Short Communication

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Phenotypic detection of metallo-β-lactamases in *Pseudomonas aeruginosa* and *Acinetobacter baumannii* isolated from hospitalized patients in São Luis, State of Maranhão, Brazil

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ABSTRACT

Introduction: Acquired metallo-β-lactamases (MβL) are emerging determinants of resistance in *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. The objectives of this study were to phenotypically detect MβL in imipenem-resistant *P. aeruginosa* and *A. baumannii*, to investigate the association between MβL-positive strains and hospitals, and to compare the resistance profiles of MβL-producing and non-MβL-producing strains. **Methods**: The approximation disk and combined disk assay methods were used in this study. **Results:** A total of 18 (38.3%) *P. aeruginosa* isolates and 1 (5.6%) *A. baumannii* isolate tested positive for the presence of MβL. **Conclusions:** These results demonstrate the need for strict surveillance and for the adoption of preventive measures to reduce the spread of infection and potential outbreaks of disease caused by MβL-producing microorganisms.

Keywords: Metallo-β-lactamases. *Pseudomonas aeruginosa. Acinetobacter baumannii*.

Metallo-β-lactamase (MβL) activity has emerged as one of the most feared resistance mechanisms because of the ability of MβLs to hydrolyze virtually all β-lactam agents, including carbapenems. However, MβLs are unable to hydrolyze monobactams because their genes are carried on highly mobile elements. The prevalence of metallo-β-lactamase-producing *Pseudomonas aeruginosa* (MPPa) causing nosocomial infections has been increasing worldwide[1].

Data from the SENTRY Antimicrobial Surveillance Program suggest 44.8% of *P. aeruginosa* isolates in Brazil are resistant to imipenem, and 43.9% of these isolates produce MβL[2].

The objectives of the present study were to phenotypically detect MβL in imipenem-resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii* isolates, to investigate the association between MβL-positive strains and the hospitals studied, and to compare the resistance profiles of MβL-producing and non-MβL-producing strains.

A total of 129 consecutive *P. aeruginosa* and 71 *A. baumannii* isolates were recovered between June and November 2008. The strains were isolated from different clinical samples obtained from 2 private hospitals (Hospital 1: 100 beds and Hospital 3: 164 beds) and 1 public hospital (Hospital 2: 121 beds) in São Luis, State of Maranhão, northeastern Brazil. All isolates were identified both by conventional techniques and by the automated Vitek 2 system (BioMérieux®, Marcy l'Etoile, France). Among those isolates, 47 (36.4%) *P. aeruginosa* isolates and 18 (25.4%) *A. baumannii* isolates were resistant to imipenem as determined by the Clinical and Laboratory Standards Institute (CLSI) disk diffusion method (inhibition zone of ≤13mm or MIC ≥16μg/mL); these isolates were characterized.

The disk diffusion method (Kirby-Bauer) was used for antimicrobial susceptibility testing according to the recommendations of the CLSI found in performance standard M100-S22 (2012). Quality control testing was performed using *P. aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922.

The disk approximation test was performed according to the method of Arakawa et al.[3], with modifications to the inhibitor volumes and concentrations. For the detection of MβL production by *P. aeruginosa*, 2 ceftazidime disks (30μg; Oxoid®, Basingstoke, England) were used as the substrates and were placed at center-to-center distances of 2.0 and 1.5cm from 2 un-impregnated disks. Next, 4μL of 2-mercaptopropionic acid (2-MPA, 1.4mM; Sigma-Aldrich®, Steinheim, Germany) was added to the first disk, and 4μL of ethylenediaminetetraacetic acid (EDTA, 400mM; Sigma-Aldrich®, Steinheim, Germany) was added to the second disk. This assay was repeated for *A. baumannii* using imipenem (10μg) as the substrate and 4μL of 2-MPA (1.4mM) and 8μL of EDTA (200mM) as inhibitors. The plates were incubated for 18-24h at 35ºC.

For the combined disk assay, the inhibitor concentrations, pipetted volumes, and cut-off values for the differentiation of producers and non-producers of MβL were determined

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according to Picão et al.[4]. Three ceftazidime (CAZ) disks were placed on the surface of Mueller-Hinton plates that were inoculated with a suspension of *P. aeruginosa*. Next, 8µL of 2-MPA (1.4mM) was added to the first disk, 8µL of EDTA (100mM) was added to the second disk, and no inhibitor was added to the third disk to determine the diameter of the halo formed in the absence of an inhibitor. This assay was repeated for *A. baumannii* using imipenem+2-MPA (4µL, 1.4mM) and imipenem+EDTA (4µL, 400mM). The plates were incubated for 18-24h at 35ºC. The diameter of the inhibition halo produced by the β-lactam disks that contained the inhibitors was compared to the halo diameter produced by the antibiotic-only disk. The result was defined as positive when, compared to the antibiotic-only disk, the increase in the diameters produced by the EDTA+CAZ or 2-MPA+CAZ disks was greater than 8 or 14mm, respectively. For *A. baumannii*, MβL production was defined as an increase in the diameter of the inhibition halo of > 4mm for imipenem+2-MPA and > for imipenem+EDTA compared to the imipenem-only disk.

Pseudomonas aeruginosa ATCC 27853 was used as the negative control. *P. aeruginosa* (PSA319) and *A. baumannii* strains provided by the Laboratory of Clinical Microbiology, Universidade Federal de São Paulo (LEMC), and the University Hospital of Universidade Federal de Santa Catarina, respectively, were used as positive controls for MβL production.

The results were analyzed using the Statistical Package for the Social Sciences (SPSS) for Windows, version 20.0 (2012). The chi-squared test for independent samples was applied to evaluate the association of MPPa or non-MβL-producing *P. aeruginosa* (NPPa) strains with each hospital and with antibiotic resistance. A level of significance of 5% ($p<0.05$) was adopted for all tests.

Strains of *P. aeruginosa* resistant to imipenem were recovered from 68 (52.7%) tracheal secretion samples, 18 (14%) urine samples, 10 (7.8%) catheter tip samples, 9 (7%) blood samples, 7 (5.4%) samples from sores/wounds, and 17 (13.1%) other biological samples. For isolates of *A. baumannii* that were resistant to imipenem, the most frequent site of strain isolation was tracheal secretion, 40 (56.3%); followed by the catheter tip, 12 (16.9%); blood, 5 (7%); urine, 5 (7%); and other biological samples, 9 (12.8%).

The imipenem resistance profiles of *P. aeruginosa* and *A. baumannii* did not differ significantly among the hospitals studied **(Table 1),** and the phenotypic detection of MβL was positive in 18 (38.3%) *P. aeruginosa* isolates and 1 (5.6%) *A. baumannii* isolate. The results from the 2 methods used were in agreement.

A total of 12 (66.7%) MPPa isolates were collected from Hospital 1, and 6 (33.3%) were collected from Hospital 3. Furthermore, there was a significant difference in the proportion of MPPa and NPPa among the 3 hospitals (p=0.0016).

The highest percentage of MPPa isolation was derived from catheter tips, 6 (33.3%); followed by urine, 5 (27.8%); tracheal secretions, 3 (16.7%); bronchoalveolar lavage fluid, 1 (5.6%); peritoneal fluids, 1 (5.6%); blood, 1 (5.6%); and nose secretions, 1 (5.6%).

In total, 62.1%, 48.3 and 89.7% of the NPPa isolates were resistant to cefepime, ceftazidime, and meropenem, respectively; all MPPa isolates were resistant to these three compounds. The resistance rate was higher among MPPa isolates, with significant differences for amikacin (p=0.0264), cefepime (p=0.0085), ceftazidime (p=0.0007), and piperacillin/tazobactam (p=0.0119). Aztreonam resistance was higher in the NPPa group (p=0.0196). None of the *P. aeruginosa* isolates were resistant to polymyxin B **(Table 2)**.

The only strain of MβL-producing *A. baumannii* (MPAb) was isolated from the blood sample of an Intensive Care Unit patient (ICU) from Hospital 1. This strain was resistant to amikacin, gentamicin, cefepime, ceftazidime, imipenem, meropenem, and ciprofloxacin and was susceptible to ampicillin/sulbactam and piperacillin/tazobactam.

Multidrug-resistant *P. aeruginosa* and *Acinetobacter* strains are becoming a worldwide problem. SENTRY data have revealed that the prevalence of antimicrobial resistance among *P. aeruginosa* isolates has been increasing in Latin American medical centers[2]. The arrival of MβL emphasizes the importance of phenotypic investigations for the presence of MβL in routine laboratory tests[5].

Monitoring programs in Brazil have indicated that the prevalence of *P. aeruginosa* isolates that are resistant to

TABLE 1 - Imipenem resistance profiles of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* strains isolated between June and November 2008, from 3 hospitals in São Luis, State of Maranhão, Brazil.

	Pseudomonas aeruginosa								*Acinetobacter baumannii*							
	resistant		intermediate		susceptible		total		resistant		intermediate		susceptible		total	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
Hospital 1	19	35.2	6	11.1	29	53.7	54	100.0	8	30.8	0	0.0	18	69.2	26	100.0
Hospital 2	19	38.8	0	0.0	30	61.2	49	100.0	1	9.1	0	0.0	10	90.9	11	100.0
Hospital 3	9	34.6	0	0.0	17	65.4	26	100.0	9	26.5	0	0.0	25	73.5	34	100.0
Total	47	36.4	6	4.7	76	58.9	129	100.0	18	25.4	0	0.0	53	74.6	71	100.0
χ^2	8.93								1.96							
P	0.0628								0.3748							

TABLE 2 - The chi-squared test for independent samples comparing the antibiotic resistance profiles of MβL-producing and non-MβL-producing *Pseudomonas aeruginosa* strains isolated between June and November 2008 from 3 hospitals in São Luis, State of Maranhão, Brazil.

Antibiotic	MPPa (%)	NPPa (%)	χ^2	p
Amikacin (30µg)	77.8	44.8	4.93	0.0264
Aztreonam (30µg)	5.6	41.4	5.44	0.0196
Cefepime (30µg)	100.0	62.1	6.92	0.0085
Ceftazidime (30µg)	100.0	48.3	11.39	0.0007
Ciprofloxacin (5µg)	94.4	75.9	2.71	0.0994
Gentamicin (10µg)	88.9	72.4	1.80	0.1797
Meropenem (10µg)	100.0	89.7	0.63	0.4257
Piperacillin/tazobactam (100/10µg)	72.2	34.5	6.33	0.0119

MPPa: metallo-β-lactamase-producing *Pseudomonas aeruginosa*; NPPa: non-metallo-β-lactamase-producing *Pseudomonas aeruginosa.*

carbapenems is between 12% and 19%, and 6% to 9% of the *Acinetobacter* spp. isolates are also resistant[6]. The proportions of MPPa and MPAb have been reported in a few studies in Brazil, but all such studies were restricted to the southeastern or southern regions of the country[5,7-10].

Epidemiological studies tracking the prevalence of MβL-producing microorganisms in northeastern Brazil are scarce[11,12]; therefore, it is necessary to adopt preventive measures and to establish effective treatment protocols that are appropriate for each hospital and region.

Machado et al.[5] detected 17.4% MPPa and 6.3% MPAb in a hospital in the State of Rio Grande do Sul, while Graf et al.[8] detected 36% MPPa in that the same state. In Hospital São Paulo, State of São Paulo, Sader et al.[9] found a prevalence of 19.7% MPPa in blood samples. Cipriano et al.[13] detected the presence of the São Paulo Metallo-β-lactamase gene (SPM) in strains of *P. aeruginosa* from the same city examined in the current study. Regarding *Acinetobacter* spp., Tognin et al.[14] found that the prevalence of MβL producers in São Paulo Hospital rose from 0% between 1993 and 1997 to 29% in 1998 and finally to 100% between 1999 and 2001. In our study, the proportion of MPPa was 38.3%, which is high compared to other states. For *A. baumannii*, we determined the rate of MβL producers was 5.6%, which is similar to the value reported by Machado et al.[5].

The higher prevalence of resistance to cefepime, ceftazidime, ciprofloxacin, meropenem, amikacin, gentamicin, and piperacillin/tazobactam observed in the MPPa group is consistent with the results of Machado et al.[5] and Zavascki et al.[7]; however, resistance in the NPPa group for our study was higher than the values reported by these authors. Normally, *P. aeruginosa* isolates are resistant to β-lactam antibiotics because of the hyperproduction of AmpC β-lactamase. In addition, the presence of an efflux pump and the alteration of the outer membrane permeability may play important roles by restricting the access of antimicrobial agents to their intracellular targets. These resistance mechanisms developed by *P. aeruginosa* may explain the high prevalence of resistance among non-MβL-producing strains that was observed in this study. The resistance of MPPa to aminoglycosides was most likely due to the spread of other resistance determinants that occur concomitantly with the dissemination of MβL, such as the expression of genes that encode aminoglycoside-modifying enzymes in class 1 integrons. These factors explain, in part, the cases of multidrug resistance[1]. There was a statistically significant difference in the proportion of resistance to the antibiotics amikacin, aztreonam, cefepime, ceftazidime, and piperacillin/tazobactam between the MPPa and NPPa groups (**Table 2**).

Although the literature states that MβL-producing microorganisms hydrolyze all commercially available ß-lactams except aztreonam, we identified 1 isolate that was completely resistant and 3 isolates that were partially resistant to this antibiotic. In addition, all isolates tested positive for MβL. The isolate identified here may be the same clone as reported by Cipriano et al.[13] because the present study was conducted in the same city (although during a different period of time).

The MβL-producing *A. baumannii* isolate detected in our study exhibited resistance to most antimicrobials, limiting the therapeutic options to ampicillin-sulbactam and piperacillin-tazobactam among the antibiotics tested. The emergence of carbapenem resistance has limited the therapeutic options for *A. baumannii* infections to polymyxins and ampicillin/sulbactam[14]. MPPa and MPAb are isolated with increasing frequency in Brazil and worldwide; therefore, understanding the prevalence and resistance of these strains has become critically important. These microorganisms are the etiological agents of most ICU-acquired infections, particularly infections of the respiratory tract; these infections are typically severe, and there are limited therapeutic options to treat them. These infections are directly responsible for the high rates of morbidity and mortality and the prolongation of hospital stays with concomitant high hospital costs. This scenario demonstrates the need for strict surveillance by infection control committees in the health care community and for the adoption of preventive measures to reduce the spread of infection and potential outbreaks caused by MβL-producing microorganisms.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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