Metallo-ß-Lactamase Detection: Comparative Evaluation of Double-Disk Synergy versus Combined Disk Tests for IMP-, GIM-, SIM-, SPM-, or VIM-Producing Isolates

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The emergence of metallo-ß-lactamase (MBL)-producing isolates is a challenge to routine microbiology laboratories, since there are no standardized methods for detecting such isolates. The aim of this study was to evaluate the accuracy of different phenotypic methods to detect MBL production among Pseudomonas spp., Acinetobacter spp., and enterobacterial isolates, including GIM, IMP, SIM, SPM, and VIM variants. A total of 46 genetically unrelated Pseudomonas aeruginosa, Pseudomonas putida, Acinetobacter spp., and enterobacterial strains producing distinct MBLs were tested. Nineteen strains were included as negative controls. The inhibition of bacterial growth and ß-lactam hydrolysis caused by MBL inhibitors (IMBL) also were evaluated. The isolates were tested for MBL production by both a double-disk synergy test (DDST) and a combined disk assay (CD) using imipenem and ceftazidime as substrates in combination with distinct IMBL. One hundred percent sensitivity and specificity were achieved by DDST using 2-mercaptopropionic acid in combination with imipenem and EDTA, with a 5.0-mm-breakpoint increase in the size of the inhibition zone. Our results indicate that both phenotypic methods to detect MBL-producing isolates should be based on the genera to be tested, regardless of the enzyme produced by such isolates, as well as on the local prevalence of MBL producers.

Since the early 1990s, new metallo-ß-lactamase (MBL)-encoding genes have been reported all over the world in clinically important pathogens, such as Pseudomonas spp., Acinetobacter spp., and members of the Enterobacteriaceae family (19, 27, 35, 40). The emergence of MBL-encoding genes is worrisome, since they usually are carried by mobile genetic structures with great ability to spread (3, 5, 18, 24, 36). Moreover, increased mortality rates have been documented for patients infected with MBL-producing Pseudomonas aeruginosa, especially due to inadequate empirical therapy (39). Therefore, early detection of MBL-producing organisms is crucial to establish appropriate antimicrobial therapy and to prevent their inter- and intrahospital dissemination (10, 35).

Several phenotypic methods based on MBL inhibition by EDTA or thiol-based compounds have been published. Although they seem to perform and cheaper than genotypic methods, they have shown discordant results depending on the employed methodology, ß-lactam substrates, MBL inhibitors (IMBL), and bacterial genus tested (11, 14, 17, 21, 26). In addition, SPM-, GIM-, and SIM-producing pathogens rarely have been evaluated by these studies.

The high diversity and prevalence of MBL-producing P. aeruginosa, Acinetobacter spp., and Enterobacteriaceae isolates have motivated the search for an accurate MBL screening test. The aim of this study was to evaluate the accuracy of the double-disk synergy test (DDST) and the combined disk (CD) assay to screen for MBL-producing isolates among P. aeruginosa, Acinetobacter spp., and selected Enterobacteriaceae isolates that are producers of either IMP, GIM, SIM, SPM, or VIM enzymes.

MATERIALS AND METHODS

Bacterial isolates. All strains tested in this study are described in Table 1.

MBL-positive controls. A total of 46 MBL-producing isolates, including Acinetobacter spp. (n = 10), P. aeruginosa (n = 28), Pseudomonas putida (n = 1), and enterobacterial isolates (n = 7), were selected as positive controls. All 46 isolates previously had their genotypes characterized by PCR and sequencing. When applicable, these isolates were molecularly typed again to ensure that they were genetically unrelated. Additionally, all isolates showed resistance or reduced susceptibility to imipenem and resistance to ceftazidime according to CLSI breakpoints (9).

MBL-negative controls. Nineteen non-MBL producers, previously screened for the presence of MBL genes (blaIMP, blaCTX, blaSIM, blaSPM, and blaVIM), were included as negative controls (23). In addition, to exclude the possible presence of other not-yet-described MBL enzymes, the imipenemase activity of cell sonicates from overnight broth cultures were determined by spectrophotometric assays using a BioMate 5 UV-visible spectrophotometer. This experiment was carried out with 150 µM imipenem as the substrate at 299 nm. All selected negative control isolates also showed reduced susceptibility to ceftazidime and/or imipenem and were genetically unrelated by random amplification of polymorphic DNA (data not shown).

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IMBL growth inhibition activity. The bacterial growth inhibition initially was evaluated by disk diffusion testing 5 μl of different concentrations of each MBL inhibitor (IMBL) against Pseudomonas aeruginosa ATCC 27853. The IMBLs used were EDTA (Sigma, Steinheim Germany), mercaptopropionic acid (MPA; Sigma); mercaptoacetic acid (MAC; Sigma); mercaptoethanol (MET; Gibco, New York, NY); and phenanthroline (PHEN; Sigma). The tested IMBL concentrations were the following: (i) EDTA, 50, 100, 300, and 500 mM; (ii) MPA, 11.2 (undiluted), 5.6 (1:2), 2.8 (1:4), and 1.4 mM; (iii) MET, 55 (undiluted), 27.5 (1:2), 13.2 (1:4), and 7.0 mM (1:8); (iv) MAC, 14.4 (undiluted), 7.2 (1:2), 3.6 (1:4), 1.2 (1:12); and (v) PHEN, 8, 4, 2, and 1 mM.

Five microliters of the IMBL solution presenting the smallest inhibition zone in the absence of β-lactam agents was selected to perform the DDST. Different amounts of the same solution were employed to perform the CD test, ranging from 2 to 10 μl of each IMBL.

The evaluation of the interference of IMBL itself on the growth of each one of the isolates used in this study was also performed by dropping the same volumes of each IMBL employed for the CD assay on a blank disk. The inhibition zones were measured after overnight incubation at 35°C, and the means of inhibition zones were calculated.

Hydrolysis tests. To assess the β-lactam hydrolysis by each IMBL, the hydrolysis rates of β-lactams were measured with a BioMate 5 UV-visible spectrophotometer. The antimicrobial powder was diluted to obtain 1.8 to 2.0 U of absorbance. Briefly, 100 μl of each selected IMBL solution was added to 900 μl of antimicrobial solution. Experiments were carried out at 299 and 260 nm for imipenem and ceftazidime, respectively.

Phenotypic detection of MBL. (i) DDST. The phenotypic tests were performed by following the CLSI recommendations for the disk diffusion method (9). Briefly, a 0.5 McFarland bacterial suspension was inoculated on a Mueller-Hinton (MH) agar plate (Oxoid, Basingstoke, England). Imipenem and ceftazidime disks were aligned around blank filter disks, which contained 5 μl of the chosen inhibitor solution added directly on the disk and already placed on the MH agar plate. The following distances between the inhibitor and the substrates were tested: 1.0, 1.5, 2.0, 2.5, and 3.0 cm (from center to center). The appearance of either an enhanced or a phantom zone between the antimicrobial agents and the inhibitor disk was considered a positive result and indicative of MBL production. The best substrate, IMBL, and distance between the antimicrobial agent and the IMBL disks to detect MBL producers were selected according to the highest sensitivity (SN) and specificity (SP) results.

(ii) CD assay. For the CD assay, ceftazidime and imipenem disks initially were placed on the inoculated MH plates with a 0.5 McFarland bacterial suspension, and 2, 4, 6, 8, or 10 μl of each inhibitor solution was added directly to the disks (1). The amounts of each IMBL solution added to the disks corresponding to 2-, 4-, 6-, 8-, and 10-μl volumes, respectively, were the following: (i) EDTA, 50, 100, 200, and 300 μM; (ii) MPA, 11.2 (undiluted), 2.8 (1:2), 1.4 (1:4), and 0.7 (1:8) μM; (iii) MET, 55 (undiluted), 27.5 (1:2), 13.2 (1:4), and 7.0 μM (1:8); (iv) MAC, 14.4 (undiluted), 7.2 (1:2), 3.6 (1:4), 1.2 (1:12); and (v) PHEN, 8, 4, 2, and 1 mM.

The inhibition zone (in millimeters) produced by each isolate was measured. The positive criteria for classifying an isolate as an MBL producer is described below.

Statistical analysis. The inhibition zone (in millimeters) produced by each isolate was measured for each tested concentration, and the differences among means (positive and negative controls) were assessed by the Student’s t-test. SN, SP, and positive and negative predictive values (PPV and NPV, respectively) were calculated for the DDST for each β-lactam/IMBL combination for the five distances evaluated: 1.0, 1.5, 2.0, 2.5, and 3.0 cm. PCR results for each MBL were considered the gold standard, and isolates were considered true MBL producers if they were positive for blaIMP, blawhcms, blaVIM, blaspa, or blavim.

SN, SP, PPV, and NPV were calculated with the formulas

\[
\text{SN} = \frac{a}{a + c}, \quad \text{SP} = \frac{d}{b + d}, \quad \text{PPV} = \frac{a}{a + b}, \quad \text{NPV} = \frac{d}{c + d},
\]

where \(a\) represents the number of isolates correctly identified as MBL producers by the DDST, \(c\) is the number of true

### Table 1. MBL-producing isolates and non-MBL-producing isolates used in this study as positive and negative controls, respectively

| Isolate group | Species (no. tested) | MBL type | Country of origin | Strain no(s). | Reference |
|---------------|---------------------|----------|------------------|---------------|-----------|
| MBL producing | Acinetobacter spp. (7) | IMP-1 | Brazil | A3035, A4861, A3880, A68, A4764, A4468, A129046 | 30 |
| | Acinetobacter spp. (2) | IMP-1 | Brazil | A695, A696 | This study |
| | Acinetobacter spp. (1) | SIM-1 | Korea | YMC 039/T104 | 18 |
| | P. aeruginosa (9) | IMP-1 | Brazil | 131, 144, 137, 98, 128, 130, 143, 145, 183 | 29 |
| | P. aeruginosa (1) | IMP-1 | Japan | PSA 320 | 13 |
| | P. aeruginosa (1) | IMP-13 | Italy | 86-10079 | 31 |
| | P. aeruginosa (1) | IMP-16 | Brazil | 101-4704 | 24 |
| | P. aeruginosa (1) | IMP-18 | Brazil | 3486 | 36 |
| | P. aeruginosa (1) | IMP-18 | Brazil | 3489 | This study |
| | P. aeruginosa (1) | GIM-1 | Germany | 73-5671 | 7 |
| | P. aeruginosa (1) | VIM-1 | Italy | 179 | This study |
| | P. aeruginosa (1) | VIM-2 | Brazil | 225 | This study |
| | P. aeruginosa (1) | VIM-7 | United States | 07-406 | 32 |
| | P. aeruginosa (1) | SPM-1 | Brazil | 48-1997 A | 33 |
| | P. aeruginosa (9) | SPM-1 | Brazil | 14, 44, 73, 75, 76, 83, 194, 196, 197 | 6 |
| | P. putida (1) | IMP-1 | Brazil | 48-12346A | 22 |
| | K. pneumoniae (2) | IMP-1 | Italy | KPN1, KPN2 | 5 |
| | Enterobacter cloacae (1) | VIM-1 | Italy | 75-10344 | 3 |
| | Enterobacter cloacae (2) | VIM-1 | Italy | ECL3, ECL4 | 5 |
| | Enterobacter cloacae (1) | IMP-1 | Brazil | 199 | 4 |
| | Serratia marcescens (1) | IMP-1 | Japan | SM 319 | 13 |
| Non-MBL producing | Acinetobacter spp. (1) | OXA-23 | Brazil | 216 | This study |
| | Acinetobacter spp. (5) | NA* | Brazil | 210, 211, 212, 213, 215 | This study |
| | P. aeruginosa (6) | NA | Brazil | 209, 227, 230, 224, 237, 238 | This study |
| | K. pneumoniae (3) | NA | Brazil | 189, 222, 223 | This study |
| | Enterobacter cloacae (1) | NA | Brazil | 221 | This study |
| | Morganella morganii (1) | NA | Brazil | 224 | This study |
| | Serratia marcescens (1) | NA | Brazil | 190 | This study |
| | Serratia marcescens (1) | SME | United States | 1065 | 12 |

* NA, not applicable.
MBL producers (positive controls) that were incorrectly assigned as non-MBL producers by DDST, d is the number of true isolates that are non-MBL producers (negative controls) that were correctly identified by DDST, and b is the number of isolates that were incorrectly identified as MBL producers.

Results from the CD phenotypic method were characterized by receiver operating characteristic (ROC) curves to choose the best cutoff values for indicating MBL production. For each IMBL, the SN and SP of all five concentrations were calculated successively according to the variation of inhibition zones of MBL-producing and non-MBL-producing isolates. The resulting SN values then were plotted against the corresponding values of 1 – SP, producing an ROC curve. The area under the ROC curve (AUC) and its standard error were calculated, and its statistical significance then was evaluated by the nonparametric method. Differences between variables’ AUCs were evaluated through a comparison of the 95% confidence intervals of the corresponding areas.

CD and DDST analyses also were stratified into seven groups according to pathogen species and MBL type produced, as follows: (i) general group, including all isolates tested; (ii) Acinetobacter spp. group; (iii) P. aeruginosa group; (iv) enterobacterial group; (v) IMP-producing isolate group; (vi) VIM-producing isolate group; and (vii) SPM-producing isolate group. For the analysis of the MBL type groups (IMP, VIM, and SPM), all 19 negative controls were included. On the other hand, only negative controls representative of the same genus/species or bacterial family were selected for the pathogen (groups ii to iv) analysis. For example, the analysis of P. aeruginosa included all 28 MBL-producing P. aeruginosa isolates and the 6 non-MBL-producing P. aeruginosa isolates.

The statistical analysis was performed using SPSS 10.0 for Windows and Epi Info (CDC), version 6.04. All reported P-values were two sided, and values below 0.05 were considered statistically significant.

RESULTS

**IMBL growth inhibition activity.** PHEN and MET showed no growth inhibition activity on P. aeruginosa ATCC 27583 at all tested concentrations. On the other hand, EDTA, MPA, and MAC inhibited the bacterial growth of P. aeruginosa ATCC 27583 differently. The IMBL concentrations of 100 mM EDTA, 1.4 mM MPA, 55 mM MET, 1.2 mM MAC, and 8 mM PHEN were chosen to be tested against the 65 genetically unrelated isolates, since they showed the lowest level of inhibition of bacterial growth (Table 2). Overall, EDTA, PHEN, and MET presented weak bactericidal activity, with an increase in the size of inhibition zones of 0 to 2 mm. In contrast, MPA produced a larger increase in the size of the inhibition zones, from 0.3 to 12.4 mm (Table 2). Despite increasing the IMBL volumes applied to the blank disks, the mean sizes of the inhibition zones were similar among MBL-producing and non-MBL-producing strains (P < 0.05), except for MAC at 10 μl (P = 0.05). Therefore, the bactericidal effect of IMBL was not influenced by the production of IMP, VIM, SPM, GIM, or SIM, making no difference to the calculation of SN and SP values.

**Hydrolysis tests.** Hydrolysis test results showed that EDTA and PHEN were not able to hydrolyze the antimicrobial agents tested, while MAC, MET, and MPA demonstrated hydrolytic activity against imipenem but not against ceftazidime (data not shown). The hydrolysis of imipenem, ceftazidime, ampicillin, and aztreonam by MPA has been observed already (14); however, the ability of other IMBLS to hydrolyze β-lactam agents has not been seen previously.

**Phenotypic MBL detection by DDST.** Table 3 shows the results of SN, SP, PPV, and NPV for DDST. According to the statistical analysis, PHEN and MET demonstrated a very poor ability to detect the tested MBL-producing strains, providing SN results for less than 20% of all 46 MBL-producing isolates (data not shown). Surprisingly, four isolates (GIM, IMP-18 [isolate 3489], a VIM-1-producing P. aeruginosa, and an SIM-1-producing Acinetobacter sp.) were categorized as MBL producers by MET, even when the distance between MET and the β-lactam disks was 3.0 cm. Further studies that included an increasing number of SIM- and GIM-producing isolates would be important to confirm the MET capability for MBL phenotypic detection.

When all 46 MBL-producing isolates (the general group) were analyzed, MPA provided the best results by using imipenem as the substrate. The SN, SP, PPV, and NPV at 1.5 cm were 93.5, 89.5, 95.6, and 85.0%, respectively. However, better SN and SP were achieved after stratifying the results into groups. The Acinetobacter sp. group had SP, SN, PPV, and NPV of 100% when imipenem was placed at 1.5 or 2.0 cm from the MPA disks. For the P. aeruginosa group, DDST results identical to those for the Acinetobacter sp. group were obtained using ceftazidime as a substrate and MPA as the IMBL at 2.0 or 2.5 cm. Figure 1 illustrates the DDST results for MBL-producing P. aeruginosa and Acinetobacter spp. isolates, using EDTA and MPA in combination with both imipenem and ceftazidime at a 2.0-cm distance between disks. Among the enterobacterial isolates, identical SN (100.0%), SP (85.7%), PPV (85.7%), and NPV (100.0%) were detected with either EDTA/imipenem or MAC/imipenem at 1.5 and 2.0 cm, respectively, by DDST (Table 3). SP results did not reach 100% due to the occurrence of false-positive DDST results, as illustrated in Fig. 1.

**Distinct results for EDTA and MPA were observed for the IMP and VIM groups (Table 3). For the IMP group, SN and SP results close to 90% were achieved only for MPA/imipenem at 1.5 cm. For VIM, 100% SN was achieved only by using**

**TABLE 2. Inhibition of bacterial growth due to MBL inhibitors**

| IMBL (vol, in μl) | Mean inhibition zone size (in mm) by strain type | P value |
|-------------------|-----------------------------------------------|---------|
|                   | MBL producer (n = 46) | Non-MBL producer (n = 19) |       |
| EDTA (2)          | 6.0†                 | 6.0             | 0.01   |
| EDTA (4)          | 6.1                  | 6.0             |        |
| EDTA (6)          | 6.4                  | 6.0             |        |
| EDTA (8)          | 6.7                  | 6.0             |        |
| EDTA (10)         | 7.0                  | 6.0             |        |
| MPA (2)           | 7.3                  | 6.0             | 0.1    |
| MPA (4)           | 10.8                 | 10.9            | 0.9    |
| MPA (6)           | 13.5                 | 14.4            | 0.78   |
| MPA (8)           | 16.1                 | 16.7            | 0.3    |
| MPA (10)          | 18.4                 | 18.2            | 0.8    |
| MAC (2)           | 6.1                  | 6.0             |        |
| MAC (4)           | 7.6                  | 7.5             | 0.76   |
| MAC (6)           | 10.6                 | 9.6             | 0.12   |
| MAC (8)           | 12.6                 | 11.7            | 0.24   |
| MAC (10)          | 14.9                 | 13.1            | 0.05   |
| MET (2)           | 6.0                  | 6.2             |        |
| MET (4)           | 6.8                  | 6.3             | 0.35   |
| MET (6)           | 7.5                  | 7.2             | 0.8    |
| MET (8)           | 8.0                  | 7.2             | 0.6    |
| MET (10)          | 8.0                  | 7.4             | 0.65   |
| PHEN (2)          | 6.0                  | 6.0             |        |
| PHEN (4)          | 6.2                  | 6.0             |        |
| PHEN (6)          | 6.5                  | 6.3             | 0.8    |
| PHEN (8)          | 6.9                  | 6.4             | 0.3    |
| PHEN (10)         | 7.6                  | 7.3             | 0.78   |

† No inhibition zone was detected, since the size of the paper disk was 6 mm.

PPV (85.7%), and NPV (100.0%) were detected with either EDTA/imipenem or MAC/imipenem at 1.5 and 2.0 cm, respectively, by DDST (Table 3). SP results did not reach 100% due to the occurrence of false-positive DDST results, as illustrated in Fig. 1.

**Distinct results for EDTA and MPA were observed for the IMP and VIM groups (Table 3). For the IMP group, SN and SP results close to 90% were achieved only for MPA/imipenem at 1.5 cm. For VIM, 100% SN was achieved only by using**
| Distance (cm) between IMBL and disk | General | Acinetobacter spp. | Pseudomonas aeruginosa | Enterobacterial | IMP | SPM | VIM |
|-----------------------------------|---------|-------------------|-----------------------|----------------|------|------|------|
| 1.0                               |         |                   |                       |                |      |      |      |
| 2.0                               |         |                   |                       |                |      |      |      |
| 3.0                               |         |                   |                       |                |      |      |      |
| Results could not be calculated because denominator values were 0.

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TABLE 3. MBL detection by DST: results of SN, SP, PPV, and NPV stratified into seven groups according to pathogen species and MBL type produced.

| Group | Ceftazidime result | Imipenem result |
|-------|---------------------|-----------------|
|       | EDTA                | EDTA            |
|       | NAC                 | NAC             |
|       | AdN                 | AdD             |
|       | Add                 | Add             |
|       | dS                  | dS              |
|       | NS                  | NS              |
|       | AdN                 | AdD             |
|       | Add                 | Add             |
|       | dS                  | dS              |
|       | NS                  | NS              |
|       | AdN                 | AdD             |
|       | Add                 | Add             |
|       | dS                  | dS              |
|       | NS                  | NS              |
|       | AdN                 | AdD             |
|       | Add                 | Add             |
|       | dS                  | dS              |
|       | NS                  | NS              |
|       | AdN                 | AdD             |
|       | Add                 | Add             |
|       | dS                  | dS              |
|       | NS                  | NS              |

AdN, AdD, dS, NS indicate resistance. Table shows MBL detection by DST: results of SN, SP, PPV, and NPV stratified into seven groups according to pathogen species and MBL type produced.
EDTA/imipenem at 1.5 cm. SPM group results were similar to those for the *P. aeruginosa* group, for which MPA/ceftazidime provided 100% SN and nearly 90% SP by using a 2.5-cm distance. The same values also were achieved for detecting SPM using the MPA/imipenem combination at 1.5 cm (Table 3). Since many SPM, VIM, and IMP isolates are *P. aeruginosa*, we also performed a second analysis, including only *P. aeruginosa* as the positive and negative controls for each MBL subgroup. For SPM-, VIM-, and IMP-producing *P. aeruginosa* isolates, 100% SN and SP were obtained for 2.0- and 2.5-cm distances by using MPA/ceftazidime (data not shown).

We also selected all IMP-producing *Acinetobacter* spp. to perform a stratified analysis. Compared to the results for non-MBL-producing *Acinetobacter* spp., SN and SP results reached 100% using MPA/imipenem at 1.5 and 2.0 cm (data not shown). These results were identical to those for the *Acinetobacter* sp. group (Table 3).

### Phenotypic MBL detection by CD test

All of the CD test results were applied in the construction of ROC curves to establish the best breakpoint (increase in millimeters) for MBL detection. Table 4 shows selected CD results. Since the study’s priority was to select a screening test that yielded the lowest number of false-negative results, only SN results greater than 80% were included in Table 4. In analyzing the general group, the CD test resulted in SN values greater than 80% for EDTA/imipenem and EDTA/MPA/ceftazidime. However, SP results varied greatly, from 31.6 to 89.5%.

The *Acinetobacter* sp. group was not accurately classified by the CD test (Fig. 2 and Table 4). The best SN and SP results were 80.0 and 100.0%, respectively, for 2 μl of MPA associated with imipenem, using a breakpoint of 0.5 mm (AUC = 0.9). However, the increase of 0.5 mm is considered a nondiscriminatory cutoff, since it cannot be adequately measured by visual inspection. All other AUCs displayed results from 0.24 to 0.54.

The *P. aeruginosa* group showed SN results close to 100% and SP results of 100% (Table 4) for ceftazidime in combination with EDTA or MPA, using 8 mm as breakpoint. Figure 3 shows the ROC graphs for ceftazidime in combination with EDTA and MPA. The best AUC (0.98) was achieved by testing 8 μl of MPA. All AUC results for EDTA and MPA were statistically significant (*P* < 0.05) (data not shown).

Surprisingly, the CD results for the enterobacterial group achieved 100% SN and 100% SP for several of the breakpoints analyzed by the ROC curve for the imipenem/EDTA combination (AUC = 1.0) (Table 4). Figure 1 shows an example of a negative CD assay for a non-MBL producer, *Klebsiella pneumoniae*, which was misclassified as an MBL producer by DDST.

Among the MBL groups, the combination of ceftazidime associated with EDTA (6 and 8 μl) or MPA (2 to 8 μl) resulted...
| Pathogen | Vol (µg/ml) | EDTA MPA MAC | SN (%) | SP (%) |
|----------|-------------|---------------|--------|--------|
| Acinetobacter spp. | 10 | 90 83 | 11.0 | 89 65 |
| | 8 | 80 70 | 11.0 | 89 65 |
| | 6 | 80 70 | 11.0 | 89 65 |
| | 4 | 80 70 | 11.0 | 89 65 |
| | 2 | 80 70 | 11.0 | 89 65 |

**TABLE 4. MBL detection by CD: results of SN, SP, PPV, and NPV stratified into seven groups according to pathogen species and MBL type produced.**

- Breakpoint is the increase in the size of the inhibition zone.
- No breakpoints yielded SN results higher than 80%.

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in 100% SN and 100% SP for detecting the SPM-producing isolates (Table 4).

**DISCUSSION**

The detection of MBL-producing isolates by PCR is expensive, requires specialized technicians and instruments, and, more importantly, is able to detect only previously described MBL-encoding genes. In addition, the cost of implementing this technique might not be justified in medical centers that have a low prevalence of MBL producers. These factors make the implementation of such tests by routine clinical microbiology laboratories difficult. However, the detection of the MBL phenotype of resistance is of crucial importance for selecting the most appropriate therapy and applying infection control measures. For these reasons, an accurate and easy-to-perform phenotypic test is desirable and urgently necessary in hospitals with a high prevalence of MBL-producing isolates (10, 35).

Many authors have published distinct phenotypic techniques for screening MBL-producing isolates; however, these studies have important limitations, such as the following: (i) the inclusion of a small number of MBL-producing isolates, sometimes harboring the same type of enzyme (2, 36); (ii) the absence of molecular typing to exclude the influence of a single clone in the interpretation of their results (28); (iii) a lack of tests for evaluating the inhibitory effect of IMBL activity on bacterial growth and β-lactam hydrolysis (2, 11, 26, 34, 37); (iv) no inclusion of SPM- or GIM-producing *P. aeruginosa* isolates or SIM-producing *Acinetobacter* spp. isolates (11, 14, 17, 21, 25); (v) a lack of results stratified according to pathogen/species, in which the SN and SP values reflect the overall performance of all isolates (2, 14, 17); and (vi) accurate statistical analysis usually is not carried out to interpret and validate their results (2, 11, 21, 37, 38).

This is the first study to assess the accuracy of phenotypic methods to detect all of the major types of mobile MBLs described (GIM, IMP, SIM, SPM, and VIM) that are produced by diverse bacterial genera with distinct imipenem susceptibility patterns. The inclusion of genetically unrelated strains isolated on three distinct continents suggests that these results would be useful for regions in which such a resistance mechanism is frequent. Depending on the tested concentration, IMBLs may possess their own bactericidal activity, which may result in expanded inhibition zones not associated with true MBL production (8). This finding may lead to the false-positive detection of MBL by both CD assay and DDST, and it has been considered a disadvantage to employ EDTA and MPA as IMBL (16). To exclude the effect of such interference on the phenotypic detection of MBL, we have tested the activity of
distinct dilutions of IMBL with respect to the growth of *P. aeruginosa* ATCC 27853. In addition, the influence of IMBL inhibitory activity on the substrate was taken into consideration in the analysis of SN and SP results for screening MBL-producing isolates. False-negative results might arise from the imipenem hydrolysis caused by thiol derivatives (14). Many studies have performed phenotypic MBL detection using *Pseudomonas* spp., *Acinetobacter* spp., and/or enterobacterial isolates, but some of them did not present SP and SN results stratified by pathogen/species, reflecting the global performance of all isolates and not for a specific pathogen (11). Other studies did stratify according to species, but it is not clear if only negative controls from the same pathogen/species were included to calculate SN and SP results (21, 37). This is a very important issue that must be taken into consideration when selecting an IMBL phenotypic test, as in this study we have observed different SP and SN values according to the group analyzed. Moreover, a rigorous statistical analysis of our data was performed.

In this study, PHEN and MET showed poor results for detecting MBL producers under the tested conditions. The IMBL derivatives of thiol compounds may possess anionic, cationic, or neutral functional groups at neutral pH. MAC and MPA are classified in the anionic group, while MET belongs to the neutral group. Siemann et al. have shown that the inhibition of MBL activity by the anionic IMBL starts immediately. In contrast, neutral IMBLs have a pronounced lag prior to the start of inhibitory activity; i.e., the best inhibitory results are achieved after preincubating the IMBL with MBL before adding the substrate. In this manner, the weakest activity of MET in this study could be explained by the absence of the preincubation step in the performance of the phenotypic detection (28).

The standardization of a phenotypic method to screen MBL-producing isolates is of crucial importance (10). Most previous studies that evaluated MBL phenotypic detection were performed under distinct experimental conditions, jeopardizing the comparison of their results to those of others (2, 11, 14, 15, 21, 25, 26). The sizes of inhibition zones produced by β-lactam/IMBL combinations may vary according to the way that the IMBL is incorporated into the β-lactam disks (1). Since the selection of an appropriate breakpoint for screening MBL-producing isolates is directly influenced by the size of the inhibition zone, the methodology used for preparing the CDs should always be described in such phenotypic reports. In the current study, we have added the IMBL solutions directly on β-lactam disks already placed on the agar plate (1), while some authors previously prepare and freeze IMBL/β-lactams disks; thus, the results of our CD assay may be comparable to those of other studies that use the same methodology.

In addition, a number of authors also have raised concerns about the influence of the bactericidal effect of IMBL alone, claiming that it could not be distinguished from the antimicrobial effect of β-lactam/IMBL (2, 8, 14, 16). Fortunately, we have addressed this issue, since we have documented that the best SN and SP results for CD assays were achieved by establishing breakpoints that were greater than the mean sizes of the inhibition zones produced by all of the IMBL themselves. When choosing the best MPA volume, we should take into consideration the inhibitory effect of MPA itself on the bacterial growth. According to Table 2, the mean increase in the size of the inhibition zone caused by MPA itself (excluding the diameter of the blank disk [6 mm]) varied from 1.3 (2 μl) to 12.4 mm (10 μl). We recommend the addition of 2 μl of MPA to the ceftazidime disk, since this volume produced a significant increase in the size of the inhibition zone (13 mm) while exerting the minimal amount of inhibitory effect of MPA itself during the bacterial growth when testing SPM-producing isolates.

A ROC curve consists of a graph containing the relationship between the SN and SP of a test, which are calculated for all possible cutoff values. Thus, in this case, each curve plots the true-positive rate against the false-positive rate for each volume of the IMBL/β-lactam combinations. Since the ROC graph is a result of SN versus 1 — SP, the most accurate test to discriminate between MBL- and non-MBL-producing isolates would pass through the upper left corner of the graph. On the other hand, the closer the curve comes to the 45-degree diagonal, the less accurate is the test. Additionally, the AUC is a measure of the test's accuracy and is useful for comparing different tests. For example, an area of 1 represents an ideal test (SN and SP of 100%), while an area of 0.5 reflects poor SN and SP results (20). The greatest advantage of using ROC curves in this case was the possibility of visualizing a wide range of breakpoints for each IMBL/β-lactam combination by the CD test. In studies of different IMBL, many authors do not consider testing a wide range of breakpoints. SN and SP results usually are calculated only for a narrow range of breakpoints that are chosen randomly or according to previously published results (38). Moreover, many studies have established the best breakpoints only by observing inhibition zones produced by the IMBL/β-lactam (11, 38). Thus, SN and SP values of all possible and different breakpoints may not be presented and are not known by the readers. In our study, a wide range of results were documented and chosen based on ROC curve results, ranging from 3 (for *Enterobacteriaceae*/imipenem/EDTA) to 18 mm (for SPM/MPA/ceftazidime). If we had tested only the breakpoint of 7 mm, for example, we would have obtained less accurate results for enterobacterial isolates (SN of 71.4% instead of 100%) and SPM-producing isolates (SP of 31.6% instead of 100%). Since higher SP results can be obtained only by jeopardizing SN values, researchers should always consider their main objective when selecting a screening method to detect MBLS. However, due to the possible clinical implications of false-negative MBL results, microbiology laboratories should always favor the selection of more sensitive methods.

### Table 5. Optimal conditions described in this study to perform phenotypic detection of MBL production among *P. aeruginosa*, *Acinetobacter* spp., and *Enterobacteriaceae* clinical isolates

| Isolate group | Test | IMBL | β-Lactam substrate | Expl condition |
|---------------|------|------|-------------------|---------------|
| Acinetobacter spp. | DDST | 5 μl MPA (1.4 mM) | Imipenem | Distance, 2.0 cm |
| P. aeruginosa | DDST | 5 μl MPA (1.4 mM) | Ceftazidime | Distance, 2.0 cm |
| Enterobacteriaceae CD | 10 μl EDTA (100 mM) | Imipenem | Breakpoint, 5 mm |

*Breakpoint is the increase in the size of inhibition zone.*
As summarized in Table 5, our results show that the DDST was the most accurate phenotypic test to detect MBL production in *P. aeruginosa* and *Acinetobacter* spp. when 1.4 mM MPA was used as the IMBL and was positioned 2 cm from the β-lactam disk. However, the choice of the best substrate depended upon the bacterial species tested: ceftazidime and imipenem for *P. aeruginosa* and *Acinetobacter* spp., respectively. By the CD assay, no breakpoint achieved 100% SN and SP for the nonfermenting gram-negative bacilli. In contrast, among the enterobacterial isolates, the CD test showed identical results (100% SN, 100% SP, and an AUC of 1) for different EDTA volumes combined with imipenem. Thus, we suggest that 10 μl of 100 mM EDTA applied to the imipenem disk is a good option to discriminate MBL-producing isolates, since it produced the largest increase in the size of the inhibition zone (5 mm). Due to the paucity of MBL-producing *Enterobacteriaceae*, only seven positive controls were included, and we believe that further studies that evaluate an increased number of MBL isolates are needed to corroborate our results.

**Concluding remarks.** By DDST, MPA proved to be an excellent IMBL choice for detecting MBL among both *P. aeruginosa* and *Acinetobacter* spp. Although the best distance varied depending on the bacterial species, a distance of 2.0 cm could be standardized, since 100% SN and 100% SP were achieved for both pathogens. Since bacterial identification is determined before susceptibility testing by routine clinical laboratories and the type of MBL is yet unknown, the same solution of MPA could be applied on a blank disk at 2.0 cm from imipenem (for *Acinetobacter* spp.) or ceftazidime (for *P. aeruginosa*). Among enterobacterial isolates, the CD test with imipenem associated with 10 μl of 100 mM EDTA was the most accurate combination for detecting MBL production with a breakpoint of 5.0 mm.

We believe that the interpretation of DDST results is more subjective than those of the CD assay (37), because the DDST depends upon the technician’s expertise to discriminate true synergism from the intersection of inhibition zones. However, the CD assay did not show a good performance for screening MBL-producing *Acinetobacter* spp.

The DDST is simple to perform and can be incorporated into the existing workflow of clinical microbiology laboratories that routinely employ disk diffusion as their preferential antimicrobial susceptibility testing method. An MPA disk could be easily placed at 2 cm from the imipenem or ceftazidime disk during the performance of antimicrobial susceptibility testing method. An MPA disk could be applied on a blank disk at 2.0 cm from imipenem (for *Acinetobacter* spp.) or ceftazidime (for *P. aeruginosa*). Among enterobacterial isolates, the CD test with imipenem associated with 10 μl of 100 mM EDTA was the most accurate combination for detecting MBL production with a breakpoint of 5.0 mm.

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**REFERENCES**

1. Andrade, S. S., R. C. Picaô, E. H. Campaña, A. G. Nicoletti, A. C. Pignatari, and A. C. Gales, 2007. Influence of disk preparation on detection of metallo-β-lactamase-producing isolates by the combined disk assay. J. Clin. Microbiol. 45:2058–2060.

2. Arakawa, Y., N. Shibata, K. Shibuya, H. Kurokawa, T. Yagi, H. Fujiiwara, and M. Goto. 2000. Convenient test for screening metallo-β-lactamase-producing gram-negative bacteria by using thiol compounds. J. Clin. Microbiol. 38:80–83.

3. Castanheira, M., R. N. Jones, H. S. Sader, R. C. Picaô, and A. C. Gales. 2005. Inter-species dissemination of an integron carrying *bla*<sub>IMP</sub> between *Pseudomonas aeruginosa* and *Enterobacter cloacae* clavulenate. Abstr. CL88-p, p. 64. Abstr. 45th Intersci. Conf. Antimicrob. Agents Chemother. American Society for Microbiology, Washing DC.

4. Castanheira, M., R. E. Mendes, R. C. Picaô, F. P. Pinto, A. M. O. Machado, T. R. Walsh, and A. Gales. 2006. Genetic analysis of a multidrug-resistant (MDR) *Enterobacter cloacae* producing IMP-1 metallo-β-lactamase (MBL), abstr. C1-63, p. 72. Abstr. 46th Intersci. Conf. Antimicrob. Agents Chemoter. American Society for Microbiology, Washington, DC.

5. Castanheira, M., R. C. Picaô, E. G. Mendes, A. C. Pignatari, E. S. Sader, and A. Gales. 2006. Discrepancy in the metallo-β-lactama-producing phenotypic tests results associated with diversity in the promoter region of class 1 integrons, abstr. P-933. Abstr. 16th Eur. Cong. Clin. Microbiol. Infect. Dis. European Society of Microbiology and Infectious Diseases, Nice, France.

6. Castanheira, M., T. R. Walsh, H. S. Sader, A. C. Pignatari, and A. C. Gales. 2005. Metallo-β-lactama-producing gene *bla*<sub>IMP</sub><sub>1</sub> evaluation of its viciquences in unrelated *Pseudomonas aeruginosa* strains isolated from distinct Brazilian hospitals, abstr. P-932. Abstr. 16th Eur. Cong. Clin. Microbiol. Infect. Dis. European Society of Microbiology and Infectious Diseases, Nice, France.

7. Castanheira, M., M. A. Toleman, R. N. Jones, F. J. Schmidt, and T. R. Walsh. 2004. Molecular characterization of a β-lactamase gene, *bla*<sub>IMP</sub><sub>1</sub>, encoding a new subclass of metallo-β-lactama. Antimicrob. Agents Chemother. 48:4654–4661.

8. Chu, Y. W., T. K. Cheung, J. Y. Ngan, and K. M. Kam. 2005. EDTA susceptibility leading to false detection of metallo-β-lactama in *Pseudomonas aeruginosa* by Etest and an imipenem-EDTA disk method. Int. J. Antimicrob. Agents 26:340–341.

9. CLSI. 2006. Performance standards for antimicrobial susceptibility testing. 20th informational supplement M100-S16. CLSI, Wayne, PA.

10. Cornaglia, G., M. Akova, G. Amicosante, R. Canton, R. Cauda, J. D. Docquier, M. Edelstein, J. M. Frere, M. Fuzi, M. Galleni, H. Giamarellou, M. Gniadkowski, R. Koncan, B. Libisch, F. Luzzaro, V. Miriagou, F. Navarro, P. Nordmann, L. Pagani, L. Peixe, L. Poirel, M. Soulé, E. Tacconelli, A. Vato-poulou, and G. M. Rossolini. 2007. Metallo-β-lactama as emerging resistance determinants in gram-negative pathogens: open issues. Int. J. Antimicrob. Agents 29:380–388.
20. Mendes, R. E., M. Castanheira, R. E. Mendes, A. P. Penteado, and A. Gales. 2006. Dissemination and diversity of metallo-beta-lactamases in Latin America: report from the SENTRY Antimicrobial Surveillance Program. Int. J. Antimicrob. Agents. 25:57–61.

25. Walsh, T. R., R. A. Mitrofanova, V. P. Syriopoulos, G. L. Daikos, G. Paneris, and E. Malamoud-Lada. 2006. Evaluation of imipenem/imipenem + EDTA disk method for detection of metallo-beta-lactamase-producing Klebsiella pneumoniae isolated from blood cultures. Microb. Drug Resist. 12:39–43.

26. Zavaschi, A. P., A. L. Barth, A. L. Goncalves, A. L. Moro, J. F. Fernandes, A. F. Martins, F. Ramos, and L. Z. Goldani. 2006. The influence of metallo-beta-lactamase production on mortality in nosocomial Pseudomonas aeruginosa infections. J. Antimicrob. Chemother. 58:387–392.