CLINICAL SCIENCE

Metallo-beta-lactamases among imipenem-resistant Pseudomonas aeruginosa in a brazilian university hospital

Maria Renata Gomes Franco, I Hélio Hehl Caiaffa-Filho, II Marcelo Nascimento Burattini, III Flávia Rossi

I Divisão de Laboratório Central, Microbiologia, Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo, São Paulo, SP, Brazil.
II Divisão de Laboratório Central, Biologia Molecular, Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo, São Paulo, SP, Brazil.
III Patologia, Faculdade de Medicina da Universidade de São Paulo, São Paulo, SP, Brazil.

INTRODUCTION: Imipenem-resistant Pseudomonas aeruginosa resulting from metallo-β-lactamases has been reported to be an important cause of nosocomial infection and is a critical therapeutic problem worldwide, especially in the case of bacteremia.

OBJECTIVES: To determine the frequency of metallo-β-lactamases among imipenem-resistant Pseudomonas aeruginosa isolates and to compare methods of phenotypic and molecular detection.

METHODS: During 2006, 69 imipenem-resistant Pseudomonas aeruginosa samples were isolated from blood and tested for metallo-β-lactamase production using phenotypic methods. Minimal Inhibitory Concentration (MIC) (μg/mL) was determined with commercial microdilution panels. Pulsed Field Gel Electrophoresis (PFGE) was performed among metallo-β-lactamase producers.

RESULTS: Of all the blood isolates, 34.5% were found to be imipenem-resistant Pseudomonas aeruginosa. Positive phenotypic tests for metallo-β-lactamases ranged from 28%-77%, and Polymerase Chain Reaction (PCR) were positive in 30% (of note, 81% of those samples were bla_SPM-1 and 19% were bla_VIM-2). Ethylenediamine tetracetic acid (EDTA) combinations for the detected enzymes had low kappa values; thus, care should be taken when use it as a phenotypic indicator of MBL. Despite a very resistant antibiogram, four isolates demonstrated the worrisome finding of a colistin MIC in the resistant range. PFGE showed a clonal pattern.

CONCLUSION: Metallo-β-lactamases among imipenem-resistant Pseudomonas aeruginosa were detected in 30.4% of imipenem-resistant Pseudomonas aeruginosa isolates. This number might have been higher if other genes were included. SPM-1 was the predominant enzyme found. Phenotypic tests with low kappa values could be misleading when testing for metallo-β-lactamases. Polymerase Chain Reaction detection remains the gold standard.

KEYWORDS: Pseudomonas aeruginosa; imipenem resistance; metallo-β-lactamases (MBL); MBL detection; SPM-1.
cerus, Aeromonas spp., and Stenotrophomonas maltophilia. Lately, there has been a dramatic increase in the detection and spread of acquired and transferrable families of these metallo-enzymes (IMP, VIM, SPM, GIM, SIM and AIM enzymes). Brazilian surveillance studies among PA MBL producers show SPM-1 as the most prevalent enzyme. Prior to the present study, we did not have information on enzyme prevalence in our institution.

MBL standardization tests using routine phenotypic detection are still controversial. This study aimed to determine the MBL frequency among IRPA isolates in our institution and to compare phenotypic and molecular methods of detection.

METHODS

This study was carried out at the Hospital das Clínicas da Universidade de São Paulo, a 2500-bed university hospital located in São Paulo, Brazil. All patients for whom blood culture (BACTEC 9240® - Becton Dickinson®; USA) results were positive for IRPA in 2006 were eligible for inclusion in the study. Each patient was included only once. Antimicrobial Susceptibility Testing (AST) was performed by the disk diffusion method, following Clinical and Laboratory Standards Institute (CLSI) recommendations. All IRPAs were stored at -70°C in TSB glycerol. MIC was determined with Microscan Neg Combo Panel Type 32 (Dade Behring®, USA), and the following drugs were analyzed: aztreonam, ceftazidime, cefepime, amikacin, gentamicin, tobramycin, imipenem, meropenem, ciprofloxacin, piperacillin, piperacillin/tazobactam and ticarcillin/clavulanic acid. MICs were read manually according to CLSI recommendations. Colistin MICs (µg/mL) were determined by Etest® (AB Biodisk®, USA) and were also interpreted according to CLSI recommendations. Agar dilution was performed in the case of MIC ≥ 2 µg/mL.

Pseudomonas aeruginosa ATCC 27853, Escherichia coli ATCC 25922 and Enterococcus faecalis ATCC 29212 were used as quality controls, again according to CLSI recommendations.

MBL phenotypic tests

Phenotypic tests for MBL were performed using three methods:

1. Double Disk Synergy (DDS) was performed according to Arakawa et al. with slight modifications taken from Mendes et al. The inoculum was adjusted to a 0.5 McFarland standard, and a 150-mm Mueller-Hinton (MH) agar plate (OXOID®, USA) was inoculated. Three blank filter paper disks were treated with 3 µL of mercaaptopropionic acid (MAA), 3 µL of 2-mercaptoethanesulfonic acid (MPS) (both using an undiluted solution), and 5 µL of EDTA (100 mM) and placed on the center of the MH plate. Imipenem (10 µg) and ceftazidime (10 µg) disks (OXOID®, USA) were placed 20 mm away from the MPA and MAA disks and 10 mm away from the EDTA disk (measured center-to-center). After overnight incubation at 35°C, any synergistic inhibition zone was interpreted to be positive by the MBL screening test.

2. Etest® MBL commercial strips (AB Biodisk®, USA) with a wide range of imipenem concentrations (MICs: 4–256 µg/mL) were overlaid with a constant EDTA concentration (MICs: 1-64 µg/mL). The Etest® strips were placed onto 150-mm MH plates that had previously been inoculated and incubated at 35°C overnight. The test was considered MBL positive when the MIC ratio of imipenem/imipenem plus EDTA was ≥ 8. The presence of a phantom zone or a deformation of the imipenem ellipse was also considered a positive result.

3. The Modified Hodge Test (MHT) was performed as described by Lee et al. The surface of a 150-mm MH agar plate (OXOID®, USA) was inoculated overnight with a suspension of Escherichia coli ATCC 25922, adjusted to a 0.5 McFarland standard. After a brief drying period, a 10-µg imipenem disk (OXOID®, USA) was placed at the center of the plate, and the PA test isolate was streaked heavily from the edge of the disk toward the edge of the plate. The MHT was considered positive if E. coli growth was observed within the inhibition zone of the imipenem disk, giving a distorted zone and interpreted as carbapenemase production.

For all three methods above, SPM-1-producing Pseudomonas aeruginosa (48-1997A) and IMP-1-producing Klebsiella pneumoniae (Kp-Br1) strains were used as positive controls, and Pseudomonas aeruginosa ATCC 27853 was used as the negative control. All MBL phenotypic methods were assessed twice by different raters.

MBL molecular detection

Polymerase Chain Reaction (PCR) analysis was performed for blaSPM-1, blaIMP-1, blaIMP-2, blaVIM-1 and blaVIM-2. The primers used were:

- **blaSPM-1** (forward: 5’ CCTACAACTCTACGGCCAC 3’, reverse: 5’ TCAGCCTGTTCCAGTATAAC 3’);
- **blaIMP-1** (forward: 5’ TACCAAGTTATCTGTATT 3’, reverse: 5’ ATGTTGCTTGGTTTTGATG 3’);
- **blaIMP-2** (forward: 5’ GGCACTGCGCTAAACAAA 3’, reverse: 5’ TAGTTACCTGTGGGTGATG 3’);
- **blaVIM-1** (forward: 5’ TATAGGAGCAACCCGATGT 3’, reverse: 5’ CAAAGCTGCCCTCAGCAG 3’);
- **blaVIM-2** (forward: 5’ AAATGTATGCGACTCACC 3’, reverse: 5’ TGCAACCTCATGTATGCGC 3’).

First, the bacterial cells were boiled to lyse the cell wall and extract the DNA, then PCR was performed. The cycling parameters for the **blaSPM-1** gene were: 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 40°C for 1 min, extension at 68°C for 1 min and a final extension at 68°C for 5 min. The parameters used for the **blaIMP-1**, **blaIMP-2**, **blaVIM-1** and **blaVIM-2** genes were: 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min and a final extension at 72°C for 7 min. The PCR products were loaded into a 2.0% agarose gel, stained with 1% ethidium bromide, electrophoresed and visualized under UV light. Positive controls were the MBL-producing strains SPM-1 (Pseudomonas aeruginosa 48-1997A), IMP-1 (Klebsiella pneumoniae Kp-Br1), IMP-2 (Acinetobacter baumannii AC-54/97), VIM-1 (Pseudomonas aeruginosa VR-143/97) and VIM-2 (Pseudomonas aeruginosa 98/10/U1315). Pseudomonas aeruginosa ATCC 27853 was the negative control.

The performance of the MBL phenotypic tests was evaluated using PCR as the gold standard. The sensitivity, specificity and positive and negative predictive values of the
phenotypic methods were calculated according to Ilstrup.\textsuperscript{17} The kappa value was calculated in order to measure the level of agreement between methods.\textsuperscript{16}

**Pulsed Field Gel Electrophoresis (PFGE)**

MBL-positive isolates were genotyped using DNA macro-restriction followed by pulsed-field gel electrophoresis (PFGE) as described by Kaufmann.\textsuperscript{19} In brief, a bacterial cell suspension was made in 100 mM Tris - 100 mM EDTA buffer. An agarose gel block was prepared from 250 μL of this suspension and 250 μL of 2% LPM agarose and was then placed in lysis buffer (0.4 M EDTA – pH 9.4, sarcosil 1%, 100 μL proteinase K) and incubated overnight at 50°C. The plugs were washed five times in CHEF-TE buffer (0.1 M Tris, pH 7.5, 0.1 M EDTA). The DNA was digested with SpeI (New England BioLabs\textsuperscript{®}, EUA) at 37°C for 12 to 20 hours and run on a 1% agarose gel with CHEF-DRII (BioRad\textsuperscript{®}, EUA) at 14°C and 6 V/cm, with pulse times of 5 and 90 seconds for 24 hours. The gels were visualized with ethidium bromide, and the results were visually analyzed according to the criteria of Tenover \textit{et al.}\textsuperscript{20}

**RESULTS**

In the study period, 238 PA blood isolates were analyzed, and 82 (34.5%) were found to be resistant to imipenem (IRPA). Thirteen IRPA isolates were excluded due to loss of viability; thus, 69 IRPA samples were included in the study. MIC (μg/mL) determinations confirmed the 100% imipenem resistance that was initially suggested by disk diffusion.

DDS results were positive in at least one of the inhibitor-substrate combinations among 53 strains (76.8%). Individual combinations of substrate and inhibitor showed distinct positivity, as can be seen in Table 1, with variable performance among the different combinations. The MBL \textit{Etest} was positive in 53 isolates (76.8%), and the MHT was positive in 19 isolates (27.5%).

PCR detection of MBL was positive in 21 strains (30.4%), 17 (81%) of which were positive for \textit{bla}\textsubscript{SPM-1} and 4 (19%) of which were positive for \textit{bla}\textsubscript{VIM-2}. The other genes (\textit{bla}\textsubscript{IMP-1}, \textit{bla}\textsubscript{IMP-2}, \textit{bla}\textsubscript{VIM-1}) were not detected.

Table 2 summarizes the sensitivity, specificity, PPV, NPV and kappa values of the phenotypic tests for the \textit{bla}\textsubscript{SPM-1} and \textit{bla}\textsubscript{VIM-2} genes according to the PCR results of the present study.

MBL-producing strains detected by PCR showed 100% resistance to ceftazidime, cefepime, gentamycin, tobramycin, ciprofloxacin and meropenem. Colistin appeared to be the most active antimicrobial agent, with MIC\textsubscript{50}, being 2 μg/mL (only two isolates had MICs of 4 and 6 μg/mL). This result was confirmed by agar dilution. Antibiogram and MIC\textsubscript{50} off all IRPA and its analysis according to MBL PCR detection were summarized in Table 3. The PFGE patterns of the SPM-1 and VIM-2 isolates were distinct. Among 17 SPM-1 isolates, 5 were indistinguishable, 11 were closely related, and 1 was possibly related. Among 4 VIM-2 isolates, 2 were indistinguishable, 1 was closely related, and 1 was different.

**DISCUSSION**

Imipenem-resistant \textit{Pseudomonas aeruginosa} (IRPA) is a current and significant concern, especially because of the limited therapeutic options for this pathogen. MBL enzymes

| Combinations        | N of positive strains | %  |
|---------------------|-----------------------|----|
| IMP / MAA           | 19/53                 | 35.8% |
| IMP / MPA           | 7/53                  | 13.2% |
| IMP / EDTA          | 52/53                 | 98.1% |
| CAZ / MAA           | 27/53                 | 50.9% |
| CAZ / MPA           | 23/53                 | 43.4% |
| CAZ / EDTA          | 47/53                 | 88.7% |

**Table 1 - Positivity of inhibitor-substrate combinations on DDS used for detecting MBL among 69 IRPA isolates.**

| Combinations | Genes      | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) | Kappa |
|--------------|------------|----------------|-----------------|---------|---------|-------|
| IMP-EDTA     | \textit{bla}\textsubscript{SPM-1} | 100            | 32.7            | 32.7    | 100     | 0.19  |
|              | \textit{bla}\textsubscript{VIM-1} | 100            | 26.2            | 7.7     | 100     | 0.03  |
| IMP-MAA      | \textit{bla}\textsubscript{SPM-1} | 100            | 96.2            | 89.5    | 100     | 0.92  |
|              | \textit{bla}\textsubscript{VIM-2} | 25             | 92              | 5.3     | 94      | -0.009|
| IMP-MPA      | \textit{bla}\textsubscript{SPM-1} | 35.3           | 98.1            | 85.7    | 82.3    | 0.42  |
|              | \textit{bla}\textsubscript{VIM-2} | 25             | 90.7            | 14.3    | 95.2    | 0.12  |
| CAZ-EDTA     | \textit{bla}\textsubscript{SPM-1} | 94.1           | 40.4            | 34      | 95.5    | 0.22  |
|              | \textit{bla}\textsubscript{VIM-1} | 100            | 33.8            | 8.5     | 100     | 0.05  |
| CAZ-MAA      | \textit{bla}\textsubscript{SPM-1} | 100            | 80.8            | 62.9    | 100     | 0.67  |
|              | \textit{bla}\textsubscript{VIM-2} | 100            | 64.6            | 14.8    | 100     | 0.17  |
| CAZ-MPA      | \textit{bla}\textsubscript{SPM-1} | 100            | 70.6            | 78.7    | 52.2    | 0.44  |
|              | \textit{bla}\textsubscript{VIM-2} | 100            | 70.8            | 70.8    | 17.4    | 0.22  |
| E-test MBL   | \textit{bla}\textsubscript{SPM-1} | 100            | 30.8            | 32.1    | 100     | 0.17  |
|              | \textit{bla}\textsubscript{VIM-2} | 100            | 30.2            | 7.5     | 100     | 0.04  |
| Hodge        | \textit{bla}\textsubscript{SPM-1} | 82.4           | 90.4            | 73.7    | 94      | 0.70  |
|              | \textit{bla}\textsubscript{VIM-1} | 75             | 75.4            | 15.8    | 98      | 0.18  |

IMP: imipenem; E-test: E-test method; MBL: metalloβ-lactamases; EDTA: etilenodiaminotetracetic acid; MAA: mercaptoacetic acid; MPA: 2-mercaptpropionic acid; PPV: positive predictive value; NPV: negative predictive value.
may play a critical role in IRPA, given that there is a high possibility of these carbapenemases being spread among nosocomial isolates. The prevalence of MBLs has been increasing significantly. MBLs now account for up to 40% of worldwide IRPA cases; furthermore, enzyme types may vary by regional areas.\textsuperscript{21,22} In the present study, 30.4% of the IRPA isolates were MBL positive, with 81% positive for SPM-1 and 19% positive for VIM-2. SPM-1 (São Paulo metallo-β-lactamase), initially described by Toleman \textit{et al.} in a 2002 case report from São Paulo, is now by far the most prevalent MBL in Brazil.\textsuperscript{16,23} Intriguingly, it is still restricted to Brazil. VIM-2 has been identified in other Latin American countries, such as Argentina, Chile, Colombia and Venezuela.\textsuperscript{16,14,23} The \textit{bla}\textsubscript{IMP-1} gene was not detected in our study, but it has previously been found in other Brazilian hospitals.\textsuperscript{24,25,26} Primers for very rare MBL enzymes that have not yet been described in our region were not included in the present study.\textsuperscript{4,24}

The prevalence of MBL-PA among other Brazilian studies has ranged from 7.5% - 44%.\textsuperscript{4,16,25,26,27} In different geographic regions, Vieira \textit{et al.} reported the lowest prevalence in the northern region.\textsuperscript{27} In 2005, Sader \textit{et al.} reported a 19.7% MBL prevalence among PA isolates, and a 43.9% IRPA prevalence, of which 55.6% were SPM-1, 30.6% were VIM-2, and 8.3% were IMP-1.\textsuperscript{4}

Phenotypic tests for MBL have not been nationally or internationally standardized. A consensus methodology for this routine laboratory method remains to be defined, and questions regarding the timing and method of MBL detection remain to be answered. Ultimately, guidelines for the reporting of such results are needed.

DDS is a feasible option for routine laboratory testing, but there is a need for different inhibitor combinations tests and performance may vary among different enzymes. In our study, MAA demonstrated the best sensitivity and specificity for detecting the SPM-1 enzyme. MPA demonstrated the best performance for detecting VIM-2. Even in the case of the Etest\textsuperscript{R} MBL strip, DDS showed a high sensitivity but low specificity for detecting both enzymes.

In another recent Brazilian study, Marra \textit{et al.} found a 69.6% false MBL detection rate with EDTA.\textsuperscript{20} Chu \textit{et al.} also reported that methods using EDTA are highly sensitive but not specific, as we observed.\textsuperscript{29} These findings suggest that caution must be taken in using only EDTA as the inhibitor agent when analyzing MBL production, as this method may lead to false positive results. Some authors have stated that the EDTA concentration is critical and that this compound may have its own bactericidal activity that leads to expanded inhibition zones (synergy) of carbapenems that are not associated with true MBL production. EDTA may also act on membrane permeability, increasing susceptibility to several antimicrobials including imipenem, which would also lead to false interpretations of MBL synergy tests.\textsuperscript{30} As another possibility of false MBL phenotypic detection, OXA enzymes that act like carbapenemases may also be affected by the EDTA inhibitory effect.\textsuperscript{31} This poor EDTA specificity is worrisome given that Etest\textsuperscript{R} MBL is a practical method for routine testing for MBL in a standard laboratory. Tests with low specificity may have a negative impact on the range of therapeutic options available and could increase the costs of nosocomial isolation precautions. False negative results of Etest\textsuperscript{R} for MBL have also been reported in isolates with an imipenem MIC < 4 μg/mL,\textsuperscript{12} but this possibility could not be assessed in our study because our isolates were all imipenem resistant (MIC > 16 μg/mL).

The positive Modified Hodge Test (MHT) is a carbapenemase-based indicator, not a MBL-specific test. That said, it may indicate enzyme production. In the present study, the MHT demonstrated good sensitivity and specificity for SPM-1 and VIM-2, reinforcing the opinions of other authors.\textsuperscript{32} The MHT is a feasible test for flagging possible production of MBL.

The IRPA strains studied had a multi-drug-resistant phenotype, and colistin was the most effective antimicrobial. In our hospital, colistin is considered a first-line drug for many severe nosocomial infections; thus, a selective pressure might explain why we found four isolates with MICs > 2 μg/mL. Aztreonam was the second most active drug, but 14.3% of the MBL-producing isolates were not susceptible to aztreonam, suggesting a possible association with other resistance mechanisms similar to Group A enzymes.\textsuperscript{33} Toleman \textit{et al.}
demonstrated that a large proportion of MBL genes are associated with one or more aminoglycoside- or β-lactam-resistant genes, partially explaining multi-drug-resistant cases.

MBL-PFGE showed a clonal-predominant genotype, reinforcing the importance of interventions to reduce cross-infection, especially once the majority of MBL-coding genes are in high-mobility genetic elements. The continued spread of such pathogens would be a clinical catastrophe, as outlined by other commentators.

CONCLUSION

A high prevalence of MBL among Pseudomonas aeruginosa is a critical problem representing a practical therapeutic challenge. Even though early recognition of MBL through routine laboratory testing is desirable, care should be taken when phenotypic tests are interpreted based on inhibitor synergy. In such cases, PCR should also be done to validate such results. The best method for MBL screening should be based on local factors such as bacterial type, MBL enzyme prevalence and the technical abilities of relevant facilities.

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