JCM
Journals.ASM.org

Evaluation of Double-Disk Potentiation and Disk Potentiation Tests Using Dipicolinic Acid for Detection of Metallo-β-Lactamase-Producing *Pseudomonas* spp. and *Acinetobacter* spp.

Dongeun Yong,[a] Yangsoon Lee,[a,b] Seok Hoon Jeong,[a] Kyungwon Lee,[a] and Yunsop Chong[a]

Department of Laboratory Medicine and Research Institute of Bacterial Resistance, Yonsei University College of Medicine, Seoul, South Korea,[a] and Department of Laboratory Medicine, National Health Insurance Corporation Ilsan Hospital, Goyang, South Korea[b]

Accurate detection of metallo-β-lactamase (MBL)-producing *Pseudomonas* spp. and *Acinetobacter* spp. became very important with the increasing prevalence of carbapenem-nonsusceptible clinical isolates. The performance of phenotypic MBL detection methods may depend on the types of MBL and the characteristics of the isolates. A high false-positive rate is a problem with EDTA-based MBL detection methods. We evaluated the performance of double-disk potentiation tests (DDPTs) and disk potentiation tests (DPTs) with dipicolinic acid (DPA) using 44 isolates of *Pseudomonas* spp. and *Acinetobacter* spp. producing IMP-1-like, VIM-2-like, and SIM-1 type MBLs. Also, we characterized *P. aeruginosa* isolates with positive imipenem (IPM)-DPA DDPT, but negative meropenem (MEM)-DPA DDPT, and determined possibility of improving a DDPT by using MacConkey agar. Among five different DDPT methods, the IPM-DPA 250-μg method showed the highest sensitivity (97.7%) and specificity (100%). Among four DPT tests, the highest sensitivity (100%) was shown by the IPM-EDTA 1,900-μg disk method, but the specificity was very low (11.4%). Five of six *P. aeruginosa* isolates with false-negative DDPTs with MEM-DPA 250-μg disks carried bla_{IMP-6}, and the high level resistance to MEM (MIC ≥ 512 μg/ml) was reduced by the presence of phenylalanine arginine β-naphtylamide. Improvement of DDPTs was observed when MacConkey agar was used instead of Mueller-Hinton agar. In conclusion, DPA is a better MBL inhibitor than EDTA for detection of *Pseudomonas* spp. and *Acinetobacter* spp. with IMP-1-like, VIM-2-like, and SIM-1-type MBLs. In DPA DDPTs, IPM disks perform better than MEM disks when the isolates are highly resistant to MEM due to the overexpression of efflux pumps.

Carbapenems are often used as "antibiotics of last resort" when patients with infections become gravely ill or are suspected of harboring resistant bacteria (14). However, carbapenem-resistant Gram-negative bacilli have been increasingly reported worldwide. In Korea, carbapenem-resistant isolates of *Enterobacteriaceae* remains very rare, but during the period of 2001 to 2009, imipenem (IPM)-resistant *Pseudomonas aeruginosa* and *Acinetobacter* spp. increased from 17 to 26% and from 5 to 51%, respectively (10). A significant proportion of the resistance in *P. aeruginosa* is due to the production of VIM-2-like and IMP-1-like metallo-β-lactamase (MBL), whereas in *Acinetobacter* spp. the majority are due to the production of OXA-type carbapenemase, although VIM-2-like, IMP-1-like, and SIM-1 enzyme-producing isolates have also been detected (8).

Various inhibitor-based methods are commonly used to detect MBLs, but these techniques are not highly sensitive or highly specific. A disk potentiation test (DPT) is also called a combined disk test. A DPT using imipenem (IPM) and EDTA (IPM-EDTA DPT) showed a high false-positive rate with *Acinetobacter* spp. due to growth inhibition by EDTA alone (20). A double-disk potentiation test (DDPT) is also known as a double-disk synergy test. To reduce false-positive results from the DDPT, we reduced the EDTA concentration from 1,900 to 760 μg and added 2 mg of sodium mercaptoacetic acid (SMA) (11), an inhibitor recommended for detecting MBL (1).

Dipicolinic acid (DPA) was reported to be the most potent inhibitor of IMP-1-type MBL, although it did not inhibit the growth of *P. aeruginosa* at <400 μg/ml (6). It was reported that a DPT using DPA was a simple method for screening for IMP-1-, VIM-1-, and VIM-2-type MBL-producing *P. aeruginosa*, although the study did not include *Acinetobacter* isolates (6). The sensitivities of DDPT with IPM and DPA (IPM-DPA DDPT) and IPM-DPA DPTs were superior to those of the EDTA-based test for the detection of VIM-2-producing *Pseudomonas* spp. and VIM-2- and IMP-1-producing *Acinetobacter* spp. (17), but the study did not include SIM-1-producing *Acinetobacter* spp. isolates, which are present in Korea (13) and China (21).

With the availability of commercial DPA tablets (Rosco Diagnostica, Taastrup, Denmark), DDPTs and DPTs have become easier to perform. Regarding the DPA content, the commercial tablet contains 250 μg, whereas Shin et al. (17) used an 835-μg disk. Recently, it was reported that a meropenem (MEM)-DPA (1,000 μg) DPT using commercial tablets (Rosco Diagnostica) showed 100% sensitivity and specificity for the detection of *Enterobacteriaceae* isolates with VIM-1- and IMP-type MBL (4). With an increasing number of isolates with multiple mechanisms of β-lactam resistance, MBL detection methods may require tailoring, depending on the local situation.

The aims of the present study were to compare the performance of DDPTs and DPTs with three different carbapenems and DPA or EDTA inhibitors for the detection of VIM-2-like, IMP-1-like, or SIM-1-type MBL-producing *Pseudomonas* spp. and *Acinetobacter* spp. We also compared the performance of Mueller-

Received 30 March 2012 Returned for modification 7 May 2012
Accepted 11 July 2012
Published ahead of print 25 July 2012
Address correspondence to Yunsop Chong, whonetkor@yuhs.ac.

doi:10.1128/JCM.00818-12

TABLE 1 Evaluation of double-disk potentiation tests for detection of MBL-producing isolates of *Pseudomonas* spp. and *Acinetobacter* spp.[a]

Organism (no. of isolates tested)	No. (%) of isolates with disks[b]									
	Method A: MEM-DPA (250 μg)		Method B: IPM-DPA (250 μg)		Method C: MEM-DPA (835 μg)		Method D: IPM-DPA (835 μg)		Method E: IPM-EDTA (760 μg) + SMA (2 mg)	
	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg
MBL positive										
P. aeruginosa (10)[c]	3	7	10	0	3	7	10	0	8	2
Pseudomonas spp. (15)[d]	14	1	15	0	11	4	15	0	13	2
Non-*baumannii Acinetobacter* (7)[e]	6	1	6	1	7	0	6	1	7	0
Non-*baumannii Acinetobacter* (7)[f]	7	0	7	0	6	1	6	1	7	0
Non-*baumannii Acinetobacter* (5)[g]	5	0	5	0	4	1	5	0	5	0
Total (44)	35 (79.5)	9 (20.5)	43 (97.7)	1 (2.3)	31 (70.5)	13 (29.5)	42 (95.5)	2 (4.5)	40 (90.9)	4 (9.1)
MBL negative										
P. aeruginosa (20)	2	18	0	20	4	16	0	20	0	20
A. baumannii (15)[h]	0	15	0	15	0	15	1	14	2	13
Total (35)	2 (5.7)	33 (94.3)	0 (0)	35 (100)	4 (11.4)	31 (88.6)	1 (2.9)	34 (97.1)	2 (5.7)	33 (94.3)

[a] IPM, imipenem; MEM, meropenem; DPA, dipicolinic acid; SMA, sodium mercaptoacetic acid; Pos, positive; Neg, negative.
[b] All carbapenem disks were products of Becton Dickinson, with the exception of the MEM tablets (Rosco) in method A. The DPA tablets in methods A and B were products of Rosco, and 835-μg DPA disks were prepared by our laboratory. The edge-to-edge distances of the two disks were 5 mm for methods A and B and 10 mm for methods C, D, and E. The following methods had statistically significant differences ($P < 0.05$ by chi-square test) for the detection of MBLs: sensitivity, method A versus methods B and D and method B versus method C; specificity, method B versus method C.
[c] With IMP-1-like MBL.
[d] Among the 15 VIM-2-like MBL-positive isolates, 13 were *P. aeruginosa,* and two were *P. putida*.
[e] With IMP-1-like MBL.
[f] With VIM-2-like MBL.
[g] With SIM-1 MBL.
[h] Eleven isolates had the IS*Aba1*-associated bla_{OXA-51}-like gene, and four had bla_{OXA-23}.

Hinton agar II (MHA) and MacConkey agar and characterized *P. aeruginosa* isolates with false-negative MEM-DPA DDPT.

MATERIALS AND METHODS

Isolates. The isolates of *Pseudomonas* spp. and *Acinetobacter* spp. used in the present study were recovered from clinical specimens. The species were identified either by conventional tests (2) or using the Vitek 32GN system (bioMérieux, Marcy l'Etoile, France). bla_{OXA-51}-like genes were detected by PCR as described previously (19) to differentiate *A. baumannii* from non-*baumannii Acinetobacter*. A total of 44 MBL-producing isolates of *Pseudomonas* spp. and *Acinetobacter* spp. used were positive for bla_{IMP-1}-like, bla_{VIM-2}-like, or bla_{SIM-1} genes by PCR (12). MBL-negative controls included 20 isolates of IPM-nonsusceptible *P. aeruginosa* and 15 isolates of OXA carbapenemase-positive (11 IS*Aba1*-associated bla_{OXA-51}-like gene-positive and four bla_{OXA-23} gene-positive) *Acinetobacter* spp.

DDPT and DPT. The antimicrobial tablets used contained 10 μg of MEM (Rosco), and disks contained 10 μg each of MEM, IPM, and ertapenem (ETP; Becton Dickinson, Sparks, MD). Inhibitor tablets contained 250 μg of DPA alone or 1,000 μg of DPA in combination with MEM (Rosco). Inhibitor disks containing 835 μg of DPA, 1,900 μg of EDTA, or 760 μg of EDTA plus 2 mg of SMA were prepared at our laboratory using chemicals from Sigma Chemical Co. (St. Louis, MO). To prepare combination disks, the inhibitors were added to the commercial IPM disks.

MHA and MacConkey agar plates were prepared at our laboratory using dehydrated products from Becton Dickinson. Freezer-stored isolates were subcultured, and fresh overnight cultures were used to prepare suspensions at a McFarland turbidity of 0.5. The plates were inoculated using cotton-tipped applicators. A DDPT using Rosco tablets was performed according to the manufacturer's instructions, by placing the two tablets 5 mm apart, edge to edge. In other DDPTs, disks were placed 10 mm apart, edge to edge (12). After a 24-h incubation of the plates, a microbiologist read the test results in a blinded fashion without prior knowledge of the MBL production of the isolates. A DDPT was interpreted as positive even if a small potentiation inhibition zone was present. In a DPT, a ≥5-mm increase in the inhibition zone caused by the presence of an inhibitor was interpreted as positive (4). A possible improvement in the sensitivity of DDPT was sought by using MacConkey agar. Oxgall (Becton Dickinson) and sucrose (Shinyo Pure Chemical Co., Osaka, Japan) were added to MHA to determine their effects on DDPTs.

Characterization of isolates with false-negative MEM-DPA DDPT. To characterize false-negative *P. aeruginosa* isolates, MICs of MEM and IPM with or without inhibitors were determined by the agar dilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines (3). Briefly, a final concentration of phenylalanine arginine β-naphtylamide (PAβN) at 30 μg/ml, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) at 2.5 μg/ml, or cloxacillin (Sigma Chemical) at 250 μg/ml was added to MHA, and then either MEM (Sumitomo, Tokyo, Japan) or IPM (Merck Sharp & Dohme, Rahway, NJ) was added to obtain final concentrations ranging from 0.12 to 512 μg/ml. *P. aeruginosa* strain ATCC 27853 was used for quality control. Also, DDPTs were performed in the presence of the aforementioned inhibitors. To estimate the prevalence of *P. aeruginosa* with false-negative MEM-DPA DDPT in Korea, the sequences of bla_{IMP-1}-like genes were analyzed as described previously (13) and compared to those in the widely disseminated *P. aeruginosa* isolates with high-level MEM resistance (16).

RESULTS

Among the six different DDPT methods compared (Table 1), method B (IPM-DPA [250 μg]) showed the highest sensitivity for MBL detection (97.7%), followed by method D (IPM-DPA [835

TABLE 2 Evaluation of the disk potentiation tests for the detection of MBL-producing isolates of *Pseudomonas* spp. and *Acinetobacter* spp.[a]

Organism (no. of isolates tested)	No. (%) of isolates[b]							
	Method A: MEM + DPA (1,000 μg)		Method B: IPM + DPA (835 μg)		Method C: IPM + EDTA (1,900 μg)		Method D: IPM + EDTA (760 μg) + SMA (2 mg)	
	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg
MBL positive								
P. aeruginosa (10)	5	5	10	0	10	0	2	8
Pseudomonas spp. (15)	15	0	15	0	15	0	11	4
Non-*baumannii Acinetobacter* (7)	7	0	6	1	7	0	2	5
Non-*baumannii Acinetobacter* (7)	7	0	6	1	7	0	3	4
Non-*baumannii Acinetobacter* (5)	4	1	4	1	5	0	1	4
Total (44)	38 (86.4)	6 (13.6)	41 (93.2)	3 (6.8)	44 (100)	0 (0)	19 (43.2)	25 (56.8)
MBL negative								
P. aeruginosa (20)	1	19	1	19	20	0	2	18
A. baumannii (15)	1	14	0	15	11	4	0	15
Total (35)	2 (5.7)	33 (94.3)	1 (2.9)	34 (97.1)	31 (88.6)	4 (11.4)	2 (5.7)	33 (94.3)

[a] For abbreviations and isolates with genes of MBL and OXA carbapenemase, see Table 1.
[b] For method A, Rosco products were used. For the other methods, laboratory-prepared inhibitors were added to imipenem disks (Becton Dickinson). The following methods had statistically significant differences ($P < 0.05$ by chi-square test) for the detection of MBLs: sensitivity, method A versus methods C and D, method B versus method D, and method C versus method D; specificity, method C versus methods A, B, and D.

μg]) (95.5%). Methods A (MEM-DPA [250 μg]) and C (MEM-DPA [835 μg]) showed sensitivities of 79.5 and 70.5%, respectively. The highest specificity (100%) was shown by method B. The specificity of method D was 97.1%, whereas that of method C was 88.6%. Among the four DPT methods compared (Table 2), the highest sensitivity (100%) was shown by method C (IPM-EDTA [1,900 μg]). The sensitivities of methods B (IPM-DPA [835 μg]) and method A (MEM-DPA [1,000 μg]) were 93.2 and 86.4%, respectively. The lowest sensitivity (43.2%) was exhibited by method D (IPM-EDTA [760 μg] + SMA [2 mg]). The highest specificity (97.1%) was achieved by method B. Both methods A and D exhibited a specificity of 94.3%. The lowest specificity (11.4%) was shown by method C.

The high false-negative rates of the MEM-DPA (250 mg) DDPT (Table 1, method A) and the MEM-DPA (1,000 μg) DPT (Table 2, method A) were due to seven and five bla_{IMP-6}-positive *P. aeruginosa* isolates, respectively. The MICs of MEM were much higher (>512 μg/ml) for these isolates than those of other isolates and were higher than those of IPM (Table 3). In the presence of 30 μg of PAβN/ml, the MICs of MEM and IPM were reduced by at least 2-fold for four of the five isolates (Table 3, group A), and the MEM-DPA DDPT became positive for two of four isolates (Fig. 1, data not shown). The effects of 2.5 μg of CCCP/ml and 250 μg of cloxacillin/ml on the MICs of MEM and IPM (Table 3) and DDPT were minimal (data not shown).

Sequencing revealed that all five bla_{IMP-1}-like genes in MEM-DPA DDPT false-negative *P. aeruginosa* isolates with a MEM MIC of >512 μg/ml were bla_{IMP-6} (Table 3, group A). However, bla_{IMP}-like genes sequenced from two and four MEM-DPA DDPT-positive *P. aeruginosa* and *Acinetobacter* spp. isolates with a MEM MIC of ≤64 μg/ml, respectively, were bla_{IMP-1} (Table 3, groups C and E). To monitor the possibility of improving DPA DDPT, the performances of three different DPA concentrations and two different media, MHA and MacConkey agar, were compared using one of the MEM-DDPT false-negative isolates. The isolate showed only a small potentiating zone with an IPM and a 500-μg DPA

TABLE 3 Effects of efflux inhibitors and CLX on the mean MICs of MEM and IPM for MEM-DPA DDPT-negative and -positive isolates[a]

Groupings based on characteristics and DDPT					Mean MIC (μg/ml) of MEM with:				Mean MIC (μg/ml) of IPM with:			
Group	Species	MBL gene	MEM-DPA/ IPM- DPA[b]	No. of isolates	None	PAβN, 30 μg	CCCP, 2.5 μg	CLX, 250 μg	None	PAβN, 30 μg	CCCP, 2.5 μg	CLX, 250 μg
A	*P. aeruginosa*	bla_{IMP-6}	Neg[c]/Pos	5	>512	>358	>512	>512	26	8	19	19
B	*P. aeruginosa*	bla_{VIM-2}	Neg/Pos	1	512	256	256	512	>128	64	128	>128
C	*P. aeruginosa*	bla_{IMP-1}	Pos/Pos	2	32	24	32	24	48	48	48	20
D	*P. aeruginosa*	bla_{VIM-2}-like	Pos/Pos	6	112	80	56	104	>64	>56	>56	>56
E	*Acinetobacter* spp.	Others[d]	Pos/Pos	6	31	17	27	27	20	11	20	11
F	Control[e]	Negative	Neg/Neg	3	16	3	11	11	7	3	18	4

[a] CLX, cloxacillin. Other abbreviations are as defined in Table 1.
[b] The patterns of DDPT with MEM-DPA (method A)/IPM-DPA (method B) are as shown in Table 1.
[c] Includes one DDPT-positive isolate with MEM-DPA (250 μg) (method A, Table 1) but negative with MEM-DPA (835 μg) (method C).
[d] Includes four isolates with bla_{IMP-1} and one each with the bla_{VIM-2} and bla_{SIM-1} genes.
[e] Includes one isolate of *P. aeruginosa* and two *Acinetobacter* spp.

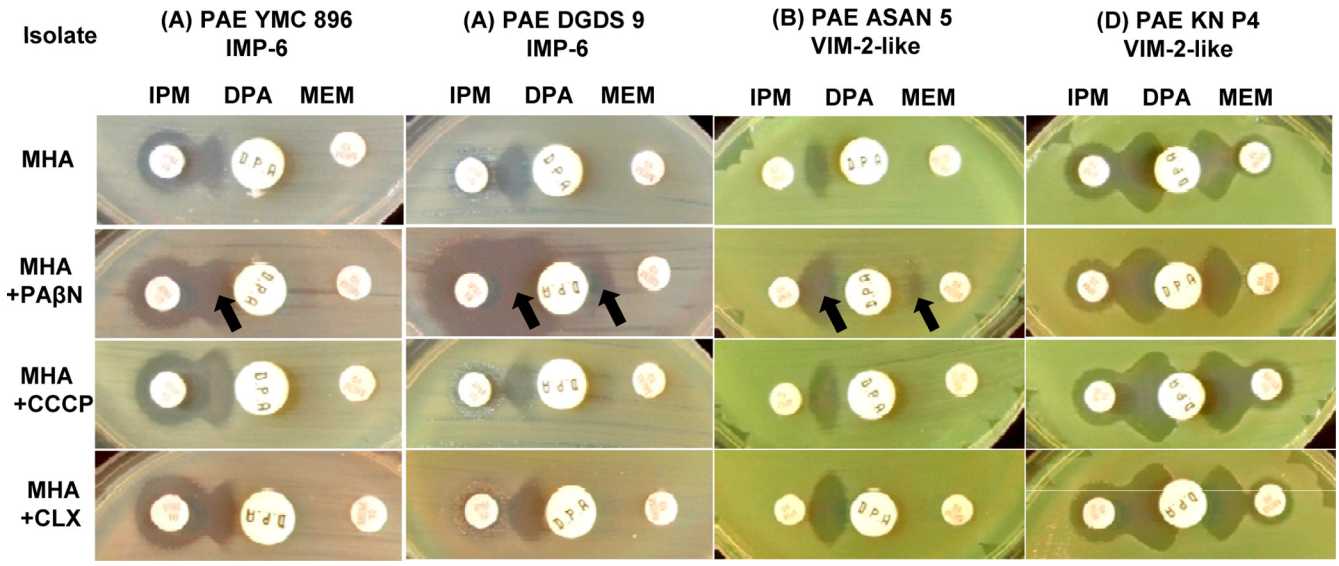

FIG 1 Effect of inhibitors of the efflux pump and AmpC β-lactamase on IPM-DPA and MEM-DPA double-disk potentiation tests using MHA for representative isolates of *P. aeruginosa* (PAE). The synergistic zones were more often enhanced by the presence of PAβN (arrows), than CCCP and cloxacillin (CLX). The letters A, B, and D in parentheses correspond to the groups in Table 3.

disk on MHA; however, even the 200-μg DPA disk showed a distinct potentiating zone on MacConkey agar (Fig. 2A). When the evaluation was expanded to include other isolates, 18 of 20 isolates showed larger IPM-DPA potentiating zones on MacConkey agar than on MHA (Fig. 2B), although all five *P. aeruginosa* isolates with bla_{IMP-6} genes remained false-negative by MEM-DPA DDPT. None of the IPM-nonsusceptible, MBL-negative isolates of *P. aeruginosa* or *Acinetobacter* spp. (10 each) showed a false-positive DPA DDPT result on MacConkey agar with IPM or MEM disks. MHA supplemented with 5% oxgall enhanced the synergistic inhibition similar to that observed with MacConkey agar, and 10% sucrose reduced the enhancing effect (Fig. 2C). Among the four MEM-DPA DPT false-negative *P. aeruginosa* isolates with bla_{IMP-6} and the one with bla_{VIM-2}, only one from each group was positive when tested with MacConkey agar (data not shown).

DISCUSSION

We consider high sensitivity to be more important than high specificity in a phenotypic MBL screening test because molecular confirmation can be performed at a reference laboratory (15). DDPTs and DPTs have been used to detect MBL-producing isolates among carbapenem-nonsusceptible, Gram-negative bacilli. A DDPT can be interpreted as positive by the presence of even a small synergistic zone, but the distance between the two disks requires adjustment depending on the characteristics of the isolates in order to achieve optimal results (5). A DPT is simple to perform and interpret for extended-spectrum β-lactamase detection in *Escherichia coli*, *Klebsiella* spp., and *Proteus mirabilis*, because the inhibitor, clavulanic acid, does not have antimicrobial activity; however, in MBL detection, enlargement of the inhibition zone could be due to an MBL inhibitor alone, which would not be recognized unless a disk containing only an inhibitor is also placed on the medium.

For DDPT, Lee et al. (11) reduced the EDTA content of a disk from 1,900 to 760 μg and added 2 mg of SMA; however, in the present study, the IPM DDPT and IPM DPT with these inhibitors showed sensitivities of only 90.9 and 43.2%, respectively (Table 1, method E; Table 2, method D). It has previously been reported that DPA had a stronger inhibitory activity on IMP-1 among the inhibitors tested (18) and that it had much less toxicity to *P. aeruginosa* (6). The high stability of the DPA disks, as is EDTA disks, is another advantage; our laboratory-prepared disks stored at room temperature for 5 years showed potentiation zone sizes similar to those of freshly prepared disks (unpublished data). The availability of commercial DPA disks could make MBL detection simpler and more accurate.

In the present evaluation of DDPT, we also included an ETP disk, but the sensitivity was the lowest (25%), although a previous evaluation of the modified Hodge test showed more distinct distortion of inhibition zone by an ETP disk (8). The sensitivity of IPM-DPA (97.7%) was higher than that of MEM-DPA (79.5%) (Table 1, methods A and B), suggesting that some test organisms overexpressed the efflux pump. It has been reported that the MICs of MEM are significantly elevated by the presence of the MexAB-OprM efflux pump in both OprD-proficient and -deficient strains (7). In the present study, the low sensitivities of MEM-DPA DDPT and MEM-DPA DPT were mostly due to bla_{IMP-6}-positive *P. aeruginosa* isolates. All five isolates carrying bla_{IMP-6} were considerably more resistant to MEM (MIC > 512 μg/ml) than to IPM (MIC = 16 to 32 μg/ml) (Table 3). One *P. aeruginosa* isolate with bla_{VIM-2} showed a false-negative MEM-DPA DDPT and was highly resistant to MEM (MIC = 512 μg/ml). If the highly MEM-resistant, bla_{IMP-6}-positive isolates had not been included in our study, the false-negative rate (20.5%) of MEM-DPA DDPT could have been much lower. We thought that if our MEM-DPA DDPT false-negative *P. aeruginosa* isolates were related to the widespread, highly MEM-resistant isolates in Korea (16), it could mean high prevalence of MEM-DPA DDPT false-negative isolates. All of our five isolates with false-negative MEM-DPA DDPT were more resistant to MEM than to IPM and carried bla_{IMP-6} (GenBank accession no. JQ764729), as were the previous reported isolates (16). These results strongly suggest high prevalence of *P.*

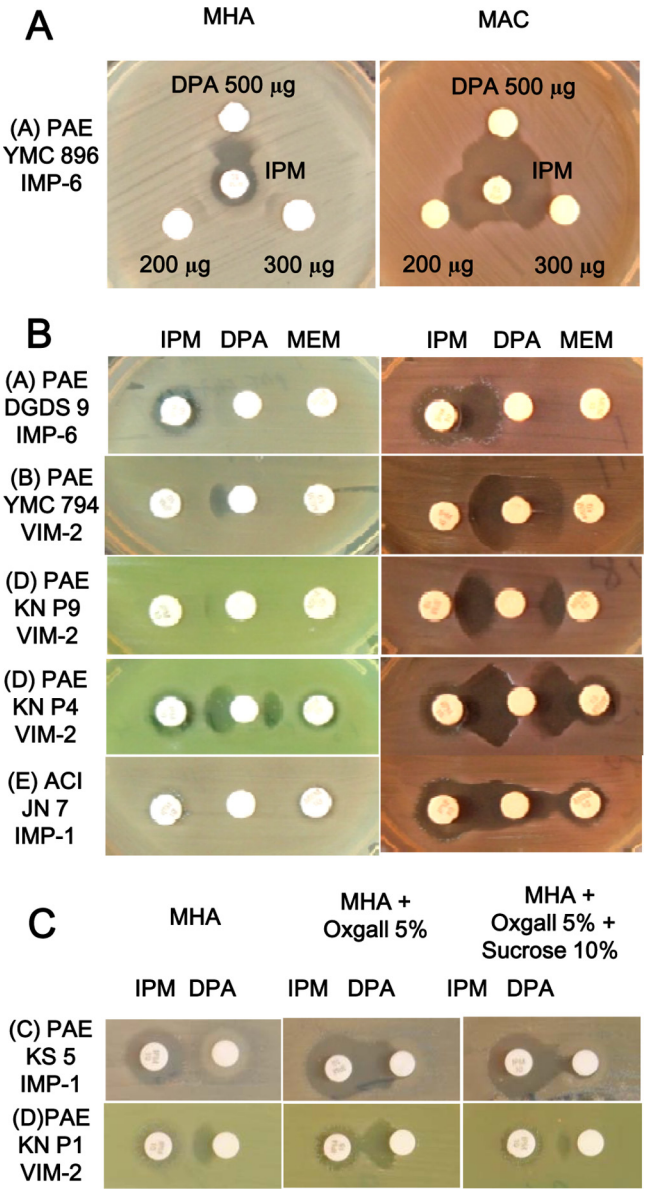

FIG 2 Improved performance of DPA DDPTs by use of IPM and MacConkey agar (MAC) instead of MEM and MHA. (A) An MEM-DPA DDPT-false-*negative P. aeruginosa* (PAE) isolate with bla_{IMP-6} shows a small inhibition zone only with disks of an IPM and a 500-μg DPA on MHA but shows a large inhibition zone even with a 200-μg DPA disk on MAC. (B) IPM-DPA DDPTs for representative isolates of PAE and *Acinetobacter* sp. (ACI) show larger synergistic zones on MAC than on MHA. (C) An improved IPM-DPA DDPT is shown on 5% oxgall-supplemented MHA. The effect is decreased when 10% sucrose is added. The letters A to E in parentheses correspond to the groups in Table 3.

aeruginosa isolates with false-negative MEM-DPA DDPT in Korea.

The MICs of MEM for all six MEM-DPS DDPT false-negative isolates (five bla_{IMP-6}- and one bla_{VIM-2}-positive *P. aeruginosa* isolates) were reduced by at least 2-fold (Table 3, groups A and B), and two isolates became positive to MEM-DPA DDPT (data not shown) in the presence of a moderate concentration (30 μg/ml) of PAβN. This indicated that the lower sensitivity of MEM-DPA DDPT was mostly due to overexpression of the efflux pump. It is important that laboratories should be aware of presence of false-negative MEM-based potentiation tests in *P. aeruginosa*. The lowest sensitivity (24.4%) of ETP-DPA DDPT can be explained by the weaker activity of this compound against *P. aeruginosa* and *Acinetobacter* spp. (14).

In a previous study, it was reported that the best results were observed when the DPA content in the disk was 835 μg for DDPTs and DPTs (17). In our study, use of a disk with 835 μg of DPA did not improve the sensitivity of the MEM-DPA DDPT test (Table 1, method A versus method C), whereas a 250-μg DPA disk achieved high sensitivity (97.7%) when used with an IPM disk (method B).

We reported previously that MacConkey agar performed better in the modified Hodge test, possibly due to the enhanced release of MBL by oxgall in the medium (9). Therefore, we were interested in elucidating the effect of MacConkey agar on the DDPT. Figure 2A shows the effects of different DPA concentrations and media on DPA DDPTs. On MHA, an MEM-DPA DDPT false-negative *P. aeruginosa* isolate with bla_{IMP-6} showed a synergistic inhibition zone only with a 500-μg DPA disk; however, on MacConkey agar, a large zone was formed even by a 200-μg disk. It was found that, on MacConkey agar, IPM-DPA synergistic zones became detectable or larger compared to those with MHA for most of the isolates tested. Nevertheless, the MEM-DPA method did not produce much improvement. Use of MacConkey agar did not yield false-positive DDPT results with 10 IPM-nonsusceptible *P. aeruginosa* and 14 OXA carbapenemase-producing *Acinetobacter* isolates (data not shown).

The MICs of IPM and MEM for *P. aeruginosa* ATCC 27853 only decreased slightly when determined using MacConkey agar rather than using MHA, from 0.75 to 0.5 μg/ml and from 0.25 to 0.19 μg/ml, respectively, although this tendency was not consistently observed for bla_{IMP-6}-positive isolates (data not shown). These findings suggest that an improved IPM-DPA DDPT test with MacConkey agar was not due to the increased activity of the carbapenems on the medium. In our present study, MHA supplemented with 5 mg of oxgall/ml (which is approximately equivalent to 1.5 mg of bile salt no. 3/ml) resulted in enhancement of the test, and this effect was reduced in the presence of 10% sucrose (Fig. 2C), suggesting that IPM, DPA, and surface-active bile salts act cooperatively.

It is apparent from the present study that the performance of DDPTs is influenced by the characteristics of the isolates. Since none of the methods could detect all MBL-producing *Pseudomonas* and *Acinetobacter* isolates, we maintain our previous view (11) that the simultaneous use of a modified Hodge test preferably on MacConkey agar (8) and a DDPT can resolve most problems encountered in detecting MBL- or class A carbapenemase-producing isolates.

In conclusion, DPA is a better MBL inhibitor than EDTA in DDPT and DPT for the detection of IMP-1-like, VIM-2-like, and SIM-1 MBL-producing *Pseudomonas* spp. and *Acinetobacter* spp. DPA (250 μg) disks performed better with IPM disks than with MEM disks when *P. aeruginosa* isolates were highly resistant to MEM due to the overexpression of efflux pumps. Laboratories should be aware of false-negative MEM-based potentiation tests. A significantly more enhanced synergistic zone can be obtained when a DPA DDPT is performed with MacConkey agar than with MHA.

ACKNOWLEDGMENTS

This study was supported by faculty research grant 6-2011-0100 from Yonsei University College of Medicine for 2011.

We thank So-ri Jong, Young Hee Seo, and Myung Sook Kim for technical assistance.

REFERENCES

1. **Arakawa Y, et al.** 2000. Convenient test for screening metallo-β-lactamase-producing gram-negative bacteria by using thiol compounds. J. Clin. Microbiol. **38**:40–43.
2. **CLSI.** 2008. Abbreviated identification of bacteria and yeast: approved guideline, 2nd ed. CLSI document M35–A2. Clinical and Laboratory Standards Institute, Wayne, PA.
3. **CLSI.** 2011. Performance standards for antimicrobial susceptibility testing; 21st informational supplement. CLSI document M100–S21. Clinical and Laboratory Standards Institute, Wayne, PA.
4. **Giske CG, et al.** 2011. A sensitive and specific phenotypic assay for detection of metallo-β-lactamases and KPC in *Klebsiella pneumoniae* with the use of meropenem disks supplemented with aminophenylboronic acid, dipicolinic acid, and cloxacillin. Clin. Microbiol. Infect. **17**:552–556.
5. **Hocquet D, Dehecq B, Bertrand X, Plesiat P.** 2011. Strain-tailored double-disk synergy test detects extended-spectrum oxacillinases in *Pseudomonas aeruginosa.* J. Clin. Microbiol. **49**:2262–2265.
6. **Kimura S, Ishii Y, Yamaguchi K.** 2005. Evaluation of dipicolinic acid for detection of IMP- or VIM-type metallo-β-lactamase-producing *Pseudomonas aeruginosa* clinical isolates. Diagn. Microbiol. Infect. Dis. **53**: 241–244.
7. **Kohler T, Michea-Hamzehpour M, Epp SF, Pechere JC.** 1999. Carbapenem activities against *Pseudomonas aeruginosa*: respective contributions of OprD and efflux systems. Antimicrob. Agents Chemother. **43**:424–427.
8. **Lee K, et al.** 2010. Improved performance of the modified Hodge test with MacConkey agar for screening carbapenemase-producing Gram-negative bacilli. J. Microbiol. Methods **83**:149–152.
9. **Lee K, et al.** 2009. Wide dissemination of OXA-type carbapenemases in clinical *Acinetobacter* spp. isolates from South Korea. Int. J. Antimicrob. Agents **33**:520–524.
10. **Lee K, et al.** 2011. Further increases in carbapenem-, amikacin-, and fluoroquinolone-resistant isolates of *Acinetobacter* spp. and *Pseudomonas aeruginosa* in Korea: KONSAR study 2009. Yonsei Med. J. **52**:793–802.
11. **Lee K, Lim YS, Yong D, Yum JH, Chong Y.** 2003. Evaluation of the Hodge test and the imipenem-EDTA double-disk synergy test for differentiating metallo-β-lactamase-producing isolates of *Pseudomonas* spp. and *Acinetobacter* spp. J. Clin. Microbiol. **41**:4623–4629.
12. **Lee K, et al.** 2005. Evaluation of Etest MBL for detection of bla_{IMP-1} and bla_{VIM-2} allele-positive clinical isolates of *Pseudomonas* spp. and *Acinetobacter* spp. J. Clin. Microbiol. **43**:942–944.
13. **Lee K, et al.** 2005. Novel acquired metallo-β-lactamase gene, bla_{SIM-1}, in a class 1 integron from *Acinetobacter baumannii* clinical isolates from Korea. Antimicrob. Agents Chemother. **49**:4485–4491.
14. **Papp-Wallace KM, Endimiani A, Taracila MA, Bonomo RA.** 2011. Carbapenems: past, present, and future. Antimicrob. Agents Chemother. **55**:4943–4960.
15. **Pitout JD, et al.** 2005. Detection of *Pseudomonas aeruginosa* producing metallo-β-lactamases in a large centralized laboratory. J. Clin. Microbiol. **43**:3129–3135.
16. **Seok Y, et al.** 2011. Dissemination of IMP-6 metallo-β-lactamase-producing *Pseudomonas aeruginosa* sequence type 235 in Korea. J. Antimicrob. Chemother. **66**:2791–2796.
17. **Shin KS, Son BR, Hong SB, Kim J.** 2008. Dipicolinic acid-based disk methods for detection of metallo-β-lactamase-producing *Pseudomonas* spp. and *Acinetobacter* spp. Diagn. Microbiol. Infect. Dis. **62**:102–105.
18. **Siemann S, et al.** 2002. IMP-1 metallo-β-lactamase: effect of chelators and assessment of metal requirement by electrospray mass spectrometry. Biochim. Biophys. Acta **1571**:190–200.
19. **Turton JF, et al.** 2006. Identification of *Acinetobacter baumannii* by detection of the bla_{OXA-51}-like carbapenemase gene intrinsic to this species. J. Clin. Microbiol. **44**:2974–2976.
20. **Yong D, et al.** 2002. Imipenem-EDTA disk method for differentiation of metallo-β-lactamase-producing clinical isolates of *Pseudomonas* spp. and *Acinetobacter* spp. J. Clin. Microbiol. **40**:3798–3801.
21. **Zhou Z, et al.** 2011. Clinical carbapenem-resistant *Acinetobacter baylyi* strain coharboring bla_{SIM-1} and bla_{OXA-23} from China. Antimicrob. Agents Chemother. **55**:5347–5349.