, 10^{-2} and 10^{-3}) with saline solution (NaCl) was prepared, and 10 μl of the well-homogenized solutions was plated in the culture media. The plates were incubated at 37°C for 24 h after inoculation. The culture media used were Tergitol-7 (Oxoid, Basingstoke, UK) agar with 0·05% triphenyltetrazolium chloride, glutamate starch phenol red agar and eosin methylene blue agar (Oxoid).

Different colonies suggesting Gram-negative bacteria strains were identified based on the following biochemical tests: oxidase test, the production of acids from glucose, lactose or sucrose, sulfate/indole/motility agar and citrate agar. Further biochemical testing to identify the species followed the guidelines in the Manual of Clinical Microbiology (Murray *et al.* 2003).

Antimicrobial susceptibility testing

The antimicrobial susceptibility of bacterial isolates was determined using the agar diffusion method according to the Clinical and Laboratory Standards Institute (CLSI 2009). The following antimicrobial discs (Oxoid) were used: amikacin (30 μg), cefalothin (30 μg), trimethoprim/sulphametoxazole (25 μg), piperacillin/tazobactam (110 μg), meropenem (10 μg), ciprofloxacin (5 μg), cefoxitin (30 μg), imipenem (10 μg), cefotaxime (30 μg), cefepime (30 μg) and ceftazidime (30 μg). A Muller-Hinton plate was swabbed with TSB (Tryptic Soy Broth) inoculated with each isolate with the turbidity of 0·5 McFarland standard. The antimicrobial agent discs were placed on the inoculated plates and were then incubated at 35°C for 18–24 h. Inhibition zone diameters (in millimetres) were measured using a ruler. The break points used to categorize isolates as resistant or susceptible for each antimicrobial agent followed the CLSI guidelines. For quality control, the ATCC standard strains *E. coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27953) and *Staphylococcus aureus* (ATCC 25923) were used.

Isolates were screened for the ESBL-producing phenotype by the standard double-disc synergy test, as described previously (Jarlier *et al.* 1988). The turbidity of the suspensions used for sensitivity testing was adjusted to 0·5 McFarland standards, and the suspensions were inoculated onto Mueller-Hinton agar medium. The following antimicrobial discs (Oxoid) were used: amoxycillin/clavulanate (30/10 μg), cefotaxime (30 μg), cefepime (30 μg), ceftazidime (30 μg) and aztrenam (30 μg). ESBL production was considered positive when an enhanced zone of inhibition was visible between the beta-lactam- and beta-lactamase inhibitor-containing discs. For quality control, the standard *Klebsiella pneumoniae* strain (ATCC 700603) was used.

Characterization of beta-lactamase-encoding genes

Genetic detection was performed using PCR with bacterial DNA, which was extracted from the isolates by boiling the bacterial suspensions. A solution with 1 μl of extracted DNA was used as a template for PCR analysis. Resistance genes were screened using *Taq* polymerase under previously reported conditions for the detection of bla_{TEM} (Hasman *et al.* 2005), bla_{SHV} (Hasman *et al.* 2005) and $bla_{CTX\text{-}M}$ (Mulvey *et al.* 2003). Beta-lactamases were detected using primers TEM-F (5′GCGGAACCCCTATT TG 3′), TEM-R (5′ACCAATGCTTAATCAGTGAG 3′), SHV-F (5′-TTATCTCCCTGTTAGCCACC-3′), SHV-R (5′-GATTTGCTGATTTCGCTCGG-3‘), CTX-M-F (5′-AT GTGCAGYACCAGTAARGTKATGGC-3′) and CTX-M-R (5′-TGGGTRAARTARGTSACCAGAAYCAGCGG-3′). Gel

electrophoresis was used for the analysis, and the PCR products were visualized using 1·5% agarose gel and ethidium bromide staining.

Statistical analysis

Data entry and analyses were perfomed using the Epi Info software ver. 3.5.1. (Centres for Disease Control and Prevention, Atlanta, GA, USA). Comparisons of the ratios of antimicrobial resistance between influent, clarifier tank effluent and chlorine contact tank effluent were made using the chi-square or Fisher's exact test as appropriate. Differences were considered significant when $P < 0{\cdot}05$.

Results

Environmental parameters

Table 1 displays the values of pH, BOD_5, COD, nitrate and ammonium ($N\text{-}NH_3$) in the water from the sewage treatment plant. In addition, concentrations of total and faecal coliforms are shown.

Bacterial diversity

A total of 646 16S rRNA sequences were obtained, 334 from the influent and 312 from treated effluent. The 16S rRNA sequences were analysed by constructing species accumulation curves and calculating diversity indices. Different groups belonging to the domain Bacteria (*Proteobacteria, Firmicutes* and *Spirochates*) were identified. Taxonomic similarities and phylogenetic relationships of common bacteria identified in both influent and treated effluent showed that most of the bacteria belonged to the *Proteobacteria*. A main phylogenetic tree, constructed containing the overall metagenomic clone sequences obtained (not shown), indicated that 38 clones retrieved from the treated effluent may belong to pathogenic bacteria. A smaller tree containing only these sequences was constructed. Clones that matched sequences belonging to potential pathogenic bacteria such as *Acinetobacter, Aeromonas, Shigella, Klebsiella* and *Stenotrophomonas* are shown in the phylogram (Fig. 1) with their nearest neighbours.

Antimicrobial susceptibility

A total of 221 Gram-negative bacteria isolates were isolated and identified using established biochemical procedures (Table 2), and the antimicrobial resistance patterns of these are shown in Table 3. Among these 221 isolates, we observed single, double and multiple resistance phenotypes. The prevalence of resistance to antimicrobial agents ranged from 0 to 83% in wastewater isolates, with most of the strains susceptible to imipenem and meropenem. The micro-organisms isolated showed higher antimicrobial resistance rates to amikacin and trimethoprim/sulphametoxazole among the non-beta-lactam antibiotics. Among antibiotics of the beta-lactam group, the highest resistance rate was found for cefalothin. Considering the presence of antimicrobial resistance in the different samples (influent, clarifier tank effluent and chlorine

Table 1 Environmental parameters obtained from the hospital wastewater

	Environmental parameters						
Wastewater samples	BOD_5 (mg l^{-1})	COD (mg l^{-1})	Ammonium (mg l^{-1})	Nitrate (mg l^{-1})	pH	Total coliforms (MPN 100 ml^{-1})	Faecal coliforms (MPN 100 ml^{-1})
Influent							
Minimum	88·0	210·0	8·03	0·43	6·5	$0{\cdot}1 \times 10^4$	$0{\cdot}1 \times 10^4$
Maximum	146·0	718·0	13·79	3644·1	8·6	$1{\cdot}0 \times 10^6$	$3{\cdot}1 \times 10^5$
Mean	100·0	379·9	11·1	1198·0	7·5	$7{\cdot}4 \times 10^5$	$0{\cdot}8 \times 10^5$
SD	19·0	163·0	2·0	1200·1	0·8	$3{\cdot}5 \times 10^5$	$1{\cdot}0 \times 10^5$
Clarifier tank effluent							
Minimum	11·0	44·0	1·95	9·57	6·0	$0{\cdot}4 \times 10^4$	$0{\cdot}1 \times 10^4$
Maximum	31·0	91·0	20·53	1161·42	7·6	$7{\cdot}6 \times 10^5$	$0{\cdot}5 \times 10^5$
Mean	19·4	64·7	6·0	220·6	6·6	$2{\cdot}0 \times 10^5$	$0{\cdot}2 \times 10^5$
SD	5·9	16·7	5·9	384·9	0·5	$2{\cdot}4 \times 10^5$	$0{\cdot}2 \times 10^5$
Chlorine contact tank effluent							
Minimum	3·0	38·0	0·25	16·86	6·1	1	1
Maximum	16·0	128·0	17·90	399·84	7·6	$1{\cdot}0 \times 10^5$	$0{\cdot}4 \times 10^5$
Mean	10·7	73·2	4·4	104·4	6·7	$0{\cdot}3 \times 10^5$	$0{\cdot}07 \times 10^5$
SD	6·2	27·4	5·7	128·1	0·5	$0{\cdot}4 \times 10^5$	$0{\cdot}1 \times 10^5$

MPN, most probable number; BOD_5, biochemical oxygen demand over 5 days; COD, chemical oxygen demand; SD, standard deviation.

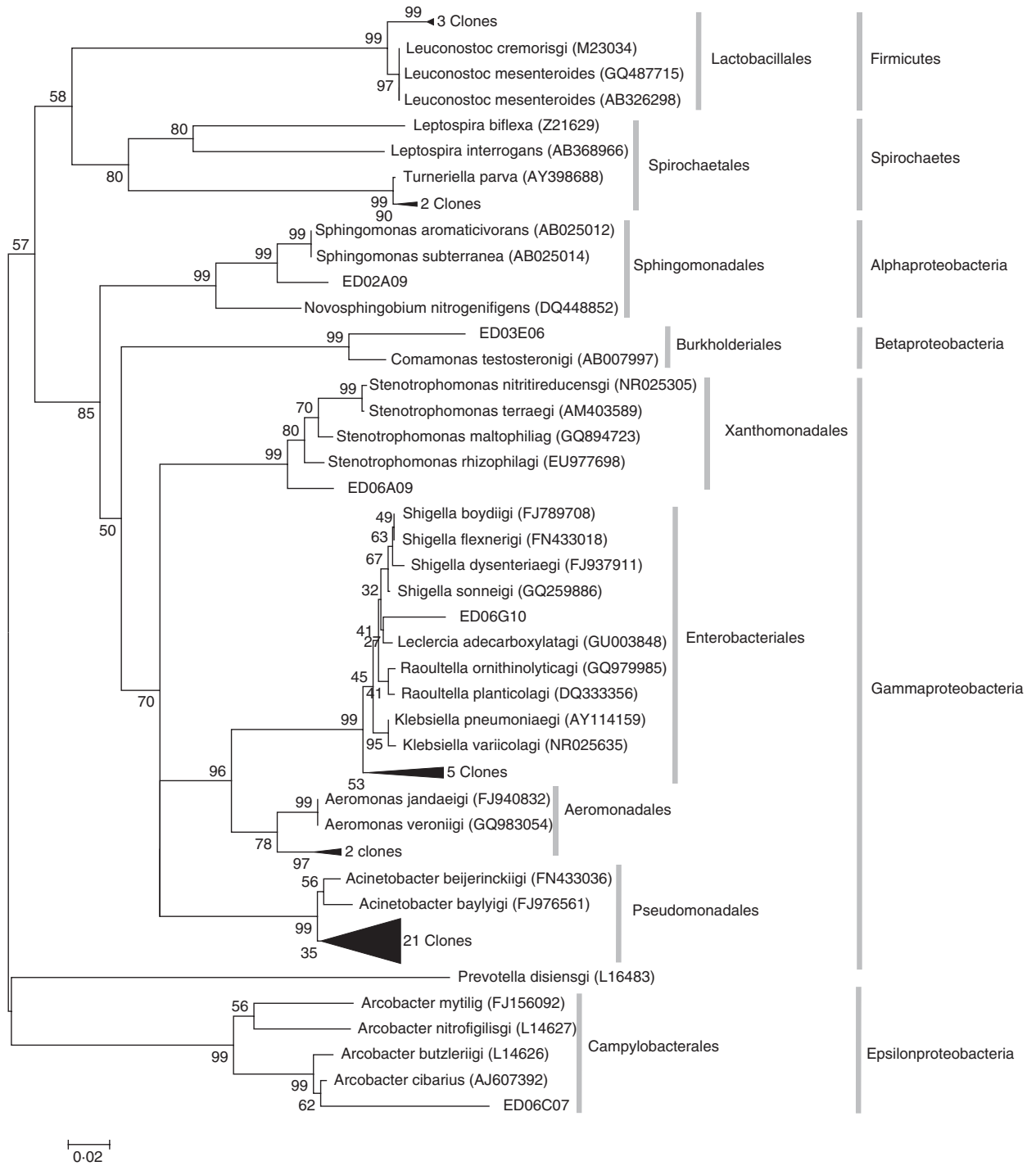

Figure 1 Phylogram of the bacterial 16S rRNA clones obtained from hospital wastewater. The clone sequences and nearest neighbours obtained were used. The phylogram was calculated with MEGA 4.0 using the neighbour-joining method and Jukes-Cantor model. Numbers at the branches show bootstrap percentages after 1000 replications of bootstrap sampling.

contact tank effluent), the chlorine contact tank effluent showed a significantly higher antimicrobial resistance rates to amikacin ($P < 0{\cdot}05$) and ceftazidime ($P < 0{\cdot}05$).

Bacterial isolates were screened for the ESBL phenotype, and 44% of bacteria isolated were characterized as ESBL producers. Distributions of the ESBL-producing

Table 2 Gram-negative bacteria isolated and identified using established biochemical procedures, from the eight sampling dates

Bacteria	Influent (*n*)	Clarifier tank effluent (*n*)	Chlorine contact tank effluent (*n*)	Total
Aeromonas spp.	3	2	0	5
Citrobacter freundii	4	1	0	5
Chromobacterium violaceum	0	3	0	3
Enterobacter asburiae	0	0	3	3
Enterobacter cloacae	15	16	1	32
Enterobacter spp.	0	4	2	6
Escherichia coli	24	8	3	35
Escherichia hermannii	1	0	0	1
Klebsiella ornithinolytica	1	0	0	1
Klebsiella oxytoca	4	3	2	9
Klebsiella pneumoniae	41	31	18	90
Klebsiella terrigena	0	1	1	2
Pantoea agglomerans	10	10	6	26
Proteus mirabilis	1	0	0	1
Serratia marcescens	1	0	0	1
Serratia rubidaceae	1	0	0	1
Total	106	79	36	221

n, Number of isolates.

isolates from influent, clarifier tank effluent and chlorine contact tank effluent were 41/106 (39%), 36/79 (46%) and 20/36 (56%), respectively. The most common ESBL-producing isolates were *K. pneumoniae* and *Enterobacter cloacae* in wastewater samples; only one *Aeromonas* sp. isolate was characterized as ESBL producers (Table 4). All ESBL-producing isolates showed a multiresistance phenotype.

Molecular analysis

The bla_{TEM}, bla_{SHV} and $bla_{CTX\text{-}M}$ genes were detected in 82, 48 and 67% of ESBL-producing isolates, respectively. Seventy-three of the isolates had accumulated other beta-lactam resistance enzymes (Table 5). The association between types TEM and CTX-M was more frequent.

Discussion

We detected pathogenic and resistant micro-organisms in the wastewater treatment plant studied, indicating a potential risk for the microbiological pollution of water resources. This potential risk should be considered possible at similar plants. In Brazil, the hospital wastewater treatment process problem is more severe than in most countries; of 127 Brazilian hospitals, only three have wastewater treatment plants (Oliveira and von Sperling 2007). Previous study showed that few plants, such as extended aeration-activated sewage plant, not would be able to present reliable performances in terms of the compliance of effluent BOD_5, COD and coliforms to discharge (Vecchia *et al.* 2009).

In this study, concentrations of COD and BOD_5 found during the stages of sewage treatment processes were lower than the concentrations in previous studies (Rezaee *et al.* 2005; Emmanuel *et al.* 2005). The wastewater comes from the hospital's units, including restaurants, laundry and the cafeteria, seem to affect the concentrations of BOD_5 and COD. The values for nitrates were high for a municipal and hospital wastewater. Certain quaternary ammonium compounds used as antimicrobials and disinfectants are discharged in wastewater treatment plant, and these possibly affected the concentrations of nitrate and ammonium and the system's performance.

Our results for the microbiological characterization of the hospital wastewater indicated that these effluents had bacteria concentrations lower than (10^8 100 ml^{-1}) those generally present in the domestic sewage system. The low MPN detected for total and faecal bacteria for the hospital is probably due to the presence of disinfectants and antibiotics (Emmanuel *et al.* 2005). Results of previous studies of the hospital wastewater reported coliform concentrations in the order of 10^2 to 10^7 100 ml^{-1} (Gallert *et al.* 2005; Reinthaler *et al.* 2010; Galvin *et al.* 2010).

In designing this study, we sought to sample multiple samples over a number of months, for the specific purposes of identifying the predominant species and possible trends in terms of microbial resistance.

Table 3 Antimicrobial resistance among bacteria wastewater isolates

	Antimicrobials										
Wastewater samples (*n*)	KF (%)	AK (%)	SXT (%)	TZP (%)	MEM (%)	CIP (%)	FOX (%)	IPM (%)	CTX (%)	FEP (%)	CAZ (%)
Influent (106)	75 (71)	28 (26)	36 (34)	16 (15)	3 (3)	18 (17)	29 (27)	1 (1)	47 (44)	9 (8)	9 (8)
Clarifier tank effluent (79)	66 (83)	18 (23)	29 (37)	14 (18)	1 (1)	21 (26)	33 (42)	0 (0)	39 (49)	8 (10)	14 (18)
Chlorine contact tank effluent (36)	30 (83)	15 (41)	15 (41)	8 (22)	1 (3)	8 (22)	18 (50)	0 (0)	20 (55)	3 (8)	10 (28)
Total (221)	171 (77)	61 (28)	80 (36)	38 (17)	5 (2)	47 (21)	80 (36)	1 (0·4)	106 (48)	20 (9)	33 (15)

n, Number of isolates; AK, amikacin; KF, cefalotin; SXT, trimethoprim/sulphametoxazole; TZP, piperacillin/tazobactam; MEM, meropenem; CIP, ciprofloxacin; FOX, cefoxitin; IPM, imipenem; CTX, cefotaxime; FEP, cefepime; CAZ, ceftazidime.

Table 4 Bacterial isolates characterized as ESBL producers

Bacterial isolates ESBL positive	Influent (*n*)	Clarifier tank effluent (*n*)	Chlorine contact tank effluent (*n*)	Total
Aeromonas spp.	1	0	0	1
Enterobacter asburiae	0	0	2	2
Enterobacter cloacae	10	12	1	23
Enterobacter spp.	0	0	2	2
Escherichia coli	5	2	0	7
Klebsiella oxytoca	4	2	2	8
Klebsiella pneumoniae	17	13	8	38
Pantoea agglomerans	4	7	5	16
Total	41	36	20	97

n, Number of isolates; ESBL, extended-spectrum beta-lactamase.

Table 5 Frequency of beta-lactamases genes detected in isolates

Beta-lactamases genes	Number of isolates (%)
bla_{CTX-M}	7 (7)
bla_{CTX-M}; bla_{TEM}	35 (36)
bla_{CTX-M}; bla_{TEM}; bla_{SHV}	22 (23)
bla_{CTX-M}; bla_{SHV}	1 (1)
bla_{TEM}	8 (8)
bla_{TEM}; bla_{SHV}	15 (16)
bla_{SHV}	9 (9)
Total	97 (100)

The increasing use of PCR-based, culture-independent methods to study microbial communities is based on the premise that such methods will help identify the nature of the bacteria (99% of the bacterial community) that cannot be cultured on bacteriological media. Given that it would have been impractical to use all the different available culture media to identify the bacterial diversity present in the water, we decided to apply a molecular-based, culture-independent method. However, we believe it is possible that our culture-based assessment may have underestimated species diversity.

In this study, we confirmed the predominance of some of the species identified via culture and identified others that were either undetected or underestimated. A minority of the species detected by the culture were also detected by the cloning method, such as *Aeromonas* and *Klebsiella*, indicating the limitations of each method. The species *Citrobacter freundii*, *Enterobacter* spp, *Escherichia* spp, *Klebsiella ornithinolytica*, *Klebsiella oxytoca*, *Klebsiella terrigena*, *Pantoea agglomerans*, *Proteus mirabilis*, *Serratia marcescens* and *Serratia rubidaceae* were identified in the culture media but were not detected by molecular approach, whereas *Klebsiella variicola* was identified by the cloning technique but not by culture methods.

Most previous studies of the antibiotic resistance profile of pathogenic microbes have been directed towards clinical isolates, and there are very few such reports for environmental strains of bacteria. Among the antimicrobials evaluated, the carbapenems (imipenem and meropenem) are the most powerful antibiotics used in antimicrobial susceptibility testing. Carbapenems such as imipenem and meropenem are recommended as therapy for severe infections caused by ESBL-producing bacteria.

Resistant bacteria were also observed in the chlorine contact tank effluent. Murray *et al.* (1984) detected a significant increase in the percentage of strains resistant to two or three antibiotics when influent was chlorinated in the laboratory and a marginal increase when influent was compared with effluent that had been treated at the sewage treatment plant. Previous study indicated the possibility that chlorination might alter wastewater populations, with the selection of chlorine-resistant bacteria, which contribute to the selection of particular resistance genes (Macauley *et al.* 2006).

The occurrence of multiresistant and ESBL-producing bacteria in hospital effluents was described in this study. Prado *et al.* (2008) previously detected the presence of *K. pneumoniae* producing ESBL (20/43) in the effluent and sludge of hospital sewage plants from Rio de Janeiro, Brazil. Given the presence of ESBL-producing bacteria in these environments, a recurring concern is the transfer of conjugative plasmids, which also carry genes of resistance to other antimicrobial agents, given the bacterial multiresistance patterns (Heuer *et al.* 2002; Paterson 2006).

The incidence of ESBL-producing bacteria is increasing globally. ESBLs have been found in 30–60% of *Klebsiella* from intensive care units in Brazil, Colombia and Venezuela. However, in recent years, there has been a wide variety of reports of true community-acquired infections with ESBL-producing organisms (Mesa *et al.* 2006; Sasaki *et al.* 2010). The prevalence of ESBL varies according to different regions and different hospitals; however, *K. pneumoniae* and *E. coli* are commonly found related to these enzymes (Freitas *et al.* 2003).

Among the beta-lactamase-encoding genes studied, bla_{TEM} was characterized in the majority of ESBL-producing isolates and was associated with TEM-type ESBL. This enzyme group and SHV beta-lactamases have long been known as an ESBL-producing group of beta-lactamases (Oliveira *et al.* 2009). However, CTX-M beta-lactamases have only recently increased in significance (Eisner *et al.* 2006). Many of the isolates harboured other beta-lactam resistance enzymes, and the association between types TEM and CTX-M was more frequent. Previous studies have shown that ESBL-mediating plasmids may carry more than one beta-lactamase gene and that they may be responsible for high-level beta-lactamase resistance

phenotypes (Kiratisin *et al.* 2008). The beta-lactamase genes in hospital wastewater micro-organisms could have opportunities for environmental dissemination and possibly human exposure and transmission, although direct links have not been shown.

In conclusion, in this study, we showed that despite the use of a hospital wastewater treatment plant, antibiotic-resistant micro-organisms may still be found at the end of the water purification process. Also, it should be remembered that many hospitals in developing countries have no wastewater treatment facilities. Urgent measures are needed to minimize the effects from the release of hospital wastewaters into water resources.

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