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Antimicrobial Susceptibility and Molecular Epidemiology of Multidrug-resistant *Pseudomonas aeruginosa* in Northeast of Brazil

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Introduction: This study aimed to investigate the antimicrobial susceptibility profile of nosocomial strains of *Pseudomonas aeruginosa* isolated from inpatients of a teaching hospital in the City of Sobral, Ceará, in Northeast of Brazil (Santa Casa de Misericórdia de Sobral - SCMS) from March/2019 to March/2020, as well as to assess the occurrence of resistance genes *bla*-$_{TEM}$, *bla*-$_{SHV}$, *bla*-$_{CTX\text{-}M\ 1/2}$, *bla*-$_{IMP\text{-}1}$, *bla*-$_{KPC}$, *bla*-$_{GES}$, *bla*-$_{SPM\text{-}1}$, *bla*-$_{NDM\text{-}1}$, *bla*-$_{VIM}$.

Methodology: Bacterial identification and antimicrobial susceptibility tests (AST) were performed using the automated system Vitek®2. Conventional polymerase chain reaction (PCR) was used to amplify genes of interest.

Results: Thirty-eight specimens of *P. aeruginosa* were collected. More than half of the isolates were resistant to imipenem (55.2%), and showed different rates of resistance to the other antimicrobials tested. In addition, intermediate susceptibility was also observed to gentamicin (7.8% of the isolates) and meropenem (10.52% of the isolates). The gene *bla*-$_{CTX\text{-}M\ 1/2}$ was the most prevalent (41.9%), while *bla*-$_{GES}$ was highly identified among the carbapenemase-producing strains (12.9%).

Conclusion: The results demonstrated considerable resistance rates to β-lactam antibiotics, which could be attributable to the indiscriminate use of these antibiotics in the analyzed hospital, whose control relies on the improvement of antimicrobial prescription policies.

Keywords: Beta-lactamases; carbapenemases; bla genes; nosocomial infection; antimicrobial resistance.

1. INTRODUCTION

Pseudomonas aeruginosa is an opportunistic, Gram-negative bacillus, and represents a major cause of healthcare-associated infections, including nosocomial pneumonia, bloodstream infections, urinary tract infections, and skin and skin structure infections [1].

Bacteria exhibit multiple resistance mechanisms to antibiotics including decreased permeability, expression of efflux systems, production of antibiotic inactivating enzymes, and target modifications. *P. aeruginosa* exhibits most of these known resistance mechanisms through both intrinsic chromosomally encoded or genetically imported resistance determinants affecting the major classes of antibiotics such as β-lactams, aminoglycosides, quinolones, and polymyxins [2].

Multidrug resistance (MDR) was defined as non-susceptibility (intermediate plus resistant [IR]) to at least one agent belonging to 3 different classes of antibiotics [3]. A noticeable ability to acquire mechanisms of resistance to several antimicrobial drugs is an important characteristic of *P. aeruginosa* [4], being the infections caused by MDR *P. aeruginosa* a therapeutic challenge and representing a rise in mortality/morbidity rates and hospitalization costs [5].

β-Lactam antibiotics have been widely used as therapeutic agents for the past 70 years, resulting in the emergence of an abundance of β-lactam-inactivating β-lactamases. β-lactamases are most important in Gram-negative bacteria, particularly in non-lactose-fermenting enteric bacteria, in which collectively they confer resistance to all β-lactam-containing antibiotics. Critical β-lactamases are those enzymes whose genes are encoded on mobile elements that are transferable among species. Major β-lactamase families include plasmid-mediated extended-spectrum β-lactamases (ESBLs), and carbapenemases now appearing globally, with geographic preferences for specific variants [6].

The carbapenems are potent β-lactams used to treat infections caused by MDR *P. aeruginosa* strains. However, the production of carbapenemases caused an increase in the resistance rates to these antimicrobial drugs, limiting their use, and reducing the therapeutic choices for their associated illnesses [7-9].

Carbapenemases are β-lactamases with versatile hydrolytic capacities. They can hydrolyze penicillin, cephalosporin, monobactams, and carbapenems. These enzymes are codified by horizontally transferable genes which are also associated with resistance determinants to other classes of antimicrobial drugs. Currently, the propagation of carbapenemase-producing organisms, especially Gram-negative bacteria, is a public health issue

that must be investigated to control its dissemination [10-13].

Beyond all the identified mechanisms of resistance detected in the *P. aeruginosa* isolates, this species can produce virulence factors that increase tissue damage, causing cell death and necrosis, as well as enable them to evade the host immune system, which further contributes to the establishment and maintenance of infectious process [14].

Thus, assessing the antimicrobial susceptibility profile and identifying genetic factors involved in the antimicrobial resistance of *P. aeruginosa* isolates are useful tools to generate epidemiological data. This information can aid health professionals in preventing the dissemination of these pathogens and choosing a suitable therapeutic approach.

This study aimed to analyze the antimicrobial susceptibility profile of nosocomial isolates of *P. aeruginosa* from inpatients of a teaching hospital (Santa Casa de Misericórdia of Sobral - SCMS) located in the city of Sobral, Ceará, in Northeast of Brazil. Additionally, the occurrence of antimicrobial drug resistance-associated genes *bla*-$_{TEM}$, *bla*-$_{SHV}$, *bla*-$_{CTX\text{-}M\ 1/2}$, *bla*-$_{IMP\text{-}1}$, *bla*-$_{KPC}$, *bla*-$_{GES}$, *bla*-$_{SPM\text{-}1}$, *bla*-$_{NDM\text{-}1}$, *bla*-$_{VIM}$ in these isolates were assessed.

2. MATERIALS AND METHODS

2.1 Bacterial Strains

Thirty-eight nosocomial strains of *P. aeruginosa* were isolated from March/2019 to March/2020 from clinical samples (blood, urine, tracheal aspirate, cutaneous catheters, wounds, anal swabs, and other non-identified sites of hospitalized patients with the diagnosis of nosocomial infection in this teaching hospital (SCMS), Ceará, Brazil. The samples were obtained from patients admitted to different hospital divisions, such as wards, neonatal intensive care unit, emergency, neurology, intensive care unit for adults, cardiology, oncology, and hemodialysis.

2.2 Bacterial Identification and Antimicrobial Susceptibility Testing

Bacterial identification and antimicrobial susceptibility testing (AST) for amikacin, cefepime, ceftazidime, ciprofloxacin, gentamicin, imipenem, meropenem, ampicillin, ampicillin/sulbactam, cefuroxime, and piperacillin/tazobactam were assessed by the automated system Vitek®2 (BioMerieux, Marcy L'Etoile, France) in the Laboratory of Microbiology of SCMS (Biovida), Ceará, Brazil. AST results were analyzed according to Clinical & Laboratory Standards Institute (CLSI, 2023).

2.3 DNA Extraction and Detection of *Bla* Genes

Seven out of thirty-eight isolates were not viable to be genetically studied because there was no bacterial growth upon the reactivation of these isolates. Thus, only thirty-one isolates of *P. aeruginosa* were subjected to DNA extraction and gene expression identification. The genomic DNA extraction was performed by using the silica microspheres technique [15] and the molecular analysis was carried out by conventional polymerase chain reaction (PCR) to detect the genes *bla*-$_{TEM}$, *bla*-$_{SHV}$, *bla*-$_{CTX\text{-}M\ 1/2}$, *bla*-$_{IMP\text{-}1}$, *bla*-$_{KPC}$, *bla*-$_{GES}$, *bla*-$_{SPM\text{-}1}$, *bla*-$_{NDM\text{-}1}$ and *bla*-$_{VIM}$. All the primers and protocols used for amplification of these genes are described elsewhere [16-20,4]. The sequence of DNA fragments to be amplified and detected are described in Table 1. For all the PCR assays, one reaction without the addition of DNA was used as a negative control for contamination and DNA samples from pre-characterized strains (CCBH27131, CCBH6556, CCBH16302, CCBH24061, CCBH4851, Pa1461, and Pa2815), kindly provided by FIOCRUZ (Oswaldo Cruz Foundation) and by Prof. Dr. Floristher Elaine Carrara - Special Laboratory for Molecular Microbiology and Antimicrobial Resistance - State University of Londrina - PR - Brazil, were included as the positive control. The products of amplification were analyzed in 1% agarose gels containing ethidium bromide (1.25 µl/100 ml).

2.4 Data Analysis

Categorical data were expressed as absolute and relative (%) frequencies and crossed with the genes detected by using Fisher's exact test or Pearson's chi-square test. The sum of antimicrobial drugs to which microorganisms were resistant was expressed as mean and standard deviation, subjected to Kolmogorov-Smirnov normality test for normality assessment, and compared to the genes by Student's t-test. All the analyses were performed using a confidence level of 95% in the software "Statistical Package for the Social Sciences (SPSS) version 20.0 for Windows.

Table 1. Primers used for amplification of antimicrobial resistance genes

Gene	Sequence (5'–3')
bla-TEM like	F: CCCTTATTCCCTTTYTTGCGG
	R: AACCAGCCAGCCWGAAGG
bla-SHV like	F: ATGCGTTATTAGTTTCGCCTGTGTATTATC
	R: TTAGCGTTGCCAGTGAGTCGATC
bla-CTX-M ½	F: ATGTGCAGYACCAGTAA
	R: CGCTGCCGGTTTTATCSCCC
bla-IMP-1	F: CTACCGCAGCAGAGTCTTTGC
	R: GAACAACCAGTTTTTGCCTTACC
bla-NDM-1	F: GCGCAACACAGCCTGACTTT
	R: CAGCCACCAAAAGCGATGTC
bla-SPM-1	F: AAAATCTGGGTACGCAAACG
	R: ACATTATCCGCTGGAACAGG
bla-KPC like	F: TCGCTAAACTCGAACAGG
	R: TTACTGCCCGTTGACGCCCAATCC
bla-GES like	F: AGCAGCTCAGATCGGTGTTG
	R: CCGTGCTCAGGATGAGTTG
bla-VIM like	F: GATGGTGTTTGGTCGCATA
	R: CGAATGCGCAGCACCAG

3. RESULTS

Eleven out of thirty-eight *P. aeruginosa* isolates were found in samples from the São Joaquin ward (medical clinics and traumatology and orthopedics division). The majority of such isolates were found in male patients (57.8%). Bloodstream was the most common origin of such isolates (26.3%), followed by urine (13.2%) and tracheal aspirate (13.2%). The distribution of the isolates by hospital division, body origin, and patient gender are presented in Table 2.

More than half of all isolates were resistant to imipenem (55.2%), and showed different rates of resistance to the other antimicrobials tested, such as, meropenem (34.2%), ceftazidime (42%), cefepime (39.4%), ciprofloxacin (31.5%), gentamicin (28.9%), and amikacin (26.31%). Of the 38 strains, only 12 (31.5%) were tested for ampicillin, ampicillin/sulbactam and cefuroxime. All of them were resistant to cefuroxime, and eleven (91.6%) were resistant to the other two antibiotics tested. Finally, twenty-eight (73.6%) isolates were tested against piperacillin/tazobactam, but only one was resistant to such antibiotics. Moreover, 2.63% of the isolates presented an intermediate susceptibility to cefepime, ceftazidime, and imipenem. Intermediate susceptibility was also observed to gentamicin (7.8% of the isolates) and meropenem (10.52% of the isolates).

The gene *bla*-*CTX-M ½* that codifies broad spectrum β-lactamases was the most prevalent one found in thirteen (41.9%) of the thirty-one isolates subjected to the molecular analyses. Moreover, *bla*-*GES* was the most common among the genes that codify carbapenemases. It was identified among four isolates (12.9%) (Table 2). Comparing the presence of the genes and the number of antibiotics to which the microorganism was resistant, it was observed that the isolates that presented *bla*-TEM genes were statistically resistant to a higher number of antibiotics (p=0.035) (Table 3).

Furthermore, six samples simultaneously presented more than one of the analyzed genes, and the gene *bla*-*CTX-M ½* was present in all of them. Such samples were isolated from inpatients admitted to the ICU and ward for patients with neurological disorders. Amongst these samples, four (66.6%) were resistant to imipenem and three (50%) were resistant to meropenem (Table 4).

The strains in which the genes *bla*-*IMP-1* and *bla*-*GES* were identified were resistant to amikacin. The only isolate in which the gene *bla*-IMP-1 was found presented resistance to 12 antibiotics. Moreover, the presence of this gene was significantly associated with resistance to amikacin (p=0.038).

No association was found between the patient's gender or origin of the isolates with the presence of the resistance genes. However, the presence of the gene *bla*-SHV like was associated with isolates from patients admitted in the neurology division, Sao Jose ward, and ICU (p<0,05) (Table 5).

Table 2. Distribution of nosocomial isolates of *P. aeruginosa* by hospital division, body origin, gender, and prevalence of the resistance genes

	n	%*
Hospital Division		
Dom Walfrido ward	1	2.6
Adult emergency	4	10.5
Hemodialysis	1	2.6
Heart hospital	2	5.3
Non-Informed	1	2.6
Neurology	4	10.5
Oncology	1	2.6
São Joaquim ward	11	28.9
São José ward	3	7.9
ICU for adults	3	7.9
Neonatal ICU	7	18.4
Body sites		
Tracheal aspirate	5	13.2
Others	11	28.9
Catheter tips	3	7.9
Bloodstream	10	26.3
Wounds	2	5.3
Anal swabs	2	5.3
Urine	5	13.2
Gender		
Female	16	42.1
Male	22	57.9
Genes		
SHV	4	12.9
TEM	6	19.4
CTXM 1/2	13	41.9
IMP 1	1	3.2
KPC	1	3.2
GES	4	12.9
SPM 1	0	0.0
NDM 1	0	0.0
VIM	0	0.0

**Data expressed as absolute and relative (%) frequencies.*

Table 3. Influence of gene expression in the antimicrobial resistance profile of nosocomial isolates of *P. aeruginosa*

	Gene		
	Absent	**Present**	**p-Value**
SHV	7.11±3.00	6.75±3.20	0.825
TEM	6.52±2.87	9.33±2.42	***0.035**
CTXM ½	6.89±3.29	7.31±2.59	0.706
IMP 1	6.90±2.88	12	0.092
KPC	7.00±3.01	9	0.518
GES	7.19±3.13	6.25±1.71	0.567

**p < 0.05, Student's t-test (mean ± SD)*

4. DISCUSSION

The infections caused by P. aeruginosa and the development of antimicrobial drug resistance of this species are becoming a worldwide worrisome problem. Brazil is a continental country with a demographic density and different traditions in different regions. The Northeast of the country is less populous than the Southeast and has fewer economic resources, so that

epidemiological data from this region may be different from other regions of the country, highlighting the need for research to establish protocols that improve patient care.

The results of our investigation showed that most of the patients diagnosed with a nosocomial infection caused by *P. aeruginosa* were male (57.89%). This finding differed from another study performed in the state of Pará, Brazil [21], whose data showed a higher prevalence in female patients (51.9%). The difference in prevalence might be due to the number of samples collected.

As described in our results, the bloodstream was the most common origin of such isolates followed by urine and tracheal aspirate, which corroborate the findings of a recent Spanish study [6]. Unlike in Taiwan, *P. aeruginosa* was identified much more commonly among isolates from lower respiratory tract infections than in urinary tract and the bloodstream [9]. The results of the Spanish group also corroborate our findings regarding the susceptibility profile once the most effective antibiotics against the isolates were colistin, amikacin, and piperacillin/tazobactam [6]. Moreover, most of the isolates presented complete resistance or intermediate susceptibility to imipenem. On the other hand, studies carried out in Brazil have shown that colistin was the most effective antimicrobial agent against *P. aeruginosa* [7,22,23].

Studies all around the world have shown an association between *P. aeruginosa* infection and patients admitted in the ICU [24,9,21], differing from our data, which showed such infections in patients admitted to other unities of the hospital. This difference might have occurred because we investigated a university hospital, where there is a higher flow of patients, healthcare professionals, students, and professors. The high number of people in and out of the hospital might lead to the dissemination of multi-resistant microorganisms.

Not a long time ago, the carbapenems had an excellent clinical performance in the treatment of *P. aeruginosa* MDR infections. However, such bacteria developed resistance mechanisms to carbapenems after being massively exposed to this and other antimicrobials [9,25]. In Taiwan, the prevalence of CRPA (carbapenem-resistant *Pseudomonas aeruginosa*) increased from 11.5%– 12.3% (2012–2015) to 19.4%–22.8% (2018–2021) [9]. In addition, a recent study showed that the prevalence of meropenem-resistant *P. aeruginosa* in Ethiopia was 15% [8]. A study of antimicrobial surveillance [26] reported a resistance rate to carbapenems that varied from 26.1 to 44.4% in *P. aeruginosa* isolated from HAIs of patients from Latin America, Europe, Oriental Mediterranean, Southeast of Asia, and Occidental Pacific. The data regarding antimicrobial resistance of this surveillance study are comparable to our results since half of the isolates were resistant to imipenem and more than a third was resistant to meropenem.

Data of antimicrobial susceptibility to *P. aeruginosa* presented by the Antimicrobial Surveillance Program regarding the period from 1997 to 2000 suggested resistance to meropenem of only 5.4%. Comparing such rates, it is possible to observe that the *P. aeruginosa* resistance to carbapenems increased significantly in the last decades [9]. Resistance rates found in Latin America are worrisome once it is higher than the one found in North America and Europe. In Brazil, the resistance rates are even higher than the ones observed in Latin America [18].

Table 4. Relationship of nosocomial isolates of *P. aeruginosa* with the coexistence of antimicrobial resistance genes and hospital division

Sample	*bla* genes detected	IMP*	MER	AMI	Division
PA 2	CTX-M1/2. SHV. KPC	R	R	S	Neurology
PA 5	CTX-M1/2. SHV. TEM	R	R	S	ICU for Adults
PA 8	CTX-M1/2. TEM. GES	R	S	S	Neonatal ICU
PA 9	CTX-M1/2. TEM. IMP	R	R	R	Neonatal ICU
PA 12	CTX-M1/2. GES	I	I	R	Neonatal ICU
PA 13	CTX-M1/2. GES	S	S	S	Neurology

**IMP: Imipenem; MER: Meropenem; AMI: Amikacin*

Table 5. Influence of hospital division, body sites, and gender in the detection of the antimicrobial resistance genes *bla* SHV, *bla* TEM, *bla* CTX-M ½.

	SHV			TEM			CTXM 1/2		
	Absent	**Present**	**p-Value**	**Absent**	**Present**	**p-Value**	**Absent**	**Present**	**p-Value**
Hospital division									
Dom Walfrido	1 (3.7%)	0 (0.0%)	0.024	1 (4.0%)	0 (0.0%)	0.284	1 (5.6%)	0 (0.0%)	0.627
Adult emergency	4 (14.8%)	0 (0.0%)		3 (12.0%)	1 (16.7%)		2 (11.1%)	2 (15.4%)	
Hemodialysis	1 (3.7%)	0 (0.0%)		1 (4.0%)	0 (0.0%)		1 (5.6%)	0 (0.0%)	
Heart hospital	2 (7.4%)	0 (0.0%)		2 (8.0%)	0 (0.0%)		2 (11.1%)	0 (0.0%)	
Non-Informed	1 (3.7%)	0 (0.0%)		1 (4.0%)	0 (0.0%)		1 (5.6%)	0 (0.0%)	
Neurology	2 (7.4%)	1 (25.0%)*		3 (12.0%)	0 (0.0%)		1 (5.6%)	2 (15.4%)	
Oncology	1 (3.7%)	0 (0.0%)		1 (4.0%)	0 (0.0%)		1 (5.6%)	0 (0.0%)	
São Joaquim ward	7 (25.9%)*	0 (0.0%)		7 (28.0%)	0 (0.0%)		3 (16.7%)	4 (30.8%)	
São José ward	0 (0.0%)	2 (50.0%)*		2 (8.0%)	0 (0.0%)		2 (11.1%)	0 (0.0%)	
ICU for adults	1 (3.7%)	1 (25.0%)*		1 (4.0%)	1 (16.7%)		1 (5.6%)	1 (7.7%)	
Neonatal ICU	7 (25.9%)*	0 (0.0%)		3 (12.0%)	4 (66.7%)		3 (16.7%)	4 (30.8%)	
Body sites									
Tracheal aspirate	3 (11.1%)	1 (25.0%)	0.902	4 (16.0%)	0 (0.0%)	0.227	3 (16.7%)	1 (7.7%)	0.349
Others	8 (29.6%)	2 (50.0%)		9 (36.0%)	1 (16.7%)		5 (27.8%)	5 (38.5%)	
Catheter tips	3 (11.1%)	0 (0.0%)		1 (4.0%)	2 (33.3%)		2 (11.1%)	1 (7.7%)	
Bloodstream	8 (29.6%)	1 (25.0%)		6 (24.0%)	3 (50.0%)		6 (33.3%)	3 (23.1%)	
Wounds	2 (7.4%)	0 (0.0%)		2 (8.0%)	0 (0.0%)		2 (11.1%)	0 (0.0%)	
Anal swab	2 (7.4%)	0 (0.0%)		2 (8.0%)	0 (0.0%)		0 (0.0%)	2 (15.4%)	
Urine	1 (3.7%)	0 (0.0%)		1 (4.0%)	0 (0.0%)		0 (0.0%)	1 (7.7%)	
Gender									
Female	15 (55.6%)	0 (0.0%)	0.101	12 (48.0%)	3 (50.0%)	0.930	11 (61.1%)	4 (30.8%)	0.095
Male	12 (44.4%)	4 (100.0%)		13 (52.0%)	3 (50.0%)		7 (38.9%)	9 (69.2%)	

**p<0.05, Fisher's exact test and Pearson's chi-square test (n, %)*

A study performed in Iran in 2010 [27] reported the occurrence of the gene *bla*-$_{TEM}$ in all samples analyzed, which differed from our results that showed that this gene was found only in one-fifth of the isolates. The gene *bla*-$_{CTX\text{-}M\ 1/2}$ was found in almost half of the isolates of our study. We have already found such genes in *Klebsiella pneumoniae* isolated from patients with nosocomial infections who were being treated at the same hospital where we performed the work presented in this report [28]. The resistance profile we have found in the isolates of the same hospital might be due to the use of different therapeutic protocols adopted by different professionals and regions. Our results also support that CTX-M as the most prevalent ESBL in Brazil [29].

Regarding the detection of the genes *bla*-$_{IMP\text{-}1}$ e *bla*-$_{KPC}$, our data corroborate findings of an investigation performed in São Paulo, Brazil [30-31]. For the detection of gene *bla*-$_{GES}$, our data corroborate an Iranian report [16,32].

Our investigation presents limitations once few specimens of *P. aeruginosa* were isolated in the hospital analyzed during the study period. Furthermore, it was not possible to identify the CTX-M group and the variants TEM, SHV, and GES carried by each strain. However, the results obtained with our work might be useful as epidemiological data that stress the need for future investigations focused on strategies to prevent the dissemination of resistant microorganisms.

5. CONCLUSION

The results demonstrated considerable rates of resistance to β-lactam antibiotics among nosocomial isolates of *P. aeruginosa* in the analyzed hospital. Moreover, our findings suggest that the CTX-M enzyme is the main ESBL responsible for the resistance phenotype of the isolates studied. Thus, our data alert to the occurrence of an endemic resistance caused by *P. aeruginosa* strains. Such a resistance profile might be due to the indiscriminate use of antimicrobial drugs, a problem that only can be controlled with the enhancement of prescription policies.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

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CONSENT

All authors declare that written informed consent was obtained from the patient for publication of this research.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee (Research and Ethics Committee of the Vale do Acaraú State University - Opinion nº 3.378.013)

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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