Further Modification of the Modified Hodge Test for Detecting Metallo-β-Lactamase-Producing Carbapenem-Resistant Enterobacteriaceae

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Background: The modified Hodge test (MHT) was designed to detect carbapenemase-producing Enterobacteriaceae (CPE). This study evaluated variables to improve the performance of MHT.

Methods: Carbapenem-resistant Enterobacteriaceae isolated from November 2010 to March 2013 at the Asan Medical Center were evaluated, including 33 metallo-β-lactamase (MBL) producers and 103 non-CPEs. MHT was performed by using two carbapenem disks (ertapenem and meropenem; Becton Dickinson, USA), three media (Mueller-Hinton agar (MHA), MacConkey agar (MAC), and zinc-enriched MHA), and two inoculums (0.5-McFarland [McF] suspension and a 10-fold dilution of it.) PCR was performed to detect β-lactamase genes of the MBL, AmpC, and CTX-M types.

Results: The sensitivity of MHT for detecting New Delhi metallo-β-lactamase (NDM) producers was highest using ertapenem and 0.5-McF, 52.0% on MHA and 68.0% on MAC, respectively. NDM-producing Klebsiella pneumoniae (NDMKP) were detected with higher sensitivity on MAC (78.6%) vs. MHA (28.6%) (P=0.016), but VIM-producing Enterobacter, Citrobacter, and Serratia were detected with higher sensitivity on MHA (78.5%) vs. MAC (14.3%) (P=0.004). MBL producers were consistently identified with lower sensitivity using meropenem vs. ertapenem, 39.4% vs. 60.6% (P=0.0156), respectively. The effects of zinc and inoculum size were insignificant. Enterobacter aerogenes producing unspecified AmpC frequently demonstrated false positives, 66.7% with ertapenem and 22.2% with meropenem.

Conclusions: The MHT should be adjusted for the local distribution of species and the carbapenemase type of MBL producers. MAC and ertapenem are preferable for assessing NDMKP, but MHA is better for VIM. Laboratory physicians should be aware of the limited sensitivity of MHT and its relatively high false-positive rate.

Key Words: Carbapenemase, Modified Hodge test, Metallo-β-lactamase, Enterobacteriaceae, Klebsiella pneumoniae

INTRODUCTION

Enterobacteriaceae have recently emerged as a significant multidrug-resistant organism comprising carbapenem-resistance in Korea [1, 2]. The most serious form of carbapenem resistance is mediated by carbapenem-hydrolyzing β-lactamases, including metallo-β-lactamases (MBLs), such as imipenemase (IMP), Verona imipenemase (VIM), New Delhi metallo-β-lactamase (NDM), Ambler class A Klebsiella pneumoniae carbapenemase (KPC), and class D oxacillinase-48 (OXA-48) [3, 4]. These en-
enzymes are usually encoded by mobile genetic elements that demonstrate a high capacity for dissemination [5]. To prevent the spread of carbapenemase genes, it is critical to differentiate carbapenemase production from other mechanisms of carbapenem resistance [6]. Detection of carbapenemases often requires special methods other than routine susceptibility testing because certain types of carbapenemases are easily overlooked owing to the low minimum inhibitory concentrations (MICs) of carbapenems [4].

To detect carbapenemase-producing Enterobacteriaceae (CPE), the CLSI currently recommends the modified Hodge test (MHT) [7]. The MHT can detect various carbapenemases by using simple techniques and can be easily applied in clinical laboratories. While the MHT demonstrates excellent sensitivity for detecting CPE producing Ambler class A KPC and class D OXA-48 carbapenemases [8], the MHT does not consistently detect NDM producers [8-10].

Carbapenemase-producers are still rare among Enterobacteriaceae identified in Korea [11-14]. However, recently NDM-producing Providencia stuartii, Serratia marcescens, Citrobacter freundii, and 6 Morganella morganii, Enterobacter cloacae, Klebsiella pneumoniae, K. pneumoniae, OXA-232-producing K. pneumoniae, and VIM-producing Enterobacteriaceae have been emerging in Korea [15, 16], and the low sensitivity of the MHT for NDM producers has become a major issue in clinical laboratories. There are a few recent studies that have modified MHT variables in order to improve its performance [8, 17]. This study aimed to optimize MHT variables in order to improve its performance for the detection of MBL producers.

METHODS

1. Bacterial isolates
Between November 2010 and March 2013, 369 Carbapenem-resistant Enterobacteriaceae (CRE) isolates were recovered from clinical specimens at Asan Medical Center, Seoul, Korea. Among these, 136 non-duplicate isolates were further characterized, including 33 MBL-producing Enterobacteriaceae and 103 non-MBL-producing isolates (7 Citrobacter freundii, 11 Enterobacter aerogenes, 29 Enterobacter cloacae, 6 Escherichia coli, 1 Klebsiella oxytoca, 74 K. pneumoniae, 1 Morganella morganii, 1 Providencia stuartii, and 6 Serratia marcescens). Non-MBL CRE isolates were consecutively isolated from October 2010 to December 2010 and from September 2011 to July 2012. CRE were defined as isolates that were not susceptible to one or more of the following carbapenems: ertapenem, meropenem, or imipenem, according to MicroScan NegCombi PanelType 44 (Siemens Healthcare Diagnostics Inc., West Sacramento, CA, USA), which follows the CLSI M100-S20 breakpoint guidelines [18]. E. coli ATCC 25922 was used as an indicator organism for MHT.

2. Modified Hodge test
The following media and carbapenem disks were used: MacConkey agar (MAC; Hanil Komed, Sungnam, Korea), Mueller-Hinton agar (MHA; Hanil Komedia, 10 μg ertapenem disks (Becton Dickinson, Sparks, MD, USA), and 10 μg meropenem disks (Becton Dickinson). The MHT carbapenem susceptible indicator organism, E. coli ATCC 25922, cultured overnight, was suspended in Mueller-Hinton broth to a turbidity of a 0.5 McFarland (McF) standard suspension (0.5-McF). An undiluted suspension or a 1:10 diluted suspension was plated onto MAC and MHA according to the CLSI disk diffusion procedure [19]. Each ertapenem or meropenem disk was placed onto the center of the plate, and test isolates were heavily streaked from the margin of the central disk to the periphery of the plate using a 10-μL disposable loop. Up to 4 isolates were tested per plate. The inhibition zone of the indicator organism was carefully examined for a cloverleaf-type indentation following growth of the test organism at the intersection with a streak of the test organism after overnight incubation at 35°C in ambient air [20]. Two levels of indentations (2 and 3 mm) were used as positive cut-off values. Five combinations of variables (media, carbapenem disks, and indicator inocula) were studied: inoculated with 0.5 McF indicator organisms, ertapenem (ETP) disks on MHA (ETP/MHA/0.5), ertapenem disks on MAC (ETP/MAC/0.5), meropenem (MEM) disks on MHA (MEM/MHA/0.5), meropenem disks on MAC (MEM/MAC/0.5), respectively, and ertapenem disks on MHA inoculated with 1:10 dilution of 0.5 McF indicator organisms (ETP/MHA/0.05). Zinc-supplemented MHA was prepared by adding 100 μg/mL ZnSO4, as described previously [8]. The MHT with zinc-supplemented MHA was performed on 22 MBL-producing isolates including 20 NDM-producing and 2 VIM-producing isolates.

3. Additional phenotypic tests and assignments
Boronic acid (BA)-ertapenem disks were prepared by adding 20 μL of a phenylboronic acid solution (20 mg/mL) to the ertapenem disks. EDTA-ertapenem disks were prepared by adding 10 μL of a 0.1M EDTA solution to the ertapenem disks. For all carbapenem-resistant isolates, disk diffusion tests were performed using 10 μg ertapenem, 10 μg meropenem, 10 μg imipenem, BA-ertapenem, and EDTA-ertapenem disks. When the inhibition zone diameters on the BA-ertapenem or EDTA-ertapenem disks increased ≥5 mm in comparison with the ertapenem
disks, it was considered that BA or EDTA inhibited carbapenemase activity. To characterize the carbapenemases further, the KPC/MBL Confirm ID kit (Rosco Diagnostica A/S, Taastrup, Denmark) was used to test the isolates with positive EDTA-er
tapenem or MHT results. MBL, KPC, and AmpC β-lactamas
tes were identified depending on the difference in inhibition zone
diameters observed with the meropenem tablet versus (vs.)
three tablets containing meropenem plus dipicolinic acid (DPA),
BA, or cloxacillin with 5 mm cut-off, as described in the manu-
facturer’s instructions.

If the isolates had carbapenemase activity inhibited by EDTA
or DPA, or MBL genes were identified using PCR, they were
classified as MBL-producing CRE. If the isolates were resistant
to ertapenem or meropenem, and the resistance was inhibited
by both BA and cloxacillin, the β-lactamase was designated as
“AmpC with carbapenem-hydrolyzing activity (AmpC-CH).” If
the isolates were negative for EDTA inhibition, BA inhibition, and
MBL gene PCR tests, they were classified as “Other CRE” than
MBL or AmpC-CH. If the other CREs were positive for MHT in
any of the variable combinations tested, the isolates were further
tested by using the OXA-48 Confirm kit comprising temocillin
tablets (Rosco Diagnostica A/S). If no zone of inhibition around
the temocillin tablet was observed, it was assumed to be caused
by an oxacillinase (OXA)-type carbapenemase.

4. PCR
DNA samples of the isolates were extracted by using the GenEl-
ute Bacterial Genomic DNA kit (Sigma-Aldrich, St. Louis, MO,
USA). For MBL, multiplex PCR targeting IMP, VIM, SPM-1, SIM-
1, and GIM-1, and monoplex PCR targeting NDM were done by
using previously described primers and conditions [21, 22]. For
AmpC β-lactamas
tes, multiplex PCR was performed targeting the
MOX β-lactamase group (including MOX-1, MOX-2, CMY-1,
and CMY-8 to CMY-11), the Citrobacter freundii-type (CIT)
β-lactamase group (including LAT-1 to LAT-4, CMY-2 to CMY-7,
and BIL-1), DHA, and ACT [23]. Multiplex PCR targeting the
CTX-M β-lactamase group 1, 2, 8, 9, and 25 genes was also
performed [24]. The amplicons of representative β-lactamase
genes were directly sequenced by using the capillary electro-
phoresis-based Sanger sequencing method.

5. Statistical analysis
The SPSS version 19.0 (SPSS, Chicago, IL, USA) statistical soft-
ware package was used for all statistical analyses. Sensitivities
of MHT to detect CPE were measured as positive rates of NDM
and VIM carbapenemases-producers, and false positive rates of
MHT were measured as positive rates of AmpC-CH or other
CRE. Sensitivities and false positive rates of MHT according to
the combination of variables were compared by using McNemar
test. The levels of indentations were compared by using Mann-
Whitney test. P values of 0.05 or less were considered statisti-
cally significant.

RESULTS

1. Characteristics of carbapenem resistance
All CPE were MBL producers in this study, and 33 isolates were
phenotypically MBL producers. A total of 25 isolates were posi-
tive for NDM-1, and a total of seven isolates were positive for
VIM-2 (Table 1). Of the MBL producers, one E. cloacae was
negative for all MBLs using PCR. According to the disk diffusion
tests with the M100-S23 CLSI breakpoint [7], all MBL-produc-
ing isolates were resistant to ertapenem. However, one NDM-
producing S. marcescens isolate was susceptible to merope-
nam, and five NDMKP, one NDM-producing K. oxytoca, and
one VIM-producing C. freundii were intermediate or susceptible
to imipenem.

Fifty-eight isolates demonstrated carbapenem-resistance that
was mediated by AmpC-CH. Among them, 19 isolates were posi-
tive for DHA, 11 E. cloacae isolates were positive for ACT, three
isolates were positive for CIT, and 25 isolates were negative for all
AmpC β-lactamases as determined by PCR. Among 58 isolates
carrying AmpC-CHs, 17 isolates (including eight E. cloacae,
eight E. aerogenes, and one K. pneumoniae) were positive in the
MHT or at least one of variable combinations (Table 1).

Forty-five isolates demonstrated carbapenem-resistance that
was not defined as MBL or AmpC-CH. By PCR, 13 isolates were
positive for CTX-M-1 or -9 groups, each of which was corre-
sponded to CTX-M-15 or CTX-14 types, respectively. 11 isolates
were positive for DHA of DHA-1 type, one E. cloacae isolate was
positive for ACT of ACT-1 type, and one K. pneumoniae was
positive for both DHA-1 and CTX-M-15. Nineteen isolates were
positive for DHA or MOX group, or MBL genes were identified using PCR, they were
classified as MBL-producing CRE. If the isolates were resistant
to ertapenem or meropenem, and the resistance was inhibited
by both BA and cloxacillin, the β-lactamase was designated as
“AmpC with carbapenem-hydrolyzing activity (AmpC-CH).” If
the isolates were negative for EDTA inhibition, BA inhibition, and
MBL gene PCR tests, they were classified as “Other CRE” than
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Table 1. β-Lactamase types and modified Hodge test results of 136 carbapenem-resistant Enterobacteriaceae isolates according to species

| Species                  | Total N | MBL carbapenemases (N = 33) | AmpC-CH (N = 58) | Other CRE (N = 45) |
|--------------------------|---------|----------------------------|-----------------|-------------------|
| Klebsiella pneumoniae    | 74      | NDM-1 (14/11)               | CIT (1/0), DHA-1 (13/0), CTX-M-15 (3/0), DHA-1/CTX-M-15 (3/0), DHA-1/CTX-M-14 (2/1), Unspecified* (3/0) | DHA-1 (8/0), CTX-M-15 (9/4), CTX-M-14 (1/0), DHA-1/CTX-M-15 (1/0), Unspecified* (16/6) |
| Enterobacter cloacae     | 29      | NDM-1 (3/3), VIM-2 (3/3), ACT-1 (11/3), DHA-1 (1/0), CTX-M-15 (3/3), ACT-1 (1/0) | - | - |
| Enterobacter aerogenes   | 11      | -                           | Unspecified* (9/8) | CTX-M-14 (1/0), Unspecified* (1/1) |
| Escherichia coli         | 6       | -                           | CIT (2/0), unspecified* (1/0) | CTX-M-15 (1/1), Unspecified* (2/0) |
| Citrobacter freundii     | 7       | NDM-1 (4/3), VIM-2 (3/3)    | - | - |
| Serratia marcescens      | 6       | NDM-1 (3/3), VIM-2 (1/1)    | - | DHA-1 (1/1), CTX-M-15 (1/0) |
| Klebsiella oxytoca       | 1       | NDM-1 (1/1)                 | - | - |
| Morganella morganii      | 1       | -                           | - | DHA-1 (1/1) |
| Providencia stuartii     | 1       | -                           | - | DHA-1 (1/0) |

*The term ‘unspecified’ means that the isolate was negative for all PCR tests performed.

Abbreviations: MBL, metallo-β-lactamase; AmpC-CH, AmpC β-lactamase with carbapenem-hydrolyzing activity; Other CRE, other β-lactamases than MBL or AmpC-CH; NDM, New Delhi metallo-β-lactamase; CIT, Citrobacter freundii-type; VIM, Verona imipenemase. CRE, carbapenem-resistant Enterobacteriaceae.

Table 2. Positive rates of the modified Hodge tests of the variable conditions among MBL-producers and non-MBL-producers

| Types of carbapenem resistance (N of isolates) | ETP/MHA/0.5 (% of positive results with two cut offs of indentation levels at each combinations of variables) | ETP/MAC/0.5 | MEM/MHA/0.5 | MEM/MAC/0.5 | ETP/MHA/0.05 |
|------------------------------------------------|---------------------------------------------------------------------------------------------------|-------------|-------------|-------------|--------------|
| MBL carbapenemases (33)                          | 20 (60.6)  13 (39.4)  19 (57.6)  14 (42.4)  13 (39.4)  9 (27.3)  19 (57.6)  10 (30.3)  16 (48.5)  14 (42.4) |
| NDM-1 (25)                                       | 13 (52.0)  6 (24.0)  17 (68.0)  13 (52.0)  6 (24.0)  4 (16.0)  16 (64.0)  9 (36.0)  8 (32.0)  7 (28.0) |
| VIM-2 (7)                                        | 6 (85.7)  6 (85.7)  2 (28.6)  1 (14.3)  6 (85.7)  5 (71.4)  3 (42.9)  1 (14.3)  7 (100)  6 (85.7) |
| Unidentified (1)                                 | 1 (100)  1 (100)  0  0  0  0  0  0  0  1 (100)  1 (100) |
| No carbapenemase (103)                           | 15 (14.6)  6 (5.8)  22 (21.4)  13 (12.6)  5 (4.9)  1 (1.0)  10 (9.7)  3 (2.9)  13 (12.6)  8 (7.8) |
| AmPC-CH (58)                                     | 8 (13.8)  4 (6.9)  14 (24.1)  9 (15.5)  2 (3.4)  1 (1.7)  4 (6.9)  1 (1.7)  7 (12.1)  6 (10.3) |
| DHA-1 (19)                                       | 0  0  1 (5.3)  1 (5.3)  0  0  1 (5.3)  0  0  0  0 |
| ACT-1 (11)                                       | 1 (9.1)  0  1 (9.1)  0  0  0  0  0  0  1 (9.1)  0 |
| CIT (3)                                          | 0  0  0  0  0  0  0  0  0  0  0  0 |
| Unspecified* (25)                                | 7 (28.0)  4 (16.0)  12 (48.0)  8 (32.0)  2 (8.0)  1 (4.0)  3 (12.0)  1 (4.0)  6 (24.0)  6 (24.0) |
| Other CRE (45)                                    | 7 (15.6)  2 (4.4)  8 (17.8)  4 (8.9)  3 (6.7)  0  6 (13.3)  2 (4.4)  6 (13.3)  2 (4.4) |

*The term ‘unspecified’ means that the isolate was negative for all PCR tests performed.

Abbreviations: ETP/MHA/0.5, ertapenem disks on Mueller-Hinton agar with 0.5-McFarland inoculums; ETP/MAC/0.5, ertapenem disks on MacConkey agar with 0.5-McFarland inoculums; MEM/MHA/0.5, meropenem disks on Mueller-Hinton agar with 0.5-McFarland inoculums; MEM/MAC/0.5, meropenem disks on MacConkey agar with 0.5-McFarland inoculums; ETP/MHA/0.05, ertapenem disks on Mueller-Hinton agar with 1:10 dilutions of 0.5-McFarland inoculums. Other abbreviations: See Table 1.
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producers, although the growth of the indicator \( E. coli \) was less confluent with ETP/MHA/0.05 (Fig. 1). While ETP/MAC/0.5 yielded higher sensitivities compared with MEM/MHA/0.5; 52.0% (95% confidence interval, 31.3-72.2) vs. 16.0% (95% confidence interval, 0.2-33.9) \((P=0.012)\) and 68.0% (95% confidence interval 46.5-85.1) vs. 24.0% (95% confidence interval, 9.4-45.1) \((P=0.016)\) using a 2 mm cut-off value, respectively (Table 2). For MBL-producing \( E. coli \) and \( C. freundii \), with 2 mm as the cut-off value, the MHT using MHA detected more than two fold positives than MAC; 85.7% vs. 14.3% for \( E. coli \), and 85.7% vs. 42.9% for \( C. freundii \), respectively, but the difference was not significant. Among AmpC-CH producers, eight out of nine \( E. aerogenes \) isolates were positive in any of the MHT combinations, and when MAC and a 2 mm cut-off value were used this false positivity increased from 44.4% to 66.7% with ETP/MHA/0.5, and 77.8% to 88.9% with ETP/MAC/0.5 when using 3 mm and 2 mm cut-off values, respectively (Table 3). The type of AmpC in 9 AmpC-CH \( E. aerogenes \) could not be determined by PCR.

**DISCUSSION**

At the highest sensitivity, the MHT only detected 60.6% of MBL producers and 68.0% of NDM producers. Our results support the previous findings that report low sensitivities of the MHT for MBL \([10, 25]\). However, the performance varied depending on the MBL and species tested. The MHT demonstrated better detection of VIM than NDM. MAC demonstrated higher sensitivity for NDM, while MHA demonstrated a higher sensitivity for VIM. Media differences had the most predominant effects on NDMKP. This is probably species-dependent, because 11 NDM-producing \( K. pneumoniae \) demonstrated higher sensitivity on MHA. A previous study recommended MAC over MHA for improving MHT performance, suggesting that the bile components of MAC enhance the release of \( \beta \)-lactamases \([17]\). However, that study included only two VIM-producing \( K. pneumoniae \) isolates, and the effect of MAC on MHT was not reproduced in a later study \([8]\). In consideration of these results, media issue requires further investigation. Our study reveals that MHT performance is enhanced by MAC only not significant \((P=0.063\) and \(P=0.375\), respectively).
in NDMKP isolates. Therefore, MAC has an advantage in circumstances where NDMKP is the major CRE problem, as is the case at our hospital.

While CLSI equally recommends ertapenem and meropenem disks for use in MHT [7], the meropenem disk demonstrates a low sensitivity. These results contrast with a previous study that reported similar performances between meropenem and ertapenem disks [4]; however, in that study, only nine MBL-producers, including six VIM- and three NDM-producing Enterobacteriaceae isolates, were tested, and two VIM-producers were weakly positive in the MHT using meropenem disks. Ertapenem and meropenem disks should be further evaluated against a larger collection of MBL-producing Enterobacteriaceae isolates.

A major limitation of this study is that most NDMKP and VIM-producing Enterobacteriaceae isolates were collected from a single institution; therefore, they were possibly composed of closely related clones. Because the epidemic NDMKP clones at our hospital were not consistently resistant to imipenem or meropenem [15], the low MIC of meropenem may affect the MHT sensitivity when using meropenem disks to test isolates.

Using MHT to detect carbapenemase-producing isolates was first described by Lee et al. [26]. In their study a “1:10 dilution”

### Table 3. Positive rates of modified Hodge tests of the variable conditions according to species distribution

| Species and carbapenemase activity (N of isolates) | ETP/MHA/0.5 | ETP/MAC/0.5 | MEM/MHA/0.5 | MEM/MAC/0.5 | ETP/MHA/0.05 |
|-------------------------------------------------|-------------|-------------|-------------|-------------|--------------|
| | ≥ 2 mm | ≥ 3 mm | ≥ 2 mm | ≥ 3 mm | ≥ 2 mm | ≥ 3 mm | ≥ 2 mm | ≥ 3 mm | ≥ 2 mm | ≥ 3 mm |
| Klebsiella pneumoniae (74) | | | | | | | |
| MBL carbapenemase (14)* | 4 (28.6) | 1 (7.1) | 11 (78.6) | 8 (57.1) | 1 (7.1) | 0 | 10 (71.4) | 4 (28.6) | 1 (7.1) | 1 (7.1) |
| AmpC-CH (25) | 0 | 0 | 1 (4.0) | 1 (4.0) | 0 | 0 | 1 (4.0) | 0 | 0 | 0 |
| Other CRE (35) | 4 (11.4) | 1 (2.9) | 4 (11.4) | 1 (2.9) | 1 (2.9) | 0 | 4 (11.4) | 1 (2.9) | 4 (11.4) | 1 (2.9) |
| Other species with NDM-1 (11)* | 9 (81.8) | 5 (45.5) | 6 (54.5) | 5 (45.5) | 4 (36.4) | 6 (54.5) | 5 (45.5) | 7 (63.6) | 6 (54.5) |
| Enterobacter cloacae (29) | | | | | | | |
| MBL carbapenemase (7) | 6 (85.7) | 5 (71.4) | 1 (14.3) | 1 (14.3) | 5 (71.4) | 3 (42.9) | 1 (14.3) | 1 (14.3) | 6 (85.7) | 5 (71.4) |
| AmpC-CH (21) | 2 (9.5) | 0 | 5 (23.8) | 1 (4.8) | 0 | 0 | 0 | 2 (9.5) | 1 (4.8) |
| Other CRE (1) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Enterobacter aerogenes (11) | | | | | | | |
| AmpC-CH (9) | 6 (66.7) | 4 (44.4) | 8 (88.9) | 7 (77.8) | 2 (22.2) | 1 (11.1) | 3 (33.3) | 1 (11.1) | 5 (55.6) | 5 (55.6) |
| Other CRE (2) | 1 (50.0) | 1 (50.0) | 1 (50.0) | 1 (50.0) | 0 | 0 | 0 | 1 (50.0) | 0 | 0 |
| Escherichia coli (6) | | | | | | | |
| AmpC-CH (3) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Other CRE (3) | 1 (33.3) | 0 | 1 (33.3) | 1 (33.3) | 0 | 0 | 1 (33.3) | 0 | 0 | 0 |
| Citrobacter freundii (7) | | | | | | | |
| MBL carbapenemase (7) | 6 (85.7) | 4 (57.1) | 3 (42.9) | 2 (28.6) | 4 (57.1) | 3 (42.9) | 5 (71.4) | 2 (28.6) | 5 (71.4) | 4 (57.1) |
| Serratia marcescens (6) | | | | | | | |
| MBL carbapenemase (4) | 3 (75.0) | 3 (75.0) | 4 (100.0) | 3 (75.0) | 3 (75.0) | 3 (75.0) | 3 (75.0) | 3 (75.0) | 3 (75.0) | 3 (75.0) |
| Other CRE (2) | 1 (50.0) | 0 | 1 (50.0) | 1 (50.0) | 1 (50.0) | 0 | 1 (50.0) | 1 (50.0) | 1 (50.0) | 1 (50.0) |
| Klebsiella oxytoca (1) | | | | | | | |
| MBL carbapenemase (1) | 1 (100) | 0 | 0 | 0 | 0 | 0 | 1 (100) | 1 (100) |
| Morganella morganii (1) | | | | | | | |
| AmpC-CH (1) | 0 | 0 | 1 (100) | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Providencia stuartii (1) | | | | | | | |
| Other CRE (1) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

*MBL carbapenemase carried by Klebsiella pneumoniae was NDM-1, and other species than Klebsiella pneumoniae carrying NDM-1 comprised three Enterobacter cloacae, four Citrobacter freundii, three Serratia marcescens and one Klebsiella oxytoca.

Abbreviations: See Table 2.
of 0.5-McF turbidity was preferred when preparing the indicator *E. coli* suspension. CLSI also recommends a 1:10 dilution of 0.5 McF as the inoculum density for indicator organisms [7]. However, in our study a 1:10 dilution augmented the level of indentations for one fourth of the tested isolates (8 of 33 isolates; 24.2%), but did not improve the sensitivity for detecting carbapenemase producers. Furthermore, less confluent growth of the indicator cells on the agar plates could result in false-negative readings in cases with a smaller level of indentation, which often occurred with the NDM producers. In another study by Lee et al. [17], low inoculum density did not consistently improve MHT performance on MAC agar plates. Therefore, laborious dilution steps could be eliminated from the MHT without significantly deteriorating its performance.

In this study, zinc supplementation did not improve the sensitivity of detecting MBL-producing isolates. Because of the low sensitivity of MHT for detecting NDM producers, divalent zinc ions, a cofactor for MBL activity, is supplemented to enhance the sensitivity of MHT [8]. A newer method to detect carbapenemase-producers, Carba-NP, also adds ZnSO\(_4\) in lysates of bacterial cells to enhance carbapenemase activity [27]. Because only 22 isolates of MBL producers were tested using zinc-supplemented MHA in this study, further evaluation of the effects of zinc supplementation is required.

This study clearly showed that MHT had limitations in sensitivity with further modification of variables and there was no single condition to achieve the best sensitivity for all types of MBL or species of tested organisms. There are alternative assays that purpose to detect CPE sensitively and specifically. Rosco KPC-MBL Confirm ID is commercially available for differentiation and detection of carbapenemases as used in this study, but it demonstrates low sensitivity for VIM-, IMP- and OXA-48 producers [10]. The sensitivity of Carba-NP is also influenced by the type of carbapenemase [28] and requires additional incubation in order to differentiate MBL from KPC [27]. More complicated alternative assays are available, such as commercial molecular tests and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry [29]. However, these techniques are too laborious and expensive for clinical laboratories. The MHT is technically more familiar and requires no other equipment or reagents than those required for disk diffusion testing. Therefore, using ertapenem disk-based MHT with appropriate media for the test organism is a practical option for characterizing CRE in the clinical laboratory.

AmpC-CH *E. aerogenes* isolates were frequently associated with the false positivity of the MHT, which was more predominant with MAC than with MHA. A previous report also suggested high false positivity of *Enterobacter* spp., but did not reveal the cause [30]. In the present study, many of *E. cloacae* were positive for ACT-1, which is a known chromosomal AmpC of *E. cloacae* carrying carbapenem hydrolyzing activity [31], but it was less frequently related to false-positive results of MHT. *E. aerogenes* could have chromosomal AmpC, which was not elucidated in this study, and the high false positivity of AmpC-CR *E. aerogenes* suggests that the unknown chromosomal AmpC \(\beta\)-lactamases might have carbapenemase-hydrolyzing activity. Unspecified AmpC was predominant among false-positive results of MHT, but other forms of AmpC, CTX-M-14, and CTX-M-15 also contributed to the false-positive results. Previous studies reported false-positive rates of up to 25% among carbapenemase non-producers in the MHT, and these outcomes have mainly been observed in CTX-M-producing isolates, including CTX-M-2, CTX-M-14, and CTX-M-15 [32, 33]. The level of indentation was usually less extensive in false-positive cases, thus the false-positive rate was markedly increased when a cut-off value of 2 mm was used in comparison with a 3-mm cut-off value. Nevertheless, it would be better to put emphasis on increase in the sensitivity rather than specificity of MHT in MBL detection.

In conclusion, MHT variables should be determined by the local distribution of species and carbapenemases. MHT using MAC and ertapenem could improve the sensitivity to detect NDM-MKP. We should be aware of the limited sensitivity of MHT and its high false-positive rate in clinical use.

**Authors’ Disclosures of Potential Conflicts of Interest**

No potential conflicts of interest relevant to this article were reported.

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