Original Article
Clinical Microbiology

Ann Lab Med 2015;35:212-219
http://dx.doi.org/10.3343/alm.2015.35.2.212
ISSN 2234-3806 • eISSN 2234-3814

ANNALS OF LABORATORY MEDICINE

Combined Use of the Modified Hodge Test and Carbapenemase Inhibition Test for Detection of Carbapenemase-Producing *Enterobacteriaceae* and Metallo-β-Lactamase-Producing *Pseudomonas* spp.

Wonkeun Song, M.D.[1], Seong Geun Hong, M.D.[2], Dongeun Yong, M.D.[3], Seok Hoon Jeong, M.D.[3], Hyun Soo Kim, M.D.[1], Han-Sung Kim, M.D.[1], Jae-Seok Kim, M.D.[1], and Il Kwon Bae, Ph.D.[4]

Department of Laboratory Medicine[1], Hallym University College of Medicine, Seoul; Department of Laboratory Medicine[2], CHA Bundang Medical Center, CHA University, Seongnam; Department of Laboratory Medicine[3], Research Institute of Bacterial Resistance, Yonsei University College of Medicine, Seoul; Department of Dental Hygiene[4], Silla University, Busan, Korea

Background: We evaluated the combined use of the modified Hodge test (MHT) and carbapenemase inhibition test (CIT) using phenylboronic acid (PBA) and EDTA to detect carbapenemase-producing *Enterobacteriaceae* (CPE) and metallo-β-lactamase (MBL)-producing *Pseudomonas* spp.

Methods: A total of 49 isolates of CPE (*15 Klebsiella pneumoniae* carbapenemase [KPC], 5 Guiana extended-spectrum β-lactamase [GES]-5, 9 New Delhi metallo-β-lactamase [NDM]-1, 5 Verona integron-encoded metallo-β-lactamase [VIM]-2, 3 imipenem-hydrolyzing β-lactamase [IMP], and 12 oxacillinase [OXA]-48-like), 25 isolates of MBL-producing *Pseudomonas* spp. (14 VIM-2 and 11 IMP), and 35 carbapenemase-negative controls were included. The MHT was performed for all isolates as recommended by the Clinical and Laboratory Standards Institute. Enhanced growth of the indicator strain was measured in mm with a ruler. The CIT was performed by directly dripping PBA and EDTA solutions onto carbapenem disks that were placed on Mueller-Hinton agar plates seeded with the test strain.

Results: Considering the results of the MHT with the ertapenem disk in *Enterobacteriaceae* and *Pseudomonas* spp., the CIT with the meropenem disk in *Enterobacteriaceae*, and the imipenem disk in *Pseudomonas* spp., three combined disk tests, namely MHT-positive plus PBA-positive, EDTA-positive, and MHT-positive plus PBA-negative plus EDTA-negative, had excellent sensitivity and specificity for the detection of KPC- (100% sensitivity and 100% specificity), MBL- (94% sensitivity and 100% specificity), and OXA-48-like-producing isolates (100% sensitivity and 100% specificity), respectively.

Conclusions: Combined use of the MHT and CIT with PBA and EDTA, for the detection of CPE and MBL-producing *Pseudomonas* spp., is effective in detecting and characterizing carbapenemases in routine laboratories.

Key Words: Modified Hodge test, Carbapenemase inhibition test, Phenylboronic acid, EDTA, *Enterobacteriaceae*, *Pseudomonas* spp.

Received: August 4, 2014
Revision received: October 6, 2014
Accepted: December 1, 2014

Corresponding author: Wonkeun Song
Department of Laboratory Medicine, Hallym University College of Medicine, Kangnam Sacred Heart Hospital, 1 Shingil-ro, Youngdeungpo-gu, Seoul 150-950, Korea
Tel: +82-2-829-5259
Fax: +82-2-847-2403
E-mail: swonkeun@hallym.or.kr

INTRODUCTION

The global spread of carbapenemase-producing gram-negative bacilli in the last decade is a serious health threat, and limited treatment options are available for such infections [1]. Rapid and accurate detection of resistance mechanisms is essential for determining appropriate antimicrobial therapy and infection control measures.

Several tests have been developed for the phenotypic detection of carbapenemases [2-4]. The modified Hodge test (MHT) is inexpensive and feasible for practically all clinical laboratories. The MHT is a CLSI-recommended phenotypic method for carbapenemase detection. This recommended method detects carbapenemase in *Enterobacteriaceae* isolates but not in *Pseudomonas* spp. Although the MHT often has high sensitivity [5-7], its interpretation is often difficult and subjective [8, 9]. Moreover, many studies have demonstrated false-positive results in the presence of extended-spectrum β-lactamases (ESBLs) and AmpC β-lactamases [10, 11]. The carbapenemase inhibition test (CIT) uses β-lactamase inhibitors, including boronic acid compounds, EDTA, dipicolinic acid (DPA), and cloxacillin (CLX) to differentiate class A carbapenemases from class B and class D carbapenemases [12-14].

In this study, we evaluated the combined use of the MHT and the CIT to more effectively detect carbapenemase-producing *Enterobacteriaceae* (CPE) and metallo-β-lactamase (MBL)-producing-*Pseudomonas* spp.

METHODS

1. Bacterial isolates

Sixty-three *Enterobacteriaceae* and 46 *Pseudomonas* spp. were used in this study; these carbapenem-non-susceptible isolates were obtained from clinical sources (Tables 1, 2). The species were identified by using the Vitek 2 system (bioMerieux Vitek Inc., Hazelwood, MO, USA). A total of 49 isolates of CPE (*Klebsiella pneumoniae* carbapenemase [KPC] [n=15], Guiana extended-spectrum β-lactamase [GES]-5 [n=5], New Delhi metallo-β-lactamase [NDM]-1 [n=9], Verona integron-encoded metallo-β-lactamase [VIM]-2 [n=5], imipenem-hydrolyzing β-lactamase [IMP] [n=3], and oxacillinase (OXA)-48-like [n=12]) and 25 isolates of MBL-producing *Pseudomonas* spp. (VIM-2 [n=14] and IMP [n=11]) were included. The remaining 35 carbapenemase-negative controls were AmpC β-lactamase-producing *Enterobacteriaceae* with porin loss (n=14) and *Pseudomonas* aeruginosa overexpressing AmpC β-lactamases (n=21). These β-lactamases were characterized by PCR and DNA sequencing [15-18]. Porin loss was detected as previously described [19]. The minimum inhibitory concentrations (MICs) of imipenem (IPM), meropenem (MEM), and ertapenem (ETP) were determined by Etest (bioMerieux, Marcy l'Etoile, France) and interpreted according to CLSI breakpoints, as updated in 2013 [20].

2. Modified Hodge test

The MHT was performed for all isolates as recommended by CLSI [20]. ETP disks were placed on Mueller-Hinton agar (MHA) (Becton-Dickinson, Cockeysville, MD, USA) plates seeded with *Escherichia coli* ATCC 25922. The isolates were inoculated in a straight line from the edge of the disk to the edge of the plate. The plates were incubated at 35°C for 16-20 hr. Enhanced growth of the indicator strain was measured in mm with a ruler. The length of the straight line from the enhanced growth obtained from the isolate to the end of inhibition zone was classified as negative (<2 mm), weakly positive (2-3 mm), and positive (≥ 4 mm). When a clear area was observed around the streak, the MHT result was considered indeterminate.

3. Carbapenemase inhibition test

The two different β-lactamase inhibitors were 30 mg/mL phenylboronic acid (PBA) (Sigma-Aldrich, St. Louis, MO, USA) solution and 30 mg/mL EDTA (Sigma-Aldrich) solution. PBA and EDTA were dissolved in dimethylsulfoxide and sterile water, respectively. A 0.5 McFarland inoculum of the organisms was prepared and spread on each MHA plate (Becton-Dickinson). Three ETP, IPM, and MEM disks were placed in rows on the MHA plate seeded with the test strain. Then, 10 μL of EDTA and 10 μL of PBA were added to the first and third disks, respectively (Fig. 1). A difference of ≥5 mm in zone diameter (around the disks) between the disks containing the PBA and EDTA solutions and that containing carbapenems alone was considered positive for PBA and EDTA, whereas an increase of <5 mm was considered negative.

The stability of the in-laboratory-prepared PBA and EDTA solutions, stored at 0°C, was tested twice weekly for 5 weeks by using 5 controls: KPC-2-producing *Klebsiella pneumoniae*, NDM-1-producing *K. pneumoniae*, VIM-2-producing *P. aeruginosa*, IMP-6-producing *P. aeruginosa*, OXA-232-producing *K. pneumoniae*, and DHA-1-producing *K. pneumoniae* with porin loss.

4. Sensitivity and specificity

The performance of the tests for the detection of carbapenemases was determined by using genotypically defined car-

Table 1. Evaluation of the MHT and CIT for the detection of carbapenemase-producing isolates of carbapenem-non-susceptible *Enterobacteriaceae*

No.	Organism	Carbapenemase	Result in MHT	Increase in zone diameter (mm) around carbapenem disks containing β-lactamase inhibitors						MIC (μg/mL) of:		
				IPM plus:		MEM plus:		ETP plus:		IPM	MEM	ETP
				PBA	EDTA	PBA	EDTA	PBA	EDTA			
1	*K. pneumoniae*	KPC-2	+	5*	-2	7	2	6	0	4	4	16
2	*K. pneumoniae*	KPC-2	+	4	0	10	2	6	0	4	4	16
3	*K. pneumoniae*	KPC-2	+	3	-1	6	0	7	1	8	8	16
4	*K. pneumoniae*	KPC-2	+	4	0	7	2	6	1	16	16	16
5	*K. pneumoniae*	KPC-2	+	6	0	8	0	8	2	4	2	8
6	*K. pneumoniae*	KPC-2	+	6	1	7	1	8	1	4	8	8
7	*K. pneumoniae*	KPC-2	+	5	0	7	0	8	0	16	16	16
8	*K. pneumoniae*	KPC-2	+	3	1	7	1	5	1	4	2	8
9	*K. pneumoniae*	KPC-2	+	4	0	7	1	7	1	16	16	16
10	*K. pneumoniae*	KPC-3	+	5	0	6	1	7	0	16	16	16
11	*K. pneumoniae*	KPC-3	+	4	0	7	1	6	0	8	4	4
12	*K. pneumoniae*	KPC-3	+	6	0	8	1	7	0	8	8	8
13	*K. pneumoniae*	KPC-3	+	2	0	8	2	8	0	8	16	16
14	*K. pneumoniae*	KPC-3	+	4	1	7	2	5	0	8	8	8
15	*K. pneumoniae*	KPC-3	+	5	0	7	1	7	0	8	16	8
16	*K. pneumoniae*	NDM-1	+/-	0	3	0	6	1	7	2	2	16
17	*K. pneumoniae*	NDM-1	+	0	7	0	14	-2	12	16	16	>32
18	*K. pneumoniae*	NDM-1	+	-1	7	-3	13	0	13	16	32	>32
19	*K. pneumoniae*	NDM-1	+	0	18	0	13	0	10	>32	>32	>32
20	*K. pneumoniae*	NDM-1	+/-	1	15	0	11	0	6	>32	>32	>32
21	*K. pneumoniae*	NDM-1	+/-	0	14	0	11	0	6	>32	>32	>32
22	*K. pneumoniae*	NDM-1	+	1	7	1	12	0	12	2	8	>32
23	*K. pneumoniae*	NDM-1	+	0	7	0	11	0	12	2	4	32
24	*K. pneumoniae*	NDM-1	+	0	14	0	11	0	8	>32	>32	>32
25	*E. cloacae*	VIM-2	+	0	5	2	7	4	2	32	16	32
26	*E. cloacae*	VIM-2	+	3	5	4	4	4	1	8	4	>32
27	*S. marcescens*	VIM-2	+	4	12	2	15	0	3	>32	>32	>32
28	*C. freundii*	VIM-2	+	0	4	2	7	2	5	2	1	2
29	*C. freundii*	VIM-2	+	0	4	0	6	1	6	1	0.5	2
30	*K. pneumoniae*	IMP-6	+	1	1	-1	5	0	5	2	2	2
31	*K. pneumoniae*	IMP-6	+	0	2	0	6	1	4	2	2	2
32	*E. cloacae*	IMP-6	+	0	3	-1	6	0	8	2	4	8
33	*K. pneumoniae*	OXA-48	+	1	1	0	0	1	1	1	0.5	2
34	*K. pneumoniae*	OXA-181	+	0	2	2	3	6	5	0.5	0.5	4
35	*K. pneumoniae*	OXA-232	+	0	2	0	2	0	2	4	32	>32
36	*K. pneumoniae*	OXA-232	+	1	1	1	1	1	0	4	16	>32
37	*K. pneumoniae*	OXA-232	+	1	0	2	2	0	1	2	8	>32

(Continued to the next page)

http://dx.doi.org/10.3343/alm.2015.35.2.212

Table 1. Continued

No.	Organism	Carbapenemase	Result in MHT	Increase in zone diameter (mm) around carbapenem disks containing β-lactamase inhibitors						MIC (μg/mL) of:		
				IPM plus:		MEM plus:		ETP plus:		IPM	MEM	ETP
				PBA	EDTA	PBA	EDTA	PBA	EDTA			
38	*K. pneumoniae*	OXA-232	+	0	0	0	0	1	0	2	8	>32
39	*K. pneumoniae*	OXA-232	+	1	1	0	0	0	0	2	16	>32
40	*K. pneumoniae*	OXA-232	+	2	0	0	0	1	0	4	16	>32
41	*K. pneumoniae*	OXA-232	+	0	-1	1	1	1	0	8	16	>32
42	*E. coli*	OXA-232	+	0	0	0	1	2	1	0.25	0.25	4
43	*E. coli*	OXA-232	+	0	0	0	0	0	0	0.5	1	16
44	*E. coli*	OXA-232	+	0	0	2	0	2	1	2	4	32
45	*K. pneumoniae*	GES-5	-	0	1	4	1	3	1	1	4	32
46	*K. pneumoniae*	GES-5	-	4	2	4	0	4	1	0.25	0.125	1
47	*K. pneumoniae*	GES-5	-	2	2	4	0	4	1	1	4	8
48	*K. pneumoniae*	GES-5	-	0	2	2	2	2	2	0.25	0.125	1
49	*K. pneumoniae*	GES-5	-	0	0	3	0	4	0	1	4	16
50	*K. pneumoniae*	None	-	2	2	3	2	4	4	1	4	>32
51	*K. pneumoniae*	None	-	2	2	2	3	2	3	1	4	>32
52	*K. pneumoniae*	None	+/-	0	2	0	0	2	2	0.25	0.25	2
53	*K. pneumoniae*	None	-	0	0	2	1	5	7	1	4	>32
54	*K. pneumoniae*	None	-	0	0	4	3	4	2	1	4	>32
55	*K. pneumoniae*	None	+/-	10	1	12	4	9	2	32	16	>32
56	*K. pneumoniae*	None	-	10	1	9	1	9	1	>32	32	>32
57	*K. pneumoniae*	None	-	10	2	5	0	11	3	>32	8	>32
58	*S. marcescens*	None	+/-	0	-2	4	2	10	5	0.25	1	4
59	*S. marcescens*	None	+/-	2	0	6	2	12	3	4	8	>32
60	*S. marcescens*	None	-	2	2	4	2	8	2	8	8	>32
61	*S. marcescens*	None	-	0	0	1	1	2	2	2	0.5	1
62	*E. cloacae*	None	-	0	1	2	2	2	0	0.25	0.125	1
63	*E. aerogenes*	None	-	0	0	2	0	4	-1	0.25	0.125	1

*Underlined values are considered positive.
Abbreviations: MHT, modified Hodge test; CIT, carbapenemase inhibition test; MIC, minimum inhibitory concentration; IPM, imipenem; MEM, meropenem; ETP, ertapenem; PBA, phenylboronic acid; KPC, *Klebsiella pneumoniae* carbapenemase; NDM, New Delhi metallo-β-lactamase; VIM, Verona integron-encoded metallo-β-lactamase; IMP, imipenem-hydrolyzing β-lactamase; OXA, oxacillinase; GES, Guiana extended-spectrum β-lactamase.

bapenemase mechanisms as the reference standard. Sensitivity was calculated from the number of true-positive isolates, whereas specificity was calculated from the number of true-negative isolates.

RESULTS

1. Modified Hodge test

The MHTs were positive for all CPEs, except five GES-5-producing *K. pneumoniae* isolates. Tests for all non-CPE isolates were negative, other than two *K. pneumoniae* and two *Serratia marcescens* isolates that showed weak positive results (Table 1). Nineteen out of 25 MBL-producing *Pseudomonas* spp. showed positive results, and all non-carbapenemase-producing *Pseudomonas* spp. showed negative (n=15) or indeterminate (n=3) results (Table 2).

Table 2. Evaluation of the MHT and CIT for the detection of MBL-producing isolates of carbapenem-non-susceptible *Pseudomonas* spp.

No.	Organism	MBL	Result in MHT	Increase in zone diameter (mm) around carbapenem disks containing β-lactamase inhibitors						MIC (μg/mL) of:	
				IPM plus:		MEM plus:		ETP plus:		IPM	MEM
				PBA	EDTA	PBA	EDTA	PBA	EDTA		
1	*P. aeruginosa*	VIM-2	-	0	10*	0	7	0	2	>32	>32
2	*P. aeruginosa*	VIM-2	-	0	9	0	6	0	3	>32	>32
3	*P. aeruginosa*	VIM-2	-	0	10	0	6	0	2	>32	>32
4	*P. aeruginosa*	VIM-2	+	1	17	1	12	0	4	32	16
5	*P. aeruginosa*	VIM-2	+	0	13	0	3	0	1	>32	>32
6	*P. aeruginosa*	VIM-2	+	0	12	0	3	0	1	>32	>32
7	*P. aeruginosa*	VIM-2	+/-	0	5	0	2	0	1	>32	>32
8	*P. aeruginosa*	VIM-2	+	2	13	3	10	4	8	16	8
9	*P. aeruginosa*	VIM-2	+	1	18	4	15	2	4	16	16
10	*P. aeruginosa*	VIM-2	+	1	11	1	11	2	7	8	4
11	*P. aeruginosa*	VIM-2	-	0	12	2	12	2	12	8	4
12	*P. putida*	VIM-2	-	0	16	0	2	0	2	>32	>32
13	*P. putida*	VIM-2	-	2	16	0	12	0	7	>32	>32
14	*P. putida*	VIM-2	+	0	17	0	3	0	2	>32	>32
15	*P. aeruginosa*	IMP-6	+	1	6	0	0	0	0	>32	>32
16	*P. aeruginosa*	IMP-6	+	-1	9	0	0	0	0	>32	>32
17	*P. aeruginosa*	IMP-26	+	6	17	0	11	0	4	16	>32
18	*P. aeruginosa*	IMP-6	+	2	5	0	0	0	0	>32	>32
19	*P. aeruginosa*	IMP-6	+	0	6	0	0	0	0	>32	>32
20	*P. aeruginosa*	IMP-6	+	1	6	0	1	0	0	32	>32
21	*P. aeruginosa*	IMP-6	+	3	10	0	1	0	1	>32	>32
22	*P. aeruginosa*	IMP-6	+	1	10	0	1	0	0	>32	>32
23	*P. aeruginosa*	IMP-6	+	3	7	0	1	0	0	32	>32
24	*P. aeruginosa*	IMP-6	+	0	7	0	0	0	1	8	>32
25	*P. aeruginosa*	IMP-6	+	2	7	0	2	0	1	16	>32
26	*P. aeruginosa*	None	-	14	1	0	3	0	1	>32	>32
27	*P. aeruginosa*	None	-	15	2	0	3	0	1	>32	>32
28	*P. aeruginosa*	None	-	15	3	3	4	0	0	>32	>32
29	*P. aeruginosa*	None	-	18	2	4	3	0	1	>32	>32
30	*P. aeruginosa*	None	ind	13	1	0	1	0	0	32	>32
31	*P. aeruginosa*	None	-	16	3	0	2	0	1	>32	>32
32	*P. aeruginosa*	None	-	16	3	0	2	0	1	>32	>32
33	*P. aeruginosa*	None	-	17	3	0	3	0	1	>32	>32
34	*P. aeruginosa*	None	-	16	3	4	2	0	1	>32	>32
35	*P. aeruginosa*	None	-	16	3	2	3	0	2	>32	>32
36	*P. aeruginosa*	None	-	15	3	0	2	0	1	>32	>32
37	*P. aeruginosa*	None	-	15	3	2	3	0	2	>32	>32
38	*P. aeruginosa*	None	-	16	3	0	0	0	0	16	>32
39	*P. aeruginosa*	None	ind	14	3	0	0	0	0	16	>32
40	*P. aeruginosa*	None	-	15	3	3	3	0	1	>32	>32
41	*P. aeruginosa*	None	-	15	3	3	0	0	0	>32	>32
42	*P. aeruginosa*	None	-	16	3	4	1	0	0	>32	>32
43	*P. aeruginosa*	None	ind	17	3	0	2	0	1	>32	>32
44	*P. aeruginosa*	None	-	18	3	3	2	0	0	>32	>32
45	*P. aeruginosa*	None	-	15	2	0	2	0	0	>32	>32
46	*P. aeruginosa*	None	-	16	1	2	2	0	1	16	32

*Underlined values are considered positive.
Abbreviations: MHT, modified Hodge test; CIT, carbapenemase inhibition test; MBL, metallo-β-lactamase; MIC, minimum inhibitory concentration; IPM, imipenem; MEM, meropenem; ETP, ertapenem; PBA, phenylboronic acid; VIM, Verona integron-encoded metallo-β-lactamase; IMP, imipenem-hydrolyzing β-lactamase.

http://dx.doi.org/10.3343/alm.2015.35.2.212

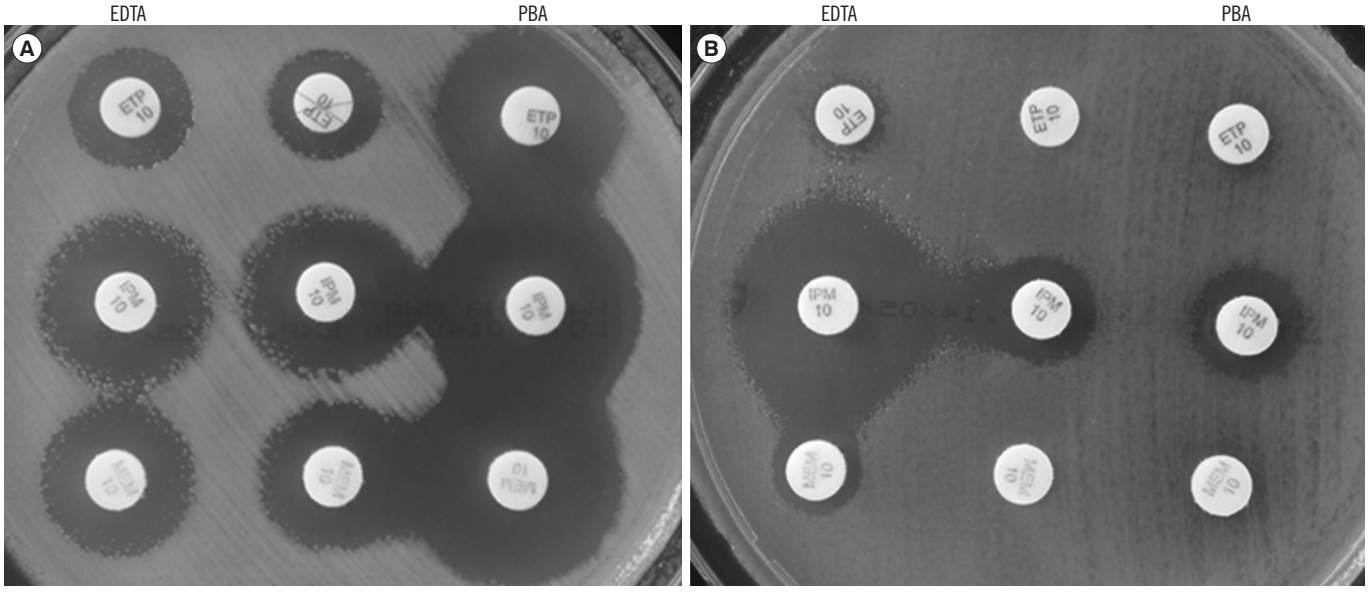

Fig. 1. Representative CIT results for the KPC-2-producing *K. pneumoniae* isolate (A) and IMP-6-producing *P. aeruginosa* isolate (B) are shown. Three horizontal lines of disks containing 3 ETP, 3 IPM, and 3 MEM were placed on a MHA plate seeded with the test strain. Then, 10 μL of EDTA (30 mg/mL) and PBA (30 mg/mL) were added along first and third vertical lines, respectively. (A) The difference in zone size in the presence and absence of PBA was ≥5 mm for ETP, IPM, and MEM, suggesting KPC production. (B) The difference in zone size in the presence and absence of EDTA was ≥5 mm for IPM, suggesting MBL production.
Abbreviations: CIT, carbapenemase inhibition test; ETP, ertapenem; IPM, imipenem; MEM, meropenem; PBA, phenylboronic acid; KPC, *K. pneumoniae* carbapenemase; MBL, metallo-β-lactamase.

Table 3. Sensitivity and specificity of MHT for ertapenem disks, CIT for meropenem disks in *Enterobacteriaceae* and imipenem disks in *Pseudomonas* spp.

Test results	Carbapenemase targeted	*Enterobacteriaceae*		*Pseudomonas* spp.	
		Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
MHT-positive plus PBA-positive	KPC*	100	100	NA	NA
EDTA-positive	MBL†	94	100	100	100
MHT-positive plus PBA-negative plus EDTA-negative	OXA-48‡	100	100	NA	NA

*KPC includes KPC-2 and -3; †MBL includes VIM-2, IMP-6, IMP-26, and NDM-1; ‡OXA-48 includes OXA-48, OXA-181, and OXA-232.
Abbreviations: MHT, modified Hodge test; CIT, carbapenemase inhibition test; PBA, phenylboronic acid; MBL, metallo-β-lactamase; NA, not available.

2. Carbapenemase inhibition test

All KPC-producing *Enterobacteriaceae* using MEM disks supplemented with PBA (MEM-PBA) tested positive. All MBL-producing *Enterobacteriaceae* were MEM-EDTA-positive except one isolate of VIM-2-producing *Enterobacter cloacae*. All OXA-48-like- and GES-5-producing *Enterobacteriaceae* were MEM-PBA-and MEM-EDTA-negative. Four of 14 non-CPE isolates were MEM-PBA-positive (Table 1). All MBL- and non-carbapenemase-producing *Pseudomonas* spp. were IPM-EDTA- and IPM-PBA-positive, respectively (Table 2).

The activities of PBA and EDTA solutions at week 1 were compared with those of freshly made solutions. Similar inhibition zones were observed for all carbapenemase-inhibitor combinations during the 5-week study period, indicating that these solutions can be prepared and kept at 0°C without losing activity (data not shown).

3. Sensitivity and specificity in the combined interpretation of MHT and CIT

We performed the MHT using ETP to screen for *Enterobacteriaceae* and *Pseudomonas* spp. and the CIT using MEM and IPM to screen for *Enterobacteriaceae* and *Pseudomonas* spp., respectively. The combined disk tests that were MHT-positive (≥4 mm) plus PBA-positive (≥5 mm), EDTA-positive (≥5 mm), and MHT-positive plus PBA-negative plus EDTA-negative, had excellent sensitivity and specificity for the detection of KPC- (100%

sensitivity and 100% specificity), MBL- (94% sensitivity and 100% specificity), and OXA-48-like-producing isolates (100% sensitivity and 100% specificity), respectively (Table 3).

DISCUSSION

The MHT is highly sensitive and suitable for the screening of carbapenemase production. However, its results are often difficult to interpret, and false-positive results are observed for strains producing ESBL or AmpC β-lactamase-with porin loss [6, 7]. Furthermore, it may be difficult for laboratories lacking experience to interpret the results, because of the subjective nature of the MHT [8, 9]. To interpret the results of the MHT objectively, we established quantitative criteria. The MHT with *E. coli* ATCC 25922 as an indicator strain previously displayed a higher percentage of indeterminate results (32%) in *P. aeruginosa* [21] than the 6.5% of indeterminate results in *Pseudomonas* spp. reported in our study.

To discriminate between the classes of carbapenemases, various CITs are commonly used, including the combined disk test (disk potentiation test) and the double disk synergy test (double disk potentiation test) [22, 23]. We used the combined disk test for carbapenem-non-susceptible strains. The CIT uses mostly dried carbapenem disks containing carbapenemase inhibitors, such as boronic acid, CLX, and DPA or EDTA. However, we selected the CIT that involved directly dripping the PBA and EDTA solutions onto carbapenem disks placed on MHA plates seeded with the test strain. This method was easier to carry out than the CIT using dried disks in routine laboratories. MEM and IPM disks were most effective for the detection of CPE and MBL-producing *Pseudomonas* spp., respectively. In a previous study, the sensitivity of IPM-DPA disks (97.7%) was higher than that of MEM-DPA disks (79.5%) for MBL-producing *Pseudomonas* spp. [24]. DPA has excellent sensitivity and specificity for the detection of MBL-producing *K. pneumoniae*, but the poorer specificity of EDTA calls into question the usefulness of this inhibitor [14]. In this study, DPA was stable for a shorter time than EDTA, at 2 weeks and 5 weeks, respectively (data not shown). Hence, we used EDTA for the detection of the MBL-producing isolates.

In our study, all isolates of KPC-producing *Enterobacteriaceae* were MEM-PBA-positive. Four of the 14 non-CPE isolates were weakly positive in MHT, but none of them were positive in MHT. It is unclear why the GES-5-producing *K. pneumoniae* isolates showed negative results for MHT and MEM-PBA. Unfortunately, these methods could not detect GES-5 class A carbapenemases. Many isolates with AmpC β-lactamase hyperproduction were both PBA-positive and CLX-positive [14]. The CIT using CLX was also needed to discriminate between KPC and AmpC β-lactamase. However, the combined use of the MHT and CIT using MEM-PBA discriminated between *Enterobacteriaceae* that produced KPC and AmpC β-lactamase. We observed weakly positive MHT results for three of nine NDM-1-producing *K. pneumoniae* isolates, but all MBL-producing *Enterobacteriaceae* isolates were MEM-EDTA-positive, regardless of the MHT results. Although there are no known specific inhibitors for class D carbapenemases, the combined use of the MHT and CIT using MEM-PBA and MEM-EDTA enabled the detection of all OXA-48-like-producing *Enterobacteriaceae*.

False-negative results of the MHT were reported for six of the 14 VIM-2-producing *Pseudomonas* spp., but all isolates of MBL-producing *Pseudomonas* spp. were IPM-EDTA-positive, regardless of the MHT results. All non-carbapenemase-producing *Pseudomonas* spp. exhibited negative MHT results but were IPM-PBA-positive, suggesting that the isolates overexpressed AmpC β-lactamases.

In conclusion, we propose a new strategy to detect carbapenemase resistance by the combined testing and interpretation of the MHT and CIT with PBA and EDTA. The combinations, namely MHT-positive (≥4 mm) plus PBA-positive (≥5 mm), EDTA-positive (≥5 mm), and MHT-positive plus PBA-negative plus EDTA-negative, had excellent sensitivity and specificity for the detection of KPC-, MBL-, and OXA-48-producing isolates, respectively. This method will facilitate the detection and characterization of carbapenemases in routine laboratories. In this study, we could include only a small number of characterized isolates, and none of the isolates that coexisted with carbapenemase production were examined; hence, further analysis is needed to validate these results.

Authors' Disclosures of Potential Conflicts of Interest

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

Acknowledgments

This study was supported by a research grant from the Korea Health Industry Development Institute in 2012 (HI12C1198). We are grateful to Patrice Nordmann for providing isolates of *K. pneumoniae*-producing OXA-48 and OXA-181.

http://dx.doi.org/10.3343/alm.2015.35.2.212

REFERENCES

1. Patel JB, Rasheed JK, Kitchel B. Carbapenemase in Enterobacteriaceae: activity, epidemiology, and laboratory detection. Clin Microbiol Newslett 2009;31:55-62.
2. Seah C, Low DE, Patel SN, Melano RG. Comparative evaluation of a chromogenic agar medium, the modified Hodge test, and a battery of meropenem-inhibitor discs for detection of carbapenemase activity in Enterobacteriaceae. J Clin Microbiol 2011;49:1965-9.
3. Jeong SH, Song W, Bae IK, Kim HS, Kim JS, Park MJ, et al. Broth microdilution methods using β-lactamase inhibitors for the identification of *Klebsiella pneumoniae* carbapenemases and metallo-β-lactamases in Gram-negative bacilli. Ann Clin Lab Sci 2014;44:49-55.
4. Nordmann P, Poirel L, Dortet L. Rapid detection of carbapenemase-producing *Enterobacteriaceae*. Emerg Infect Dis 2012;18:1503-7.
5. Anderson KF, Lonsway DR, Rasheed JK, Biddle J, Jensen B, McDougal LK, et al. Evaluation of methods to identify *Klebsiella pneumoniae* carbapenemase in *Enterobacteriaceae*. J Clin Microbiol 2007;45:2723-5.
6. Pasteran F, Mendez T, Guerriero L, Rapoport M, Corso A. Sensitive screening tests for suspected class A carbapenemase production in species of *Enterobacteriaceae*. J Clin Microbiol 2009;47:1631-9.
7. Pasteran F, Mendez T, Rapoport M, Guerriero L, Corso A. Controlling false-positive results obtained with the Hodge and Masuda assays for detection of class a carbapenemase in species of enterobacteriaceae by incorporating boronic acid. J Clin Microbiol 2010;48:1323-332.
8. Carvalhaes CG, Picão RC, Nicoletti AG, Xavier DE, Gales AC. Cloverleaf test (modified Hodge test) for detecting carbapenemase production in *Klebsiella pneumoniae*: be aware of false positive results. J Antimicrob Chemother 2010;65:249-51.
9. Pournaras S, Poulou A, Tsakris A. Inhibitor-based methods for the detection of KPC carbapenemase-producing Enterobacteriaceae in clinical practice by using boronic acid compounds. J Antimicrob Chemother 2010;65:1319-21.
10. Doumith M, Ellington MJ, Livermore DM, Woodford N. Molecular mechanisms distrupting porin expression in ertapenem-resistant *Klebsiella* and *Enterobacter* spp. clinical isolates from the UK. J Antimicrob Chemother 2009;63:659-67.
11. Hirsch EB and Tam VH. Detection and treatment options for *Klebsiella pneumoniae* carbapenemases (KPCs): an emerging cause of multidrug-resistant infection. J Antimicrob Chemother 2010;65:1119-25.
12. Tsakris A, Themeli-Digalaki K, Poulou A, Vrioni G, Voulgari E, Koumaki V, et al. Comparative evaluation of combined-disk tests using different boronic acid compounds for detection of *Klebsiella pneumoniae* carbapenemase-producing Enterobacteriaceae clinical isolates. J Clin Microbiol 2011;49:2804-9.
13. Birgy A, Bidet P, Genel N, Doit C, Decré D, Arlet G, et al. Phenotyping screening of carbapenemases and associated β-lactamases in carbapenem-resistant Enterobacteriaceae. J Clin Microbiol 2012;50:1295-302.
14. Giske CG, Gezelius L, Samuelsen Ø, Warner M, Sundsfjord A, Woodford N. A sensitive and specific phenotypic assay for detection of metallo-β-lactamases and KPC in *Klebsiella pneumoniae* with the use of meropenem disks supplemented with aminophenylboronic acid, dipicolinic acid and cloxacillin. Clin Microbiol Infect 2011;17:552-6.
15. Lee K, Yong D, Yum JH, Lim YS, Bolmström A, Qwärnström A, et al. Evaluation of Etest MBL for detection of bla_{IMP-1} and bla_{VIM-2} allele-positive clinical isolates of *Pseudomonas* app. and *Acinetobacter* spp. J Clin Microbiol 2005;43:942-4.
16. Yigit H, Queenan AM, Anderson GJ, Domenech-Sanchez A, Biddle JW, Steward CD, et al. Novel carbapenem-hydrolyzing β-lactamase, KPC-1, from a carbapenem-resistant strain of *Klebsiella pneumoniae*. Antimicrob Agents Chemother 2001;45:1151-61.
17. Poirel L, Castanheira M, Carrër A, Rodriguez CP, Jones RN, Smayevsky J, et al. OXA-163, an OXA-48-related class D β-lactamase with extended activity toward expanded-spectrum cephalosporins. Antimicrob Agents Chemother 2011;55:2546-51.
18. Solé M, Pitart C, Roca I, Fàbrega A, Salvador P, Muñoz L, et al. First description of an *Escherichia coli* strain producing NDM-1 carbapenemase in Spain. Antimicrob Agents Chemother 2011;55:4402-4.
19. Song W, Suh B, Choi JY, Jeong SH, Jeon EH, Lee YK, et al. In vivo selection of carbapenem-resistant *Klebsiella pneumoniae* by OmpK36 loss during meropenem treatment. Diagn Microbiol Infect Dis 2009;65:447-9.
20. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing. 23rd Informational supplement, M100-S23. Wayne, PA: CLSI, 2013.
21. Pasteran F, Veliz O, Rapoport M, Guerriero L, Corso A. Sensitive and specific modified Hodge test for KPC and metallo-β-lactamase detection in *Pseudomonas aeruginosa* by use of a novel indicator strain, *Klebsiella pneumoniae* ATCC 700603. J Clin Microbiol 2011;49:4301-3.
22. Arakawa Y, Shibata N, Shibayama K, Kurokawa H, Yagi T, Fujiwara H, et al. Convenient test for screening metallo-β-lactamase-producing gram-negative bacteria by using thiol compounds. J Clin Microbiol 2000;38:40-3.
23. Lee K, Lim YS, Yong D, Yum JH, Chong Y. Evaluation of the Hodge test and the imipenem-EDTA double-disk synergy test for differentiating metallo-β-lactamase-producing isolates of *Pseudomonas* spp. and *Acinetobacter* spp. J Clin Microbiol 2003;41:4623-9.
24. Yong D, Lee Y, Jeong SH, Lee K, Chong Y. Evaluation of double-disk potentiation and disk potentiation tests using dipicolinic acid for detection of metallo-β-lactamase-producing *Pseudomonas* spp. and *Acinetobacter* spp. J Clin Microbiol 2012;50:3227-32.