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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*

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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-

zymes 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 *Klebsiella pneumoniae* (NDMKP), KPC-producing *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 NegCombo 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 Komed), 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-ertapenem or MHT results. MBL, KPC, and AmpC β-lactamases 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 manufacturer'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 GenElute 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-lactamases, 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 electrophoresis-based Sanger sequencing method.

5. Statistical analysis

The SPSS version 19.0 (SPSS, Chicago, IL, USA) statistical software 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 statistically 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 positive 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-producing isolates were resistant to ertapenem. However, one NDM-producing *S. marcescens* isolate was susceptible to meropenem, 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 positive 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 corresponded 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 negative for all AmpC and CTX-M as determined by PCR. A total of 14 isolates were positive in the MHT of at least one of variable combinations, and they were tested with OXA-48 Confirm kit. None of them were compatible with the OXA carbapenemases type. Among them, nine isolates were positive for either of CTX-M-15, DHA or MOX group β-lactamases (Table 1).

2. Effects of modified Hodge test variables

Using a cut-off value of 2 mm, ETP/MHA/0.5, was more sensitive for the MBL producers than MEM/MAC/0.5; 60.6% (95%

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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	β-Lactamase types by PCR (N of isolates/N of MHT-positive isolates in at least one of variable combinations)		
		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), Unidentified (1/1)	ACT-1 (11/3), DHA-1(1/0), CTX-M-15 (3/3), Unspecified* (6/2)	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)	N (%) of positive results with two cut offs of indentation levels at each combinations of variables									
	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
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	1 (100)	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
ACT -1 (11)	1 (9.1)	0	1 (9.1)	0	0	0	0	0	1 (9.1)	0
CIT (3)	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.

confidence interval, 42.1-77.1) vs. 39.4% (95% confidence interval, 22.9-57.9) (P=0.0156) (Table 2) 103 non-MBL CRE isolates, yielded the highest false positive results, using ETP/MAC/0.5 (21.4%) and the lowest using MEM/MHA/0.5 (4.9%) (Table 2). Using a cut-off value of 3 mm, MBL producers demonstrated less positive results from 27.3% to 42.4% and other CRE yielded less false positive results from 1.0% to 12.6% (Table 2). Meropenem combinations usually demonstrated smaller indentations than ertapenem for MBL producers (mean 1.7 mm vs. 2.5 mm); however this difference was not significant (P=0.083). ETP/MHA/0.05 and ETP/MHA/0.05 were similar in the level of indentations, with an average of 2.6 mm for MBL

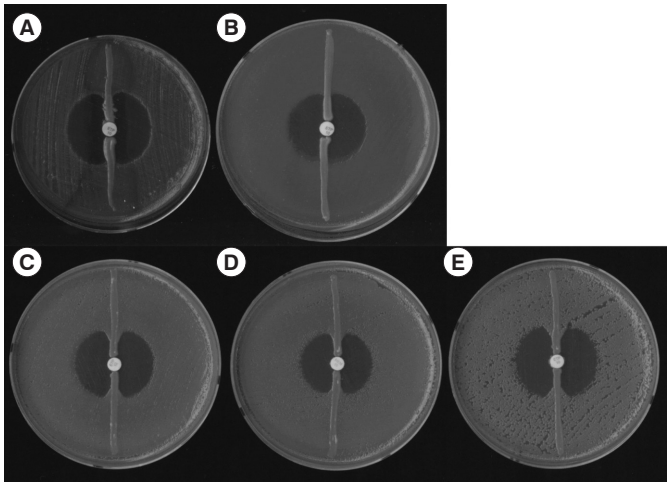

Fig. 1. Modified Hodge test for NDM-producing *Klebsiella pneumoniae* using ertapenem disks on MacConkey agar with 0.5-McFarland inoculum (A) and ertapenem disks on Mueller-Hinton agar with 0.5-McFarland inoculum (B). The tests on MacConkey agar produced positive results, and the tests on Mueller-Hinton agar produced negative results. Modified Hodge test for a NDM-producing *Enterobacter cloacae* using ertapenem disks on MacConkey agar with 0.5-McFarland inoculum (C), meropenem disks on MHA with 0.5-McFarland inoculum (D), and ertapenem disks on MHA with a 1:10 dilution of 0.5-McFarland inoculum (E). Meropenem (D) demonstrated unclear margins of inhibition in *Escherichia coli*, and the indentation was small. The 1:10 dilution (E) demonstrated similar results as the 0.5-McFarland suspension (C).

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, 4.5-36.1) (P=0.012) and 68.0% (95% confidence interval, 46.5-85.1) vs. 24.0% (95% confidence interval, 9.4-45.1) (P=0.003) for NDM-producing isolates, using 3- and 2 mm cut-off values, respectively, combination of MHA consistently yielded the higher sensitivity than those of MAC for seven VIM-producing isolates; 6 positive by ETP/MHA/0.5 and 5 positive by MEM/MHA/0.5 vs. 1 positive by ETP/MAC/0.5 and 1 positive by MEM/MAC/0.5, therefore, 78.5% (11 of 14) (95% confidence interval, 49.1-95.3) for MHA-based tests vs. 14.3% (2 of 14) (95% confidence interval, 1.78-42.6) for MAC-based tests (P=0.004) using 3-mm cut-off values (Table 2).

Evaluation of the effect of zinc supplementation with twenty-two MBL-producing isolates demonstrated that ETP/MHA/0.5 and ETP/MHA/0.05 with zinc supplementation produced positive results for six isolates and eight isolates, respectively, while ETP/MHA/0.5 without zinc supplementation was positive for 11 of the MBL-producing isolates. However, these differences were not significant (P=0.063 and P=0.375, respectively).

3. Bacterial species and modified Hodge test

Of 14 NDMKP, ETP/MAC/0.5 demonstrated higher sensitivity than ETP/MHA/0.5, 57.1% (95% confidence interval, 28.9-82.3) vs. 7.1% (95% confidence interval, 0.2-33.9) (P=0.016) using a 3-mm cut-off value and 78.6% (95% confidence interval 49.2-95.3) vs. 28.6% (95% confidence interval 8.4-58.1) (P=0.016) using a 2 mm cut-off value, respectively (Table 3). However, in 11 NDM-producing *Enterobacteriaceae* other than *K. pneumoniae*, ETP/MHA/0.5 detected more positive than ETP/MAC/0.5, but the difference was not significant; 81.8% vs. 54.5% (P=0.375) when using 2 mm as the cut-off value (Table 3). For MBL-producing *E. cloacae* and *C. freundii*, with 2 mm as the cut-off value, the MHT using MHA detect more than two fold of positives than MAC: 85.7% vs. 14.3% for *E. cloacae*, 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 *Enterobacteriaceae* other than *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 β-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

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

Species and carbapenemase activity (N of isolates)	N (%) of positive results with two cut offs of indentation levels at each combinations of variables									
	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)	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	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)	1 (50.0)	0	0	0	1 (50.0)	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	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.

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"

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 β-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 NDMKP. 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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