

Metallo- β -lactamase and genetic diversity of *Pseudomonas aeruginosa* in intensive care units in Campo Grande, MS, Brazil

ABSTRACT

Infection by *Pseudomonas aeruginosa* has spread worldwide, with limited options for treatment. The purpose of this study was to investigate metallo- β -lactamase-producing *P. aeruginosa* strains and compare their genetic profile using samples collected from patients in intensive care units. Forty *P. aeruginosa* strains were isolated from two public hospitals in Campo Grande, Mato Grosso do Sul State, from January 1st, 2007 to June 31st, 2008. Profiles of antimicrobial susceptibility were determined using the agar diffusion method. Metallo- β -lactamase was investigated using the double-disk diffusion test and PCR. Molecular typing was performed by pulsed-field gel electrophoresis (PFGE). Respiratory and urinary tracts were the most common isolation sites. Of the 40 samples tested, 72.5% (29/40) were resistant to ceftazidime and 92.5% (37/40) to imipenem, whereas 65% (26/40) were resistant to both antimicrobials. Fifteen pan-resistant samples were found. Five percent (2/40) of samples were positive for metallo- β -lactamase on the phenotype test. No metallo- β -lactamase subtype was detected by PCR. Macrorestriction analysis revealed 14 distinct genetic patterns. Based on the superior accuracy of PCR, it can be inferred that *P. aeruginosa* isolates from the investigated hospitals have alternative mechanisms of carbapenem resistance. The results also suggest clonal spread of *P. aeruginosa* between the studied hospitals.

Keywords: *Pseudomonas aeruginosa*; drug resistance, multiple; beta-lactamases; electrophoresis; gel; pulsed-field.

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INTRODUCTION

Pseudomonas aeruginosa, a non-fermenting Gram-negative rod of great clinical and epidemiological relevance in hospital-acquired infections, is more frequently found in intensive care units (ICUs)¹ and is associated with high morbimortality.²

The selective pressure exerted by the indiscriminate use of antimicrobial agents in the last decades might have contributed to the emergence of microorganisms that are resistant to different classes of antibiotics and is currently a matter of worldwide concern.³ Several mechanisms are involved in *P. aeruginosa* resistance to antimicrobial agents, such as chromosomal expression, β -lactamase production, efflux pumps and decrease in membrane permeability.⁴

The currently observed increased resistance to carbapenem has stimulated studies aiming at a better understanding of the resistance mechanisms of *P. aeruginosa*.^{3,5,6} The emergence and dissemination of metallo- β -lactamases (MBL)

have contributed to the high rate of resistance among the *P. aeruginosa* strains isolated in the last decades. The MBL production knowledge is crucial to implement preventive measures that can curb the expansion of bacteria presenting this potent resistance gene.³

The objective of the present study was to investigate metallo- β -lactamase and genetic similarity among *P. aeruginosa* strains isolated in ICUs of two hospitals in the city of Campo Grande, state of Mato Grosso do Sul, Brazil.

MATERIALS AND METHODS

Research site and period

The study was carried out with *P. aeruginosa* strains from patients admitted at adult ICUs, coronary care units (CCUs) and pediatric ICUs (PICUs) of two public hospitals in the state of Mato Grosso do Sul, Brazil, from 01/01/2007 to 06/31/08. Hospital A is a 250-bed university hospital and hospital B is a 360-bed tertiary hospital.

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We declare no conflict of interest.

Inclusion criteria

P. aeruginosa strains resistant to ceftazidime and/or imipenem, isolated from several types of biological material were included in the study. One sample was obtained from each patient and stored in a strain bank at the hospitals. These strains were randomly selected without distinction regarding infection or colonization and were not obtained from surveillance cultures.

Bacterial identification and susceptibility to antibiotic agents

P. aeruginosa isolates were identified using conventional methodology.⁷ The antimicrobial susceptibility was determined by the agar diffusion method, according to the Clinical Laboratory Standard Institute (CLSI) recommendations.⁸ The tested antibiotics were polymyxin – POL (10 µg), tazobactam + piperacillin – TZP (100/10 µg), imipenem – IPM (10 µg), meropenem – MER (10 µg), amikacin – AMI (30 µg), gentamicin – GEN (10 µg), aztreonam – ATM (30 µg), cefepime – CPM (30 µg), ceftazidime – CAZ (30 µg) and ciprofloxacin – CIP (5 µg). *P. aeruginosa* strains sensitive to polymyxin B only were considered pan-resistant.

MBL detection

MBL was detected using a phenotypic disc method as described by Arakawa *et al.*,⁹ with the modifications suggested by Picão *et al.*¹⁰ The samples were suspended in saline solution (0.5 in the McFarland turbidity scale) and seeded with a sterile swab on an Oxoid Mueller-Hinton Agar dish. The ceftazidime (30 µg) and imipenem (10 µg) discs were placed at a 2-cm distance from a disc without antibiotic agent, which received 5 µL of 2-mercaptopropionic acid solution diluted 1:8. After incubation at 37°C for 16 to 18 hours, samples that presented an increase in halo size around the ceftazidime or imipenem discs were considered positive. A standard strain of *P. aeruginosa* (SPM-1, P1088) was used as positive control, which was kindly provided by ALERTA Laboratory - UNIFESP.

P. aeruginosa strains positive for MBL production at the phenotypic test were submitted to conventional PCR.¹¹ The starter oligonucleotides used were: *bla*SPM-1 F (5' CCT ACA ATC TAA CGG CGA CC3') and R (5' TCG CCG TGT CCA GGT ATA AC3'), *bla*IMP-1 F (5' CTA CCG CAG CAG AGT CTT TTG3') and R (5' AAC CAG TTT TGC CTT ACC AT3') *bla*IMP-2 F (5' GTT TTA TGT GTA TGC TTC CTT TGT AGC3') and R (5' CAG CCT GTT CCC ATG TAC G3'), *bla*VIM-1 F (5' GTT TGG TCG CAT ATC GCA AC3') e R (5' AGA CCG CCC GGT AGA CC3'), *bla*VIM-2 F (5' ATG AAA GTG CGT GGA GAC3') and R (5' CTA CTC AAC GAC TGA GCG ATT TGT3').^{12,13}

Pulsed field gel electrophoresis

Genotype determination was carried out by pulsed field gel electrophoresis (PFGE), according to Romao *et al.*¹⁴ Chromosomal DNA was prepared using the *in situ* technique in agarose blocks. A bacterial suspension at the exponential growth phase

was added to a 400 µL of BSC solution (Tris 1 M, pH 8.0, EDTA 0.5 M). After that, 5 µL of proteinase K (Sigma) and 200 µL of 1% agarose (low melting) were added to the cell suspension. The mixture was distributed in molds. The blocks were transferred to a lyse solution (TRIS 1 M, pH 8.0; EDTA 0.5 M, pH 8.0 and 10% N-lauryl sarcosine + 5 µL of proteinase K) and incubated at 50°C for 24 hours. Subsequently, the molds were washed six times with TE buffer at 37°C and incubated with enzyme buffer for one hour at 5°C. After buffer removal, the molds were incubated with restriction enzyme *SpeI* (Invitrogen) at 37°C for 2 hours. The electrophoresis procedure was carried out by PFGE in 1% agarose gel and run with a buffer containing 0.4X Tris Borate EDTA in the CHEF DR II system (BioRad, California). The gel run consisted in a crescent pulse time of 5 to 25s, for 18 hours at 6 V/cm at a temperature of 14°C, with an angle of 120°. The fragments were stained with ethidium bromide and photographed. The band profile was analyzed using the Gel Compar III system (Applied Maths, Belgium). The dendrogram was generated using the UPGMA algorithm.

RESULTS

A total of 40 strains of *P. aeruginosa* were analyzed, of which 12 were isolated from hospital A and 28 from hospital B. Of the total number of samples, 36 came from the adult intensive care unit (ICU), 3 from the coronary care unit (CCU) and 1 from the pediatric ICU. The samples were isolated from several sites: tracheal aspirate (17; 42.5%), urine (13; 32.5%), catheter tip (4; 10%), blood (2; 5%) and others (4; 10%), such as pressure ulcer secretion, sacral ulcer and trochanteric ulcer.

Table 1 shows the percentage of resistance in the presence of the tested antimicrobial agents. Of the total strains, 72.5% (29/40) were resistant to ceftazidime, 92.5% (37/40) to imipenem and 65% (26/40) had simultaneous resistance to both antimicrobials. Approximately 37.5% of the strains were considered pan-resistant. All samples were sensitive *in vitro* to polymyxin B.

Table 1. Antimicrobial resistance of *P. aeruginosa* in ICUs, Campo Grande/MS

Antimicrobial agents	Resistance % (n = 40)
Cefepime	95
Imipenem	92.5
Ciprofloxacin	90
Gentamicin	85
Aztreonam	85
Amikacin	72.5
Ceftazidime	72.5
Meropenem	70
Tazobactam + piperacillin	70
Polymyxin	00

ICUs, intensive care units.

Table 2 shows 17 different bacterial resistance patterns: six were found in hospital A and 14 in hospital B. Three resistance patterns (numbers 1, 8 and 15) were found in both hospitals studied.

The phenotypic test to detect MBL was positive in 5% (2/40) of *P. aeruginosa* isolates; however, none of the genes in-

vestigated (*bla*-IMP, *bla*-VIM and *bla*-SPM) was identified by PCR. The molecular analysis generated by the PFGE disclosed 14 distinct genetic patterns (A to N). Table 2 shows some common profiles (A, B, E, F, J, K) at the two hospital institutions. Profile A was the most frequent (n = 9), of which one strain was from hospital A and eight from hospital B (Table 3).

Table 2. Phenotypic and molecular profile of *P. aeruginosa*, Campo Grande/MS

Profile	Patterns of resistance	Hosp	PFGE	Number of samples
01	PPT/AMI/ATM/CPM/CAZ/CIP/GEN/IPM/MER	HB	A/B/D/E	07/1/1/1
		HA	A/B/J	1/2/1
02	PPT/AMI/ATM/CAZ/CIP/GEN/IPM/MER	HB	G	1
03	PPT/CPM/ATM/CAZ/CIP/GEN/IPM	HB	A	1
04	CIP/GEN/IPM	HB	C	1
05	AMI/CPM/CIP/GEN/IPM	HB	C/E	1/1
06	CPM/GEN/CIP/IPM	HB	C	1
07	PPT/AMI/ATM/CPM/CAZ/CIP/GEN	HB	C	1
08	AMI/ CPM/ ATM/ CIP/ GEN/ IPM/ MER	HB	B/C/K	1/1/1
		HA	B	2
09	PPT/ ATM/CPM/CAZ/IPM	HB	I	1
10	PPT/ ATM/CPM/CAZ/GEN/IPM	HB	D	3
11	PPT/ ATM/CPM/CAZ/GEN	HB	M	1
12	PPT/AMI/CPM/CAZ/CIP/GEN/IPM	HB	J	1
13	ATM/CPM/CAZ/CIP/IPM/MER	HA	N	1
14	PPT/ATM/CPM/CAZ/IPM/MER	HB	C	1
15	PPT/AMI/ATM/CPM/CIP/GEN/IPM/MER	HB	F/H	1/1
		HA	F	1
16	AMI/CPM/CAZ/CIP/GEN/IPM/MER	HA	L	1
17	AMI/ATM/CPM/CAZ/CIP/GEN/IPM/MER	HA	E/K	1/1

PFGE, pulsed field gel electrophoresis; AMI, amikacin; ATM, aztreonam; CAZ, ceftazidime; CIP, ciprofloxacin; CPM, cefepime; GEN, gentamicin; IPM, imipenem; MER, meropenem; PPT, piperacillin + tazobactam; POL, polymyxin; Hosp, hospital; HA, hospital A; HB, hospital B.

Table 3. Period and hospital where *P. aeruginosa* was isolated with PFGE profile A

Register	Hospital	Date	Clinic	Underlying disease	Material
1777	HB	09/01/06	Adult ICU	Kidney disease	U
1694	HB	21/02/06	Adult ICU	Neurological disease	TA
1695	HB	07/03/06	Adult ICU	Neurological disease	TA
1696	HB	17/04/06	Adult ICU	Diabetes <i>mellitus</i>	U
1770	HA	26/6/06	Adult ICU	Heart disease	U
1701	HB	11/07/06	CCU	Respiratory disease	TA
1712	HB	27/11/06	Adult ICU	Liver disease	BL
1718	HB	23/03/07	Adult ICU	AIDS	CT
1726	HB	08/11/07	Adult ICU	Respiratory disease	TA

PFGE, pulsed field gel electrophoresis; ICU, intensive care unit; CCU, coronary care unit; AIDS, acquired immune deficiency syndrome; U, urine; TA, tracheal aspirate; BL, blood; CT, catheter tip; HA, hospital A; HB, hospital B.

DISCUSSION

Patients with preexisting comorbidities, long hospital stay and those submitted to invasive procedures such as catheters and mechanical ventilation make the ICUs propitious places for *P. aeruginosa* dissemination,^{15,16} very often associated with high levels of morbimortality.¹⁷

P. aeruginosa was isolated mainly from materials such as tracheal aspirate and urine, corroborating the literature data reporting that this agent is more often isolated from the lower respiratory and urinary tracts.¹⁸⁻²⁰

Resistance to different classes of antibiotics has been attributed to the combination of multiple mechanisms,⁴ which might explain the diversity of resistance patterns shown in Table 2.

The use of the lipopeptide class, such as polymyxin B and colistin, was abandoned for a while due to the high toxicity, but recently, it has once again become an important therapeutic option for multiresistant microorganism infections.²¹ Supporting this concept at the present investigation, all *P. aeruginosa* strains were sensitive to this drug.

The analysis of the susceptibility profile *in vitro* demonstrated higher levels of resistance in the presence of cefepime, imipenem and ciprofloxacin, drugs used in the treatment of severe *Pseudomonas* infections. On the other hand, as shown in Table 1, tazobactam in combination with piperacillin, meropenem and ceftazidime were the drugs that demonstrated the lowest resistance, suggesting that these drugs, as well as polymyxin, can be used as therapeutic option for *P. aeruginosa* infection in both institutions. The higher resistance of cefepime when compared to ceftazidime might indicate the presence of OXA-31, an oxacillinase that has cefepime as its preferential substrate.²²

The dissemination capacity of MBL that hydrolyses expanded-spectrum β -lactams, including cephalosporins and carbapenems, has been a matter of great concern in the world. Studies in several parts of the world have demonstrated varied rates of MBL production by *P. aeruginosa*, such as 6.2% in Korea,⁶ 35% in Canada,⁵ and 62% in Greece.²³ In Brazil, the SPM subtype has been described as the most prevalent in several regions of the country. National data have reported different rates of MBL production, which vary according to the institution and the studied region. As an example, one could cite rates of 10.9% in hospitals in Rio Grande do Sul,²⁴ 20% in Rio de Janeiro – RJ,²⁵ and 41.9% in the state of Goiás.²⁶ Figueiredo-Mendes *et al.*²⁷ described higher rates of this enzyme production (77.8%) among *P. aeruginosa* strains previously resistant to carbapenems in hospitals in São Paulo and Brasília.

Different from what is observed in other Brazilian regions, our series did not show the presence of MBL-producing *P. aeruginosa*. Further studies with larger sample sizes are necessary to detect the emergence of this important resistance gene in hospitals in the state of Mato Grosso do Sul.

The fact that the genes *bla*-SPM, *bla*-IMP and *bla*-VIM were not detected even in samples with a positive phenotypic test for MBL suggests the unreliability of the phenotypic test that was applied.

The finding of identical genetic profiles generated by PFGE in the two hospitals suggests an inter-hospital dissemination of *P. aeruginosa*. The high rate of healthcare professional and patient turnover between the hospitals and hospital wards might have contributed to these results.

The distribution of *P. aeruginosa* with A profile (Table 3) suggests inter-hospital transmission, as it was initially found in the adult ICU and it was subsequently found at the CCU of hospital B. This clone (A profile) was also isolated at different periods, which might indicate that it is endemic in this hospital. It is noteworthy the fact that many strains with this profile were sensitive only to polymyxin B, which shows high resistance of strains with this genetic pattern.

CONCLUSION

The results suggest that the resistance of *P. aeruginosa* to carbapenem agents in the present study is due to resistance mechanisms other than MBL production. The inter-hospital and intra-hospital clonal dissemination is a matter of concern and must be contained through preventive hospital infection control measures. Considering the emergent characteristic of MBL, its surveillance becomes important to control the transmission of this resistance mechanism.

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