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Antibiotic resistance pattern and prevalence of *qacEΔ1* and *sul1* genes in *Pseudomonas aeruginosa* from hospital wastewater

Perfil de resistência aos antibióticos e prevalência dos genes *qacEΔ1* e *sul1* em *Pseudomonas aeruginosa* de efluente hospitalar

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ABSTRACT

Introduction: Hospital effluents may pose great environmental risk due to the presence of pathogenic microorganisms, drugs and chemical components. *Pseudomonas aeruginosa* is an opportunistic pathogen frequently found in hospital environments. **Objective:** To evaluate the resistome of *P. aeruginosa* from the hospital wastewater treatment plant (HWTP) in a hospital complex of Rio de Janeiro city. **Method:** Twenty isolates from the five stages of the HWTP were identified as *P. aeruginosa* by 16S rRNA gene sequencing analysis. Susceptibility to antibiotics was determined according to CLSI and *qacEΔ1* and *sul1* genes were detected by PCR. Sulphonamide residues were investigated by high performance liquid chromatography coupled to sequential mass spectrometry. **Results:** Sulfamethoxazole was observed at a level below 50 ng L-1. Sulfonamide resistance (80%) was followed by quinolones (50%) and 13 susceptibility patterns to antimicrobials. The *qacEΔ1-sul1* genes were detected in 100% of isolates suggesting the presence of class 1 integrons in the whole HWTP. **Conclusions:** The results indicated limitations of the HWTP and propagation of resistance genes in all stages of the HWTP. These data also contribute to the environmental sanitary surveillance in the design of prevention actions against negative impacts on public health.

KEYWORDS: Hospital Sewage; *Pseudomonas aeruginosa*; Multidrug-Resistant Bacteria; Class 1 Integrons

RESUMO

Introdução: Efluentes hospitalares representam riscos à saúde pública e ambiental devido à presença de microrganismos patogênicos, drogas e produtos químicos. *Pseudomonas aeruginosa* é um patógeno oportunista frequentemente encontrado no ambiente hospitalar. **Objetivo:** Avaliar o resistoma de isolados de *P. aeruginosa* da estação de tratamento de esgoto hospitalar (ETEH) de um complexo hospitalar na cidade do Rio de Janeiro. **Método:** Vinte isolados dos cinco estágios da ETEH foram identificados como *P. aeruginosa* pelo sequenciamento do gene 16S rRNA. A suscetibilidade aos antibióticos foi determinada segundo o CLSI e os genes *qacEΔ1* e *sul1* foram detectados pela PCR. Resíduos de sulfonamidas foram pesquisados por cromatografia líquida de alta eficiência acoplada à espectrometria de massas sequencial. **Resultados:** Foi demonstrada a presença de sulfametoxazol em nível inferior a 50 $ng \cdot L^{-1}$, resistência às sulfonamidas (80%) seguida pelas quinolonas (50%) e 13 perfis de suscetibilidade aos antimicrobianos. Os genes *qacEΔ1-sul1* foram detectados em 100% dos isolados, sugerindo a presença de integrons de classe 1 em toda a ETEH. **Conclusões:** Os resultados sinalizaram limitações no tratamento e a propagação de genes de resistência nas etapas da ETEH. Esses dados contribuem com órgãos competentes no desenho de ações preventivas frente aos impactos negativos à saúde pública.

PALAVRAS-CHAVE: Efluente Hospitalar; *Pseudomonas aeruginosa*; Bactérias Multirresistentes; Integron de Classe 1

INTRODUCTION

The development and spread of antibiotic resistance among bacteria threatens human and environmental health. Many studies have demonstrated the importance of environmental elements like water and soil in the cycle of resistance to antibiotics in nature[1,2].

Hospital wastewater sent to hospital wastewater treatment plants (HWTP) contains high concentrations of bacteria, nutrients, oxygen, chemicals, heavy metals, antimicrobial agents and other non-metabolized drugs[3]. This environment increases selective pressure and thereby the horizontal transfer and spread of resistance genes and resistant organisms to the outer environment[4,5,6].

Among the bacteria found in hospital wastewater, *Pseudomonas aeruginosa* stands out as an opportunistic pathogen usually involved in hospital infections[7]. It is known not only for its versatile metabolism, but also for its extraordinary ability to adapt and colonize a wide variety of environments (waters, soil, rhizosphere and animals). Furthermore, its natural (intrinsic) and acquired resistance to a wide range of antimicrobial agents results in the emergence of multidrug resistant strains[8,9]. This multidrug resistance has been extensively described and involves different mechanisms like efflux pumps, low membrane permeability, antimicrobial agent target change, modifications in outer membrane proteins, production of ß-lactamases and other enzymes[6,10].

In Brazil, some studies about antimicrobial susceptibility patterns of bacterial isolates of hospital wastewater found multidrug resistant strains in all stages of the system and even after the treatment[1,6,11,12]. In addition, another study revealed extended spectrum ß-lactamase producer strains, *Klebsiella pneumoniae* carbapenemases (KPC) and metallo-ß-lactamases (MBL)[13]. Miranda et al.[11] detected *bla*SHV, *bla*CTX-M-1, *bla*CTX-M-2, *bla*TEM, *bla*VIM, *bla*SPM and *bla*KPC genes in clinical isolates and in the HWTP of the same hospital. Gene *bla*SPM-1 is the most prevalent in Brazil. It was found in *P. aeruginosa* isolates obtained from environmental samples collected in hospital wastewater and in the surface water of the river where it is discharged[14].

Problems related to low microbial antibiotic susceptibility dates back to a long time ago. Sulfonamide resistance appeared shortly after its introduction in clinical practice (1930) and may have resulted from mutations at the target site or by the acquisition of antisynthetase resistance genes (*sul*)[15,16]. This resistance is primarily mediated by the *sul1*, *sul2* and *sul3* genes, which encode *dihydropteroate synthetase* (DHPS) and have little affinity with sulfonamides[17,18]. Several bacterial species have these genes, which are located in transposons or in auto-transferable or mobilizable plasmids with a wide range of hosts; they have multiple antibiotic resistance, which is cosselected with sulphonamides[19,20].

The *sul1* gene is part of the conserved 3' segment of the class 1 integron, while the *sul2* gene appears associated with streptomycin resistance genes. In some studies, 70% of sulfonamide resistance could be conferred to these genes[21]. Subsequently, the spread of *sul 3*, detected in a strain of *Escherichia coli* in Switzerland, seemed to be related with transposons[22]. The integron/cassette system is considered one of the best examples of capture and expression of new genes[23,24] and is important to antibiotic resistance and biocides research[25,26].

Bacterial antibiotic resistance is a global problem that has been increasing progressively with the indiscriminate and growing use of antimicrobial substances. On the other hand, resistance to biocides is an emerging issue, as these substances are widely used in decontamination, disinfection and sterilization to control the spread of microorganisms[27,28].

Among these substances, quaternary ammonium compounds (QAC) are routinely used as antiseptics and disinfectants in domestic, veterinary, industrial and hospital environments[29,30]. These compounds are biodegradable under aerobic conditions and, thus, their concentrations in the various environments may oscillate continuously. In sewage, surface water, sediments and treatment plant effluents, they are usually found in subinhibitory concentrations. This fact makes these environments selective and causes the emergence and spread of microorganisms with decreased susceptibility to these compounds among different microbial genera, including pathogens of medical interest[31].

Among the QAC resistance mechanisms, the expression of efflux pumps by *P. aeruginosa* may include multidrug efflux systems, including the QacE and QacEΔ1 proteins. The *qacEΔ1* gene is located in the conserved 3' segment of the class I integron, initially described as a variant of the *qacE* gene[32].

To date, several *qac* genes have been described, like *qacA*, *qacB*, *smr* (former *qacC* and *qacD*), *qacE*, *qacF*, *qacG*, *qacH*, *qacJ* and *qacZ*. In Gram-negative bacteria, like enterobacteria and *P. aeruginosa*, the *qacE* gene (including the attenuated variant *qacEΔ1*) is widely spread. These genes can also be found in other species, like *Aeromonas* spp., *Vibrio* spp. and *Acinetobacter* spp[33]. This is due to the high prevalence of class I integrons, which in Gram-negative bacteria usually include the *qacEΔ1* gene[34]. Thus, there is the concern that the exposure of microorganisms to quaternary ammonium compounds may select isolates resistant to several antimicrobial agents and favor the emergence of multiresistant strains[27,35]. Recent papers describe an increase in resistance levels associated with *qac* genes and a decreased efficacy of compounds like benzalkonium chloride and chlorhexidine[36,37]. In addition, the *qacEΔ1* and *qacE* genes have been detected in surface water[38] and in environments contaminated with QAC[39].

This study aimed to evaluate the antibiotic resistance patterns in *P. aeruginosa* isolated from a HWTP and to investigate the presence of genes that generate resistance to quaternary ammonium

compounds and sulfonamides (*qacE*, *qacEΔ1* and *qacEΔ1-sul1*) associated with the class I integron gene cassette.

METHODS

Study site and sample collection

The study was carried out at the HWTP that serves a hospital complex located in Barra da Tijuca, city of Rio de Janeiro, state of Rio de Janeiro, Brazil (22°59'42.36''S, 43°21'49.62''O). This complex is formed by two health units with 322 beds and 30,000 monthly medical appointments. The treatment plant of this complex has the capacity to treat 220 m^3 of sewage per day. The collection of samples (500 ml) was performed in December 2010 from five points of every step of the wastewater treatment (Figure 1). Samples were collected in sterile polyethylene vials, refrigerated at 4° C and taken to the laboratory.

Dosages of physical and chemical parameters

The physical and chemical parameters of temperature, pH, conductivity, dissolved oxygen (DO), turbidity, salinity and chlorine of the samples were analyzed using the *Water Quality Checker* U-10 portable equipment (Horiba) and a chlorine measuring device (Homis), respectively.

Detection of antimicrobials through chromatography

The sulfonamide extraction methodology was based on the official method of the United States Environmental Protection Agency (US EPA) - Method 1694[40] and on the method described by Monteiro et al.[41] An aliquot of 25 mL of the filtered sample was acidified with concentrated HCl to adjust the pH to 2.5. Next, 25 mg of Na_2EDTA were added and then we proceeded to the solid phase extraction step, when we used Oasis HLB 500 mg/6 mL cartridges (Waters, Milford, MA) previously conditioned with 5 mL of methanol, 5 mL of ultrapure water and 5 mL of water acidified with concentrated HCl to pH 2.5. After washing it with two portions of 2.5 mL of ultrapure water to eliminate interferents, we applied vacuum for 2 minutes to dry the cartridge at a pressure of approximately 35 kPa and then the analytes were eluted with two portions of 3 mL of methanol and then with two portions of 2 mL of a methanol: acetone solution (1:1, v/v). Five mL of the eluate were separated for sulfonamide analysis and evaporated to dryness with N_2 at a maximum temperature of 47.5° C. The dried extract contained in the centrifuge tube was reconstituted with 1 mL of the reconstitution solvent (80:20 [v/v] of 0.1% v/v of formic acid in water and methanol). Then, we shook it in vortex, filtered it with a 0.2 µm glass fiber/nylon filter and transferred it to vial for injection in the system of High Performance Liquid Chromatography Coupled to Sequential Mass Spectrometry (LC-MS/MS)[41]. The LC-MS/MS system is composed of a *Shimadzu Prominence* high performance liquid chromatograph (with the following modules: LC-20AD quaternary pump, DGU-20A5 membrane degasser, SIL-20AC auto-sampler, CTO-20AC column oven and a CBM-20A controller) and an API5000 Applied Biosystems/MDS Sciex detector with Turbo V® source and TurbolonSpray® probe, controlled by the software Analyst version 1.4.2 from the same manufacturer. The antimicrobials were separated at 25° C on C18 column (Pursuit™ RS, 2 mm x 100 mm x 3 µm - Agilent) with guard column of the same type (2 mm x 3 µm). The mobile phases used in the gradient elution program were 0.1% v/v of formic acid in water (mobile phase A), 0.1% v/v of formic acid in acetonitrile (mobile phase B) and 0.1% v/v formic acid in methanol (mobile phase C). The gradient elution program and the mass spectrometer parameters are described in Monteiro et al.[41]

Isolation and biochemical identification of P. aeruginosa

After homogenization, aliquots of 2 mL of every HWTP point were inoculated in 5 mL of nutrient broth and incubated for 24 hours at 37° C. The aliquot of the first HWTP stage was also inoculated in Letheen liquid culture medium (DIFCO™) and incubated for 72 hours at 37° C. Then, bacterial cultures were seeded by streaking in nutrient agar and cetrimide agar (DIFCO™) and incubated under the same conditions. Gram staining and standard biochemical tests were performed to identify the strains according to the Bergey's Manual[42]. The following biochemical tests were performed with the Gram-negative bacilli (GNB): Sulfide, Indole and Motility (SIM), catalase, oxidase, OF-glucose, xylose, mannitol, lactose, lysine, arginine, ornithine decarboxylase, gelatin and incubation at 42° C.

DNA extraction and molecular identification

The isolates, previously phenotypically identified, were certified through molecular methodology. Extraction of the genomic DNA was performed using the *Dnaeasy® Blood &*

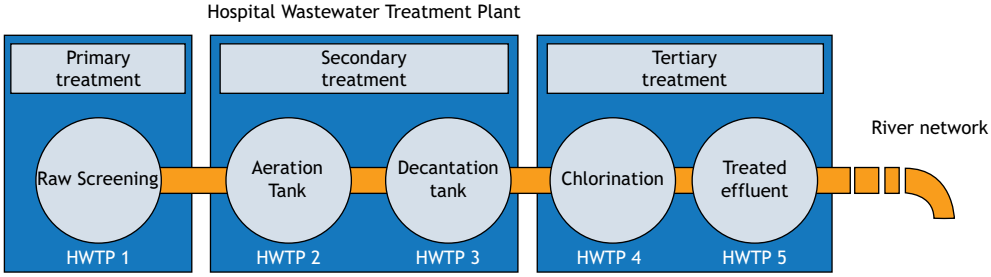


Figure 1. Scheme of the sewage treatment plant of the hospital complex (Lourenço Jorge Municipal Hospital and Leila Diniz Maternity Hospital).

Tissue (Qiagen GmbH, Hilden, Germany) kit according to the manufacturer instructions. Identification of isolates was confirmed by amplification of the 16S subunit encoding gene of *P. aeruginosa* specific rRNA. Reactions were performed with 50 µL of Polymerase Chain Reaction (PCR) mixture under the following conditions: 50 ng of template DNA, 50 pmol of each primer (PA-SS-F and PA-SS-R), as described by Spilker et al.[43], 0.2 mmol.l^{-1} of every deoxyribonucleotide triphosphate (dNTPs), 1X PCR buffer (pH 9.0), 2.5 mmol.l^{-1} of $MgCl_2$ and 2U of *Taq Platinum DNA Polymerase* (Invitrogen) (Table 1). *P. aeruginosa* INCQS 0024 (ATCC 29336) and *E. coli* INCQS 0033 (ATCC 25922) reference strains were used as PCR control. PCR products were analyzed through one hour of electrophoresis at 50 V on 1% agarose gel in 0.5X Tris-Borate-EDTA (TBE) buffer, that was stained with ethidium bromide (3 mg/mL). The 100 bp DNA *ladder* (Invitrogen) was used as the molecular weight standard. After electrophoresis, the gel image was visualized by ImageQuant 300 video documentation software (GE Healthcare). The specificity of primers and PCR were previously established with reference strains. The fragment was purified with the QIAquick PCR Purification kit (Qiagen) and sequenced with the *BigDye® Terminator Cycle Sequencing Standard Version* 3.1 kit (Applied Biosystems™) through capillary electrophoresis on the ABI 3730 DNA *Analyzer* (Applied Biosystems™), in the Platform of the Technology Development Program of Health Inputs of the Oswaldo Cruz Foundation (PDTIS/Fiocruz). Sequence similarity analysis was performed by the BLASTn software (http://www.ncbi.nlm.nih.gov/BLAST/) in GenBank.

Susceptibility to antibiotics

The antibiotic susceptibility pattern was determined through the disk-diffusion technique (Kirby-Bauer method) according to the criteria established by the *Clinical Laboratory Standards Institute* (CLSI)[44]. The turbidity of the suspensions used for sensitivity tests was adjusted in sterile saline solution (0.85% NaCl) to obtain the 0.5 turbidity standard on the McFarland scale. Then, these suspensions were inoculated in Mueller-Hinton agar medium (DIFCO). The isolates obtained were tested for resistance against 13 antimicrobials (CEFAR®). The antibiotics used were: Piperacillin/tazobactam (PPT-110 µg), ticarcillin/clavulanic acid (TIC-85 µg), ceftazidime (CAZ-30 µg), cefepime (CPM-30 µg), imipenem (IPM-10 µg), meropenem (MER-10 µg), aztreonam (ATM-30 µg), gentamicine (GEN-10 µg), tobramycin (TOB-10 µg), sulfonamide (SUL-300 µg), ciprofloxacin (CIP-5 µg), norfloxacin (NOR-10 µg) and polymyxin B (POL-300 UI). Quality control of antibiotic disks was performed using *P. aeruginosa* INCQS 00099 (ATCC 853), *Staphylococcus aureus* INCQS 00015 (ATCC 25923) and *E. coli* INCQS 00033 (ATCC 25922) reference strains. Three resistance patterns were established (Sensitive - S, Resistant - R and Multidrug resistant - MDR) for the isolates analyzed[45].

Detection of qacE, qacEΔ1 and qacEΔ1-sul1 genes

The PCR mixture achieved the final volume of 50 µL containing the following reagents: 50 ng template DNA, 50 pmol of every primer (Table 1), 0.2 mmol.l^{-1} of every dNTP, 1X PCR buffer (pH 9.0), 1.5 mmol.l^{-1} of $MgCl_2$ and 1U of *Taq Platinum DNA Polymerase* (Invitrogen). The programs used for every primer are described in Table 1. *P. aeruginosa* 531-95 [27] (Genbank GU182337) was used as positive control of the gene *qacE*Δ1. PCR products were analyzed through one hour of electrophoresis at 50 V on 1% agarose gel in 0.5X Tris-Borate-EDTA (TBE) buffer, that was stained with ethidium bromide (3 mg/mL). The 100 bp DNA ladder (Invitrogen) was used as the molecular weight standard. After electrophoresis, the gel image was visualized by ImageQuant 300 video documentation software (GE Healthcare).

Table 1. Primers and amplification programs used.

Target Gene	Primers	Sequence	Program		Fragment Size	Reference
16S rRNA *P. aeruginosa*	PA-SS_F PA-SS_R	5'-GGGGATCTTCGACCTCA-3' 5'-TCCTTAGAGTGCCCACCG-3'	95° C - 2' 94° C - 20'' 55° C - 1' 72° C - 1' 72° C - 7'	25X	956 bp	43
qacE	F1N R2B	5'-GCCCTACACAAATTGGGAGA-3' 5'-TACTACACCACTAACTATGA-3 '	94° C - 3' 94° C - 30'' 57° C - 90'' 72° C - 1' 72° C - 10 '	30X	319 bp	46 27
qacEΔ1	FQ1 FQ2	5'-CCCGAATTCATGAAAGGTGG-3 ' 5'-TATAAGCTTTCACCTGGCG-3'	94° C - 3' 94° C - 30'' 57° C - 90'' 72° C - 1' 72° C - 10'	35X	350 bp	35
qacEΔ1 and sul1	P1_*Qdelta1* *Sul1 R*	5'-TAGCGAGGGCTTTACTAAGC-3' 5'-GCCGATCGCGTAAGTTCCG-3'	94° C - 5' 94° C - 30'' 60° C - 1' 72° C - 2' 72° C - 7'	35X	800 bp	This study 47

bp: base pairs

Table 2. Physicochemical parameters

	Collection points of the Hospital Wastewater Treatment Plant					
Parameter	**Conama Resolution nº 430/2011**	**Point 1**	**Point 2**	**Point 3**	**Point 4**	**Point 5**
pH	5.0 to 9.0	7.6	7.9	8.2	8.4	8.4
Conductivity (mS/cm)	-	0.82	0.38	0.38	0.48	0.35
Turbidity (NTU[a])	< 100	10	99	6	4	7
Dissolved oxygen (mg/L)	-	4.5	9.2	9.3	9.2	3.7
Temperature (°C)	< 40	29	29	29	29	29
Salinity (%)	-	0	0	0	0	0
Chlorine (ppm)	-	1.0	1.0	1.0	< 10	0.01

[a] Nephelometric Turbidity Units; Conama: Brazilian Council for the Environment; ppm: parts per million.

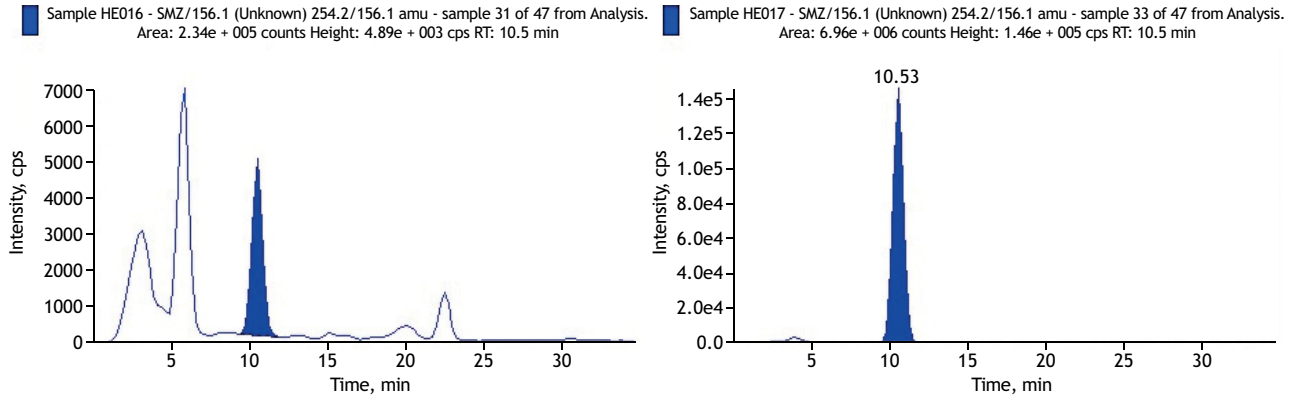


Figure 2a. Sulfamethoxazole detection in the HWTP influent (Sample HE016) and in the treated effluent (Sample HS017).

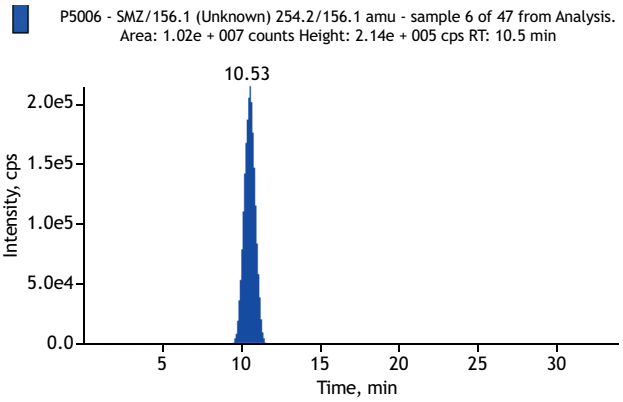


Figure 2b. Sulfamethoxazole detection in standard solution.

RESULTS

Dosages of the physical and chemical parameters

The pH of the samples varied between 7.6 and 8.4. The conductivity varied between 0.82 mS/cm and 0.35 mS/cm. The turbidity index presented a higher index at point 2 (99 NTU) because of the O_2 injection. The dissolved oxygen (DO) index started at 4.5 mg/L, then varied from 9.2 to 9.3 mg/L and reduced to 3.7 mg/L in the treated wastewater. The active chlorine concentration started at 1 ppm, and had a significant increase at the point where the chemical compound was added. The temperature and salinity level remained the same at all points (Table 2).

Detection of antimicrobials through chromatography

The antimicrobial sulfamethoxazole was identified in two samples, one from the HWTP influent water and the other from the treated effluent, both at concentrations lower than 50 $ng.L^{-1}$ (quantification limit of the method) (Figure 2a). Figure 2b shows the injection of a standard solution containing the sulfamethoxazole that was also injected into LC-MS/MS, for comparison purposes.

Isolation of P. aeruginosa

21 mobile GNB strains were isolated; H_2S, indole and lactose negatives; xylose variables; oxidative; mannitol, lysine, arginine, ornithine, gelatin, catalase and growth at 42° C positives. PCR of the *P. aeruginosa* strains resulted in the amplification of a 956 bp fragment in 95% (20/21) of the strains.

Antimicrobial susceptibility

Susceptibility to antibiotics presented higher percentages of resistance to sulfonamides and quinolones, followed by the other three classes analyzed (Figure 3). Analysis of antibiogram data showed 13 distinct resistance patterns among the isolates (Table 3). Forty-five percent had the MDR phenotype, 44% of which were from the chlorination stage of the treatment plant (Figure 4).

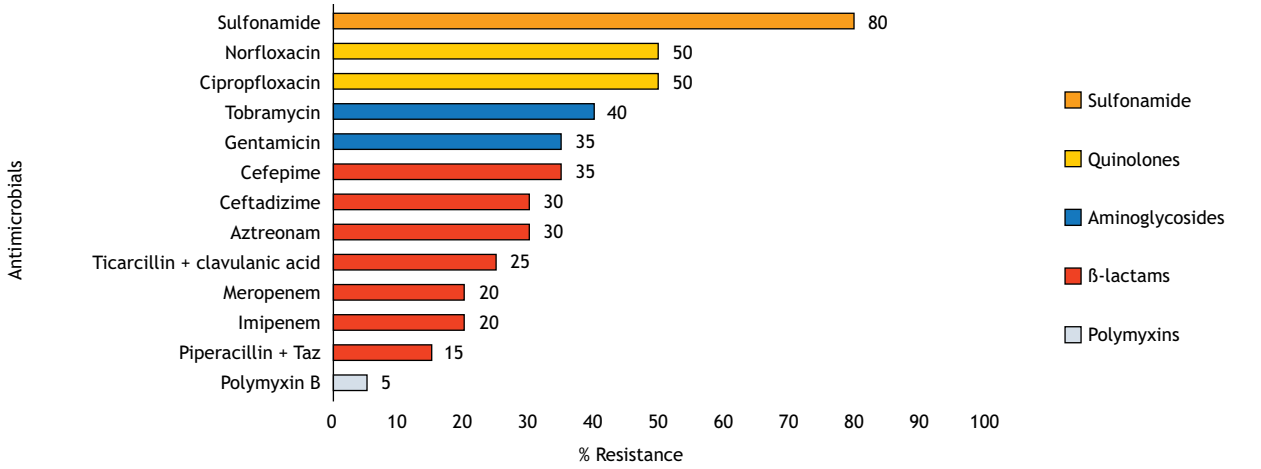
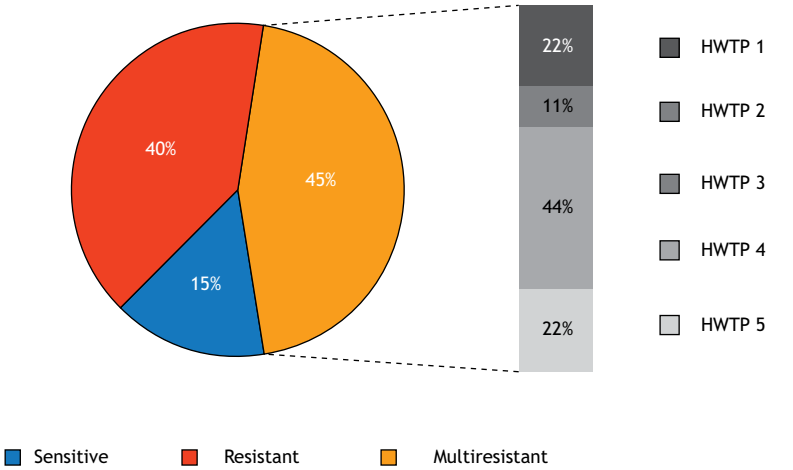


Figure 3. Susceptibility to antibiotic classes in *P. aeruginosa* strains.

Table 3. Antimicrobial resistance pattern of *P. aeruginosa* strains according to the collection point.

Pattern	Resistance	Number of isolates	Origin
I	SUSCEPTIBLE TO ALL	2	HWTP 2, 3, 5
II	SUL	6	HWTP 2, 5
III	TOB, CIP	1	HWTP 3
IV	CAZ, CPM	1	HWTP 5
V	GEN, NOR, SUL	1	HWTP 2
VI	TOB, CIP, NOR, SUL	1	HWTP 1
VII	TIC, ATM, CIP, NOR, SUL	1	HWTP 1
VIII	CPM, GEN, TOB, ATM, CIP, NOR, SUL	2	HWTP 5
IX	TIC, CAZ, CPM, ATM, CIP, NOR, SUL	1	HWTP 1
X	CAZ, GEN, TOB, IPM, MER, CIP, NOR, SUL	1	HWTP 4
XI	PPT, TIC, CAZ, CPM, GEN, TOB, IPM, MER, CIP, NOR, SUL	1	HWTP 4
XII	PPT, TIC, CAZ, CPM, GEN, TOB, ATM, IPM, MER, CIP, NOR, SUL	1	HWTP 4
XIII	PPT, TIC, CAZ, CPM, GEN, TOB, ATM, IPM, MER, CIP, NOR, SUL, POL	1	HWTP 4

PPT: Piperacillin + Tazobactam; TIC: Ticarcillin + clavulanic acid; CAZ: Ceftazidime; CPM: Cefepime; GEN: Gentamicin; TOB: Tobramycin; ATM: Aztreonam; IPM: Imipenem; MER: Meropenem; CIP: Ciprofloxacin; NOR: Norfloxacin; SUL: Sulfonamide; POL: Polymyxin. HWTP: Hospital wastewater treatment plant: HWTP 1: sewage intake; HWTP 2: aeration tank; HWTP 3: decantation tank; HWTP 4: chlorination; HWTP 5: treated effluent.



HWTP 1: sewage intake; HWTP 2: aeration tank; HWTP 3: decantation tank; HWTP 4: chlorination; HWTP 5: treated effluent.

Figure 4. Antimicrobial susceptibility phenotypes of *P. aeruginosa* isolates.

Detection of qacE, qacEΔ1 and qacEΔ1-sul1 genes

PCR was performed on the 20 *P. aeruginosa* strains to check for the presence of the *qacE, qacEΔ1 and qacEΔ1-sul1* genes. The *qacE* gene was not detected in any of the isolates. However, the *qacEΔ1* gene was found in 90% (18/20) of the analyzed isolates and presented a 400 bp fragment while the *qacEΔ1-sul1* genes amplified together presented a single fragment of approximately 800 bp in 100% (20/20) of the strains. The *P. aeruginosa* P5520 strain fragment, used as the PCR positive control, was sequenced and demonstrated 96% - 98% of similarity with the database sequences. This sequence was deposited in GenBank under access number MF801598.

DISCUSSION

Hospitals are considered ecological niches for bacteria resistant to antibiotics and play an important role in the emergence and spread of resistance. These bacteria leave hospitals through colonized patients and also through wastewater treatment systems[48].

In this study, strains of *P. aeruginosa* were isolated from all collection points of the hospital sewage treatment plant, including the final stage, in which the wastewater was already treated and able to be released to the environment. An intriguing fact was the absence of viable cells of *P. aeruginosa* in the first stage of the treatment plant (arrival of the hospital sewage). However, the occurrence of bacterial growth after cultivation in culture medium containing inhibitors of some active principles of antiseptics and disinfectants allowed us to verify that the microorganisms were under bacteriostatic action of these products and other antimicrobials. QACs are one of the most used surfactants in disinfection of hospital environments and when they are released in aquatic environments, they present antimicrobial action at relatively low concentrations[49,50]. In addition to bactericidal and/or bacteriostatic activity, these products may increase selective pressure and resistance to antibiotics and biocides[51].

Our results showed high diversity in the resistance patterns and high frequency of MDR strains in the different stages of the hospital sewage treatment. The highest amount of strains with MDR phenotype at the chlorination point is probably related to the increase in the transfer of resistance plasmids during the chlorination process, a critical part of the treatment. Since the 1970s, studies about the effect of chlorination on antibiotic resistant bacteria describe a considerable increase in bacteria resistance to multiple antibiotics in water and sewage[52,53,54]. In addition to presenting the highest number of MDR strains, at the point HWTP 4 an isolate resistant to all tested antibiotics was found, including to polymyxin B, that is considered highly effective against strains of *P. aeruginosa*, according to the Epidemiological Surveillance Program (SENTRY - 2001 to 2004)[55].

The presence of MDR strains in the stage that the wastewater had already been treated demonstrates a certain limitation of the treatment in eliminating these pathogens. This limitation may be associated with the size of the initial microbial population, the different development stages of the microorganism, the disinfectant concentration, the wastewater pH and the time of exposure to the disinfectant[56]. The evaluation of antibiotic susceptibility of *Pseudomonas* spp. in influents and effluents of a sewage treatment plant showed strains resistant to carbapenem only in the treated effluent[57].

Our results clearly demonstrated that the physicochemical conditions of the environment and the presence of antimicrobials probably affected the antibiotic susceptibility phenotypes of these bacteria. Furthermore, the fact that antibiotics at subinhibitory concentrations may have an impact on cellular functions and alter the expression of virulence factors or the transfer of antibiotic resistance genes is known[58].

On the other hand, the low susceptibility to sulfonamides (80%), followed by ciprofloxacin and norfloxacin (50%), deserves attention. Although we did not have information about the therapeutic prescriptions of these hospitals, the presence of a sulfonamide, sulfamethoxazole, both in raw and treated sewage, observed in our data suggests that the use of high concentrations of these antimicrobials may induce mutations in the DNA gyrase enzyme or in the overexpression of the efflux system and contribute to this resistance[17,59]. Sulfonamides can also strongly resist natural biodegradation as seen in the high frequency of their detection in aquatic environments. Researchers from the US Geological Service (USGS) detected sulfamethoxazole in rivers and streams with frequencies up to 27%[60]. In addition, their low tendency to partition in sediments allows them to be transported over long distances in running water. Perez et al.[61] evaluated the degradability of sulfamethazine, sulfamethoxazole and sulfathiazole in surface water samples. After more than a month, the sulfonamides were not degraded by surface water microorganisms in a batch reactor.

Considering the composition of the hospital sewage and the low susceptibility to sulfonamides in our isolates (60%), we decided to investigate the presence of *sul1*, *qacE* and *qacEΔ1* genes, which generate sulfonamide and QAC resistance, respectively. We then verified that the detection of the *sul1* gene did not match the results showed by the phenotype, in which 80% of the isolates presented resistance to sulfonamide and 100% of them had the *sul1* gene. This resistance percentage may have occurred due to the absence of expression of the *sul1* gene. Grape et al.[62] showed that among 105 clinical isolates of the *Enterobacteriaceae* family, 64 were resistant to sulfonamides. The *sul1* gene was found in 14 isolates, the *sul2* gene in 23 isolates, the *sul1* and *sul2* genes were simultaneously found in 25 isolates and *sul3* was found only in two isolates.

The distribution of the *qacE* and *qacEΔ1* genes was evaluated in Gram-negative isolates of clinical and environmental origin by Kazama et al.[46] The percentage of positive strains for these two genes in clinical isolates of *P. aeruginosa* (n = 63) was 65% for *qacEΔ1* and 24% for *qacE*. In environmental strains of *P. aeruginosa* (n = 5) the distribution was 40% for *qacEΔ1* and none for *qacE*. Another study also detected *qacEΔ1* in 48% of the clinical isolates of *P. aeruginosa* (n = 60), 88% of which

found in MDR strains and 35% in non-multiresistant strains. This finding reinforces the relation of this gene with antimicrobial multidrug resistance[27].

The present study found *qacEΔ1-sul1* in 100% of the hospital sewage isolates (45% MDR, 40% R and 15% S), but no *qacE* gene was detected. This absence is probably due to the insertion of the segment containing the sulfonamide resistance gene (*sul1*) near the 3' region of *qac3*, which becomes *qacEΔ1*[32].

The *qacEΔ1 and sul1* genes are often associated with class 1 integrons[63,64,65]. The association of these genes with class 1 integrons was detected in 97% of the isolates of bacteria of the genus *Salmonella* in Portugal[66]. Assuming that the presence of these genes strongly suggests the presence of a class 1 integron, we can conclude that these elements are widely distributed in the different stages of this hospital sewage treatment plant system.

QAC-based compounds are often found in cationic biocide formulations. Therefore, these products may play an important but indirect role in the selection of bacteria resistant to antibiotics[67]. QAC resistance genes, mainly related to efflux pumps, are widely found in human and animal healthcare facilities. Specific efflux or multidrug pumps are important determinants of intrinsic and/or acquired resistance to antimicrobials. Some of these elements generate resistance to both antibiotics and biocides. This fact, not yet well understood, can lead to the selection of antibiotic-resistant organisms via biocide selection[31,68].

Exposure to antibiotics, biocides or heavy metals and many other environmental factors increases the amount of cells containing integrons[39,69,70]. Additionally, exposure to different antibiotics (aminoglycosides, ß-lactams, fluoroquinolones, among others) facilitates recombination of gene cassettes, occasionally involving the overexpression of the IntI1 integrase triggered by the SOS system which induces the deactivation of LexA[71,72]. The current trend to use biocides in a wider and more indiscriminate manner may lead to the emergence of new genetic elements with unpredictable consequences for human welfare[2].

It is clear that, although there is a general understanding of the origins, there are still many details to be clarified about the acquisition and dissemination mechanisms of antibiotic resistance genes in microbial populations. These poorly understood aspects largely occur because the studies focus mainly on the properties of pathogens from clinical origin and little effort has been made to understand the behavior of environmental strains.

CONCLUSIONS

Based on our results, we concluded that the presence of antibiotic resistant *P. aeruginosa* in all stages of the HWTP is important to evaluate the efficiency of the biological treatment of hospital effluents. The detection of the highest percentage of MDR strains in the chlorination stage suggests increased horizontal transfer and the spread of resistant genes and resistant organisms.

The presence of resistant strains of *P. aeruginosa*, including MDR, in the treated effluent indicates that the treatment did not completely eliminate these pathogens and alerts to the risks to the local community, since the final destination of this effluent is a lagoonar complex used as a recreation site and subsistence fishing area.

Although limited because of the small number of strains studied, our results are impressive, since we found microbial strains resistant to antibiotics used to treat serious infections, like fourth generation cephalosporins, carbapenems and even polymyxin, one of the last resources in the antimicrobial therapeutic arsenal.

Data obtained in this study help us interpret the efficiency of hospital wastewater treatment. Furthermore, they may contribute to environmental health surveillance regarding the design of actions to prevent the negative impact of these effluents on aquatic systems and public health.

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Conflict of Interest

Authors have no potential conflict of interest to declare, related to this study's political or financial peers and institutions.