Comparison of Test Methods for Detecting Metallo-\(\beta\)-Lactamase-Producing Gram-Negative Bacteria

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(Received May 23, 2013. Accepted July 18, 2013)

INTRODUCTION

Metallo-\(\beta\)-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 \(\beta\)-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 \(\beta\)-lactams, including the oxyimino cephalosporins, cephemycins, and carbapenems, and are not inhibited by any therapeutically utilized \(\beta\)-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-type enzymes (4,5), and some of the NDM-1 producers demonstrate low resistance to carbapenems (MIC, <2 \(\mu\)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 developed 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 \(\beta\)-lactamas (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 \(\beta\)-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...
MLB-producing strains (n = 24)  
Plural classes of β-lactamases (n = 3)  
- NDM-1, CTX-M-1, E. coli  
- CMY-4 and TEM-1  
- NDM-1 and TEM-1, K. pneumoniae  
- IMP-1 and CTX-M-2, K. pneumoniae  

IMP- or VIM-type β-lactamase (n = 21)  
- IMP-1, E. coli  
- IMP-1, K. pneumoniae  
- IMP-1, Acinetobacter sp.  
- IMP-1, P. aeruginosa  
- IMP-1, P. putida  
- VIM-1, P. aeruginosa  
- VIM-2, P. aeruginosa  

Non-MLB-producing strains (n = 32)  
- CMY-type β-lactamase (n = 5)  
  - CMY-2, E. coli  
  - CMY-8, K. pneumoniae  
  - CMY-9, E. coli  
  - CMY-9, K. pneumoniae  
- CTX-M-type β-lactamase (n = 15)  
  - CTX-M-15, E. coli  
  - CTX-M-2, E. coli  
  - CTX-M-14, E. coli  
  - CTX-M-8, E. coli  
- KPC-type β-lactamase (n = 1)  
  - KPC-3, K. pneumoniae  
- Non-β-lactamase producing strains (n = 11)  
  - P. aeruginosa  
  - Acinetobacter sp.  

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

<table>
<thead>
<tr>
<th>Isolate (n = 56)</th>
<th>No. of isolates</th>
<th>MIC (µg/ml)CAZ IPM</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBL-producing strains (n = 24)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NDM-1, CTX-M-1, E. coli</td>
<td>1</td>
<td>128 &lt; 32</td>
</tr>
<tr>
<td>CMY-4 and TEM-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NDM-1 and TEM-1, K. pneumoniae</td>
<td>1</td>
<td>128 &lt; 4</td>
</tr>
<tr>
<td>IMP-1 and CTX-M-2, K. pneumoniae</td>
<td>1</td>
<td>32 0.5</td>
</tr>
<tr>
<td>IMP- or VIM-type β-lactamase (n = 21)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IMP-1, E. coli</td>
<td>3</td>
<td>128 &lt; 4-8</td>
</tr>
<tr>
<td>IMP-1, K. pneumoniae</td>
<td>3</td>
<td>128 &lt; 4-64</td>
</tr>
<tr>
<td>IMP-1, Acinetobacter sp.</td>
<td>6</td>
<td>128 &lt; 32-128 &lt;</td>
</tr>
<tr>
<td>IMP-1, P. aeruginosa</td>
<td>6</td>
<td>128 &lt; 64-128 &lt;</td>
</tr>
<tr>
<td>IMP-1, P. putida</td>
<td>1</td>
<td>128 &lt; 128 &lt;</td>
</tr>
<tr>
<td>VIM-1, P. aeruginosa</td>
<td>1</td>
<td>128 &lt; 128 &lt;</td>
</tr>
<tr>
<td>VIM-2, P. aeruginosa</td>
<td>1</td>
<td>128 &lt; 128 &lt;</td>
</tr>
<tr>
<td>Non-MLB-producing strains (n = 32)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CMY-2, E. coli</td>
<td>2</td>
<td>128 &lt; 0.5-1</td>
</tr>
<tr>
<td>CMY-8, K. pneumoniae</td>
<td>1</td>
<td>64 0.25 &gt;</td>
</tr>
<tr>
<td>CMY-9, E. coli</td>
<td>1</td>
<td>128 &lt; 0.5</td>
</tr>
<tr>
<td>CTX-M-15, E. coli</td>
<td>5</td>
<td>8-32 0.25-0.5</td>
</tr>
<tr>
<td>CTX-M-2, E. coli</td>
<td>5</td>
<td>0.5-32 0.25</td>
</tr>
<tr>
<td>CTX-M-14, E. coli</td>
<td>4</td>
<td>0.5-8 0.25</td>
</tr>
<tr>
<td>CTX-M-8, E. coli</td>
<td>1</td>
<td>1 0.25</td>
</tr>
<tr>
<td>KPC-3, K. pneumoniae</td>
<td>1</td>
<td>128 &lt; 128 &lt;</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>6</td>
<td>32-128 &lt; 8-64</td>
</tr>
<tr>
<td>Acinetobacter sp.</td>
<td>5</td>
<td>2-16 0.25-0.5</td>
</tr>
</tbody>
</table>

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 Standards 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 (MEMP; 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 MEMP 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 (2) 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 MBL-negative when no nitrocefin hydrolysis was observed with the Cica-Beta I test or when nitrocefin hydrolysis was observed with both the Cica-Beta I and Cica-Beta MBL tests.

Dry-plate DPDI test: Dry-plate DPDI 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-
MEPM, and IPM, were evaluated when the edge-to-
tations (23) in the presence of 175
CAZ and/or IPM was reduced by three doubling dilu-
isolates were considered MBL-positive when MIC of

tive (cottons wab. A nM E P Md i s k( 1 0
dardN o . 0 . 5a n ds p re a do n a nM Ha g a rp l a t eu s i n ga
adjusted to one-tenth the turbidity of McFarland stan-

tive and negative predictive values were also calculated
number of true negative bacteria. In addition, the posi-
tive bacteria, whereas the Sp was calculated from the

bapenemase production.

growth inhibition zone was interpreted as positive car-

Statistical analysis: The sensitivity (Se) of each pheno-
typic test was calculated from the number of true posi-
tive bacteria, whereas the Sp was calculated from the
number of true negative bacteria. In addition, the posi-
tive 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 ap-
approximately 5, 10, 15, and 20 mm. In the IMP- or VIM-
type MBL producers (21 isolates), the clearest appear-
ance of a growth-inhibition zone was observed when the
distance between the two disks was maintained at ap-
approximately 5 mm so that the combination of CAZ-
SMA (Se, 100%) yielded a better result than other com-
binations (Table 2). No distinct change in the appear-
ance of the growth-inhibition zone was observed for
strains producing only the KPC- or CMY-type β-lac-
tamase 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 inter-
pretation criteria compared, those based on alteration in
the shapes of growth-inhibition zones toward the anti-
microbial 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-inhibi-
tion 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 interpreta-
tion criteria using the expansion (≥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 com-
binations (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-

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, iso-
lates 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.
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-lactamase (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-lact-

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**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 disk¹ | | DDSTs using EDTA disk¹ |
|-------|---------------------------|------------------------||------------------------|
|       | 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 | | |
| 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.

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**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)**

<table>
<thead>
<tr>
<th>Criteria</th>
<th>EDTA-CAZ (5 mm)</th>
<th>EDTA-MEPM (5 mm)</th>
<th>EDTA-IPM (5 mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expansion (mm)</td>
<td>Shape of zone²</td>
<td>Expansion (mm)</td>
</tr>
<tr>
<td></td>
<td>≥ 5¹</td>
<td>≥ 2.5</td>
<td></td>
</tr>
<tr>
<td>Se (%)</td>
<td>66.7</td>
<td>76.2</td>
<td>76.2</td>
</tr>
<tr>
<td>Sp (%)</td>
<td>87.5</td>
<td>78.1</td>
<td>34.3</td>
</tr>
</tbody>
</table>

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.
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 μg/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, nitrocefin 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 μg/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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Combination of antimicrobial agent and inhibitor1)</th>
<th>CAZ-SMA Distance (mm)</th>
<th>CAZ-EDTA Distance (mm)</th>
<th>MEPM-SMA Distance (mm)</th>
<th>MEPM-EDTA Distance (mm)</th>
<th>IPM-SMA Distance (mm)</th>
<th>IPM-EDTA Distance (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>10</td>
<td>5</td>
<td>10</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>NDM-1-producing E. coli</td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>NDM-1-producing K. pneumoniae</td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>IMP-1 and CTX-M coproducing K. pneumoniae</td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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</tbody>
</table>

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.

The use 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
Acknowledgments
This study was supported by the Ministry of Health, Labour and Welfare of Japan (Grant no. H21-Shinkou-Ippan-008 and H24-Shinkou-Ippan-010).

Conflict of interest
None to declare.

REFERENCES

<table>
<thead>
<tr>
<th>Screening test method</th>
<th>All strains (n = 56)</th>
<th>IMP- or VIM-producers (n = 21)</th>
<th>Plural classes of ( \beta )-lactamas producers (n = 3)</th>
<th>non-MBL-producers (n = 32)</th>
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<tbody>
<tr>
<td></td>
<td>Se (%)</td>
<td>Sp (%)</td>
<td>PPV (%)</td>
<td>NPV (%)</td>
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<td><strong>DDSTs (5 mm)</strong></td>
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<td>SMA-CAZ</td>
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<td><strong>DDSTs (5 mm)</strong></td>
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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 \( \beta \)-lactamas.