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Detection of metallo-beta-lactamase-producing genes *bla*$_{SPM}$ and *bla*$_{NDM}$ in *Pseudomonas aeruginosa* isolated from wastewater in Southern Brazil

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ABSTRACT

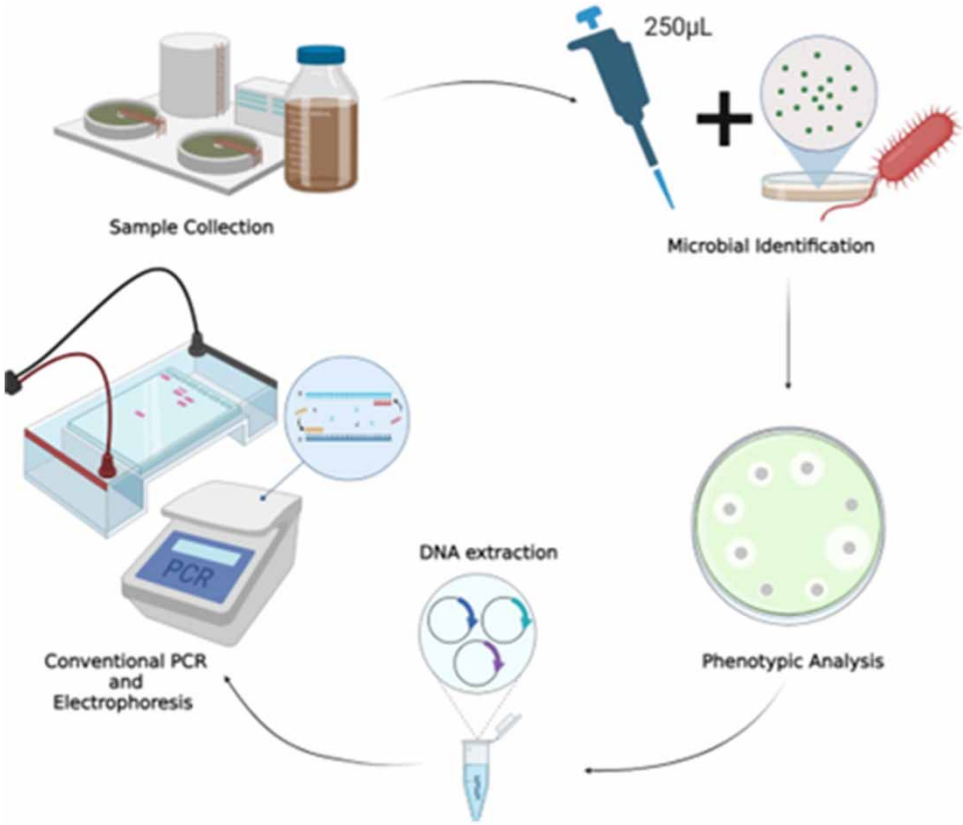
Pseudomonas aeruginosa is commonly associated with the ability to acquire antimicrobial resistance. The surveillance of resistance genes in various environmental matrices has gained prominence in recent years, being seen as a potential threat to public health. The objective of this study was to investigate genes encoding metallo-beta-lactamases (MBLs), which confer resistance to carbapenems, in wastewater. Fifteen isolates of *P. aeruginosa* were collected for five months from samples obtained from a municipal wastewater treatment plant in Rio Grande do Sul. These isolates were subjected to disk diffusion testing using 10 different antimicrobials. Phenotypic enzymatic tests for MBLs were conducted, and positive isolates underwent DNA extraction and gene detection using the polymerase chain reaction. The resistance rate to ceftazidime was 100%, cefepime 73.3%, piperacillin–tazobactam 66.67%, imipenem 53.30%, levofloxacin 46.67%, tobramycin 40%, and ciprofloxacin and amikacin 13.33%. Both meropenem and aztreonam resistances were rare accounting for 6.60% of the tested isolates. Among these isolates, 20% were classified as multidrug-resistant and were found to carry the *bla*$_{NDM}$ and *bla*$_{SPM}$ genes. The results suggest that evaluating resistance genes in bacteria from urban raw sewage can provide data that assist in surveillance, as this environment can stimulate increased bacterial resistance.

Key words: antimicrobial resistance, carbapenemase, *Pseudomonas aeruginosa*, sewage, surveillance

HIGHLIGHTS

- Twenty per cent of *P. aeruginosa* isolates were classified as multidrug-resistant (MDR).
- Urban sewage, which is associated with hospital sewage contributes to the spread of antimicrobial resistance genes (ARGs).
- Three isolates carrying genes encoding MBLs presented resistance to beta-lactam antibiotics.
- *P. aeruginosa* showed resistance characteristics to different classes of antibiotics and may have suffered stress of anthropogenic origin.

GRAPHICAL ABSTRACT



1. INTRODUCTION

Pseudomonas aeruginosa is widely distributed in the environment and is frequently found in hospital moist settings (Brooks *et al.* 2014). Its broad environmental distribution is facilitated by its simple growth characteristics and nutritional versatility, which enhances its adaptability to environmental changes and its omnipresence in water (Stover *et al.* 2000; Wailan *et al.* 2016).

Infections caused by *P. aeruginosa* are commonly associated with high mortality rates in hospitals, which are attributed to its intrinsic resistance to many classes of antimicrobials and its ability to rapidly acquire resistance through mutation and the horizontal transfer of resistance determinants (Potron *et al.* 2015). Common resistance mechanisms involve mutations in outer membrane proteins, efflux pumps, and the production of beta-lactamase enzymes such as the carbapenemases SPM (São Paulo metallo-beta-lactamase, MBL) and NDM (New Delhi MBL), which hydrolyze carbapenems. These enzymes are of great concern as carbapenems are considered last-resort antibiotics to combat infections caused by multidrug-resistant (MDR) bacteria (Kumar *et al.* 2012).

Recent data indicate that the Coronavirus disease (COVID-19) pandemic has led to a 32% increase in the circulation of MDR bacteria in the United States, resulting in 2,500 deaths in 2020. The weighted average of deaths from *P. aeruginosa* infections with resistance in the European population was 3,210 in the same year. Approximately 700,000 people die each year globally as a consequence of antimicrobial resistance (AR) infections (WHO 2019; CDC 2022; WHO/ECDC 2022).

Research evaluating the human impact on the entire ecosystem has gained prominence in recent years. Antimicrobial resistance genes (ARGs) have already been isolated in aquatic environments since there is a dynamic presence of these agents in urban sewage, river wastewater, as well as marine and polar environments. The dispersion of these agents in this complex matrix, comprising a vast microbiota of environmental bacteria, can change in its composition through selection processes, altering the entire ecosystem (Brum *et al.* 2015; Gao *et al.* 2018).

The wastewater forms a complex environment that can be widely used for surveillance, providing an abundance of nutrients, where bacterial communities can be influenced and exhibit resistance characteristics. Among these influences are

abiotic conditions such as ambient temperature, the structure of the present organisms, and especially, selective pressure factors such as heavy metals and antimicrobial residues (Novo *et al.* 2013). Among the methodologies used by various studies, molecular analyses can complement surveillance studies, offering a potentially useful tool for genetic variability analysis in a single sample or based on isolates (Aarestrup & Woolhouse 2019; Hendriksen *et al.* 2019).

Community sewage receives a significant contribution of antimicrobial residues and is recognized as a major source and dissemination route for resistance determinants, with hospital sewage being a contributor (Aggarwal *et al.* 2020). Furthermore, the CDC (2022) outlined several benefits of monitoring antibiotic resistance, as a single sample can represent thousands of people in a wastewater reservoir. In fact, the antimicrobials used by the population are not completely degraded, and their metabolites are excreted, negatively impacting effluent pollution and public health.

While community effluent constitutes the largest proportion of treated sewage, hospital sewage exerts the greatest influence on bacterial resistance. This is not only due to the release of strains previously colonized in healthcare settings, but also because of the higher rates of antimicrobial residues present in this type of effluent (Pazda *et al.* 2019). In municipal wastewater treatment plants (WWTPs), the adoption of activated sludge-based treatment methods is common. This configuration significantly intensifies the possibilities of resistance transfer among microbial communities, concentrating both urban and hospital sewage (Asfaw *et al.* 2017).

A previous study (Fuentefria *et al.* 2009) describes the potential of wastewater to disseminate *P. aeruginosa*-carrying SPM genes, which has been reported in some cities in the state of Rio Grande do Sul (RS). However, more recent studies have observed the continuity of the NDM gene in other bacterial isolates in the state. This gene has been associated with high levels of resistance to carbapenems in South America (Ramalho *et al.* 2022).

In this context, the present study aimed to conduct the surveillance of MDR *P. aeruginosa* in wastewater from the city of São Leopoldo/RS through the phenotypic and molecular evaluation of the bla_{SPM} and bla_{NDM} genes. This is the first time that the molecular resistance panel has been analyzed in the Vale dos Sinos region. This raises the hypothesis that the origin of community and hospital sewage will impact the diversity of ARGs and their prevalence in the state.

2. METHODS

2.1. Study area description

The Vicentina WWTP is situated in the Vicentina district of São Leopoldo, where only 12.45% of the city's sewage is collected and treated. This WWTP can treat up to 150 liters of sewage per second, benefiting approximately 54,000 inhabitants. The WWTP serves several neighborhoods including the city center and also receives sewage from the Public Hospital (SEMAE 2021). The selection of this particular wastewater treatment plant was based on its ability to serve approximately 23% of the population affected in the city, particularly in terms of receiving hospital sewage. Additionally, it is situated in a city within the Vale do Rio dos Sinos region. This region is characterized by the passage of a river, which serves as the main water resource for the Vale do Sinos region. The river plays a vital role in supporting the fauna, flora, and the quality of life of the nearby residents.

2.2. Sample collection

Samples were collected at the inlet point of the WWTP. These samples represent raw wastewater and were collected using sterile 500 mL glass containers. The samples were collected from the water surface (10 cm) and immediately placed in thermal transport boxes (with ice) to retain their temperature during transportation to the bacteriology laboratory at the Integrated Center for Health Specialties (CIHS) of Feevale University. Sampling occurred approximately every 15 days over a period of five months from November 2022 to March 2023 for monitoring purposes. Physicochemical data such as pH, water, and ambient temperature were kindly provided by the treatment plant at the time of collection.

2.3. Microbial identification

In the laboratory, the samples were homogenized, and 250 μL was spread onto Petri dishes with Cetrimide agar (Difco™) using the surface spread plate method. After incubation at 35 °C for 24 h, green colonies suggestive of *P. aeruginosa* were subcultured by streaking onto Cetrimide agar. The resulting growth was tested for oxidase (+) reaction, Gram-staining (Gram-negative bacilli), extracellular pigment production (pyoverdine and pyorubin), and characteristic odor (grape-like smell), following the description of Brooks *et al.* (2014).

2.4. Phenotypic resistance testing

The standard Kirby–Bauer disk diffusion method (Bauer *et al.* 1966) was employed, following the Brazilian Committee on Antimicrobial Sensitivity Testing (BrCAST 2022) guidelines. To characterize the phenotypic antibiotic susceptibility profile, the method recommended by the Brazilian Health Regulatory Agency (ANVISA) Technical Norm No. 01/2013 for investigating MBLs was used in combination. A bacterial suspension equivalent to the 0.5 McFarland standard was prepared in sterile 0.9% saline and uniformly spread onto the surface of Mueller–Hinton agar (Himedia) in a sterile 15×150 mm Petri dish.

After excess moisture evaporated, antibiotic disks were applied, and the plates were incubated for 18 h at 36 ± 1 °C in an incubator. For the screening phenotypic test, the following antibiotics were used: amikacin (30 μg), aztreonam (30 μg), cefepime (30 μg), ceftazidime (30 μg), ciprofloxacin (5 μg), imipenem (10 μg), meropenem (10 μg), levofloxacin (5 μg), tobramycin (10 μg), piperacillin–tazobactam (30–6 μg), and the combination discs with ethylenediamenetetraacetic acid (EDTA), imipenem/EDTA (10 μg/10 μL), and meropenem/EDTA (10 μg/10 μL). The discs combined with 10 μL of 0.1 M EDTA were prepared on the day of testing and applied to the plates after drying. The classification of MDR, extensively drug-resistant (XDR), and pan drug-resistant (PDR) strains followed the criteria of Magiorakos *et al.* (2012), and the degree of risk in the contamination zone was assessed by calculating the multiple antimicrobial resistance (MAR) index that is determined by dividing the number of resistant antimicrobials by the total number of antimicrobials tested. A MAR index greater than 0.2 indicates high-risk contamination (Krumperman 1983).

2.5. DNA extraction

The samples were transported to the Laboratory of Molecular Microbiology (LMM) at Feevale University. Resistant bacterial isolates showing a halo of ≥5 mm for the MBL test (ANVISA 2013) were subjected to thermal lysis extraction. To achieve this, our group used the following extraction method. The isolates were suspended in ultrapure water (400 μL) in a 1.5 mL DNase-free microtube, triturated with a pipette tip, incubated at 95 °C for 15 min in a thermoblock, followed by an ice bath at −20 °C, vortexed, and repeated twice. Subsequently, the samples were centrifuged for 10 min at 10,000 rpm, and the supernatant containing bacterial DNA was extracted and transferred to another microtube containing 100 μL of TE buffer (Tris–HCl–EDTA, pH 8). To assess the quality and quantity of the extracted DNA, 1 μL was pipetted onto a Thermo Scientific™ NanoDrop™ One spectrophotometer. The extraction result was compared with a similar method that achieved a maximum of 1,263.8 ng for bacterial DNA (Ribeiro *et al.* 2016) and pure DNA (ratio ~1.8) using the ThermoFisher technical protocol (Thermo Scientific 2012). After extraction, the DNA was stored at −20 °C until amplification.

2.6. Molecular identification

Molecular identification was performed using primer sequences described by Poirel *et al.* (2011). In the current study, reactions for the target gene identification were conducted using both the conventional polymerase chain reaction (PCR) and the multiplex PCR. The PCR mix was composed of 5× Green GoTaq buffer (10 mmol/L Tris–HCl (pH 8.5) and 50 mmol/L KCl), 1.5 mmol/L $MgCl_2$, 0.125 mmol/L of each deoxynucleotide triphosphate, 0.1 μmol/L of each primer (in the same multiplex PCR or in the conventional PCR), and 1.25 U of GoTaq DNA Polymerase. The PCR conditions for the bla_{SPM} and bla_{NDM} genes were as follows: initial denaturation at 94 °C for 10 min, followed by 30 cycles of denaturation at 94 °C for 40 s, annealing at 57 °C for 40 s, and extension at 72 °C for 1 min, with a final extension step at 72 °C for 5 min.

The amplified DNA products were analyzed using 2% agarose gel in 1% TBE buffer (Tris/Borate/EDTA) with ethidium bromide as an intercalating agent. A 100 bp DNA ladder marker was used as a reference scale. The gels were stained with SYBR GREEN® and visualized under UV transillumination.

3. RESULTS AND DISCUSSION

3.1. Physicochemical parameters

Among the physicochemical parameters analyzed in the wastewater samples, the average pH value was 7.39, ranging from 7.7 to 7.29, and the sample temperature was 27.8 °C, ranging from 22 to 29.9 °C. The average ambient temperature recorded on the sampling dates was 29.2 °C, varying from 17.3 to 34.8 °C. Statistical analysis using Pearson's correlation coefficient showed a significant correlation between ambient temperature and sample temperature ($p > 0.05$).

3.2. Antimicrobial susceptibility profile

All 15 analyzed bacterial isolates exhibited biochemical characteristics consistent with *P. aeruginosa*. The bacterial isolates were categorized as resistant (R), susceptible (S), or susceptible increased exposure (I) to antibiotics including imipenem, cefepime, aztreonam, piperacillin–tazobactam, ciprofloxacin, and levofloxacin. None of the isolates were classified as wild type, with a halo of >50 mm (BrCast 2022). Among the *P. aeruginosa* isolates (15/15), all were resistant to ceftazidime. A total of 11/15 (73.3%) were resistant to cefepime, whereas 10/15 (66.67%) were resistant to piperacillin–tazobactam. In terms of resistance to other antibiotics, 8/15 (53.30%) *P. aeruginosa* isolates were resistant to imipenem, followed by 7/15 (46.67%) resistant to levofloxacin, 6/15 (40%) resistant to tobramycin, and 2/15 (13.33%) isolates resistant to ciprofloxacin and amikacin. Resistance to meropenem and aztreonam was rare among the tested isolates, with only 1/15 (6.60%) isolate showing resistance to aztreonam and another 1/15 (6.60%) isolate being classified as sensitive with increased exposure to meropenem (Figure 1).

Evaluating the association between tested antibiotic classes and resistant and non-resistant samples revealed a significant difference ($p = 0.020$), with a Chi-square result of 7.73.

A significant correlation ($p < 0.05$) was observed among beta-lactams, with the most pronounced statistical difference observed between meropenem and aztreonam compared to ceftazidime, followed by cefepime, and finally piperacillin–tazobactam (Figure 2).

Of the 15 isolates tested, three (20%) isolates showed resistance to three or more antimicrobials. These three isolates were classified as MDR. No XDR or PDR isolates were found in this study (Figure 3). The MAR index had an average of 0.4 for the collected samples.

3.3. Molecular characterization

The average concentration of extracted bacterial DNA from the isolates was 1,253.8 ng, with a variance in the 260/280 ratio of approximately 1.8. Out of the 15 samples analyzed, six (40%) showed an increased halo using the ANVISA (Brazilian Health Regulatory Agency) method and were therefore selected for molecular evaluation. Among these six, three (50%) carried the genes, with one (33.33%) harboring bla_{NDM} and two (66.67%) carrying bla_{SPM}. The multiplex PCR was validated using a lower primer concentration compared to the article, with the same band size as reported in the literature (Figure 4).

The three positive isolates for the SPM and NDM genes exhibited a resistance profile similar to the tested beta-lactams. However, there was sensitivity to meropenem in two findings (Table 1). According to the statistical analysis between the presence of genes and ambient temperature for both SPM ($p = 0.933$) and NDM ($p = 0.733$), as well as sample temperature for SPM ($p = 0.838$) and NDM ($p = 0.933$), there was no statistical correlation.

3.4. Discussion

In this study, resistance profiles in *P. aeruginosa* were analyzed in raw urban wastewater samples. Resistant isolates were detected in all of the studied samples, indicating that dissemination through the sewage system is likely, originating from

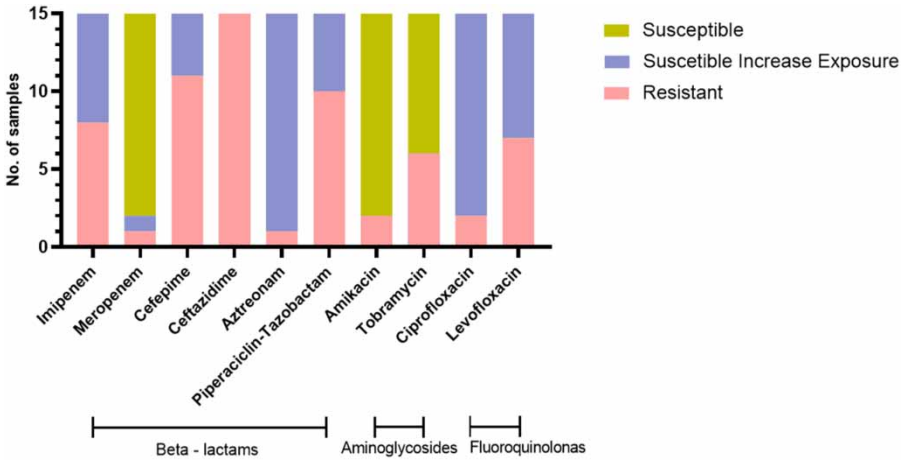


Figure 1 | Antimicrobial susceptibility of *P. aeruginosa* to the evaluated antibiotics.

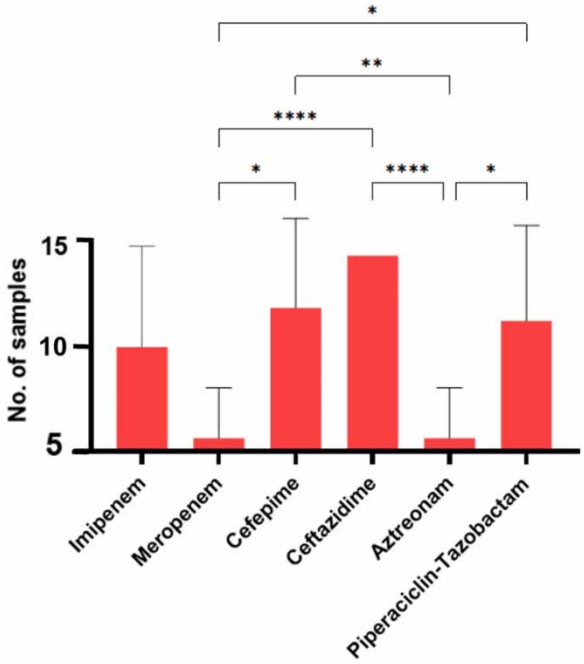


Figure 2 | Significance relationship between beta-lactams.

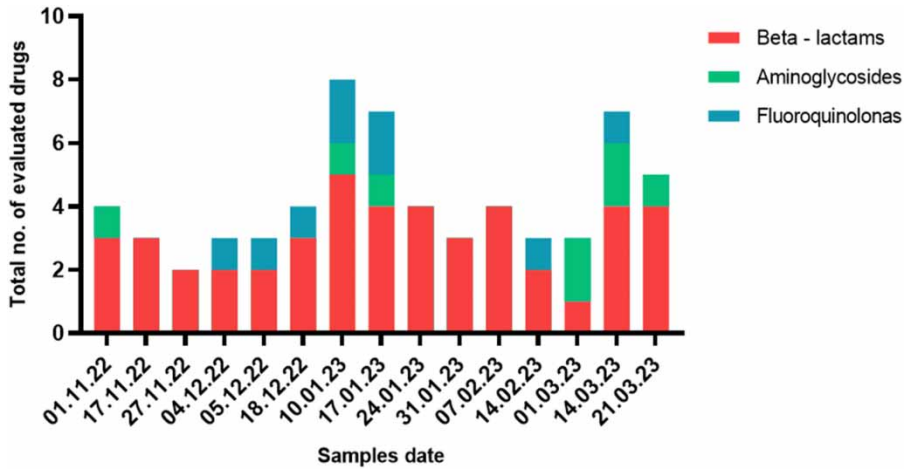


Figure 3 | Resistance profile by antibiotic classes over the evaluated period.

both hospital and urban sources. The variation in resistance results (Figure 3) can be attributed to fluctuations in antimicrobial residue concentrations during the collection week, the presence of ions in the environment, and the duration of exposure to these factors. Isolates may have undergone selective pressure, yet the occurrence of resistance was significant.

The pH data of the samples fell within the range conducive to the growth of *P. aeruginosa*, with an average value of 7.39 across the samples, and the average water temperature of 27.8 °C facilitated the growth of mesophilic bacterial groups. The bacterial DNA extraction via thermal lysis corresponds to the similar mass found by Ribeiro *et al.* (2016) using the same principle, and the ratio was consistent with the quality of pure DNA as described in the Thermo Scientific™ NanoDrop™ One technical bulletin (Thermo Scientific 2012).

In this study, there was a predominant resistance to cephalosporins (ceftazidime and cefepime), as shown in Figure 1. Among the tested cephalosporins, ceftazidime is classified as a third-generation antibiotic, while cefepime is classified as a

Figure 4 | Multiplex and single PCR for target genes. Columns 1: 100 bp molecular weight marker; 2: multiplex PCR; 3: negative control; 4: sample 10/01; 5: sample 17/01; 6: sample 14/03.

Table 1 | Phenotypic profile of gene-carrying samples

Sample	Gene	Imipenem	Meropenem	Cefepime	Ceftazidime	Aztreonam	Piperacillin–tazobactam
10/01	SPM	R	R	R	R	I	R
17/01	NDM	R	S	R	R	I	R
14/03	SPM	R	S	R	R	I	R

fourth-generation antibiotic. Both of these are well known for their anti-*Pseudomonas* activity. Resistance to these antibiotics implies therapeutic complications, as they are among the first-line treatments for *Pseudomonas* infections. An explanation for the high rate of cephalosporin resistance may lie in the presence of potential efflux pumps, which reduce the intracellular concentration of the antibiotic, changes in entry porins and permeability, as well as modifications in the intracellular targets of cephalosporins. In *P. aeruginosa*, the most frequently observed mechanism is the potential overproduction of the chromosomal beta-lactamase *Amp*C. The three homologs of the *Amp*D protein are recognized for positively regulating the chromosomal cephalosporinase, and this enzyme has a direct impact on the hydrolysis of cephalosporins when overexpressed (Strateva & Yordanov 2009; Potron *et al.* 2015).

A study conducted in South Africa by Mapipa *et al.* (2021) highlighted the overexpression of *Amp*C beta-lactamase and found that ceftazidime (63%) and cefepime (35%) showed the highest levels of resistance in *P. aeruginosa* isolated from hospital wastewater. The growth of cephalosporin resistance can be influenced by cultural and social factors. In regions with high antibiotic consumption and less effective healthcare infrastructure in pathogen control, there is a tendency for resistance development, and this phenomenon may vary across different locations. In a study conducted in the southern region of Brazil, specifically in the Tramandaí lagoon, isolates resistant to ceftazidime and imipenem were identified. This finding strengthens the notion that naturally occurring bacterial microbiomes are susceptible to anthropogenic stresses. These stresses can be triggered by a variety of factors stemming from human activities, spanning from clinical to agricultural and industrial domains. Such factors encompass environmental pollutants, such as chemical waste, heavy metals, and pesticides, as well as alterations in soil and water composition. It is noteworthy that antimicrobial residues stand out as prominent examples in the formation of mutated bacterial microbiomes (LEITE *et al.* 2019).

The notable resistance to penicillins (66.67%) in *P. aeruginosa* can be attributed to various mechanisms that may coexist or combine in diverse ways. Due to its remarkable adaptability, this bacterial species can adjust the expression of these resistance mechanisms in response to specific environmental conditions. Such mechanisms include the synthesis of penicillin-binding proteins with possible reduced affinity for some β-lactams, changes in the permeability of the outer membrane (including the loss of *Opr*D proteins), and the overexpression of efflux systems such as *Opr*M and *Opr*J, presenting broad substrate profiles (Strateva & Yordanov 2009). Research conducted by Hosu *et al.* (2021) detected a high rate of resistance to beta-lactams such as piperacillin and piperacillin/tazobactam (47.2%) in surface waters downstream of slaughterhouses.

The relatively low resistance to aztreonam in environmental isolates was observed recently by Roulová *et al.* (2022), where only 8.5% of the 59 isolates were resistant to the antibiotic. In this study, one isolate demonstrated resistance to aztreonam (Figure 2). This can be explained by the fact that aztreonam shows relative resistance to efflux pumps or has had lower previous exposure to structurally similar antibiotics in the environment and aztreonam is stable in the presence of MBL enzymes, which gives it the ability to overcome this resistance mechanism compared to some other beta-lactams (Leite *et al.* 2019; Khan *et al.* 2021). When *Amp*C is overproduced, it can not only hydrolyze β-lactam antibiotics but may also be linked to the cell wall renewal process, which is regulated by the *Amp* operon. The hydrolysis of peptidoglycan by *Amp*C can interfere with the normal recycling process, leading to a greater inability of Monobactam penetration (Berrazeg *et al.* 2015).

In our study, fluoroquinolones showed relatively low levels of resistance (Figure 1), except for levofloxacin (46.67%). Fluoroquinolones primarily act by inhibiting DNA gyrase or topoisomerase IV, affecting bacterial DNA replication, cell division, and the transcription of essential enzymes, potentially leading to programmed cell death (Strateva & Yordanov 2009). However, single-point mutations in relevant *Gyr*A genes have been shown to cause ciprofloxacin resistance in wastewater isolates (Schwartz *et al.* 2006). In Passo Fundo, RS, Fuentefria *et al.* (2011) highlighted ciprofloxacin resistance only in hospital effluents, possibly due to drug exposure, including the presence of *Gyr*A genes and the hyperexpression of efflux pumps. Another study found isolates from a stream in Manaus that showed greater sensitivity to ciprofloxacin compared to levofloxacin. However, these isolates also exhibited high resistance to the antibiotic (Magalhães *et al.* 2016). Clinically, the use of both fluoroquinolones (ciprofloxacin and levofloxacin) depends on the type of infection being treated, as both have a broad spectrum of activity and clinically similar results (BrCast 2022). In this study, ciprofloxacin (13.33%) showed higher efficiency compared to levofloxacin, which could be due to prior exposure to antimicrobial residues in the environment or alterations in resistance in the isolate's conformation.

The mechanism of the action of tobramycin involves binding to the 30 S and 50S ribosome regions, preventing the formation of the 70S complex. However, mutations in *orf*N, *fus*A, and *pmr*B have been shown to confer resistance in *P. aeruginosa* isolates (Sanz-García *et al.* 2018). On the other hand, enzymes modified through biochemical processes such as O-phosphorylation, O-adenylation, or even 16S rRNA methylases can confer high levels of resistance to aminoglycosides (Neves *et al.* 2011). The resistance proportion to tobramycin (40%), when compared to amikacin (13,33%) as shown in Figure 1, has been observed in Rio de Janeiro, where 20% of isolates in a hospital wastewater treatment network were resistant to tobramycin and 2% to amikacin (Miranda *et al.* 2015). Among the results, aminoglycosides exhibited better performance against the isolates (Figure 1), which aligns with clinical studies demonstrating greater efficacy of the drug amikacin against *P. aeruginosa* (Pires *et al.* 2009).

The observed resistance (Figure 2) rate in imipenem is commonly reported in *P. aeruginosa* due to the presence of *Opr*D, which significantly influences the loss of outer membrane porins or high *Amp*C expression. *Opr*D is capable of facilitating imipenem diffusion more effectively than meropenem (Young *et al.* 2019).

In Porto Alegre, in the year 2013, the first case of NDM gene presence was confirmed in *Providencia rettgeri* and *Enterobacter cloacae*. Since then, this resistance has been spreading to different regions of Brazil (ANVISA 2013). In Dhaka, Bangladesh, a study conducted by Islam *et al.* (2017) compared ARGs from hospitals and community sewage and showed that 71% of samples were positive for NDM-1 in hospital sewage. They also demonstrated the presence of the NDM gene in the community, with warmer seasons favoring plasmid transfer. New resistance genes are frequently described. For example, a new variant called NDM-33 has been identified in *Escherichia coli* isolated from hospital sewage. This variant is associated with an IncX3-type plasmid (Wang *et al.* 2021). The ongoing evolution of NDM enzymes can lead to the emergence of new variants with different beta-lactam hydrolysis activities, potentially being transported through sewage.

SPM-1 was first discovered in Brazil in 1997 from a clinical isolate of *P. aeruginosa* (Toleman *et al.* 2002), and its presence has since been confined to South America, contributing to the growth of carbapenemase cases in Brazil. The SPM-1 gene is chromosomal- or plasmid-encoded, possibly associated with the common insertion sequence 4 (ISCR4) region in its acquisition, expression, and likely transposition through a replication mechanism (Hong *et al.* 2015).

A regional study conducted by Fuentefria *et al.* (2009) investigated hospital effluents in RS, specifically at the São Vicente de Paulo Hospital (HSVP) in Passo Fundo and the Divina Providência Hospital (HDP) in Porto Alegre. The study found high resistance to imipenem and/or meropenem in both hospitals, with the bla_{SPM} gene present in five isolates from the HDP and four isolates from the HSVP. It is worth noting that there were discrepancies between the phenotypic tests where many isolates tested positive in the enzymatic screening test but negative in the PCR. In this study, it was the first time that the genes

were analyzed in wastewater in the Vale dos Sinos region, in São Leopoldo, raising the fact of their continued presence in isolates from the state over these years.

Among the isolates, six were phenotypically positive using the screening test for MBLs, but only three were genetically positive (Figure 4). Therefore, these isolates may carry another type or allelic variant of the MBL gene, or they may have another mechanism of resistance to carbapenems, as phenotypic data can differ from molecular data, which is currently considered the gold standard (Beig *et al.* 2021). Furthermore, EDTA can provide false-positive results due to increased permeability of the cell wall and, through zinc chelated by EDTA, accelerate the decomposition of imipenem and decrease the expression of *Opr*D of *P. aeruginosa* (Conejo *et al.* 2003).

Among the isolates that harbored the genes (Table 1), resistance to beta-lactams, especially cephalosporins and piperacillin–tazobactam, was observed, while aztreonam remained stable in the presence of MBLs synthesized by NDM and SPM genes (Khan *et al.* 2021). One of the discrepancies was observed in meropenem resistance, where two isolates were susceptible to the drug. These results can vary due to other genetic and phenotypic characteristics of the isolates, and other studies have reported the genetic presence of MBL producers that are susceptible to meropenem (Picão *et al.* 2012; Pascale *et al.* 2019).

In Brazil, a study conducted by Ramalho *et al.* (2022) highlighted the presence of bla_{TEM} and *tet*B genes in water for human consumption, and $\text{bla}_{\text{KPC-2}}$, $bla_{\text{NDM-1}}$, and mcr-1 in effluents. According to the study, the summer season was characterized by high concentrations of tetracyclines, which are commonly used in agriculture. These antibiotics have greater stability in the environment compared to other antibiotics like beta-lactams. Other studies emphasize the importance of monitoring these potable waters, as other MBL-encoding genes have been found (Mombini *et al.* 2019).

Finally, the average MAR index of 0.4 obtained (Figure 3) is concerning, as public sewage, with hospital sewage being a potential major contributor, may act as a zone of the concentration of resistance genes for other bacteria, posing a risk to the health of the local population. In our study, an analysis with a larger sample size could better confirm our results. Additionally, it is important to evaluate in future studies not only the molecular mechanisms of beta-lactamases but also changes in permeability, efflux pumps, and factors in the specific environment that contribute to the emergence of mutated microbial communities.

4. CONCLUSIONS

Resistant isolates of *P. aeruginosa* were found in all studied raw sewage samples, with factors such as temperature and pH contributing to the growth of this bacterial species. In this study, 20% of isolates demonstrated resistance to three or more antimicrobials and were classified as MDR. The results unveiled significant resistance to important antibiotics such as cephalosporins (ceftazidime and cefepime), which are considered first-line choices in *P. aeruginosa* infection therapy, suggesting potential *Amp*C hyperproduction and/or alterations in entry porin permeability. The release of resistant bacterial isolates of anthropogenic origin into municipal wastewater systems may result in extensive horizontal gene exchange, especially concerning carbapenem resistance. Genes like MBL-type beta-lactamases pose a public health threat, and the findings of this study align with epidemiological data on the dissemination of SPM and NDM genes in the country.

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AUTHOR CONTRIBUTIONS

V.M.B. built the work, performed methodology, carried out data curation, and wrote the original draft. B.S.R. and J.F.S. found resources and performed methodology. J.R. did data analysis and reviewed and edited the manuscript. C.R. and S.U.P. supervised and conceptualized the work, did a critical review, and edited the original draft.

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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