〈Research Report〉

# Evaluation of the MASTDISCS *combi Carba plus* for differentiation of carbapenemase-producing *Enterobacteriaceae*

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The MASTDISCS *combi Carba plus* is a set of inhibitor combination discs that differentiates carbapenemases from AmpC *β*-lactamases. We evaluated the performance of this disc set in discriminating *β*-lactamase genotypes using 36 clinical and 5 reference strains, as well as discriminating clinical isolates using phenotypic assays. The results for all *Enterobacteriaceae* strains producing 21 carbapenemases (16 clinical isolates and 5 reference strains), including 14 IMP-1, 1 VIM-1, 2 NDM, 2 OXA-48, and 2 KPC carbapenemases, using the MASTDISCS *combi Carba plus* were consistent with the genotypes identified by multiplex PCR. Sixteen carbapenemase-producing *Enterobacteriaceae* clinical isolates were positive for carbapenemase production according to the modified carbapenem inactivation methodology, and 13 MBL-producing strains were positive for carbapenemase production according to the sodium mercaptoacetic acid double disc synergy test. Twenty-one of the AmpC *β*-lactamase- and ESBL-producing strains were identified as AmpC- or non-carbapenemase-producing *Enterobacteriaceae* using the MASTDISCS *combi Carba plus*. Ten AmpC *β*-lactamase-producing clinical strains were all positive for the aminophenyl boronic acid double disc synergy test. Overall, the MASTDISCS *combi Carba plus* allowed easy identification and successful typing of major carbapenemases using routine microbiology methods.

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## Introduction

Carbapenemase-producing *Enterobacteriaceae* (CPE) are resistant to carbapenems and broad-spectrum *β*-lactam antibiotics, and detection of CPE in clinical specimens has increased over the last decade[1,2]. CPE have been detected among species including *Escherichia coli*, *Klebsiella* spp., and *Enterobacter* spp. These organisms are major causes of community-associated and healthcare-associated infections, which pose a threat especially to immunocompromised patients[3].

There are limited antimicrobial therapeutic options for patients with infections caused by CPE. To control infections, rapid detection and prevention of the spread of CPE in healthcare settings are important[1]. Among the four classes of *β*-lactamases defined by the Ambler classification system, the carbapenemases produced by CPE belong to class A (*Klebsiella pneumoniae* carbapenemases [KPC]), class B (metallo-*β*-lactamases [MBL], e.g., IMP, VIM, and NDM), or class D (OXA-48-like carbapenemases).

The Clinical and Laboratory Standards Institute (CLSI) recommends the modified carbapenem inactivation method (mCIM) for screening carbapenemase production[4]. This method can be used to screen almost all carbapenemases, but it cannot classify every type. Although the double disc synergy test (DDST) using specific inhibitors is commonly based on phenotypic assays for identifying *β*-lactamases, *β*-lactamases must be examined individually according to their presumed CPE type[5,6]. Determination of carbapenemase genes using PCR is also performed in some reference microbiology laboratories.

MASTDISCS *combi Carba plus* is a set of inhibitor combination discs used to differentiate KPC, MBL, OXA, and AmpC *β*-lactamases using five discs that contain specific inhibitors of each *β*-lactamase[7]. The purpose of this study was to evaluate the performance of the MASTDISCS *combi Carba plus* for identifying carbapenemases in *Enterobacteriaceae* clinical isolates.

## Materials and Methods

### Bacterial strains

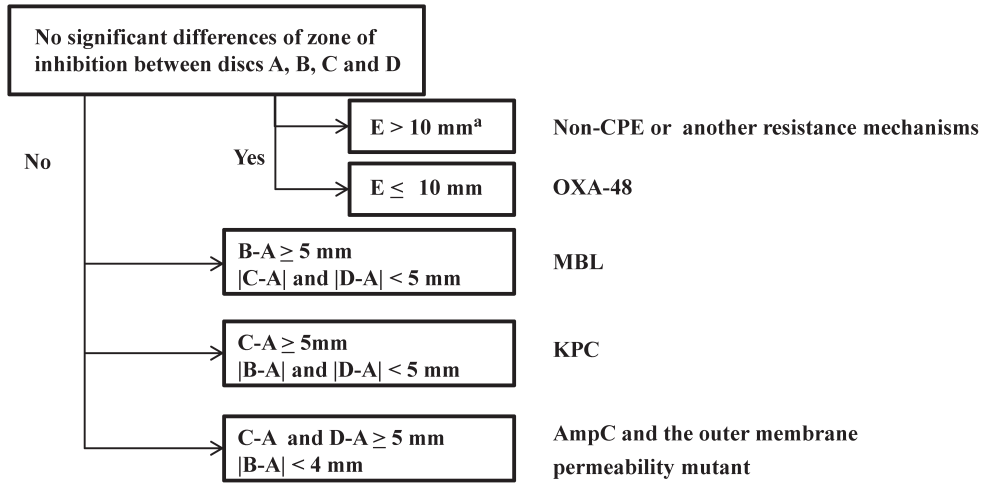
In total, 36 drug-resistant clinical isolates, including 10 *Enterobacter* spp., 9 *Escherichia coli*, 10 *Klebsiella* spp., 5 *Citrobacter* spp., 1 *Leclercia* sp., and 1 *Serratia* spp., were analyzed from 2012 to 2017 at the Juntendo University Hospital. The CPE included IMP-1, NDM, KPC, and OXA-48, based on PCR, and the non-CPE included AmpC *β*-lactamase and extended-spectrum *β*-lactamase (ESBL), as determined by phenotypic methods in a routine microbiology laboratory. Five carbapenemase-producing organisms, *E. coli* BAA-2469, *K. pneumoniae* NCTC 13442, *K. pneumoniae* NCTC 13440, *K. pneumoniae* NCTC 13438, and *K. pneumoniae* NCTC

13809, were used as positive controls. All clinical isolates were identified by the MALDI Biotyper (Bruker Daltonics, Billerica, MA, USA). An antimicrobial susceptibility test was conducted using the MicroScan 96 Plus with the NENC1J panel (Beckman Coulter, Brea, CA, USA), and the minimum inhibitory concentrations (MICs) were determined based on the CLSI criteria[8].

**MASTDISCS *combi Carba plus***

The MASTDISCS *combi Carba plus* (Mast Group, Liverpool, UK) was used according to the manufacturer's instructions as follows. A 0.5 McFarland inoculum was prepared and inoculated on Mueller–Hinton II agar plates (Nippon Becton Dickinson, Tokyo, Japan). The MASTDISCS *combi Carba plus* consists of five discs (A–E) containing the follow drugs: A, 10 $\mu$g faropenem; B, 10 $\mu$g faropenem with an MBL inhibitor disc; C, 10 $\mu$g faropenem with a KPC inhibitor disc; D, 10 $\mu$g faropenem with an AmpC inhibitor disc; and E, 30 $\mu$g temocillin with an MBL inhibitor disc. Each disc was placed on the Mueller–Hinton II agar plates, ensuring sufficient distance between the discs, and incubated at 35℃ for 18–24 h. The diameter (in mm) of the zone of inhibition was measured. Discs showing no zone of inhibition were assigned a diameter of 6 mm. A flowchart depicting the identification of carbapenemases using the MASTDISCS *combi Carba plus* is shown in Fig. 1. The diameter of the zone of inhibition on the penem disc (A) was compared with that on each penem plus inhibitor disc (B, C, or D). If disc B only showed a diameter difference ≥5 mm compared with disc A (the differences for discs C vs. A and D vs. A should be <5 mm), the strain was regarded as an MBL producer. If disc C only showed a diameter difference ≥5 mm compared with disc A (the differences for discs B vs. A and D vs. A should be <5 mm), the strain was regarded as a KPC producer. If discs C and D both showed significant di-

**Fig. 1. Flowchart for identification of carbapenemase using MASTDISCS Combi Carba plus**




[a] Each millimeter indicates zone diameter or zone difference between discs.

ameter differences (≥5 mm) compared with disc A (B vs. A should be <4 mm), the strain was regarded as a AmpC β-lactamase producer with porin loss (impermeability). If disc E showed a zone of inhibition diameter difference of <10 mm, and insufficient difference was obtained among discs A, B, C, and D, the strain was regarded as an OXA-48 producer. If an equivocal result was generated but resistance on disc A was shown, the organism was considered to express a carbapenemase enzyme.

**Phenotypic confirmation of β-lactamase production**

A phenotypic assay to detect β-lactamases was performed using the mCIM, recommended by the CLSI[4], and DDSTs. DDSTs were performed using a commercially available sodium mercaptoacetic acid (SMA) disc (metallo-β-lactamase SMA Eiken; Eiken Chemical, Tokyo, Japan) as an inhibitor of MBL (SMA-DDST), and 50 mg/mL 3-aminophenyl boronic acid (APBA; Tokyo Chemical Industry, Tokyo, Japan) was used as the inhibitor of AmpC β-lactamases (APBA-DDST). The SMA-DDST was performed using both imipenem and ceftazidime discs (Sensi-Disc; Nippon Becton Dickinson) according to the manufacturer's instructions. If SMA with imipenem and/or with ceftazidime was positive, it was interpreted as SMA-DDST positive. ABPA-DDST was performed using ceftazidime and cefmetazole discs (Sensi-Disc; Nippon Becton Dickinson) with or without 300 μg APBA (6 μL 50 mg/mL solution) according to a previously reported method[6].

**PCR detection of β-lactamase genes**

Bacterial DNA extraction and PCR of carbapenemase genes were performed using the Cica Geneus DNA Extraction Reagent and Cica Geneus Carbapenemase Genotype Detection Kit (Kanto Chemical, Tokyo, Japan), respectively, according to the manufacturer's instructions. The following carbapenemase genes were amplified by multiplex PCR: $bla_{NDM}$, $bla_{KPC}$, $bla_{OXA-48}$, $bla_{GES}$, $bla_{IMP-1}$, and $bla_{VIM}$. AmpC β-lactamase and ESBL genes were detected using multiplex PCR using a previously published method[9,10]. PCR was performed using the TaKaRa PCR Thermal Cycler Dice® Gradient (TaKaRa Bio, Kusatsu, Japan). The amplified PCR products were electrophoresed on 1% agarose gels (Agarose S; FUJIFILM Wako Pure Chemical, Osaka, Japan) using the Mupid-exU (TaKaRa Bio, Kusatsu, Japan) at 100 V for 40 min. The gels were stained for 30 min using GelRed Nucleic Acid Stain (Biotium, Fremont, CA, USA) and visualized using UV light.

## Results

The β-lactamase genotypes, MASTDISCS *combi Carba plus*, mCIM, and DDST for 36 strains from clinical specimens and five reference strains are shown in Table 1. Among the 36

clinical strains, 16 contained carbapenemase genes, as determined by PCR (IMP-1 in 14, NDM in 1, and OXA-48 carbapenemase in 1). Two strains contained genes encoding the following plasmid-mediated AmpC *β*-lactamases (DHA in one and CIT in one). Ten strains contained genes encoding CTX-M-9 *β*-lactamases. The results of the MASTDISCS *combi Carba plus*, mCIM, and DDST were compared with the *β*-lactamase genotypes. The types of carbapenemase-positive cases according to the MASTDISCS *combi Carba plus* are shown in Fig 2. Number of strains identified as MBL producers by the MASTDISCS *combi Carba plus* were 14 strains and all were positive for the mCIM tests. Of these 14 strains, 13 had a positive result on the SMA-DDST using either the imipenem or ceftazidime disc, but one *K. pneumoniae* was negative for the SMA-DDST, which is the IMP-1 types. One OXA-48 type strain was identified as a OXA-48 producer by the MASTDISCS *combi Carba plus* and had a positive result on the mCIM.

Of the 36 strains, 20 had no detectable carbapenemase genes that include the DHA type and CIT type identified as an AmpC producer and non-CPE by the MASTDISCS *combi Carba plus*, respectively. Ten CTX-M-9-producing strains were identified as non-CPE by the MASTDISCS *combi Carba plus*. Of the remaining eight strains with no detectable carbapenemase or AmpC *β*-lactamase genes, five were identified as AmpC producers and three as non-CPE by the MASTDISCS *combi Carba plus*. These three non-CPE strains were positive according to APBA-DDST. All five carbapenemase-producing reference strains showed agreement between the MASTDISCS *combi Carba plus* results and the *β*-lactamase genotypes.

## Discussion

The MASTDISCS *combi Carba plus* is a unique disc set that differentiates the three major carbapenemase types and AmpC *β*-lactamases based on phenotype. It is easy to perform this method using the same procedure as the disc diffusion method.

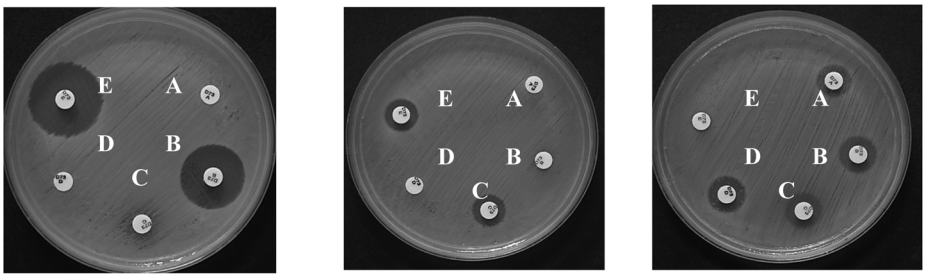
We evaluated the performance of this disc set for identifying carbapenemases produced by 41 clinical and reference *Enterobacteriaceae* strains. Fourteen clinical and 5 reference strains which were identified as MBL producers by the MASTDISCS *combi Carba plus*, all were consistent with the carbapenemase genotypes identified by PCR. Twenty CPE strains including MBL, OXA-48, and KPC-producing strains were correctly differentiated using the MASTDISCS *combi Carba plus.* Identification of carbapenemases from *Enterobacteriaceae* using the MASTDISCS *combi Carba plus* has been described previously[7], and our results were very similar to those of this report. Evaluation of inhibitor combination discs using meropenem as the inhibitor, similar to the MASTDISCS *combi Carba plus*, for identifying CPE has been reported previously[11~13]. However, meropenem-based disc sets are less sensitive for detecting CPE strains, showing low meropenem MIC values. The MASTDISCS *combi Carba plus* uses faropenem, which is more

**Teble 1. Results of MASTDISCS combi carba plus and phenotypic assay for detction of $\beta$-lactamase of clinical and reference strains**

| Group | Species (Formerly genus) | Genotype | Zone diameter of each disc and difference than disc A (mm) | | | | | | | | | Phenoytpic assay | | | | MIC of carbapanem | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | A | B | B-A | C | C-A | D | D-A | E | Result | mCIM[a] | SMA-DDST[b] | APBA-DDST[c] | Result | IPM[d] | MEPM[e] |
| CPE | *Citrobacter freundii* | IMP-1 | 6 | 18 | 12 | 6 | 0 | 6 | 0 | 20 | MBL | + | + | - | MBL | 2 | 2 |
| | *Enterobacter cloacae* | IMP-1 | 6 | 16 | 10 | 6 | 0 | 6 | 0 | 24 | MBL | + | + | - | MBL | 2 | >2 |
| | *Enterobacter cloacae* | IMP-1 | 6 | 17 | 11 | 6 | 0 | 13 | 7 | 28 | MBL | + | + | - | MBL | 2 | >2 |
| | *Enterobacter cloacae* | IMP-1 | 6 | 14 | 8 | 6 | 0 | 6 | 0 | 18 | MBL | + | + | - | MBL | 2 | >2 |
| | *Enterobacter cloacae* | IMP-1 | 6 | 14 | 8 | 6 | 0 | 6 | 0 | 22 | MBL | + | + | - | MBL | 2 | 2 |
| | *Enterobacter cloacae* | IMP-1 | 6 | 14 | 8 | 6 | 0 | 6 | 0 | 22 | MBL | + | + | - | MBL | 2 | 2 |
| | *Enterobacter cloacae* | IMP-1 | 6 | 11 | 5 | 6 | 0 | 6 | 0 | 17 | MBL | + | + | - | MBL | 2 | >2 |
| | *Enterobacter cloacae* | IMP-1 | 18 | 17 | 1 | 18 | 0 | 18 | 0 | 24 | ND | + | - | - | CPE | >2 | >2 |
| | *Escherichia coli* | IMP-1 | 6 | 18 | 12 | 6 | 0 | 9 | 3 | 23 | MBL | + | + | - | MBL | >2 | >2 |
| | *Escherichia coli* | IMP-1 | 6 | 17 | 11 | 6 | 0 | 6 | 0 | 21 | MBL | + | + | - | MBL | ≤1 | ≤1 |
| | *Escherichia coli* | IMP-1 | 6 | 16 | 10 | 6 | 0 | 6 | 0 | 20 | MBL | + | + | - | MBL | ≤1 | ≤1 |
| | *Klebsiella pneumoniae* | IMP-1 | 6 | 18 | 12 | 6 | 0 | 6 | 0 | 21 | MBL | + | + | - | MBL | >2 | >2 |
| | *Klebsiella pneumoniae* | IMP-1 | 6 | 18 | 12 | 6 | 0 | 6 | 0 | 19 | MBL | + | - | - | CPE | >2 | >2 |
| | *Lacleria adecarboxylata* | IMP-1 | 6 | 17 | 11 | 6 | 0 | 6 | 0 | 30 | MBL | + | + | - | MBL | 2 | >2 |
| | *Escherichia coli* | NDM | 6 | 20 | 14 | 6 | 0 | 6 | 0 | 23 | MBL | + | + | - | MBL | >2 | ≤1 |
| | *Klebsiella pneumoniae* | OXA-48 | 10 | 11 | 1 | 11 | 1 | 11 | 1 | 6 | OXA-48 | + | - | - | CPE | 2 | >2 |
| Non-CPE | *Citrobacter freundii* | Not detected | 6 | 15 | 9 | 18 | 12 | 18 | 12 | 17 | AmpC | - | - | + | AmpC | ≤1 | ≤1 |
| | *Citrobacter freundii* | Not detected | 15 | 15 | 0 | 18 | 3 | 20 | 5 | 18 | ND | - | - | + | AmpC | ≤1 | ≤1 |
| | *Enterobacter cloacae* | DHA | 6 | 9 | 3 | 11 | 5 | 15 | 9 | 15 | AmpC | - | - | + | AmpC | 2 | 2 |
| | *Enterobacter cloacae* | Not detected | 7 | 11 | 4 | 18 | 11 | 18 | 11 | 18 | AmpC | - | - | + | AmpC | ≤1 | ≤1 |
| | *Enterobacter cloacae* | Not detected | 6 | 6 | 0 | 6 | 0 | 13 | 7 | 9 | AmpC | - | - | + | AmpC | 2 | 2 |
| | *Escherichia coli* | CIT | 21 | 20 | 1 | 20 | 1 | 20 | 1 | 21 | ND | - | - | + | AmpC | ≤1 | ≤1 |
| | *Escherichia coli* | Not detected | 24 | 22 | 2 | 22 | 2 | 26 | 2 | 25 | ND | - | - | + | AmpC | ≤1 | ≤1 |
| | *Klebsiella* (*Enterobacter*) *aerogenes* | Not detected | 6 | 6 | 0 | 12 | 6 | 15 | 9 | 6 | AmpC | - | - | + | AmpC | >2 | >2 |
| | *Klebsiella* (*Enterobacter*) *aerogenes* | Not detected | 16 | 15 | 1 | 19 | 3 | 19 | 3 | 21 | ND | - | - | + | AmpC | ≤1 | ≤1 |
| | *Serratia marcescens* | Not detected | 6 | 6 | 0 | 6 | 0 | 13 | 7 | 6 | AmpC | - | - | + | AmpC | 2 | ≤1 |
| | *Citrobacter freundii* | CTX-M-9 group | 6 | 14 | 8 | 16 | 10 | 16 | 10 | 19 | ND | | | | ND | ≤1 | ≤1 |
| | *Citrobacter freundii* | CTX-M-9 group | 12 | 12 | 0 | 15 | 3 | 19 | 7 | 16 | ND | | | | ND | ≤1 | ≤1 |
| | *Escherichia coli* | CTX-M-9 group | 24 | 24 | 0 | 24 | 0 | 24 | 0 | 24 | ND | | | | ND | ≤1 | ≤1 |
| | *Escherichia coli* | CTX-M-9 group | 19 | 19 | 0 | 19 | 0 | 19 | 0 | 18 | ND | | | | ND | ≤1 | ≤1 |
| | *Escherichia coli* | CTX-M-9 group | 24 | 22 | 2 | 22 | 2 | 26 | 2 | 24 | ND | | | | ND | ≤1 | ≤1 |
| | *Klebsiella oxytoca* | CTX-M-9 group | 21 | 21 | 0 | 21 | 0 | 21 | 0 | 25 | ND | | | | ND | ≤1 | ≤1 |
| | *Klebsiella oxytoca* | CTX-M-9 group | 20 | 20 | 0 | 20 | 0 | 20 | 0 | 21 | ND | | | | ND | ≤1 | ≤1 |
| | *Klebsiella oxytoca* | CTX-M-9 group | 24 | 24 | 0 | 24 | 0 | 24 | 0 | 26 | ND | | | | ND | ≤1 | ≤1 |
| | *Klebsiella pneumoniae* | CTX-M-9 group | 21 | 22 | 1 | 22 | 1 | 22 | 1 | 24 | ND | | | | ND | ≤1 | ≤1 |
| | *Klebsiella pneumoniae* | CTX-M-9 group | 23 | 23 | 0 | 23 | 0 | 23 | 0 | 25 | ND | | | | ND | ≤1 | ≤1 |
| Reference | *Escherichia coli* ATCC BAA-2469 | NDM | 6 | 18 | 12 | 6 | 0 | 6 | 0 | 19 | MBL | + | + | - | MBL | >2 | >2 |
| | *Klebsiella pneumoniae* NCTC 13440 | VIM-1 | 6 | 18 | 12 | 6 | 0 | 6 | 0 | 18 | MBL | + | + | - | MBL | >2 | ≤1 |
| | *Klebsiella pneumoniae* NCTC 13438 | KPC | 6 | 6 | 0 | 12 | 6 | 6 | 0 | 10 | KPC | + | - | + | MBL | >2 | 2 |
| | *Klebsiella pneumoniae* NCTC 13809 | KPC | 6 | 6 | 0 | 12 | 6 | 6 | 0 | 16 | KPC | + | - | + | MBL | >2 | >2 |
| | *Klebsiella pneumoniae* NCTC 13442 | OXA-48 | 6 | 6 | 0 | 6 | 0 | 6 | 0 | 6 | OXA-48 | + | - | - | CPE | 2 | 2 |

Abbreviations: +, positive; -, negative; ND, not determined; slanted line, not tested.
[a]Modified carbapenemase inactivation method.
[b]Sodium mercaptoacetic acid double-disk synergy test with imipenem or ceftazidime.
[c]Aminophenyl boronic acid double-disk synergy test.
[d]Imipenem.
[e]Meropenem.

**Fig. 2. Representative results of MASTDISCS *Combi Carba plus.* (2-1) IMP-1type, (2-2) KPC type and (2-3) OXA-48 type**




**2-1** **2-2** **2-3**

sensitive for detecting carbapenemase production[14].

We compared the results of mCIM and SMA-DDST with those of the MASTDISCS *combi Carba plus* for detection of carbapenemase producers. The mCIM was able to identify carbapenemase production from all strains containing carbapenemase genes. One of *E. cloacae* and one of *K. pneumoniae* harboring IMP-1 were mCIM-positive, but not SMA-DDST. The *E. cloacae* strain showed large zone of inhibition with disc A of the MASTDISCS *combi Carba plus*, possessing lost the drug-resistant gene. Another strain of *K. pneumoniae* might be highly resistant to carbapenems, with the expansion of zone by SMA could not be clearly observed. Fourteen MBL producers were identified by SMA-DDST using imipenem and ceftazidime discs. The first report of IMP-1-producing *Enterobacteriaceae* isolated from *Serratia marcescens* was in Japan[15]. Because the hydrolytic activity of this *β*-lactamase was lower toward imipenem than ceftazidime, it was considered a false-negative for imipenem. All strains containing AmpC *β*-lactamase and ESBL genes were identified as AmpC producers or non-CPE strains by the MASTDISCS *combi Carba plus*. The APBA-DDST identified 10 AmpC *β*-lactamase producers, but AmpC *β*-lactamase genes were detected in only two of these strains. In this study, resistance mechanisms among these eight strains remain to be identified. We suspected that chromosomal AmpC *β*-lactamase might be involved.

Our results showed that the MASTDISCS *combi Carba plus* is useful for accurately differentiating carbapenemase types, as well as evaluating their production. There were some limitations to this study. The KPC carbapenemase-producing strains have been tested only against reference strains, and the IMP carbapenemase-producing strain was not tested for the IMP-6 type, which has recently increased in prevalence in Japan[16]. Further studies are therefore required to evaluate the performance of this disc set with a larger number of KPC and IMP-6 type CPE strains. GES-type producer is relatively rare CPE in Japan, but has not been evaluated in this study[17]. Therefore, it is also necessary to evaluate the detection and differentiation of this type of CPE in the future.

In conclusion, the MASTDISCS *combi Carba plus* enables convenient screening and differentiation of the major carbapenemases, and this method provides useful information for antimicrobial therapies and control of infections. However, in routine microbiology laboratory, it is important that MASTDISCS *combi Carba plus* is rational use for differential test for CPE by the first-line test such as mCIM, although it lacks speed.

**Conflicts of interest**

There is no conflict of interest.

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