Antibiotic resistance profile and occurrence of AmpC between Pseudomonas aeruginosa isolated from a domestic full-scale WWTP in southeast Brazil

Luiza Gerçossimo Oliveira, Leticia Gonçalves Resende Ferreira, Andrea Maria Amaral Nascimento, Mariana de Paula Reis, Marcela França Dias, William Gustavo Lima and Magna Cristina Paiva

ABSTRACT

Wastewater treatment plants (WWTPs) represent an important reservoir of antibiotic resistance determinants. Although many studies have been conducted to evaluate resistance profiles in Enterobacteriaceae isolates from this setting, the dynamics of this phenomenon are poorly known to the bacterium Pseudomonas aeruginosa. Here we aimed to evaluate the resistance profiles and the production of AmpC β-lactamase in P. aeruginosa isolates from a domestic full-scale WWTP. Samples of the raw sewage and effluent were collected and the bacterium P. aeruginosa was isolated on cetrimide agar. Susceptibility to β-lactams, fluoroquinolones and aminoglycosides was evaluated by the disc diffusion method, and the presence of AmpC β-lactamase was investigated phenotypically and by molecular method. We recovered 27 isolates of P. aeruginosa. Of these, 81.5% were susceptible to all antimicrobials tested. However, a considerable rate of resistance to carbapenems (11%) was found among the isolates. Twenty-two isolates were positive in the phenotypic test for inducible AmpC β-lactamase but the blaampC gene was only identified in four isolates, suggesting the presence of other independent resistance mechanisms besides this β-lactamase. In summary, we have shown that P. aeruginosa isolates from a domestic WWTP represents a potential reservoir of blaampC genes and other resistance determinants, including those that result in low susceptibility to carbapenems and aminoglycosides.

Key words | Aminoglycosides resistance, blaampC, carbapenem resistance, effluent, Pseudomonas aeruginosa, raw sewage

INTRODUCTION

Pseudomonas aeruginosa is an aerobic, motile and non-glucose-fermenting Gram-negative bacilli that can proliferate in various environments, at nutrient levels that may be low (water) or high (sewage and the human body) (Moradali et al. 2017). It is considered an important healthcare-associated opportunistic pathogen, especially affecting patients who are carriers of HIV/AIDS, cancer, burn wounds and cystic fibrosis (Tsao et al. 2017). P. aeruginosa is responsible for 10–25% of the nosocomial infections worldwide (Hedfi et al. 2016). Bloodstream infections caused by P. aeruginosa are important in the hospital environment and have high mortality rates, ranging from 20% to 38% of infected patients (Suárez et al. 2010). Besides being intrinsically resistant to many antimicrobial agents, the bacterium P. aeruginosa also shows a high degree of versatility in acquiring resistance, meaning that the infections caused by this microorganism are often a major challenge for health services (El-Mahdy 2014).

Resistance to β-lactams is notable in P. aeruginosa, being principally caused by the acquisition of genes that encode enzymes with the ability to hydrolyze these antibiotics such as extended-spectrum β-lactamases (ESBLs, e.g. SHV, TEM, PER, VEB, BEL, GES, CTX-M), metallo-β-lactamases (MBLs, e.g. IMP, VIM, SPM, GIM, and AIM), oxacilllnas
and AmpC β-lactamase (Tankhiwale 2016). Originally encoded by \( \text{bla}_{\text{ampC}} \), AmpC is the most common β-lactamase in the bacterium \( P. \text{aeruginosa} \) (Jacoby 2009; Tankhiwale 2016). With the exception of those in \( E. \) coli, \( S. \) spp. and \( A. \) baumannii, chromosomal AmpC β-lactamase is usually produced at a low level, but its expression is induced by mutations (involving the \( \text{bla}_{\text{ampD}}, \text{bla}_{\text{ampR}} \) and \( \text{bla}_{\text{ampG}} \) genes) or by inducing agents such as β-lactams, specially cefoxitin and imipenem (Jacoby 2009). The overexpression of these enzymes can confer resistance to broad-spectrum cephalosporin (except cefepime and cepirome), penicillins, aztreonam and β-lactamase inhibitors (clavulanate, sulbactam and tazobactam) (Jacoby 2009; Tankhiwale 2016). Besides their chromosomal locations, AmpC β-lactamase coding genes have also been detected on plasmids in \( P. \text{aeruginosa} \) clinical isolates, although this is more frequent in members of the Enterobacteriaceae. Thus, horizontal transfer of plasmids by conjugation may contribute to a rapid spread of AmpC β-lactamase among different Gram-negative species (Shahid et al. 2003).

Domestic sewage and activated sludge have contributed to the dissemination of antibiotic resistance determinants since constitute major reservoirs of antibiotic-resistant bacteria and resistance genes (Chen & Zhang 2013). Conventional sewage treatment methods are not completely effective in the elimination of antimicrobial residues, thus, effluents released into the natural environment contribute to the spread of antibiotic resistance, constituting a critical global public health problem (Michael et al. 2014). Antibiotic-resistant bacteria resident in the environment may contribute in the number of infections with multidrug-resistant (MDR) pathogens (Heft et al. 2016). MDR-\( P. \) aeruginosa highlights in this context because it can remain in aquatic environments for long periods, creating dissemination routes and environmental reservoirs of antibiotic resistance genes (Pappa et al. 2016). However, despite the large number of antibiotic-resistant \( P. \) aeruginosa continuously discharged into natural water through sewage, most studies on resistance profile in the environment have focused on Enterobacteriaceae (Devarajan et al. 2017). Furthermore, antibiotic resistance has been extensively studied in wastewater treatment plants (WWTPs) in Europe and the USA, but the expansion of this phenomenon has been little explored in developing countries such as Brazil (Rafraf et al. 2016).

Thus, considering the limited studies about antibiotic resistance between non-glucose-fermenting Gram-negative bacilli from domestic WWTPs and the unknown impact this environment on dissemination of \( \text{bla}_{\text{ampC}} \), we aimed to investigate the occurrence of antibiotic resistance and AmpC β-lactamase production in \( P. \) aeruginosa isolates from of a full-scale WWTP localized in southeast Brazil.

**MATERIALS AND METHODS**

Sample collection

This study was carried out in the city of Divinópolis (MG), localized in southeast Brazil (232,945 inhabitants). Samples of raw sewage (RS) and effluent (EF) were collected at the Rio Pará WWTP (geographical coordinates: 20° 08′ 20” S and 44° 53′ 02″ W) on the 8th June 2015. The studied WWTP serves approximately 24,000 people and uses the conventional activated sludge method to treat domestic sewage and the treated effluent is discharged into the river. All water samples (1 L) were placed into sterilized polypropylene bottles and taken to the laboratory by refrigerated transport. Samples were processed within 2 h of collection at the Laboratório de Diagnóstico Laboratorial e Microbiologia Clínica at the Universidade Federal de São João Del-Rei (Divinópolis-MG/Brazil). The sample collection was authorized by Companhia de Saneamento de Minas Gerais (Copasa), a publicly owned company responsible for the collection and treatment of sewage and water supply in the state of Minas Gerais (BR).

Cultures and \( P. \) aeruginosa isolation

\( P. \) aeruginosa was isolated by plating 100 μL of RS and EF on cetrimide agar, directly and after serial dilution in 0.9% sterile saline solution (Himedia, Brazil). The plates were incubated at 37 °C for up to 48 h and the total number of colony forming units (CFU) was determined. Colonies with macroscopic characteristics of bacterium \( P. \) aeruginosa (large colonies and production of a diffusible green pigment) (Mossel & Indacochea 1971) were selected, inoculated in Brain Heart Infusion broth (BHI; Difco, The Netherlands), and incubated at 37 °C for 24 h. Subsequently, the isolates were repeatedly streaked onto the same agar to check their purity and to confirm the production of specific pigments. Furthermore, production of the enzyme cytochrome oxidase and growth capacity at 42 °C were verified to confirm the species identification (Parr et al. 1975; Oberhofer 1979). The isolates were stored in nutrient broth with 25% glycerol at –80 °C until further use.
**Bacterial clearance rates**

The clearance rate at the WWTP was determined through the equation described by Slekovc et al. (2012):

\[
\text{Mean bacterial or } P. \text{aeruginosa load in raw sewage} \\
- \text{Mean bacterial or } P. \text{aeruginosa load in effluent} \\
\times 100
\]

Bacterial load experiment was realized in triplicate and the results were expressed as the mean value.

**Antibiotic susceptibility test and MDR classification**

All isolates were tested for antimicrobial susceptibility using the standard disc diffusion method according to the guidelines of the Clinical Laboratory Standards Institute (CLSI 2017). The following antimicrobials were tested: uredopenicillins: piperacillin-tazobactam (PTZ); aminoglycosides: gentamicin (GEN) and amikacin (AMI); fluoroquinolones: ciprofloxacin (CIP) and norfloxacin (NOR); carbapenems: imipenem (IPM) and meropenem (MEM); third generation cephalosporins: ceftazidime (CAZ); and fourth generation cephalosporins: cephepime (CPM). *P. aeruginosa* ATCC 27853 was used as a quality control strain. The isolates were classified as MDR when they were resistant to at least one antimicrobial in each of three different classes (Kataoka et al. 2015).

**AmpC β-lactamase and blaAmpC gene detection**

Inducible AmpC β-lactamase production was investigated in all isolates by the phenotypic test of antagonism with imipenem and ceftazidime, according to Cantarelli et al. (2007). A reduction in the halo inhibition of ceftazidime adjacent to the imipenem disk indicates a positive result for AmpC β-lactamase production (Figure 1).

Molecular identification of *blaAmpC* was realized using polymerase chain reaction (PCR). DNA was extracted from *P. aeruginosa* isolates following the method of Pérez-Pérez & Hanson (2002). The quantity and quality of the total DNA extracted was determined using a NanoDrop spectrophotometer (Life Technologies, USA). The *blaAmpC* primer set used was: 5’-CCCGCTTATAGGCGACAC3’ (634 bp) (Forward) and 5’-TCAATGCGACTTACACCC-3’ (Reverse). The PCR reaction was performed in a total volume of 25 μL using an amplification cycle with a denaturation step at 95 °C for 15 min, followed by 35 cycles of 94 °C for 60 s, 58 °C for 2 min, 72 °C for 3 min and a final cycle at 72 °C for 10 min (Pérez & Hanson 2002). *P. aeruginosa* ATCC 27853 and *Citrobacter freundii* (Paiva et al. 2017) were used as negative and positive controls, respectively.

**Statistical analysis**

Pairwise comparisons were conducted using Fisher’s exact test and categorical variables were compared using Pearson’s Chi-squared test. Compared values were considered statistically significant when *p* values were less than 0.05.

**RESULTS AND DISCUSSION**

WWTPs are the main anthropogenic source of discharge of antibiotic resistance genes into the environment (Fuentefería et al. 2011; Chen & Zhang 2013; Michael et al. 2014; Miranda et al. 2015; Magalhães et al. 2016). Sewage treatment methods, such as the activated sludge process, as well as being ineffective in removing resistance determinants, create a favorable environment for the horizontal transmission of resistance genes between microorganisms (Michael et al. 2014). In this context, genes coding for AmpC β-lactamase, are particularly important because they have the capacity for rapid dissemination among different bacterial communities (Jacoby 2009). WWTPs are an important component in this process because they are an environment with a big bacterial community and rich in resistance genes (Chen & Zhang 2013). However, despite the potential for dissemination of AmpC β-lactamase, there is little available information concerning its impact on non-fermenting Gram-negative bacilli (Rafraf et al. 2016; Devarajan et al. 2017). Furthermore, few studies have
evaluated the influence of domestic WWTPs on antimicrobial resistance in developing countries, and the dynamics of this phenomenon remain to be fully elucidated in these regions (Fuentefria et al. 2011; Rafraf et al. 2016). In Brazil several studies have been conducted on *P. aeruginosa* susceptibility from hospital wastewater treatment (Fuentefria et al. 2011; Miranda et al. 2015; Magalhães et al. 2016), in contrast to domestic sewage and its effluent.

Thus, in this paper, we aim to evaluate the resistance profile and detect by molecular approach and phenotypic test the production of AmpC β-lactamase in *P. aeruginosa* isolated from a domestic full-scale WWTP in southeast Brazil. After collection and bacteriological analysis of the samples, a bacterial load of $1.2 \times 10^2$ CFU/mL and $0.8 \times 10^2$ CFU/mL from RS and EF, respectively, were found (Figure 2). The sewage treatment process resulted in a global clearance rate of 33.3%, which was considerably lower compared to other studies that show a rate above 90% (Bréchet et al. 2014). A total of 27 colonies the grown (23 from RS and four from EF) were identified as *P. aeruginosa*, according to previously described criteria (Mossel & Indacochea 1971). Here, in contrast to the low depuration of global bacterial charge, the clearance rate of bacterium *P. aeruginosa* was high (82%; $p < 0.0001$).

Similarly, Slekovec et al. (2012) showed that a WWTP in eastern France (Besançon City; 130,000 inhabitants) was able to reduce *P. aeruginosa* isolates by 94% compared to untreated sewage. In another study, the wastewater treatment process decreased the population of *Acinetobacter* spp., another genus of non-glucose-fermenting Gram-negative bacilli, by three orders of magnitude in a WWTP in Michigan (USA; Ann Arbor City; 210,000 inhabitants) (Zhang et al. 2009). However, it is worth noting that WWTPs in the USA usually chlorinate the effluent, which might be the reason for the huge reduction in bacterial load (Naidoo & Olaniran 2014). Already in the present study, the reduction of the *P. aeruginosa* population, it may be related to the retention time of the effluent. With increasing retention times, the cell surface properties (i.e. zeta potential, hydrophobicity, charge density) of the bacteria present in the effluent are profoundly affected, thereby compromising their survival (Li et al. 2015).

The antimicrobial susceptibility profile to β-lactams (ureidopenicillin, carbapenems and cephalosporin), aminoglycosides and fluoroquinolones was determined for all *P. aeruginosa* isolates. As shown in Figure 2, the proportion of isolates susceptible to all antimicrobials was high (81.5%, 22/27, 19 from RS and three from EF). Similarly, high sensitivity rates have been reported for *P. aeruginosa* isolated from domestic WWTPs localized in medium-sized cities (from 100,000 to 500,000 inhabitants) in France (76.5% of isolates were sensitivity to all the antibiotics tested) (Slekovec et al. 2012) and South Africa (74.3% of isolates were sensitivity to all the antibiotics tested) (Odjadjare et al. 2012). In contrast, *P. aeruginosa* isolates from hospital wastewater treatment (HWWTP) exhibited high resistance profiles, possibly due to the selective pressure of antibiotic, to which they are continuously exposed in the hospital environment. In Brazil, for example, the frequency of MDR-*P. aeruginosa* isolates in HWWTP was 85.4% in Passo Fundo/RS (Fuentefria et al. 2011), 82% in Rio de Janeiro/RJ (Miranda et al. 2015), and 60% in Manaus/AM (Magalhães et al. 2016).

In the present study, the highest resistance rate was observed for carbapenems (11%). Three isolates (6_RS, 13_RS and 23_RS) obtained from raw sewage were resistant to both, meropenem and imipenem (Table 1). Considering that this class of antibiotic is widely used in the treatment of infections caused by Gram-negative bacteria with the MDR phenotype, resistance to carbapenems represents a great clinical concern (Suárez et al. 2010). Here, we report the highest rate of resistance to carbapenems in *P. aeruginosa* isolates from domestic WWTPs found to date. Łuczkiwicz et al. (2015) showed that all *P. aeruginosa* isolates obtained from a full-scale WWTP located near the Baltic Sea in northeastern Poland were sensitive to meropenem and imipenem. However, in *P. putida* isolates the carbapenem resistance was more common, with 6.5% and 8.5% of isolates resistant to imipenem and meropenem, respectively (Łuczkiwicz et al. 2015). In this study, resistance to aminoglycosides (amikacin and gentamicin) was found in a single isolate (8_RS) obtained from RS, in contrast to other studies where resistance to this class was

---

**Figure 2** | Bacterial load from raw sewage and effluent of a full-scale WWTP from the southeast of Brazil. The isolates identified as *P. aeruginosa* (black) and the proportion of isolates resistant to one or more antibiotics (gray) are shown.
absent (Odjadjare et al. 2012; Luczkiewicz et al. 2015). In Brazilian sanitary legislation, the use of carbapenems and aminoglycosides is restricted to the hospital environment. However, the presence of resistant isolates to these drugs in the community WWTP can be explained because it is common in country hospital wastewater to be drained into the municipal wastewater system (Fuentefria et al. 2014).

Although rates of resistance to fourth generation cephalosporins frequently reach 30–35% of P. aeruginosa isolates from WWTPs (Odjadjare et al. 2012; Slekovec et al. 2012; Luczkiewicz et al. 2015), in this study only one isolate (8_RS, 3.7%) showed this phenotype.

Isolates resistant to carbapenems and aminoglycosides were not found in EF. Considering the four isolates obtained in the effluent, only one was resistant to a third-generation cephalosporin (ceftazidime). All isolates were sensitive to fluoroquinolones and piperacillin/tazobactam and no MDR-P. aeruginosa was identified in the raw sewage or effluent. Previous studies have also reported the susceptibility of P. aeruginosa isolates to fluoroquinolones and piperacillin/tazobactam, suggesting that these sensitive isolates are ubiquitous in domestic sewage (Odjadjare et al. 2012; Slekovec et al. 2012; Luczkiewicz et al. 2015). In addition, MDR-P. aeruginosa has been found mainly in HWWTPs, with only one study reporting the presence of these microorganisms in domestic WWTPs, in South Africa (Odjadjare et al. 2012).

Inducible AmpC β-lactamase-positive Gram-negatives are clinically relevant since they can be resistant to a wide variety of β-lactam drugs (Tankhiwale 2016). Thus, several phenotypic tests have been proposed for the detection this β-lactamase in order to target a more specific and successful therapies and reduce laboratory costs (Jacoby 2009; Fuentefria et al. 2011). However, specific guidelines are not available for AmpC β-lactamase detection and molecular tests are being considered the gold standard, since they present high sensibility and specificity (Jacoby 2009). In this study, AmpC β-lactamase was determined by molecular approach and phenotypic test in order to elucidate the dissemination of this enzyme among the bacterium P. aeruginosa in domestic sewage. We observed that 22 out of 27 P. aeruginosa isolates (19 from RS and three from EF) were positive for inducible AmpC β-lactamase, according to the phenotypic test (antagonism with IPM and CAZ) (Figure 1). By contrast, only four isolates (1.1%) harbored the blaAmpC gene, all from RS (Table 1). This result indicates that the phenotypic test was affected by other additional mechanism of resistance to β-lactams.
present in the isolates, which resulted in a false-positive detection of AmpC β-lactamase (Tankhiwale 2016). In fact, Jacoby (2009) has shown that a combination of resistance mechanisms is often found in β-lactam-resistant P. aeruginosa, especially involving overproduction of efflux pumps, decreased production of porins, target site or outer membrane alterations and/or acquisition of another β-lactamases (e.g. ESBL, MβL, OXA). In all blaAmpC-positive isolates, sensibility to cephepine was shown, confirming that the AmpC β-lactamase-producing strains did not inactivate this compound (Jacoby 2009). However, we also observed isolates harboring blaAmpC that were fully sensitive to all tested antimicrobials, including β-lactams that are known substrates for AmpC. This fact suggests the possibility that the expression of the enzyme is not occurring or occurs at baseline levels in these specimens.

CONCLUSION

In summary, the present study highlights the presence of P. aeruginosa strains that are resistant to clinically relevant antimicrobials, and the production of AmpC β-lactamase by these isolates, in a domestic full-scale WWTP. Here is shown for the first time that domestic sewage is a possible route of dissemination of AmpC β-lactamase-positive P. aeruginosa to the clinical setting in Brazil. Further, this study showed that domestic WWTPs, which can also carry hospital wastewater, are an important source of carbapenem-resistant P. aeruginosa and can contribute to the environmental dissemination of resistance to this important class of antibiotics.

AUTHOR CONTRIBUTIONS

All authors contributed to the development, analysis and drafting of this article.

CONFLICTS OF INTEREST

The authors have reported no conflicts of interest.

ETHICAL APPROVAL

Not required.

ACKNOWLEDGEMENTS

The authors would like to thank the Universidade Federal de São João del Rei for its support during the research. W.G.L. is grateful to Fundação de Amparo à Pesquisa do estado de Minas Gerais (FAPEMIG) for a fellowship.

REFERENCES


Identification of new bacteria harboring qnrS and aac(6’)-Ib-cr and mutations possibly involved in fluoroquinolone resistance in raw sewage and activated sludge samples from a full-scale WWTP. Water Res. 110, 27–37.


