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Imipenem-EDTA Disk Method for Differentiation of Metallo-β-Lactamase-Producing Clinical Isolates of *Pseudomonas* spp. and *Acinetobacter* spp.

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Rapid detection of metallo-β-lactamase (MBL)-producing gram-negative bacilli is necessary to prevent their dissemination. The method using a disk with imipenem plus 750 μg of EDTA differentiated all MBL-producing pseudomonads, and the sensitivity and specificity for acinetobacters were 95.7 and 91.0%, respectively. The imipenem-EDTA disks were stable for 12 and 16 weeks at 4 and −20°C, respectively.

An increasing prevalence of carbapenem resistance mediated by acquired metallo-β-lactamases (MBLs) is being reported, particularly for *Pseudomonas aeruginosa* clinical isolates in several countries (4, 6, 8, 9, 11–14, 17, 18). In Korea, approximately 10 and 50% of imipenem resistance in *P. aeruginosa* (8) and *Acinetobacter* spp. (19), respectively, are due to MBL production. The resistance may spread rapidly to various species of gram-negative bacilli, as the MBL genes reside in mobile gene cassettes inserted in integrons (3). The rapid detection of MBL-positive gram-negative bacilli is necessary to aid infection control and to prevent their dissemination (5). A PCR method was simple to use in detecting MBL-producing isolates initially (16), but it became more difficult with the increased number of types of MBLs.

MBL activity is inhibited by chelating agents. Double-disk synergy tests using a ceftazidime disk and a 2-mercaptopropionic acid disk (1), or an imipenem disk and an EDTA disk (7), have been reported as a simple method to detect MBL-producing clinical isolates. However, occasional adjustment of the distance between the two disks is required to obtain optimal results (1, 7), as is the case with the double-disk test for the detection of extended-spectrum β-lactamase-producing isolates (2). For the phenotypic confirmation of extended-spectrum-β-lactamase-producing isolates, inhibition zones are compared by using both ceftazidime and cefotaxime disks with and without clavulanic acid (10). The aim of this study was to determine the feasibility of using an imipenem disk with added EDTA to confirm MBL-producing clinical isolates of *Pseudomonas* spp. and *Acinetobacter* spp.

The MBL-producing gram-negative bacilli used in this study were 102 isolates of *P. aeruginosa*, 14 of *Pseudomonas putida*, 20 of *Acinetobacter baumannii*, and 3 of *Acinetobacter* genomospecies 3. All of the isolates were VIM-2 MBL producers except for one isolate of *P. aeruginosa* and five isolates of acinetobacters, which were IMP-1 MBL producers. MBL genes were detected by PCR, and MBL production was detected by the imipenem-EDTA double-disk synergy test as described previously (8). Imipenem-resistant or -intermediate but non-carbapenemase-producing isolates were included for comparison.

Test organisms were inoculated onto plates of Mueller-Hinton agar (Becton Dickinson, Cockeysville, Md.) as recommended by the National Committee for Clinical Laboratory Standards (10). A 0.5 M EDTA solution was prepared by dissolving 186.1 g of disodium EDTA · $2H_2O$ (Junsei Chemical, Tokyo, Japan) in 1,000 ml of distilled water and adjusting it to pH 8.0 by using NaOH. The mixture was sterilized by autoclaving (15). Two 10-μg-imipenem disks (Becton Dickinson) were placed on the plate, and appropriate amounts of an EDTA solution were added to one of them to obtain the desired concentration. The inhibition zones of the imipenem and imipenem-EDTA disks were compared after 16 to 18 h of incubation in air at 35°C.

To test the stability of the EDTA-added imipenem disks, an EDTA solution was added to 10-μg-imipenem disks to obtain a concentration of 1,000 μg. The disks were dried immediately in an incubator and stored at 4 or at −20°C in airtight vials without desiccant. The inhibition zones produced for MBL-positive and -negative isolates were compared after storage of the disks.

A preliminary study showed that a disk with imipenem plus 150 μg of EDTA could increase the mean inhibition zone diameter by 12 mm for four MBL-positive *P. aeruginosa* isolates, but the increase was only 6 mm for three MBL-positive *Acinetobacter* isolates. A disk with imipenem plus 1,500 μg of EDTA increased the mean inhibition zones for three MBL-negative isolates by 7 mm. Therefore, 750- and 1,000-μg EDTA concentrations were chosen for further study.

Figure 1 shows inhibition zones for MBL-positive and -negative imipenem-resistant pseudomonads and acinetobacters produced by disks with imipenem alone and disks with imipenem plus 750 μg of EDTA (data for the disk with imipenem

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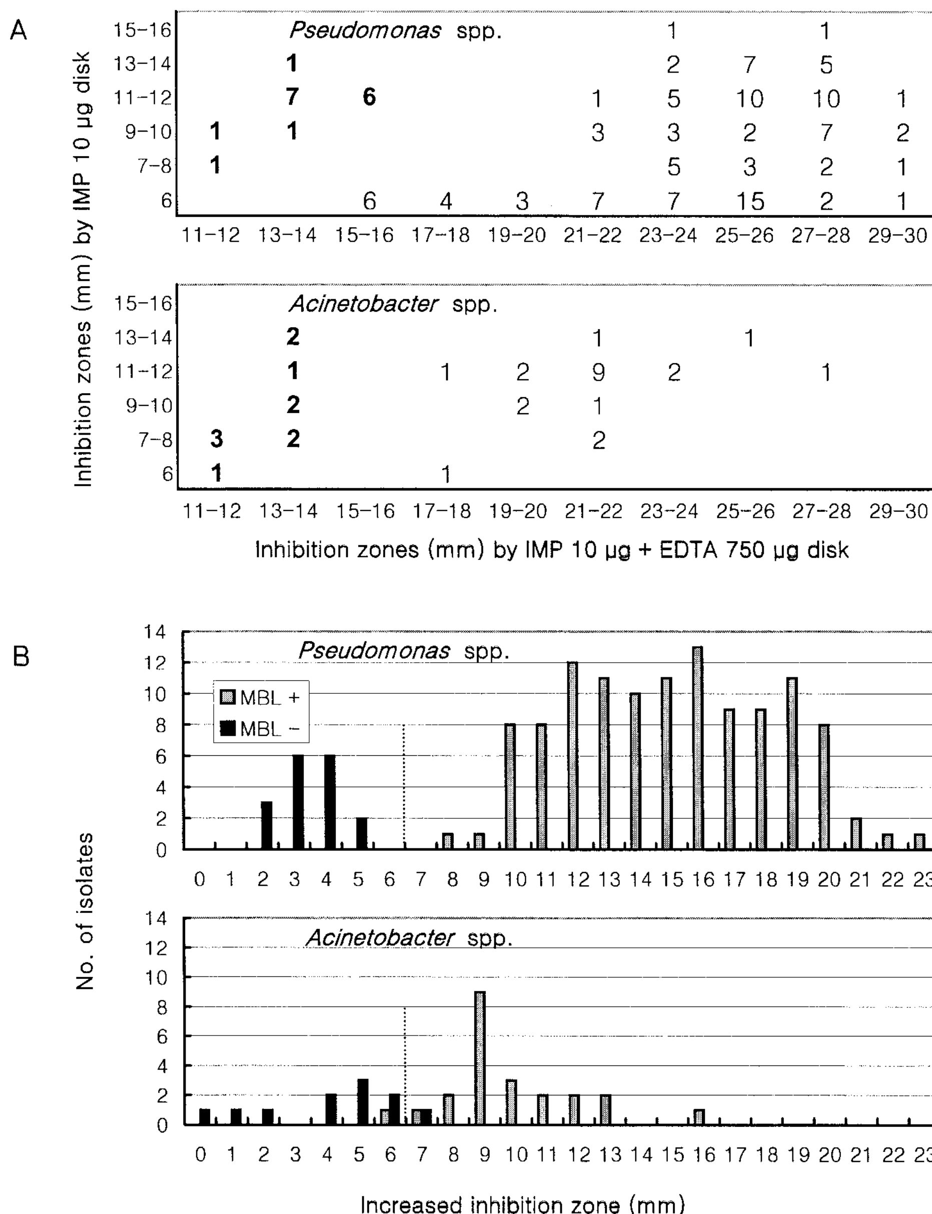

FIG. 1. Comparison of inhibition zone diameters produced by disks with imipenem and imipenem plus 750 μg of EDTA. (A) Scattergram comparing inhibition zone diameters. Boldface indicates imipenem-resistant or -intermediate, but MBL-negative isolates, and lightface indicates MBL-positive isolates. (B) Increased inhibition zones with imipenem-EDTA disks. IMP, imipenem.

plus 1,000 μg of EDTA are not shown). For MBL-positive isolates of *Pseudomonas* spp., disks with imipenem plus 750 and 1,000 μg of EDTA increased inhibition zones by 8 to 15 mm (mean, 10.5 mm) and 9 to 16 mm (mean, 11.5 mm), respectively, while the increases for MBL-negative isolates were 1 to 5 mm (mean, 3.8 mm) and 2 to 6 mm (mean, 5.0 mm), respectively (data not shown). The inhibition zone diameter for MBL-positive *Acinetobacter* spp. increased by 6 to 13 mm (mean, 9.4 mm) and 7 to 14 mm (mean, 10.4 mm), respectively, while that for MBL-negative isolates increased by 1 to 7 mm (mean, 4.0 mm) and 3 to 9 mm (mean, 6.0 mm), respectively. The disks with imipenem plus 750 μg of EDTA and those with imipenem plus 1,000 μg of EDTA produced comparable results, and therefore, a disk containing 750 μg of EDTA was chosen.

With *Pseudomonas* spp., all of the MBL-positive isolates were well separated from MBL-negative isolates by the criterion of a ≥7-mm increase of inhibition zone with the disks to which 750 μg of EDTA was added (Fig. 1B). However, by the same criterion, 1 of 23 (4.3%) MBL-positive and 1 of 11 (9.1%) MBL-negative acinetobacters showed false-negative and false-positive results, respectively (Fig. 1B). The size of inhibition zones was helpful for resolving this problem with equivocal isolates. The inhibition zones with imipenem-EDTA disks were ≤14 mm for the MBL-negative isolates, while they were ≥17 mm for the MBL-positive isolates (Fig. 1A).

TABLE 1. Stability of imipenem disks with 1,000 μg of EDTA added during storage as determined by change of inhibition zone diameter

Storage (wk)	Diam (mm) of:										
	IMP[b]-EDTA disk zone										IMP disk zone, *P. aeruginosa* ATCC 27853[a]
	IMP resistant, MBL positive				IMP resistant, MBL negative				*P. aeruginosa* ATCC 27853		
	P. aeruginosa		*A. baumannii*		*P. aeruginosa*		*A. baumannii*				
	4°C	−20°C	4°C	−20°C	4°C	−20°C	4°C	−20°C	4°C	−20°C	
Immediate	24	24	23	23	15	15	17	17	24	24	27
1	23	23	22	23	12	13	15	15	22	23	24
4	21	22	22	22	13	13	14	16	21	22	25
8	20	21	22	23	13	12	15	15	20	21	25
12	21	24	23	23	11	13	14	16	20	22	22
16	20	23	23	25	11	13	9	15	19	22	25
24	18	23	22	23	11	12	12	13	19	22	25
Mean	21.0	22.9	22.4	23.1	12.3	13.0	13.7	15.3	20.7	22.3	24.7

[a] Routine quality control results with disks stored as recommended by the National Committee for Clinical Laboratory Standards. The acceptable imipenem disk zone diameter is 20 to 28 mm.
[b] IMP, imipenem.

A ceftazidime disk method was reported elsewhere to be more sensitive than an imipenem disk method for the detection of MBL by the double-disk synergy test (1). However, the use of a 2-mercaptopropionic acid-containing ceftazidime disk was not considered in this study, because the chemical is volatile and the MICs of imipenem for all of our MBL-producing isolates were ≥8 μg/ml.

An EDTA stock solution is stable, but addition of the solution at each performance of the test is time-consuming. To determine the stability of imipenem disks containing 1,000 μg of EDTA, the dried disks were stored at 4 or at −20°C without desiccant to simulate the most unfavorable laboratory condition. The inhibition zones with the imipenem-EDTA disks did not decrease much for one each of MBL-positive or -negative, imipenem-resistant *P. aeruginosa* and *A. baumannii* isolates for 12 and 16 weeks at 4 or at −20°C, respectively (Table 1). The imipenem-susceptible *P. aeruginosa* ATCC 27853 showed only slightly smaller inhibition zones with the imipenem-EDTA disk than with imipenem disks for routine use. The inhibition zone for the National Committee for Clinical Laboratory Standards control strain was within the acceptable range (10) for at least 24 weeks.

In conclusion, the method using a disk with imipenem plus 750 μg of EDTA is simple to perform and highly sensitive in differentiating MBL-producing isolates. The specificity was excellent for pseudomonads and good for acinetobacters. The imipenem-EDTA disks can be stored at −20°C without significant loss of activity for at least 16 weeks.

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