Original Article

Comparison of Test Methods for Detecting Metallo- β -Lactamase-Producing Gram-Negative Bacteria

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SUMMARY: The recent increase in gram-negative bacteria coproducing multiple classes of β -lactamases has made it difficult to accurately identify metallo- β -lactamase (MBL) producers. In the present study, six methods for detecting MBL producers were compared using 56 gram-negative bacterial isolates that produce various β -lactamases. Sodium mercaptoacetic acid (SMA) and EDTA were used as inhibitors for a double-disk synergy test (DDST), and antimicrobial agents, including ceftazidime (CAZ), imipenem, and meropenem (MEPM), along with the distance between the disks, were compared. The Etest®, dry-plate DPD1®, Cica-Beta®, and modified Hodge test were also compared. Among the six methods compared, DDST using the SMA disk showed the highest sensitivity (Se) and specificity (Sp). In DDST, the clearest appearance of growth inhibition was observed when the distance between the disks was maintained at approximately 5 mm. A combination of CAZ and SMA successfully detected only MBL-producing isolates (Se, 87.5%; Sp, 100%), and MEPM exhibited the best performance in combination with SMA in the detection of MBL producers coproducing other classes of β -lactamases such as CTX-M- and CMY-type enzymes (Se, 79.1%; Sp, 100%). DDST using SMA and CAZ and/or MEPM is a simple, specific, and cost-effective method to screen MBL producers in routine clinical laboratory testing.

INTRODUCTION

Metallo- β -lactamases (MBLs) have been globally isolated from numerous bacterial species; more than 80 distinct types have been identified worldwide, with over 75% occurring as plasmid-encoded enzymes (1). The major families of acquired MBLs, including IMP- and VIM-type β -lactamases, were initially found in *Serratia marcescens* and *Pseudomonas aeruginosa* isolates (2,3) but have since been encountered in various bacterial species belonging to the genus *Pseudomonas* and the family *Enterobacteriaceae* (1). MBLs are worrisome because they virtually hydrolyze all β -lactams, including the oxyimino cephalosporins, cephamycins, and carbapenems, and are not inhibited by any therapeutically utilized β -lactamase inhibitors. Moreover, a growing concern is the rapid spread of a novel MBL group (NDM-types), which are often coproduced with CTX-M- and/or CMY-enzymes (4,5), and some of the NDM-1 producers demonstrate low resistance to carbapenems (MIC, $<2 \mu\text{g/ml}$). In the early stage of admission, exact identification of MBL producers by routine microbiological tests is therefore sometimes difficult.

To date, several different methods have been devel-

oped to detect MBL-producing gram-negative bacteria, including the following three tests that are available in Japan: a double-disk synergy test (DDST) using inhibitors, such as sodium mercaptoacetic acid (SMA) or ethylenediaminetetraacetic acid (EDTA), a microdilution test using 2,6-pyridinedicarboxylic acid (dipicolinic acid [DPA]) as an inhibitor, and the Etest using EDTA as an inhibitor. However, these tests are based on different combinations and concentrations of antimicrobials and inhibitors, as well as interpretation criteria; thus, no standardized protocol for reliable detection of MBL producers is available. In addition, recent emergence and global dissemination of gram-negative bacteria producing new MBLs, such as NDM-1, together with CTX-M-type extended spectrum β -lactamases (ESBLs) and CMY-type cephalosporinases, have muddled the interpretation of the results of these detection methods (6).

In an era where the epidemiology of MBL is constantly changing, it remains unclear which detection methods are the most reliable and which inhibitors are the most suitable. Therefore, the aim of this study was to compare test methods that can be routinely applied in microbiological laboratories for detecting MBL-producing gram-negative bacteria, particularly bacterial isolates that coproduce plural classes of β -lactamases.

MATERIALS AND METHODS

Bacterial strains: The bacterial strains tested in the present study are listed in Table 1 and included genetically well-characterized strains that produce various

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Table 1. Control strains with well-characterized resistance mechanisms used for evaluating test methods in this study

Isolate (<i>n</i> = 56)	No. of isolates	MIC ($\mu\text{g/ml}$) ¹⁾		
		CAZ	IPM	
MBL-producing strains (<i>n</i> = 24)				
Plural classes of β -lactamases (<i>n</i> = 3)				
NDM-1, CTX-M-1, CMY-4 and TEM-1	<i>E. coli</i>	1	128 <	32
NDM-1 and TEM-1	<i>K. pneumoniae</i>	1	128 <	4
IMP-1 and CTX-M-2	<i>K. pneumoniae</i>	1	32	0.5
IMP- or VIM-type β -lactamase (<i>n</i> = 21)				
IMP-1	<i>E. coli</i>	3	128 <	4–8
IMP-1	<i>K. pneumoniae</i>	3	128 <	4–64
IMP-1	<i>Acinetobacter</i> sp.	6	128 <	32–128 <
IMP-1	<i>P. aeruginosa</i>	6	128 <	64–128 <
IMP-1	<i>P. putida</i>	1	128 <	128 <
VIM-1	<i>P. aeruginosa</i>	1	128 <	128 <
VIM-2	<i>P. aeruginosa</i>	1	128 <	128 <
Non-MBL-producing strains (<i>n</i> = 32)				
CMY-type β -lactamase (<i>n</i> = 5)				
CMY-2	<i>E. coli</i>	2	128 <	0.5–1
CMY-8	<i>K. pneumoniae</i>	1	64	0.25 >
CMY-9	<i>E. coli</i>	1	128 <	0.5
CMY-9	<i>K. pneumoniae</i>	1	128 <	0.5
CTX-M-type β -lactamase (<i>n</i> = 15)				
CTX-M-15	<i>E. coli</i>	5	8–32	0.25–0.5
CTX-M-2	<i>E. coli</i>	5	0.5–32	0.25
CTX-M-14	<i>E. coli</i>	4	0.5–8	0.25
CTX-M-8	<i>E. coli</i>	1	1	0.25
KPC-type β -lactamase (<i>n</i> = 1)				
KPC-3	<i>K. pneumoniae</i>	1	128 <	128 <
Non- β -lactamase-producing strains (<i>n</i> = 11)				
	<i>P. aeruginosa</i>	6	32–128 <	8–64
	<i>Acinetobacter</i> sp.	5	2–16	0.25–0.5

¹⁾: Antimicrobial susceptibility test was performed using the agar dilution method as recommended by the Clinical and Laboratory Standards Institute (CLSI), and the interpretation of minimal inhibitory concentration (MIC) results was in accordance with CLSI criteria in document M7-A9.

CAZ, ceftazidime; IPM, imipenem.

MBLs, including IMP-type, VIM-type, and NDM-1-type. Clinical isolates producing CTX-M-type ESBLs, CMY-type cephalosporinase, or KPC-3 carbapenemase, which belong to different classes of β -lactamases, and non- β -lactamases-producing isolates with decreased ceftazidime (CAZ) susceptibility were also used to examine specificities (Sps) of six methods as the negative control strains.

DDSTs: DDST was performed using SMA or EDTA as inhibitors as described previously (7). A colony of each bacterial strain was suspended and adjusted with Mueller-Hinton (MH) broth (Becton Dickinson, Sparks, Md., USA) to the turbidity of McFarland standard No. 0.5 and then spread on an MH agar plate (Becton Dickinson) using a cotton swab in accordance with the protocol of the Clinical and Laboratory Stan-

dards Institute (CLSI) (8). After the agar surface dried, a CAZ disk (30 μg /disk), an imipenem disk (IPM; 10 μg /disk), or a meropenem disk (MEPM; 10 μg /disk) (Eiken Co., Ltd., Tokyo, Japan) was placed on the agar plate together with a SMA disk (Eiken) or a blank filter disk. The distance between the two antimicrobial disks was maintained at approximately 4–5 cm, and the SMA disk or blank filter disk was placed in close proximity of one of the antimicrobial disks within an edge-to-edge distance of 5, 10, 15, or 20 mm. Twenty microliters of 0.5 M EDTA-2Na solution (Nacalai Tesque, Inc., Kyoto, Japan) was added to the blank filter disk placed on the agar plate, which was then incubated for 18 h at 35°C. In DDST with the SMA disk, strains were considered to be MBL-positive when an expansion (≥ 5 mm) of the growth-inhibition zone was observed around the antimicrobial disk near the SMA disk compared with the growth-inhibition diameter around the control disk containing the same antimicrobial agent (Fig. 1A–a). In the test with EDTA disk, the bacterial strain was considered to be an MBL producer when the result met at least one of the following three interpretation criteria: (i) the observed growth-inhibition zone was 5 mm greater than that of the control antimicrobial disk, similar to DDST using the SMA disk; (ii) the growth-inhibition zone was 2.5 mm greater than that of the control antimicrobial disk (Fig. 1B–b) based on the mean expansion of the growth-inhibition zones of 2.5 mm for IMP-1 producers (19 isolates) in the present study; and (iii) when no apparent expansion of the growth-inhibition zone was observed, the strain was considered MBL-positive if the shape of the growth-inhibition zone expanded toward the CAZ, IPM, or MEPM disk or when a characteristic growth-inhibition zone was observed (Fig. 1B–c).

Etset MBL IP/IPI: Etset (bioMerieux, Lyon, France) was performed using MH agar plates according to the manufacturer's guidelines and interpretation criteria, and the isolates were considered MBL-positive when a reduction in MIC by three doubling dilutions (2³) was observed in the presence of EDTA (i.e., MIC ratio ≥ 8).

Cica-Beta test MBL: Cica-Beta test MBL (Kanto Chemical, Inc., Tokyo, Japan) was performed according to the manufacturer's guidelines, which was based on hydrolysis of the chromogenic cephalosporin and nitrocefin on paper strips. Two strips were needed: a control strip with no inhibitor (Cica-Beta I) and a second with SMA to detect the inhibition of MBL function (Cica-Beta MBL). Results were obtained within 15 min using colonies grown on agar plates by these two tests. Isolates were considered to be MBL-positive when nitrocefin hydrolysis was observed with the Cica-Beta I test but not the Cica-Beta MBL test. Isolates were deemed as MBL-negative when no nitrocefin hydrolysis was observed using the Cica-Beta I test or when nitrocefin hydrolysis was observed with both the Cica-Beta I and Cica-Beta MBL tests.

Dry-plate DPD1 test: Dry-plate DPD1 test was performed according to the manufacturer's guidelines. This method is based on the microdilution test using DPA as an MBL inhibitor. A colony of each bacterial strain was suspended in MH broth and adjusted to the turbidity of McFarland standard No. 1.0. A 25- μl ali-

Fig. 1. (Color online) Interpretation criteria of double-disk synergy tests (DDSTs) for detecting MBL production. (A) CAZ and SMA disk. Isolates were considered MBL-positive when the inhibition zone of the antimicrobial disk placed near the SMA disk was at least 5 mm greater than that of corresponding antimicrobial disk (a). (B) CAZ and EDTA disk. Isolates were considered as MBL-positive when they satisfied interpretation criteria as following: (b) The mean expansion of the growth-inhibition zone for IMP-1 producers was 2.5 mm in this study. Then, isolates were considered MBL-positive if the growth-inhibition zone was 2.5 mm greater than that of corresponding antimicrobial disk. (c) If no expansion of the growth-inhibition zone was observed, isolates were considered MBL-positive when the shape of the growth inhibition had changed, or when a characteristic shaped-zone was observed. CAZ, ceftazidime; EDTA, ethylenediaminetetraacetic acid; SMA, sodium mercaptoacetic acid.

quot of the bacterial suspension was added to 12 ml of MH broth, followed by addition of a 100- μ l aliquot of the test suspension to each well of the dry-plate DPD1, which was then incubated for 18 h at 35°C. Bacterial isolates were considered MBL-positive when MIC of CAZ and/or IPM was reduced by three doubling dilutions (2^3) in the presence of 175 μ g/ml of DPA.

Modified Hodge test: The modified Hodge test was performed according to the CLSI protocol (8). *Escherichia coli* ATCC 25922 was used as an indicator strain. The *E. coli* culture suspension to be tested was adjusted to one-tenth the turbidity of McFarland standard No. 0.5 and spread on an MH agar plate using a cotton swab. An MEPM disk (10 μ g/disk) was placed at the center of the MH agar plate, and both a positive (*Klebsiella pneumoniae* ATCC BAA-1705) and a negative (*K. pneumoniae* ATCC BAA-1706) control strain were heavily streaked from the edge of the MEPM disk to the periphery of the MH agar plate, which was then incubated for 18 h at 35°C. Appearance of a distorted growth inhibition zone was interpreted as positive carbapenemase production.

Statistical analysis: The sensitivity (Se) of each phenotypic test was calculated from the number of true positive bacteria, whereas the Sp was calculated from the number of true negative bacteria. In addition, the positive and negative predictive values were also calculated as described previously (9).

RESULTS AND DISCUSSION

The Se and Sp of DDST using SMA or EDTA as an inhibitor and three kinds of β -lactams, including CAZ, MEPM, and IPM, were evaluated when the edge-to-edge distances between the disks containing a β -lactam and an inhibitor, respectively, were maintained at approximately 5, 10, 15, and 20 mm. In the IMP- or VIM-type MBL producers (21 isolates), the clearest appearance of a growth-inhibition zone was observed when the distance between the two disks was maintained at approximately 5 mm so that the combination of CAZ-

SMA (Se, 100%) yielded a better result than other combinations (Table 2). No distinct change in the appearance of the growth-inhibition zone was observed for strains producing only the KPC- or CMY-type β -lactamase so that DDST using SMA provided the highest Sp (100%) among the non-MBL producers (32 isolates) (Table 2). In DDST using EDTA, interpretation criteria substantially affected Se and Sp. Among the three interpretation criteria compared, those based on alteration in the shapes of growth-inhibition zones toward the antimicrobial agent disk yielded the highest Se (Table 3). Meanwhile, when the distance between the two disks was maintained at approximately 5 mm, the criteria yielded false-positive results in multidrug-resistant *P. aeruginosa* strains that did not produce MBL, which was likely because the expansion of the growth-inhibition zone was determined to be greater than that of the control antimicrobial disk by larger alterations in the shape of the inhibition zone. As a result, the interpretation criteria using the expansion (≥ 2.5 mm) of the growth-inhibition zone presented the most reproducible results so that the combination of EDTA-IPM yielded better results (Se, 81.0%; Sp, 87.5%) than other combinations (Table 3). However, DDST using EDTA failed to detect IMP-1-producing *Acinetobacter* sp. because EDTA itself disrupted the growth of this organism (Fig. 2B), resulting in a decreased Se compared with those of SMA. Moreover, the use of EDTA yielded false-positive results in the CMY-type β -lactamase-producing *E. coli* strains because EDTA probably disrupted the growth of this strain by chelating biologically important metals in the MH agar (Fig. 3B). These results indicated that EDTA is not an appropriate inhibitor for detecting MBL producers. EDTA behaves as a strong chelator of biologically important cations, including zinc, in the medium, but it is not a specific MBL inhibitor. However, SMA reportedly binds to the active center of MBLs (10) and blocks their functions, whereas EDTA indirectly inhibits the functions of metallo-enzymes, including MBL, by reducing the free-zinc concentration in the MH agar. In this case, EDTA would merely dis-

Table 2. Summary of sensitivities and specificities of the DDSTs using of SMA and EDTA in the phenotypic detection of IMP- or VIM-producing strains

Agent	Distance between disks (mm)	DDSTs using SMA disk ¹⁾						DDSTs using EDTA disk ¹⁾					
		MBL-producing strains (n = 21)		non-MBL-producing strains (n = 32)		Se (%)	Sp (%)	MBL-producing strains (n = 21)		non-MBL-producing strains (n = 32)		Se (%)	Sp (%)
		Positive	Negative	Positive	Negative			Positive	Negative	Positive	Negative		
CAZ	5	21	0	0	32	100	100	16	5	7	25	76.2	78.1
	10	20	1	0	32	95.2	100	11	10	0	32	52.4	100
	15	20	1	0	32	95.2	100	1	20	0	32	4.8	100
	20	5	16	0	32	23.8	100	0	21	0	32	0	100
MEPM	5	16	5	0	32	76.2	100	15	6	5	27	71.4	84.4
	10	16	5	0	32	76.2	100	3	18	1	31	14.3	96.9
	15	14	7	0	32	66.7	100	0	21	2	30	0	93.8
	20	3	18	0	32	14.3	100	0	21	2	30	0	93.8
IPM	5	18	3	0	32	85.7	100	17	4	4	28	81.0	87.5
	10	18	3	0	32	85.7	100	8	13	0	32	38.1	100
	15	17	4	0	32	81.0	100	1	20	0	32	4.8	100
	20	1	20	0	32	4.8	100	0	21	0	32	0	100

¹⁾: Isolates were considered MBL-positive if the growth-inhibition zone was 2.5 mm larger than that of corresponding antimicrobial disk. MBL, metallo- β -lactamase; DDSTs, double-disk synergy tests; Se, sensitivity; Sp, specificity; CAZ, ceftazidime; MEPM, meropenem; IPM, imipenem; SMA, sodium mercaptoacetic acid; EDTA, ethylenediaminetetraacetic acid.

Table 3. Comparison of sensitivity and specificity of the DDSTs using EDTA obtained by three interpretation criteria in IMP- or VIM-producing strains (21 strains)

Criteria	EDTA-CAZ (5 mm)			EDTA-MEPM (5 mm)			EDTA-IPM (5 mm)		
	Expansion (mm)		Shape of zone ³⁾	Expansion (mm)		Shape of zone	Expansion (mm)		Shape of zone
	$\geq 5^{1)}$	$\geq 2.5^{2)}$		≥ 5	≥ 2.5		≥ 5	≥ 2.5	
Se (%)	66.7	76.2	76.2	61.9	71.4	81.0	57.1	81.0	100
Sp (%)	87.5	78.1	34.3	90.6	84.4	78.1	93.8	87.5	68.8

DDSTs were performed using EDTA as inhibitor, and the distance between the two disks was maintained at approximately 5 mm.

¹⁾: Isolates were considered MBL-positive when the growth-inhibition zone was observed 5 mm larger than that of corresponding antimicrobial disk.

²⁾: Isolates were considered MBL-positive when the growth-inhibition zone was observed 2.5 mm larger than that of corresponding antimicrobial disk.

³⁾: Isolates were considered MBL-positive when the shapes of growth inhibition were changed or characteristic shaped-zone was observed.

Abbreviations are in Table 2.

rupt nonspecific bacterial growth in some strains or species through the shortage of metals in the media.

When the distance between the disks maintained at 15 or 20 mm as recommended by the manufacturer, two NDM-1 producers isolated in Japan were not detected regardless of the antimicrobial agents used as the indicator. The combination of SMA and CAZ failed to detect NDM-1 producers because the NDM-producers coproduced CAZ-hydrolyzing enzymes, such as CTX-M-15, CMY-type cephalosporinases, and/or SHV-12 (Fig. 4A). However, the combination of SMA and MEPM yielded positive results when the distance between the two disks was maintained at approximately 5 mm (Fig. 4C; Table 4) because MEPM is barely hydrolyzed by SHV-derived ESBLs, CTX-M-type ESBLs, and CMY-type cephalosporinases. Moreover, under the same experimental conditions, the best appearance of a growth-inhibition zone was observed in the *K. pneumoniae* isolates that coproduced IMP-1 and CTX-M-2 β -lac-

tamase (Table 4). Thus, MEPM showed the best performance in combination with SMA in detecting MBL-producing strains that coproduce CTX-M-type and/or CMY-type enzymes. The combination of EDTA and MEPM also produced positive results in NDM-1-producing *E. coli* and *K. pneumoniae* isolates. However, EDTA disrupted growth of these NDM-1-producers to some extent, and ambiguous growth-inhibition zones appeared for both isolates of NDM-1-producing *E. coli* and *K. pneumoniae* (Fig. 4D). Thus, when two disks containing MEPM and EDTA were placed in close proximity of each other at a distance of approximately 5 or 10 mm, it became difficult to interpret the expansion of the growth-inhibition zones due to the formation of an apparent growth-inhibition zone around the EDTA disk. Therefore, unlike DDST using SMA, it should be noted that the combination of EDTA and MEPM may occasionally overlook MBL-producing strains coproducing MBL and other classes of β -lac-

Fig. 2. (Color online) Appearance of growth-inhibition zone in IMP-1-producing *Acinetobacter* sp. The DDSTs using EDTA failed to detect IMP-1-producing *Acinetobacter* sp. because EDTA disrupted the growth of this organism. Abbreviations are in Fig. 1.

Fig. 3. (Color online) Appearance of growth-inhibition zone in CMY-producing strains. In DDSTs using SMA, no distinct change in the appearance of the growth-inhibition zone was observed for strains producing CMY. However, the use of EDTA yielded false-positive results in the CMY-type β -lactamases producing strains, since EDTA itself disrupted the growth of this organism. Abbreviations are in Fig. 1.

tamases, particularly when the disk distance is maintained at approximately 5 mm (Table 4).

The DDST results of 56 strains were compared with four other test methods. The DPD1 plate test provided the highest Se (87.5%) and Sp (96.9%) among the four compared screening methods (Table 5). In contrast, the Etest showed false-negative results in IMP-1-producing *E. coli* and *K. pneumoniae* strains isolated in Japan; the Cica-Beta test produced false-negative results in IMP-1-producing *Acinetobacter* sp.; and the modified Hodge test also showed false-negative results in IMP-1-producing *P. aeruginosa*, resulting in Se values of 58.3%, 66.7%, and 70.8%, respectively (Table 5). These results were due to the fact that MIC of IPM (i.e., the substrate used in the MBL Etest) is occasionally low (MIC, <2 $\mu\text{g/ml}$) for the IMP-1-producing *E. coli* and *K. pneumoniae* strains (11). Meanwhile, both the Cica-Beta I and Cica-Beta MBL tests yielded positive results in IMP-1-producing *Acinetobacter* strains, indicating false-negative results. Because many *Acinetobacter baumannii* strains coproduce OXA-type carbapenemases, such as OXA-23-like and OXA-58-like enzymes, together with or without production of intrinsic OXA-51-like enzymes, nitrocefim hydrolysis by the OXA-type carbapenemases may yield false-negative results in the Cica-Beta MBL test despite MBL inhibition by SMA. In addition, as a matter of course, both the Etest and Cica-

Beta test failed to specify carbapenemase types produced by each of three strains coproducing plural classes of β -lactamases, and the modified Hodge test also failed to detect NDM-1-producing *E. coli*. Our results support the findings of previous studies; which found that the modified Hodge test occasionally lacks Se (e.g., weak detection of NDM producers) (11). Both the Etest and Cica-Beta test are indeed simple and rapid screening methods, which are easy to implement into a clinical laboratory, although they are not specific for the detection of MBL producers, particularly strains coproducing plural classes of β -lactamases. The modified Hodge test is also a simple and inexpensive assay, but it may produce false-positive results in ESBL-producing clinical isolates (12,13) and negative or weakly positive results in NDM producers. Although MEPM showed the best performance in combination with SMA in detecting MBL-producing strains coproducing other classes of β -lactamases, the combination of SMA and CAZ among 56 strains provided the highest overall Se (87.5%) and Sp (100%) for the detection of MBL producers. CAZ appears to be the most suitable substrate at present for the identification of only MBL-producing isolates because almost all isolates usually demonstrated high resistance to CAZ (MIC, >64 $\mu\text{g/ml}$), even if the IPM MIC was low, as reported previously (14,15). As shown in the present study, addi-

Fig. 4. (Color online) Appearance of growth-inhibition zone in NDM-1-producing strains. The combination of SMA and CAZ failed to detect NDM-1 producers (A). The combination of SMA and MEPM gave positive results when the distance between the two disks was maintained at approximately 5 mm (C). The combination of EDTA and MEPM also produced positive results in NDM-1 producers. However, EDTA disrupted the growth of this organism, and the ambiguous growth-inhibition zones appeared for NDM-1 producers (D). MEPM, meropenem. Other abbreviations are in Fig. 1.

Table 4. Detection of MBL producers by DDSTs in bacteria coproducing plural classes of β -lactamases

Strain	Combination of antimicrobial agent and inhibitor ¹⁾									
	CAZ-SMA		CAZ-EDTA		MEPM-SMA		MEPM-EDTA		IPM-SMA	
	Distance (mm)		Distance (mm)		Distance (mm)		Distance (mm)		Distance (mm)	
	5	10	5	10	5	10	5	10	5	10
NDM-1-producing <i>E. coli</i>	–	–	–	–	+	–	+	+	+	–
NDM-1-producing <i>K. pneumoniae</i>	–	–	+	+	+	–	+	+	–	–
IMP-1 and CTX-M coproducing <i>K. pneumoniae</i>	–	–	–	–	+	+	–	–	–	–

¹⁾ Isolates were considered MBL-positive if the growth-inhibition zone was 2.5 mm larger than that of corresponding antimicrobial disk. Abbreviations are in Table 2.

tion of the MEPM disk was effective for successful detection of MBL-producing isolates coproducing other classes of cephalosporin-hydrolyzing enzymes while performing DDST, although such strains remain rare in Japan. The DPD1 plate test also showed fine results similar to those of DDST using SMA and CAZ in 56 strains (Se, 87.5%; Sp, 96.9%), as shown in Table 5, suggesting that SMA and DPA may be the most suitable inhibitors for detecting MBL producers. Thus, both the SMA disk method and DPD1 plate test are easy to perform and interpret and can easily be incorporated into the daily workflow of clinical microbiological laboratories. However, the DPD1 plate test is too expensive for

use in routine testing.

In conclusion, we demonstrated that CAZ and MEPM showed the best performance in combination with SMA when the distance between two disks was maintained at approximately 5 mm, even though the clinical isolates produced MBL, together with broad-spectrum serine β -lactamases, such as CMY- and CTX-M-type enzymes. A combination of CAZ with SMA successfully detected isolates producing only MBL, and MEPM showed the best performance in combination with SMA in the detection of MBL-producing strains coproducing other classes of β -lactamases. DDST using SMA and CAZ and/or MEPM is a simple, specific, and

Table 5. Comparison of sensitivities and specificities of six screening test methods to detect MBL producers

Screening test method		All strains (<i>n</i> = 56)				IMP- or VIM- producers (<i>n</i> = 21)	Plural classes of β -lactamases producers (<i>n</i> = 3)	non-MBL- producers (<i>n</i> = 32)
		Se (%)	Sp (%)	PPV (%)	NPV (%)	TP/FN ³⁾	TP/FN	FP/TN ³⁾
DDSTs (5 mm) ¹⁾								
	SMA-CAZ	87.5	100	100	91.4	21/0	0/3	0/32
	SMA-MEPM	79.1	100	100	100	16/5	3/0	0/32
	SMA-IPM	79.1	100	100	94.1	18/3	1/2	0/32
DDSTs (5 mm) ^{1,2)}								
	EDTA-CAZ	70.8	78.1	70.8	92.5	16/5	1/2	7/25
	EDTA-MEPM	70.8	84.4	77.2	96.4	15/6	2/1	5/27
	EDTA-IPM	79.1	87.5	82.6	96.6	17/4	2/1	4/28
Dry-plate DPD1 test		87.5	96.9	95.4	91.2	19/2	2/1	1/31
Etest MBL IP/IPI		58.3	96.9	93.3	75.6	14/7	0/3	1/31
Cica-Beta test MBL		66.7	100	100	80.0	14/7	2/1	0/32
Modified Hodge test		70.8	100	100	82.1	16/5	1/2	0/32

¹⁾: In DDSTs, the distance between the two disks was maintained at 5 mm from edge-to-edge.

²⁾: Isolates were considered MBL positive if the growth-inhibition zone was 2.5 mm larger than that of corresponding antimicrobial disk.

³⁾: Results shown are number of isolates that yielded true-positive (TP), true-negative (TN), false-positive (FP), and false-negative (FN) results, respectively.

PPV, positive-predictive value; NPV, negative-predictive value. Other abbreviations are in Table 2.

cost-effective method that is feasible for screening MBL-producing clinical isolates in routine clinical laboratory analysis. Because the data analyses in the present study were performed with a limited number of strains, we plan to further evaluate these methods using additional clinical isolates that produce multiple classes of β -lactamases.

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Conflict of interest None to declare.

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