Metallo-β-lactamase-producing *Pseudomonas aeruginosa* in two hospitals from Southern Brazil

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This study determined the prevalence of metallo-β-lactamase (MBL)-producing *Pseudomonas aeruginosa* in two hospitals located in the Southern part of Brazil and compare the performance of two different phenotypic tests. Thirty-one non-repetitive *Pseudomonas aeruginosa* isolates from various clinical samples from patients admitted to two hospitals located in Rio Grande do Sul, Brazil (twenty-three from a hospital in Porto Alegre City and eight isolates from a hospital in Vale dos Sinos Region). All strains suggestive of possessing MBLs by phenotypic methods were included in this study. Phenotypic detection of MBLs was carried out simultaneously by using both the MBL Etest® and disk approximation test using 2-mercaptopropionic acid close to a ceftazidime disk. Strains positive were further confirmed using molecular techniques for bla_{VIM}, bla_{IMP} and bla_{SPM-1}. The prevalence of MBLs from samples of inpatients from the hospital located in Porto Alegre was 30.4% and that of inpatients from Vale dos Sinos hospital was only 3.1%. Only MBL type SPM-1 was detected in these samples by molecular analysis and all were detected by the Etest® MBL strips. The prevalence of *P. aeruginosa* that produce MBLs can be markedly different in distinct geographical areas, even among different hospitals in the same area. In our study, the EDTA-based method was the only method able to detect all strains harboring the SPM-1 enzyme.

Key-Words: Metallo-beta-lactamase, *Pseudomonas aeruginosa*, multiresistance.

Pseudomonas aeruginosa is a Gram-negative rod unable to ferment glucose and is widely known as an opportunistic organism, frequently involved in infections of immunosuppressed patients, and also causes outbreaks of hospital-acquired infections [1]. *P. aeruginosa* is very commonly isolated from nosocomial infections and is the most prevalent bacterial agent isolated from cases of pneumonia in Brazilian hospitals. This organism is clinically important since it possesses several virulence factors and is intrinsically resistant to most antimicrobial and disinfectant agents, a feature that is also responsible for the difficulty in treating infected patients [2].

Carbapenems are generally used as an option to treat serious infections caused by *P. aeruginosa*, since these drugs present a good spectrum of activity and are stable to hydrolysis by most β-lactamases, including the extended spectrum b-lactamases (ESBL). However, the use of carbapenems has been hampered by the emergence of strains that produce metallo-β-lactamase (MBL), an enzyme that is able to hydrolyze and inactivate this class of antibiotics. Moreover, the spread of MBL-producing clones, even over distant regions, has been reported [2].

Among β-lactamases, MBLs are unique in requiring the presence of zinc ion in the active site of the enzyme, and are, thus, inhibited by chelating agents such as EDTA [3].

Since the early 90s, new genes coding for distinct MBLs have been described in clinical important pathogens like *Pseudomonas* spp., *Acinetobacter* spp. and even among members of the family *Enterobacteriaceae*. These genes are usually inserted in mobile elements facilitating the exchange of these resistance genes among several bacterial species [4]. Several MBLs were reported, including: IMP, VIM, SPM, GIM and, more recently, SIM-1, encoded by the bla_{SIM-1} gene in isolates of *A. baumannii* from South Korea [5].

Prompt detection and recognition of the MBLs is important to implement adequate counter-measures to control the spread of the organisms bearing these enzymes, and proper treatment of infections caused by MBL-producing microorganisms. A few phenotypic methods have been published for MBL detection; however, the results have shown that no method alone is able to detect all these enzymes, probably due to the genetic variability of these enzymes [6]. Molecular methods are more sensitive and specific for the detection of MBLs, but they may still be technically demanding and non available in most clinical microbiology laboratories.

We sought to determine the prevalence of MBL in *P. aeruginosa* strains from two South Brazilian hospitals and compare the efficacy of two different phenotypic methods to detect MBLs in this organism.

Material and Methods

Thirty-one clinical isolates of carbapenem-resistant *P. aeruginosa* were collected from two hospitals in Southern Brazil. Identification of the isolates was confirmed using conventional techniques [7] and disk diffusion susceptibilities were determined according to the CLSI methodology [8]. Only one isolate per patient was included and those isolates from cystic fibrosis patients were excluded from this study.

The following antimicrobial agents were included: aztreonam (ATM), cefepime (CPM), ceftazidime (CAZ) and imipenem (IPM), all from Cecon, São Paulo, SP, Brazil. Strains classified as resistant or intermediate to these drugs were included in this study. The production of MBL was screened for using two different methods, simultaneously: (i) using the

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approximation test with CAZ disk close to a 2-mercaptopropionic acid (2-MPA, Sigma-Aldrich**,** Buchs, United States) and 3 μL of the original reagent was used to impregnate a blank disk, exactly as described by Arakawa et al. (2000) [9]. The 2-MPA-containig disk was placed 25 mm apart (center to center) from a ceftazidime disk on a Mueller-Hinton agar plate. An inhibition zone observed close to these disks were considered positive for the presence of MBL.

The second method consisted of an Etest® MBL-detection strip (AB Biodisk**,** Dalvägen, Solna, Sweden) placed onto the same agar plate, which was considered positive for MBL whenever the minimal inhibitory concentration (MIC) of imipenem plus EDTA decreased at least three dilutions as compared to the imipenem MIC alone [10]. A strain of *P. aeruginosa* known to produce SPM-1 metallo-beta-lactamase was used as a positive control in all cases. This strain has previously been characterized in Laboratório Weinmann (Porto Alegre, Brazil) by PCR with primers specific for $bla_{SPM,}$ as described by Gales et al. (2003) [2], and further confirmed by direct DNA sequencing of the generated PCR products. A MBL negative strain (ATCC 27853; distributed by Oxoid, Basingstoke, Hampshire, England) was also used as a negative control.

All strains suggestive of possessing MBLs by either of the phenotypic tests were further confirmed using molecular techniques. SPM-1 was detected using the polymerase chain reaction (PCR) and specific primers as described above. Two other clinical strains of *P. aeruginosa* positive for MBL and confirmed to harbor bla_{VIM} and bla_{IMP} by specific PCR, as described previously by Pitout et al. [11], and further checked by DNA sequencing. All PCR tests were conducted in appropriated rooms to prevent cross-contamination and proper positive and negative controls were used all the time.

Results

Eight *P. aeruginosa* strains isolated from the Vale dos Sinos hospital fulfilled the criteria for inclusion in this study: resistance to CAZ, CPM and/or IPM. Among these 3 (37.5%) were considered resistant and 1 (12.5%) intermediate to ceftazidime, 4 (50%) were resistant to cefepime, 2 (25%) resistant to aztreonam and only 1 (12.5%) showed resistance to imipenem.

The frequency of MBL in this institution was considered low (3.1%), and the single MBL-positive strain was detected by the Etest-MBL® alone. None of the isolates were from intensive care unit (ICU) patients. The isolates were from diverse clinical samples, such as urine (50%), sputum (25%), tracheal aspirates (12.5%) and skin ulcers (12.5%). The MBL-positive *P. aeruginosa* strain was from a skin ulcer and presented classical phenotypical features compatible with the presence of MBL, i. e., resistance to CAZ, CPM and IPM and susceptibility to ATM.

Among the 23 strains isolated in Porto Alegre, 7 (30.4%) showed phenotypic evidences of MBL production and all were detected only with the E-test method (Table 1). Thirteen strains were isolated from ICU patients (56.5%), and among these, 5 were positive for MBL production; however, this finding was not considered statistically significant by the Qui-square test.

The strains of *P. aeruginosa* isolated in Porto Alegre were from clinical samples such as tracheal aspirates (69.6%), sputum (17.4%), urine (8.7%) and one was from an unknown source (4.3%).

Interestingly, none of the 7 MBL-producing strains could be detected by the CAZ/2-MPA disks, as proposed by Arakawa et al. (2000) [9]. However, all strains were considered positive for MBL production by the E-test strip.

According to Marra et al. (2006) [12] and Magalhães et al. (2005) [13], the SPM-1 enzyme, which is highly prevalent in Brazil, could be equally detected by the 2-MPA method and the EDTA-based method. Zavascki et al. (2005) [14] reported that 77.1% of their *P. aeruginosa* isolates, resistant to IPM, could be detected by 2-MPA and 87.5% of the strains carried the bla_{SPM-1} gene. Similarly, another study by Zavascki et al. (2006) [15] reported the detection of 28.9% of MBL-positive *P. aeruginosa*, containing the bla_{SPM-1} gene, when the 2-MPA method was used to screen for the presence of this enzyme. In our study, 19.3% (6 strains) were positive for the presence of SPM-1, as confirmed by PCR analysis, but none could be detected using the phenotypic test proposed by Arakawa et al. (2000) [9]. Interestingly, in the remaining 2 strains, which were clearly MBL positive by phenotypic test (Etest), no other beta-lactamase gene could be found by PCR as specific primers ($bla_{SPM-1,}$ bla_{IMP} or bla_{VIM}), suggesting that maybe another kind of MBL gene(s) may be involved in these strains. It is known that EDTA may increase bacterial cell-wall permeability and that zinc (chelated by EDTA) accelerates imipenem decomposition and decreases OprD expression of *P. aeruginosa* [16]. Another alternative would be the presence of OXA-10 and/or OXA-14 stabilized in the dimeric form by metal ions (Zn^{++2}). Dimeric forms are more active than the monomeric form, increasing its enzymatic activity turning the enzyme more efficient to inactivate carbapenems and other beta-lactam antibiotics. However, in the presence of a chelator agent, such as EDTA, the OXA class of enzymes is converted to their monovalent forms, which are less efficient in their activity against carbapenems. For these reasons, it is advisable to be cautious when interpreting the results of tests that use EDTA, as the E-Test MBL [17].

Detailed molecular knowledge of the MBLs involved may be necessary to better understand the reasons for success or failure of the phenotypic methods used for MBL screening.

Considering the susceptibility profile of our isolates, all MBL-positive samples were resistant to IPM, CAZ and CPM, including three of these samples also resistant to ATM. The possible explanation for this event is the concomitant presence of another extended-spectrum beta-lactamase (Such as the Ambler's class A). Similar results were found in a work by Marra and coworkers (2006) [12], where, among four *P. aeruginosa* isolates positive for SPM-1, only two were resistant to ATM, while all strains were resistant to CPM and IPM. In this study, the authors also found isolates harboring the IMP-16, which were equally resistant to ATM.

Table 1. Clinical and microbiological characteristics of imipenem-resistant clinical isolates of *Pseudomonas aeruginosa.*

Strain	Hospital	Sample	Diffusion disk[a] ATM/CAZ/CFP/IMP	DDT[b]	E-Test[c] IP/IP+EDTA≥8	PCR *bla* gene
1	VS	skin ulcer	S/R/R/R	-	+	SPM
2	PA	tracheal aspirate	R/R/R/R	-	+	SPM
3	PA	tracheal aspirate	R/R/R/R	-	+	SPM
4	PA	sputum	S/R/R/R	-	"Ghost zone"	SPM
5	PA	tracheal aspirate	R/R/R/R	-	+	SPM
6	PA	tracheal aspirate	S/R/R/R	-	+	SPM
Control	-	-	S/R/R/R	+	+	SPM

[a]ATM: aztreonam; CAZ: ceftazidime; CFP: cefepime; IMP: imipenem. S: susceptible, R: resistant.[b]Double-disk test (DDT) was performed using disks containing the 2-mercaptopropionic acid and ceftazidime as inhibitor and substrate, respectively. (-) negative; (+) positive.[c]The MBL presence is considered when MIC IP/IP+EDTA =8. Ghost zone: suggestive of MBL production.
VS: Vale dos Sinos; PA: Porto Alegre.

In the present study, among the strains negative for the presence of MBLs, two were resistant only to IMP. The susceptibility to the others β-lactamic agents, such as CAZ, and resistance to IMP could be explained by a mechanism involving porin loss. According to Troillet et al. (1997) [18], this discrepancy can be explained by the fact that carbapenems use a specific porin to enter the bacterial cell (porin OprD), while other β-lactamic agents use other kinds of porins. In this way, the loss of OprD alone would result in an increased MIC for IMP only. In this case, it would be possible to use the results of the susceptibility test to guide the therapeutic approach used to treat the infected patient.

Conclusion

In conclusion, we demonstrated here that some strains of *P. aeruginosa* producing the SPM-1 type of MBL are not detected by phenotypic tests based on the disk-approximation method (e.g. CAZ plus 2-MPA), suggesting that the use of EDTA-based tests, or a combination of tests, would be preferred to increase the sensitivity of these methods to detect the presence of MBL among clinical isolates of *P. aeruginosa*.

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