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Carbapenem-resistant *Pseudomonas aeruginosa*: association with virulence genes and biofilm formation

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ABSTRACT

Pseudomonas aeruginosa is an opportunistic pathogen that causes frequently nosocomial infections, currently becoming more difficult to treat due to the various resistance mechanisms and different virulence factors. The purpose of this study was to determine the risk factors independently associated with the development of bacteremia by carbapenem-resistant *P. aeruginosa*, the frequency of virulence genes in metallo- β -lactamases producers and to evaluate their ability to produce biofilm. We conducted a case-control study in the Uberlândia Federal University – Hospital Clinic, Brazil. Polymerase Chain Reaction was performed for metallo- β -lactamases and virulence genes. Adhesion and biofilm assays were done by quantitative tests. Among the 157 strains analyzed, 73.9% were multidrug-resistant, 43.9% were resistant to carbapenems, 16.1% were phenotypically positive for metallo- β -lactamases, and of these, 10.7% were positive for *bla*_{SPM} gene and 5.3% positive for *bla*_{VIM}. The multivariable analysis showed that mechanical ventilation, enteral/nasogastric tubes, primary bacteremia with unknown focus, and inappropriate therapy were independent risk factors associated with bacteremia. All tested strains were characterized as strongly biofilm producers. A higher mortality was found among patients with bacteremia by carbapenem-resistant *P. aeruginosa* strains, associated independently with extrinsic risk factors, however it was not evident the association with the presence of virulence and metallo- β -lactamases genes.

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Introduction

Healthcare-associated infections (HAIs) like bacteremia caused by multidrug-resistant *Pseudomonas aeruginosa* strains result in an increased morbidity and mortality, prolonging the

hospitalization, and higher costs compared to those infections caused by susceptible strains.¹⁻³ Infections caused by these resistant microorganisms are often associated with age, cancer, heart disease, diabetes, intensive use of antibiotics, and invasive procedures such as hemodialysis, mechanical ventilation catheter, tracheostomy, and others.⁴

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The increasing incidence of multidrug-resistant *P. aeruginosa* as a cause of nosocomial infection is a global problem, a consequence of the ability of this microorganism to develop resistance to almost all other antimicrobial agents during antimicrobial chemotherapy, either by selection of mutations in chromosomal genes or by horizontal gene transfer.^{5,6} In Brazil, this problem is even more significant, since there is a very high density of antibiotic use, especially β -lactams, carbapenems, and fluoroquinolones.^{7,8} The resistance in *P. aeruginosa* to carbapenems is up to 60% in some Brazilian hospitals^{9,10} and mainly occurs by production of metallo- β -lactamases (MBL).

Ten subclasses of the MBL enzymes are known: IMP (Imipenemase), VIM (Verona Imipenemase), SPM-1 (São Paulo MBL), GIM (German Imipenemase), SIM-1 (Seul Imipenemase),¹¹ AIM-1 (Australian Imipenemase),¹² KHM (Kyorin University Hospital),¹³ NDM-1 (New Delhi MBL),¹⁴ DIM-1 (Dutch Imipenemase),¹⁵ and TMB (Tripoli MBL).¹⁶ In Brazil, the most prevalent subclasses are IMP-1 and SPM-1.¹¹

Several other virulence genes as well as biofilm formation may contribute to the pathogenicity of severe infections, particularly including those associated with invasive procedures.¹⁷ Among the major virulence factors described in the literature, we highlight those related to the adherence of microorganisms to host cells through the flagella, fimbriae, and alginate¹⁸ and those that facilitate the disruption of epithelial integrity and interfere with the immune system, such as elastase, phospholipase C and protease alkaline, further exotoxin A, pyocyanin, and pyoverdine.¹⁹ The biofilm production is particularly associated with the difficulty of antibiotics to penetrate the cells, since they secrete a polymeric matrix composed of polysaccharides, proteins, and DNA.^{20,21}

The aim of this study was to identify the risk factors associated with bacteremia caused by carbapenem-resistant *P. aeruginosa* as well as the production of MBL. Additionally, we investigated the frequency of virulence genes and their ability to form biofilm.

Materials and methods

Patients and hospital

The *P. aeruginosa* strains were recovered from patients admitted to the Uberlândia University Hospital (Brazil), Federal University of Uberlândia (HC-UFU), and obtained from the Microbiology Laboratory of the HC-UFU, during the period from May 2009 to December 2012, considering only the first episode of infection.

Study design

We conducted a case (patients with bacteremia due to *P. aeruginosa* resistant to carbapenems) versus control (patients with bacteremia caused by *P. aeruginosa* susceptible to carbapenems) study to identify risk factors among patients infected with *P. aeruginosa* resistant to carbapenems. Additionally, we evaluated secondary outcomes, including periods of hospitalization, admission to the intensive care unit (ICU), and use of

invasive procedures. Demographic, clinical, and epidemiological characteristics of each patient included in the study were recovered from the clinical records.

Definitions

Healthcare-associated infections (HAIs) are defined as any infection acquired after a patient's admission to the hospital. HAIs may manifest during hospitalization or after discharge, since they are related to hospitalization or procedures performed during the hospitalization.²² Bacteremia, according to the Centers for Disease Control and Prevention,²³ can be defined as the presence of viable bacteria in the blood documented by a positive blood culture result. Bacteremia was classified as primary when it was unrelated to another focus of infection or when it was related to an intravenous catheter, and secondary when it was clinically related to infection in another anatomic site.⁹ Multidrug-resistance is defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories.²⁴ The antimicrobial therapy was considered inappropriate when an isolated microorganism presented *in vitro* resistance to antimicrobials used for treating the patient and/or a lack of treatment for 24 h after diagnosis of microbial infections.²⁵

Clinical microbiological and molecular testing

Cultures were collected according to the protocol used by the Microbiology Laboratory of the HC-UFU and were processed using the automated system BACT/Alert[®] (BioMérieux, Durham, USA). The identification and antimicrobial susceptibility tests were performed by automation using the VITEK II system and the strains that showed intermediate susceptibility were considered resistant. Quality-control protocols were used according to the standards of the Clinical and Laboratory Standard Institute.²⁶⁻²⁸ The carbapenem-resistant *P. aeruginosa* isolates were phenotypically screened for MBL production using double-disc synergy tests, as previously described.^{29,30} In addition, to assess the presence of MBL genes in *P. aeruginosa* strains, a multiplex PCR was performed, as described previously.³¹ The cycling conditions were: 94 °C for 5 min, followed by 30 denaturation cycles at 94 °C for 30 s, annealing at 53 °C for 45 s and extension at 72 °C for 30 s, followed by final extension at 72 °C at 10 min, all in a MasterCycler personal (Eppendorf). Detection of virulence genes codifying alkaline protease (*aprA*), elastase A (*lasA*), elastase (*lasB*),³² haemolytic phospholipase C (*plcH*), non-haemolytic phospholipase C (*plcN*), exotoxin A (*toxA*) and alginate (*algD*)³³ were determined by uniplex PCR, using the following protocol: 94 °C for 3 min, 30 cycles at 94 °C for 30 s, 55 °C for 1 min and 72 °C for 1 min and 30 s and 72 °C for 5 min.

Initial adhesion assay³⁴

In order to evaluate the initial adhesion, 200 μ L of a cell suspension containing 1×10^7 cells/mL prepared in TSB was added to 96-well polystyrene plates. Initial adhesion was allowed to occur for 2 h at 37 °C with rotation at 120 rpm. Bacteria adhered in 96-well polystyrene plates were washed twice with a 0.9% NaCl solution and harvested by scraping the

wells for 90 s. The cell suspension obtained was plated on TSA for colony-forming unit (CFU) enumeration. All experiments were done in triplicate in three independent experiments. The strain ATCC15692 (PAO1) was used as a positive control and TSB without bacteria was used as a negative control.

Biofilm formation assay³⁵

200 μ L of a cell suspension containing 1×10^7 cells/mL prepared in TSB was added to 96-well polystyrene plates. Biofilm formation was allowed to occur for 24 h at 37 °C with rotation at 120 rpm. Bacteria grown in 96-well polystyrene plates were washed twice with a 0.9% NaCl solution and left to dry in an inverted position. The total biomass was measured by methanol (Merck) fixation, crystal violet (Merck) staining, and acid acetic (Merck) elution as previously described. The eluted dye was removed from each well and placed in a new 96-well microtiter plate, and its absorbance was read on an ELISA plate reader (BioTek Instruments Inc., Vermont, USA) at 570 nm. The experiments were done with eight replicates for each strain in three independent experiments. TSB without bacteria was used as a negative control. The optical density cut-off value (ODc) was established as three standard deviations (SD) above the mean of the optical density (OD) of the negative control: $ODc = \text{average OD of negative control} + 3 \times \text{SD of negative control}$. For easier interpretation of the results, strains were divided into the following categories according to optical density (ODi): $ODi \leq ODc$ or $ODc < ODi < 2 \times ODc$ = non-biofilm producer/weak biofilm producer; $2 \times ODc < ODi < 4 \times ODc$ = moderate biofilm producer; $4 \times ODc < ODi$ = strong biofilm producer.

Biofilm cell concentration³⁶

The biofilm cell concentration was determined by CFU enumeration. After biofilm formation, the biofilms were washed twice with a 0.9% NaCl solution and harvested after scraping the wells for 90 s. The cell suspension obtained was plated onto TSA plates. All experiments were done in triplicate, on three independent occasions.

Statistical analysis

Statistical analysis was performed using GraphPad Prism v.5 (GraphPad Software, San Diego, CA). Quantitative assays were compared using the Kruskal–Wallis, applying Dunn's multiple comparison test. All tests were performed with a confidence level of 95% and statistical significance was defined as $p < 0.05$.

Ethical approval

The research Ethics Committee of the Federal University Uberlândia evaluated and approved our study design.

Results

From May 1, 2009, to December 31, 2012, a hospital surveillance of the incidence of *P. aeruginosa* bacteremia was performed in the microbiology laboratory of the University Hospital.

Table 1 – Distribution of patients infected with *Pseudomonas aeruginosa* in different units of the Clinical Hospital of the Federal University of Uberlândia from May/2009 to December/2012.

Clinics	Total N = 157 (%)	Carbapenem-resistant <i>Pseudomonas aeruginosa</i> N = 69 (%)
Surgery	35 (22.3)	16 (23.2)
Medical clinic	24 (15.3)	10 (14.5)
Oncology	14 (8.9)	2 (2.9)
Emergency room	9 (5.7)	5 (7.3)
ICU ^a	50 (31.9)	31 (44.9)
Neonatal ICU	2 (1.3)	0
Pediatric ICU	2 (1.3)	1 (1.5)
Transplant	2 (1.3)	0
Others ^b	3 (1.9)	0

^a Intensive care unit.
^b Burneds, thoracic pain unit.

In this period, 157 non-repetitive patients with *P. aeruginosa* bacteremia were included in the study. The detailed information on factors associated with the development of bacteremia and the clinical and demographic characteristics, as well the distribution of patients by wards are shown in **Tables 1 and 2**. The primary bacteremia occurred in 75.8% and those with unknown focus accounted in 62.4% of the cases. Secondary bacteremia accounted for 24.2% of patients, where the respiratory tract was the main focus of infection (17.2%). Furthermore, 43.9% of patients had bacteremia with carbapenem-resistant *P. aeruginosa*, and most of these patients were admitted to the ICU. Most patients were male (66.8%), with an average hospital stay of 63.2 ± 80.17 days and an average age of 52.01 ± 20.24 years.

The previous use of antibiotics (78.3%), invasive procedures (88.5%), comorbidities (77.7%), and prior surgery (43.9%) were common. It was found that 31.2% of patients received inadequate treatment, and the death rate was 58.6%. Several intrinsic and extrinsic factors for the development of bacteremia by strains of *P. aeruginosa* resistant to carbapenems were detected by univariate analysis. However, only mechanical ventilation, enteral/nasogastric tubes, primary bacteremia with unknown focus, and inappropriate therapy were risk factors independently associated with the development of carbapenem-resistant *P. aeruginosa* bacteremia.

MBL production was analyzed for 56 carbapenem-resistant *P. aeruginosa* isolates. Nine (25.0%) isolates were phenotypically positive and a total of 16.1% ($n = 9/56$) were consistent with the amplicons MBL genes, being 10.71% *bla*_{SPM-1} and 5.3% *bla*_{VIM} genes. In general, the strains showed a multidrug resistance profile. For other virulence genes evaluated (*aprA*, *plcH*, *plcN*, *lasA*, *lasB*, *toxA*, and *algD*), all strains showed a high frequency (88%).

Among the 88 carbapenem-susceptible *P. aeruginosa* strains, 21 (23.9%) were resistant to other β -lactams. For 69 strains resistant to carbapenems, the resistance profile was high: β -lactam 50/69 (72.5%), fluoroquinolones 48/69 (69.6%), and aminoglycosides 50/69 (72.5%) (**Table 3**).

In addition to MBL research, five strains were selected (two containing SPM-1, one containing VIM, and two

Table 2 – Risk factors associated with carbapenem resistance in patients with bacteremia caused by *Pseudomonas aeruginosa*.

Risk factors	Total N = 157 (%)	Carbapenem resistant N = 69 (%)	Multisensitive ^a N = 65 (%)	Univariate		Multivariate	
				OR ^b (CI ^c 95%)	p ^d	OR (CI 95%)	p
Age-mean (years)	52.01 ± 24.49	59.01 ± 20.24	43.18 ± 27.56	–	0.0009 [*]	–	–
Male/female	105/52 (66.88/33.12)	45 (65.22)/24 (34.78)	43 (66.15)/22 (33.85)	0.9593 (0.4698–1.959)	0.9092	–	–
Time of hospitalization, mean (days)	63.2 ± 80.17	60.01 ± 48.97	68.86 ± 110.4	–	0.3477	–	–
Intensive care unit	74 (47.13)	38 (55.07)	22 (33.85)	2.396 (1.190–4.822)	0.0135	–	–
Surgery	69 (43.95)	37 (53.62)	20 (30.77)	2.602 (1.281–5.282)	0.0075 [*]	–	–
Invasive procedures (72 h)	139 (88.53)	64 (92.75)	54 (83.08)	2.607 (0.8527–7.973)	0.0843	–	–
Mechanical ventilation	89 (56.69)	48 (69.56)	28 (43.08)	3.020 (1.485–6.144)	0.002 [*]	3.0674 (1.22–7.73)	0.0175
Tracheostomy	70 (44.58)	43 (62.32)	17 (26.15)	4.670 (2.234–9.759)	<0.0001 [*]	–	–
Urinary catheter	101 (64.33)	52 (75.36)	34 (52.31)	2.789 (1.340–5.804)	0.0054 [*]	–	–
Venous central catheter	125 (79.62)	59 (85.51)	48 (73.85)	2.090 (0.8761–4.984)	0.0926	–	–
Surgical drain	24 (15.29)	14 (20.29)	7 (10.77)	2.109 (0.7918–5.618)	0.1297	–	–
Probes enteral/gastric nutrition	109 (69.43)	54 (78.26)	40 (61.54)	2.250 (1.053–4.810)	0.0345 [*]	3.8347 (1.43–10.26)	0.0074
Hemodialysis	39 (24.84)	27 (39.13)	8 (12.31)	4.580 (1.892–11.09)	0.0004 [*]	–	–
Parenteral nutrition	25 (1.92)	14 (20.29)	8 (12.31)	1.814 (0.7052–4.664)	0.2125	–	–
Comorbidity conditions	122 (77.70)	53 (76.81)	49 (75.38)	1.082 (0.4886–2.394)	0.8465	–	–
Heart failure	35 (22.29)	21 (30.43)	12 (18.46)	1.932 (0.8597–4.343)	0.1079	–	–
Cancer	32 (20.38)	8 (11.59)	17 (26.15)	0.3703 (0.1473–0.9308)	0.0306	–	–
Diabetes mellitus	21 (13.37)	9 (13.04)	7 (10.75)	1.243 (0.4341–3.558)	0.6849	–	–
Chronic renal failure	40 (25.48)	21 (30.43)	16 (24.61)	1.340 (0.6249–2.873)	0.4514	–	–
Human immunodeficiency virus	10 (6.37)	5 (7.25)	3 (4.61)	1.615 (0.3699–7.048)	0.7191	–	–
ASIS ^e score ≥ 4	81 (51.59)	35 (50.72)	33 (50.77)	0.9982 (0.5068–1.966)	0.9959	–	–
Primary bacteremia	119 (75.79)	45 (65.22)	57 (87.69)	0.2632 (0.1080–0.6413)	0.0023 [*]	–	–
Central line catheter related	21 (13.37)	11 (15.94)	7 (10.75)	1.571 (0.5693–4.338)	0.3802	–	–
Unknown	98 (62.42)	34 (49.27)	50 (76.9)	0.2914 (0.1383–0.6143)	0.0009	0.2363 (0.08–0.73)	0.0127
Secondary bacteremia	38 (24.20)	24 (34.78)	8 (12.31)	3.800 (1.559–9.260)	0.0023 [*]	–	–
Respiratory tract	27 (17.19)	17 (24.64)	5 (7.69)	3.923 (1.353–11.37)	0.0081 [*]	–	–
Urinary tract	6 (3.82)	4 (5.79)	1 (1.54)	3.938 (0.4282–36.22)	0.3666	–	–
Inappropriate therapy	49 (31.21)	32 (46.37)	8 (12.31)	6.162 (2.560–14.83)	<0.0001 [*]	8.5534 (3.28–22.32)	<0.0001
Previous use of antibiotics	123 (78.34)	59 (85.51)	43 (66.15)	3.019 (1.297–7.025)	0.0086 [*]	–	–
Mortality	92 (58.59)	48 (69.56)	32 (49.23)	2.357 (1.163–4.779)	0.0165 [*]	2.3660 (1.15–4.85)	0.0186

^a Multisensitive strains were considered as those who presented susceptibility to all antimicrobials tested.

^b Odds ratio.

^c Confidence interval.

^d p value.

^e Average severity of illness score.

* Statistically significant (P ≤ 0.05).

Table 3 – Antimicrobial resistance profiles in samples of *Pseudomonas aeruginosa* isolated from blood.

Antimicrobial	Carbapenems		p (OR)
	Resistant group N = 69 (%)	Susceptible group N = 88 (%)	
Ceftazidime	51 (73.9)	10 (11.4)	<0.0001 (9.446–51.71)
Cefepime	45 (65.2)	17 (19.3)	<0.0001 (3.792–16.17)
Piperacillin/tazobactam	37 (53.6)	12 (13.6)	<0.0001 (3.386–15.84)
Fluorquinolones (ciprofloxacin and levofloxacin)	48 (69.6)	17 (19.3)	<0.0001 (4.568–19.95)
Aminoglicosides (amikacin and gentamicin)	50 (72.5)	12 (13.6)	<0.0001 (7.442–37.32)

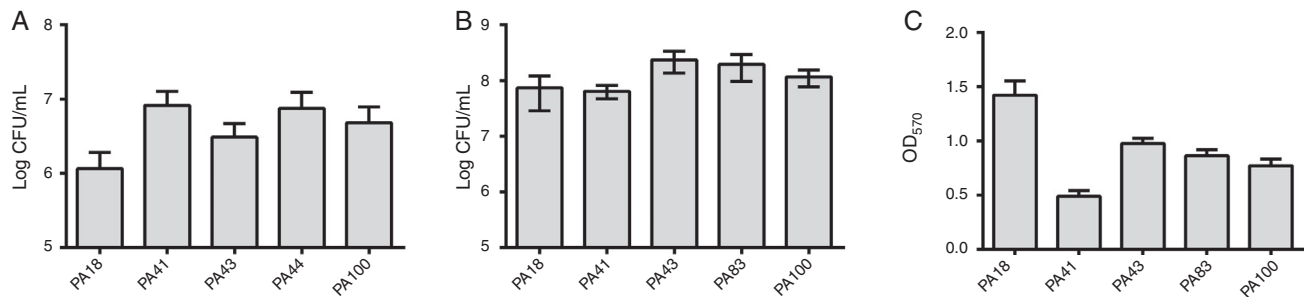


Fig. 1 – (A) Number of cells adhered to a polystyrene surface after 2 h, expressed as log CFU/mL for 5 samples of *Pseudomonas aeruginosa* isolated from blood. (B) Number of viable cells in the biofilm (log CFU/mL). (C) Biomass of biofilm expressed as optical density of crystal violet (OD_{570nm}).

phenotypically positive for MBL) to the evaluation of the biofilm formation using quantitative assays of total biomass by staining with crystal violet. Considering the presence of MBL genes, all strains were identified as strongly biofilm producers with an average of 0.90 ± 0.61 of biomass. Differences in initial adhesion of individual strains were not observed. However, there was a lower OD for the strain containing the *bla*_{VIM} gene (Fig. 1).

Discussion

Hospital bacteremia caused by multiresistant microorganisms, whether Gram-positive or Gram-negative, has often been described as a significant health problem that increases hospital costs and makes it difficult to establish an appropriate antimicrobial therapy, which results in a worse prognosis.³⁷ The high proportion of hospital bacteremia caused by *P. aeruginosa* resistant to carbapenems indicates the importance of this organism as a significant cause of this infection in our hospital. The high proportion of hospital bacteremia caused by *P. aeruginosa* resistant to carbapenems indicates the importance of this organism as a significant cause of this infection in our hospital. The results of different studies have suggested that intrinsic risk factors such as mechanical ventilation, use of a nasogastric tube, and prior use of antibiotics increase the risk of bacteremia development caused by *P. aeruginosa* resistant to carbapenems, as well as the risk of morbidity and mortality.³⁸

In this study, more than 50% of the risk factors were associated with infection by this microorganism according to univariate analysis. However, only mechanical ventilation, enteral/gastric feeding tubes, primary bacteremia of unknown origin, and inappropriate treatment were considered independent risk factors by multivariate analysis.

A central venous catheter is one of the most significant risk factors for acquired bacteremia in the hospital, as it was associated with more than 90% of these infections.³⁹ Although it was not a significant risk factor in this study, 85.5% of patients with bacteremia with carbapenem-resistant *P. aeruginosa* had this invasive procedure.

Often, bacteremia caused by *P. aeruginosa* resistant to antibiotics has a higher mortality rate due, in particular, to the administration of inappropriate antibiotic therapy.⁴⁰ In this study, we investigated a cohort of 157 patients with bacteremia caused by *P. aeruginosa* strains, with 69 of them infected by carbapenem-resistant *P. aeruginosa* isolates. Total mortality was higher in the carbapenem-resistant group; however, the presence of resistant strains was significantly associated with inappropriate antimicrobial therapy, proving that this group usually has a poorer prognosis. Similar to our findings, previous studies showed that *P. aeruginosa* isolated from patients who received inappropriate therapy had a worse prognosis, with rates of 46.1%⁴¹ and 53.8%.⁴²

Besides to be associated with severe infections, resistance to carbapenems in *P. aeruginosa* often results in the production of MBL.⁴³ Our results showed that the carbapenem-resistant *P. aeruginosa* were prevalent throughout the hospital with genes that encoding these enzymes in 16.1% of the strains, being 10.7% of genotype *bla*_{SPM-1} and 5.4% of genotype *bla*_{VIM}. Since the frequency of multidrug-resistant strains was high, these results suggest that other resistance mechanisms coexist in these strains, such as efflux pumps and impermeability of the membrane.⁴⁴

The prevalence of MBL as a resistance mechanism has increased, particularly in Latin America.⁴⁵ In Brazil, the prevalence of MBL-producing *P. aeruginosa* varies between different regions and between hospitals, with rates ranging from 7.5% to 44%.¹¹

SPM-1 enzyme is considered the most common in Brazil, followed by IMP-1,^{11,46} however, there has also been an increase in the frequency of *P. aeruginosa* isolates containing VIM enzyme.^{45,47} In our study, the SPM-1 enzyme was detected in 16.7% of phenotypically producing samples of MBL, followed by VIM enzyme, which was detected in 8.3% of cases. These results indicate a significant spread of MBL-encoding genes in our region.

Considering the presence of MBL and biofilm production, 100% of the strains were classified as strong producers. Another study in Brazil found that 40% of *P. aeruginosa* classified as strong biofilm-producing were also MBL producers.³⁵ We believe that these results are important once these characteristics (biofilm production and MBL) overlap and infections caused by these bacteria are difficult to treat. This is justified, in part, because the growth of bacteria in the biofilm is about 64 times more resistant to antimicrobial.⁴⁸

P. aeruginosa has a large number of extracellular virulence factors that also contribute to the pathogenicity and severity of these infections, such as that encoded by *aprA*, *plcH*, *plcN*, *lasA*, *lasB*, *toxA*, and *algD* genes.^{32,33} Our data showed that the presence of a multiresistant profile, in the most of the strains (88%) were positive for the *aprA*, *plcH*, *plcN*, *lasA*, *lasB*, *toxA*, and *algD* virulence genes, except in one strain that did not show the *toxA* and *plcH* genes, which can further contribute to a worse prognosis associated with severe infections.

Our results confirm previous findings regarding risk factors for the development of *P. aeruginosa* carbapenem-resistant bacteremia, as well as the dissemination of MBL-producing strains of SPM-1 type. However, this study contributes for further evidence of the spread of MBL-producing strains, particularly the VIM type, in highly virulent strains and strongly biofilm producers.

Conflicts of interest

The authors declare no conflicts of interest.

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