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

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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.
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 carbapenem-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: |
|-----|----------------|---------------|---------------|-------------------------------------------------------------|-----------------|
|     |                |               | PBA | EDTA | PBA | EDTA | PBA | EDTA | IPM | MEM | ETP |
| 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   | 0   | 2   | 0   | 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)
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 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 |
| 38  | K. pneumoniae     | OXA-232       | +            | 0       | 0        | 0        | 1   | 0   | >32 |
| 39  | K. pneumoniae     | OXA-232       | +            | 1       | 1        | 0        | 0   | 0   | >32 |
| 40  | K. pneumoniae     | OXA-232       | +            | 2       | 0        | 0        | 0   | 0   | >32 |
| 41  | K. pneumoniae     | OXA-232       | +            | 0       | -1       | 1        | 1   | 0   | >32 |
| 42  | E. coli           | OXA-232       | +            | 0       | 0        | 0        | 1   | 2   | 4   |
| 43  | E. coli           | OXA-232       | +            | 0       | 0        | 0        | 0   | 0   | >32 |
| 44  | E. coli           | OXA-232       | +            | 0       | 0        | 2        | 0   | 2   | 4   |
| 45  | K. pneumoniae     | GES-5         | -            | 0       | 0        | 0        | 1   | 4   | 32  |
| 46  | K. pneumoniae     | GES-5         | -            | 0       | 0        | 0        | 1   | 4   | 32  |
| 47  | K. pneumoniae     | GES-5         | -            | 0       | 0        | 0        | 4   | 1   | >32 |
| 48  | K. pneumoniae     | GES-5         | -            | 0       | 0        | 0        | 4   | 1   | >32 |
| 49  | K. pneumoniae     | GES-5         | -            | 0       | 0        | 0        | 4   | 1   | >32 |
| 50  | K. pneumoniae     | None          | -            | 0       | 0        | 0        | 2   | 4   | 32  |
| 51  | K. pneumoniae     | None          | -            | 0       | 0        | 0        | 2   | 4   | 32  |
| 52  | K. pneumoniae     | None          | +/-          | 0       | 2        | 0        | 2   | 2   | 0.25 |
| 53  | K. pneumoniae     | None          | -            | 0       | 0        | 0        | 2   | 4   | >32 |
| 54  | K. pneumoniae     | None          | -            | 0       | 0        | 0        | 4   | 1   | >32 |
| 55  | K. pneumoniae     | None          | +/-          | 10      | 1        | 12       | 9   | 2   | 32  |
| 56  | K. pneumoniae     | None          | -            | 10      | 1        | 9        | 9   | 1   | >32 |
| 57  | K. pneumoniae     | None          | -            | 10      | 2        | 5        | 0   | 11  | >32 |
| 58  | S. marcescens     | None          | +/-          | 0       | -2       | 4        | 2   | 10  | 5   |
| 59  | S. marcescens     | None          | +/-          | 0       | 2        | 6        | 2   | 12  | 3   |
| 60  | S. marcescens     | None          | -            | 2       | 2        | 4        | 2   | 8   | 2   |
| 61  | S. marcescens     | None          | -            | 0       | 0        | 1        | 2   | 2   | 0.25 |
| 62  | E. cloacae        | None          | -            | 0       | 1        | 2        | 2   | 0   | >32 |
| 63  | E. aerogenes      | None          | -            | 0       | 0        | 2        | 0   | 4   | 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.
| No. | Organism     | MBL   | Result in MHT | Increase in zone diameter (mm) around carbapenem disks containing β-lactamase inhibitors | MIC (µg/mL) of: |
|-----|--------------|-------|---------------|-------------------------------------------------------------------------------------|-----------------|
|     |              |       |               | IPM plus: PBA | MEM plus: PBA | ETP plus: EDTA | MEM plus: EDTA | IPM | MEM |
| 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            | 3            | 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-6 | +             | 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    | >32  | >32  |
| 25  | *P. aeruginosa* | IMP-6 | +             | 2            | 7              | 0            | 2            | 0    | 1    | >32  | >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    | >32  | >32  |
| 39  | *P. aeruginosa* | None  | ind           | 14           | 3              | 0            | 0            | 0    | 0    | >32  | >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 integrin-encoded metallo-β-lactamase; IMP, imipenem-hydrolyzing β-lactamase.
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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 (%)   |
| MHT-positive plus PBA-positive                     | KPC*                   | 100                | 100              |
| EDTA-positive                                     | MBL†                   | 94                 | 100              |
| MHT-positive plus PBA-negative plus EDTA-negative  | OXA-48‡                | 100                | 100              |

*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; KPC, K. pneumoniae carbapenemase; MBL, metallo-β-lactamase.

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 CIs 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 CI test uses mostly dried carbapenem disks containing carbapenemase inhibitors, such as boronic acid, CLX, and DPA or EDTA. However, we selected the CI 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 CI 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 CI test using CLX was also needed to discriminate between KPC and AmpC β-lactamase. However, the combined use of the MHT and CI 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 CI 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 CI 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.

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