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Adjustment of Modified Carbapenem Inactivation Method Conditions for Rapid Detection of Carbapenemase-Producing *Acinetobacter baumannii*

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Background: The existing modified carbapenem inactivation methods (mCIMs) recommended by the CLSI for detecting carbapenemase production have not been applicable for *Acinetobacter baumannii*. We evaluated the influence of matrices used in mCIMs and CIMTris on the stability of the disks for detecting carbapenemase producers and suggested optimal mCIM conditions for detecting carbapenemase-producing *A. baumannii*.

Methods: Seventy-three *A. baumannii* isolates characterized for antimicrobial susceptibility and carbapenemase encoding genes were tested for carbapenemase production using mCIM and CIMTris. The influence of the matrices (Tryptic soy broth [TSB] and Tris-HCl) used in these methods on the stability of the meropenem (MEM) disk was also evaluated. The mCIM conditions were adjusted to enhance screening sensitivity and specificity for detecting carbapenemase-producing *A. baumannii*.

Results: The matrices had an impact on the stability of the MEM disk after the incubation period (two or four hrs). TSB nutrient broth is an appropriate matrix for mCIM compared with Tris-HCl pH 7.6, which leads to the loss of MEM activity in CIMTris. The sensitivity and the specificity of the optimal mCIM were both 100%.

Conclusions: We established optimal mCIM conditions for simple, accurate, and reproducible detection of carbapenemase-producing *A. baumannii*.

Key Words: Carbapenemase, *Acinetobacter baumannii*, Modified carbapenem inactivation method, CIMTris

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INTRODUCTION

The emergence of carbapenem-resistant *Acinetobacter baumannii* is of particular concern worldwide as infections with this pathogen have high mortality rates and limited treatment options [1-4]. *A. baumannii*'s growing carbapenem resistance is mainly due to the production of acquired carbapenemases [5]. A rapid and reliable phenotypic method for detecting these carbapenemase producers in clinical samples is indispensable for therapeutic and infection control reasons. Although the number of non-molecular methods described for rapid detection of carbapenemase activity in gram-negative bacteria has increased over the past few years [6, 7], phenotypic screening of carbapenemase in *A. baumannii* remains a challenge for clinical microbiologists. The OXA-type β-lactamase enzymes harbored by some carbapenem-resistant *A. baumannii* hydrolyze carbapenem less

efficiently than many class A and B carbapenemases, but can still lead to high-level carbapenem resistance when present together with other resistance mechanisms such as porin mutations or efflux [8]. Various procedures have been developed to optimize the method for *A. baumannii*; however, each has led to undesirable trade-offs between sensitivity and specificity [9].

According to the 26th edition of the CLSI recommendations (2016), the modified Hodge test should be applied to only *Enterobacteriaceae*, and the Carba NP test should be applied for all *Enterobacteriaceae, Pseudomonas aeruginosa*, and *Acinetobacter* spp. [10]. These two phenotypic methods were maintained in the 27th edition with the same procedures described in 2016, but another method, the modified carbapenem inactivation method (mCIM), was introduced for only *Enterobacteriaceae* [11]. However, the Carba NP test was no longer recommended for *Acinetobacter* spp. in the 28th edition (2018) [12, 13]. Moreover, the mCIM was further modified to detect carbapenemases in *P. aeruginosa* but remained inapplicable for *Acinetobacter* spp. [12, 13]. Furthermore, the modified Hodge test was no longer included in the 28th edition, even for *Enterobacteriaceae*. In 2017, a research group in Japan described another revised version of the carbapenem inactivation method, CIMTris, for detecting carbapenemase production in *Acinetobacter* and *Pseudomonas* spp. [14]; they used Tris-HCl buffer instead of tryptic soy broth (TSB) to extract the carbapenemases, resulting in higher sensitivity but lower specificity than the mCIM [14]. However, to date, no data exist that illustrate the interactions between the matrix and carbapenem stability and/or the effects of matrices on the enzyme-substrate reaction of carbapenem hydrolysis, which may cause the lower specificity for CIMTris [14].

We evaluated the influence of the matrices used in mCIM and CIMTris on the stability of the meropenem (MEM) disk for detecting carbapenemase producers. We also suggest optimal mCIM conditions for detecting carbapenemase-producing *A. baumannii*.

METHODS

Bacterial isolates and antimicrobial susceptibility test (AST)

A total of 68 *A. baumannii* isolates were retrospectively selected from the frozen stocks kept in skim milk at -80°C. The stocks were recovered from the respiratory, urine, and blood samples requested for culture from 2016 to 2017 at Severance Hospital, Seoul, Korea. This study does not include any patient information; hence, approval from the Institutional Review Board was not necessary. Additionally, five isolates with carbapenemase genes (bla_{IMP-4}, bla_{OXA-25}, bla_{OXA-26}, bla_{OXA-27}, and bla_{OXA-58}) were provided by Professor D. M. Livermore, University of East Anglia, UK. The identification and antimicrobial susceptibility of the bacterial isolates were determined using the VITEK MS system (bioMérieux, Marcy-l'Étoile, France) and the VITEK 2 system (bioMérieux, Durham, NC, USA).

In addition, the disk diffusion method was performed for all strains on Mueller-Hinton agar (MHA; Becton, Dickinson and Company, Sparks, MD, USA) with imipenem (IPM) and MEM disks (Becton, Dickinson and Company) to confirm susceptibility to carbapenem, according to the CLSI M100-S20 [15].

Molecular testing for carbapenemase-encoding genes

Multiplex PCR was conducted to detect four groups of OXA-carbapenemase genes, as previously described [16]. Primer sets IS*Aba*1F/OXA-23R, IS*Aba*4F/OXA-23R, and IS*Aba*OXA-51R were used to detect the presence of IS*Aba*1 and IS*Aba*4 upstream of the bla_{OXA-23}-like genes, and IS*Aba*1 upstream of the bla_{OXA-51}-like genes, respectively, as previously described [17].

mCIM and CIMTris

Two different mCIMs, one using a 1-μL loopful of *Enterobacteriaceae* colonies (Procedure A) and another using a 10-μL loopful of *P. aeruginosa* colonies (Procedure B) in 2 mL TSB (Becton, Dickinson and Company), were performed separately according to the 28th edition of the CLSI guidelines (2018) [13]. For CIMTris, the carbapenem inactivation step of the mCIM was modified by preparing a 10-μL loopful of bacteria emulsified in 400 μL of 0.5 M Tris-HCl buffer (pH 7.6) (Sigma-Aldrich Co., LLC, St. Louis, MO, USA), and decreasing the incubation time for the MEM disk in the buffer from four hours to two hours [14]. The steps performed after the two-hour incubation corresponded to those used in the mCIM. Three negative controls were also tested using both methods by performing the same procedure without a bacterial isolate. The methods were conducted in triplicate for every isolate as well as negative control.

Measurement of remaining MEM disk potency and matrices after incubation

The effects of the matrices on MEM were measured without any bacteria at different time points of incubation. Specifically, an MEM disk was directly added to each of four tubes containing different volumes of matrix and no bacteria: (a) 400 μL TSB, (b) 2 mL TSB, (c) 400 μL Tris-HCl, and (d) 2 mL Tris-HCl. Each set of four tubes was incubated at 35±2°C for two different time periods: two and four hours. After two or four hours of incubation, the MEM disk was removed from each tube to measure

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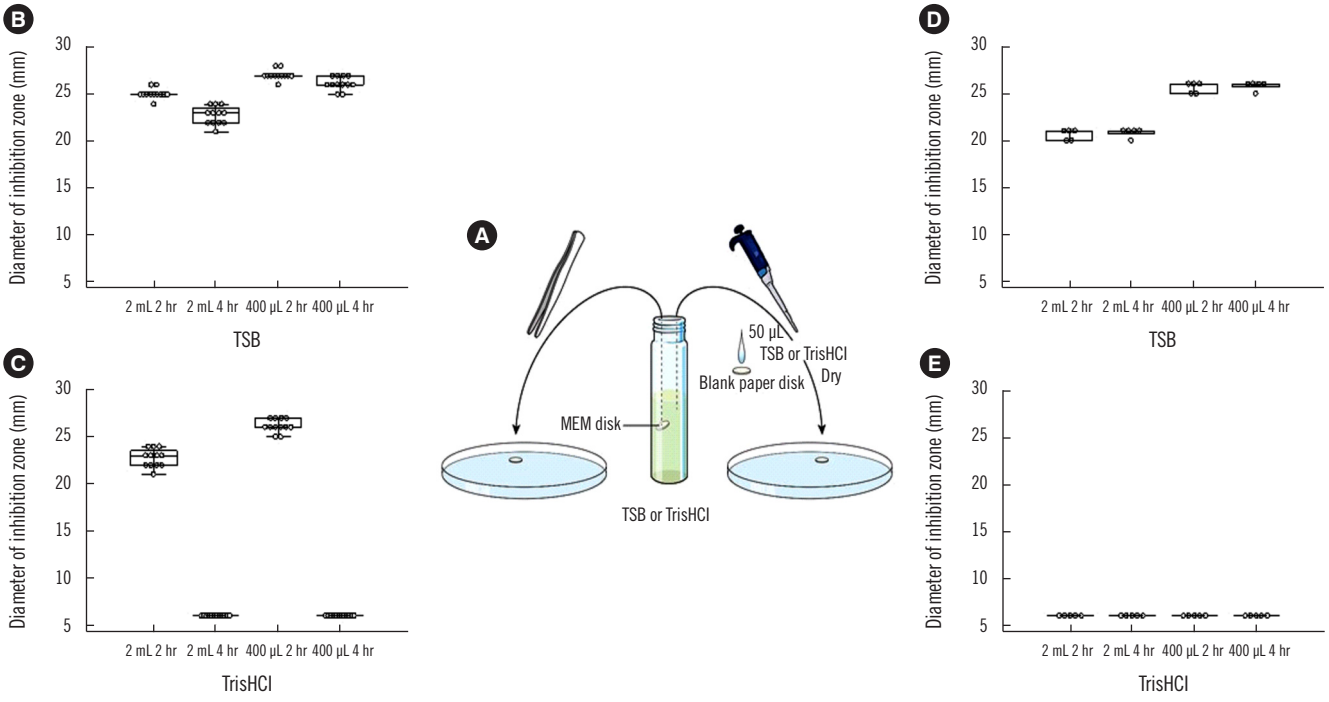

Fig. 1. (A) Schematic drawing of remaining potency of MEM disks in two types of buffers (TSB and Tris-HCl) after two or four hours of incubation. Diameter of inhibition zone with (B, C) incubated MEM disk depending on type and volume of matrix and duration of incubation, (D, E) MEM dissolved in TSB or Tris-HCl buffer. Tris-HCl weakened MEM potency, which was not maintained in either the buffer or disk.
Abbreviations: MEM, meropenem; TSB, tryptic soy broth.

disk potency. The disks were placed on an MHA plate with a lawn of the MEM-susceptible *Escherichia coli* ATCC 25922 indicator strain. The MHA plates were then incubated at 35±2°C for 18–24 hours. Following incubation, the zone of inhibition was measured (Fig. 1). After removing the MEM disks from the tubes, a 50-µL aliquot of the residual matrix was added to a blank disk, and the diameters of the inhibition zones created by those disks were then measured and recorded.

Adjustment of mCIM conditions

To enhance sensitivity, the mCIM was adjusted using different inoculum sizes and broth volumes. First, the inoculum size was increased up to two 10-µL loopfuls of test strain colonies, while the broth volume was maintained as for normal mCIM (2 mL TSB). Further, to increase the density and accelerate the carbapenemase reaction, the volume of TSB was reduced from 2 mL to 400 µL. Next, three different inoculum sizes were tested: (i) one 10-µL loopful, (ii) one and a half 10-µL loopfuls, and (iii) two 10-µL loopfuls of bacteria from an overnight blood agar plate. The subsequent steps corresponded to those used in the mCIM.

Statistical analysis

Sensitivity and specificity were calculated to compare results of this test method with phenotypic AST and molecular test for carbapenemase-encoding genes. Mann-Whitney tests were performed, and the box and whisker plots were created using MedCalc Statistical Software version 18 (MedCalc Software bvba, Ostend, Belgium). In Fig. 1, the central box demonstrates the lower to upper quartile (25th to 75th percentile). The middle horizontal line represents the median value.

RESULTS

Of the 73 bacterial isolates tested, 46 (63.0%) were resistant to both IPM and MEM. Of these, 38 isolates (82.6%) were positive for both bla_{OXA-23}-like and bla_{OXA-51}-like genes; 37 isolates had IS*Aba*1 upstream of the bla_{OXA-23}-like gene; and the remaining single isolate (2.1%) was positive for the bla_{OXA-51}-like gene and had an IS*Aba*1 element upstream of this gene (Table 1). Of the 73 isolates, 27 that showed susceptibility to carbapenem by VITEK 2 were confirmed using the disk diffusion method, with inhibition zones ≥18 mm for MEM and ≥22 mm for IPM.

Procedure A of the mCIM was modified from a screening me-

Table 1. Carbapenemase genes detected in *A. baumannii* isolates and their CIMTris and mCIM results

Carbapenem susceptibility	Isolates (N)	Positive isolates (N)													
		OXA-51-like	OXA-23-like	OXA-24-like	OXA-25-like	OXA-26-like	OXA-27-like	OXA-58-like	IMP-1	IMP-4	SIM-1	VIM-2	ISAba1-bla_{OXA-51}	ISAba1-bla_{OXA-23}	mCIM procedure A*	mCIM procedure B†	CIMTris
Resistant	46	38	37	0	1	1	1	2	1	1	1	1	1	37	6	30	46
Susceptible	27	9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2

*1-μL loopful of bacteria in 2 mL TSB; †10-μL loopful of bacteria in 2 mL TSB.
Abbreviations: mCIM, modified carbapenem inactivation method; TSB, tryptic soy broth.

Table 2. Number of isolates according to mCIM matrix, volume, and inoculum size

Matrix, volume, and bacteria inoculum size		Carbapenem resistant (N=46)			Carbapenem susceptible (N=27)		
		Positive (%)	Indeterminate (%)	Negative (%)	Positive	Indeterminate	Negative (%)
TSB, 2 mL	Two 10-μL loopful	39 (84.7)	4 (8.7)	3 (6.5)	0	0	27 (58.7)
TSB, 400 μL	(i) One 10-μL loopful	36 (78.3)	2 (4.3)	8 (17.4)	0	0	27 (58.7)
	(ii) One and a half 10-μL loopful	44 (95.6)	2 (4.3)	0	0	0	27 (58.7)
	(iii) Two 10-μL loopful	46 (100)	0	0	0	0	27 (58.7)

Abbreviations: TSB, tryptic soy broth; mCIM, modified carbapenem inactivation method.

thod initially called CIM [6, 7]. Of the 46 carbapenem-resistant bacterial isolates tested using mCIM procedure A, three (6.5%) were positive and two (4.3%) were indeterminate (data not shown). In addition, 27 carbapenem-susceptible isolates were negative using procedure A. Following procedure B, the mCIM showed higher sensitivity; 30 carbapenem-resistant isolates (65.2%) were carbapenemase producers. Furthermore, CIMTris showed that all 46 (100%) carbapenem-resistant isolates were carbapenemase falsely positive; two (7.4%) and one (3.7%) of the 27 carbapenem-susceptible isolates were positive and indeterminate, respectively (Table 1).

We next tested whether the incubation buffer inactivates MEM activity in the absence of carbapenemase-producing organism. An inhibition zone diameter of 30±1 mm was measured after an overnight incubation and was termed the original inhibition zone (repeated 12 times). The results after incubating the MEM and IPM disk in the matrix showed that the inhibition zone surrounding the incubated disk was smaller than that around the non-incubated disk (repeated five times).

Similar results were observed in both the mCIM and CIMTris. Noticeably, the inhibition zone in CIMTris was significantly smaller ($P<0.001$) than that in the mCIM and the original inhibition zone mentioned above (Fig. 1B and C). In addition, we evaluated the influence of the matrices on the MEM disk; we measured the inhibition zone diameters dependent on the following three factors: (1) type of matrix, (2) volume of matrix, and (3) duration of incubation (Fig. 1D and E).

When incubated in the same volume for the same time period, the MEM disk maintained higher stability in TSB broth than in Tris-HCl buffer. In addition, when the volume was increased and the incubation time was extended, the diameter of the inhibition zone surrounding the MEM disk incubated in TSB changed slightly, while the change in Tris-HCl was greater. Moreover, the MEM disk incubated in Tris-HCl buffer for four hours could not inhibit the growth of indicator organism *E. coli* ATCC 25922 and gave false-positive results, with a 6 mm diameter of inhibition zone in both 400 μL and 2 mL (Fig. 1C). No inhibition zone >6 mm in diameter was observed for disks incubated in Tris-HCl (Fig. 1E).

Based on the modification of the mCIM with 400 μL TSB and (i) one 10-μL loopful of bacteria, 36 (78.3%) of the 46 isolates were detected as carbapenemase producers, while the remaining two (4.3%) and eight (17.3%) isolates were identified as indeterminate and negative, respectively (Table 2). When the mCIM with 400 μL TSB and (ii) one and a half 10-μL loopfuls of bacteria was examined, eight more isolates were detected as carbapenemase-

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producing, resulting in 44 (95.7%) carbapenem-resistant isolates, and the two remaining isolates were identified as indeterminate; with (iii) two 10-μL loopfuls of bacteria, all 46 (100%) isolates were detected as carbapenemase-producing *A. baumannii*. Our experiment showed that the optimal mCIM with two 10-μL loopfuls of bacteria incubated in 400 μL TSB had markedly high sensitivity and specificity (100% and 100%, respectively).

DISCUSSION

We determined that (1) incubation in either matrix, even in the absence of the test bacterial isolates, leads to some of the MEM and IPM dissolving into the matrix, resulting in a reduced amount of active MEM in the disk at the end of incubation; (2) both a longer duration of incubation and an increased volume of matrix reduce active MEM in the disk at the end of incubation; (3) a longer incubation causes a more pronounced loss of active carbapenem from the disk when the incubation takes place in Tris-HCl; and (4) incubation in Tris-HCl actually leads to not only dissolution of MEM into the matrix but also its inactivation. We demonstrated that Tris-HCl inactivates MEM activity in the absence of an organism. This might be caused by the effects of the matrix on the MEM disk during incubation. CIMTris differed from mCIM in the following three aspects: (1) type of matrix, (2) volume of matrix, and (3) duration of incubation. These results indicate that an increased volume of matrix and an extended incubation time lead to an elevated amount of dissolved MEM and IPM (Fig. 1D and E).

Our results support a previous report that CIMTris shows a high percentage of false-positive results after four hrs of optimal incubation time [14]. The aforementioned two carbapenem-susceptible isolates that showed positive results in CIMTris are considered false-positive. The false-positive results suggest that MEM might be not only dissolved but also inactivated by Tris-HCl following incubation. Therefore, Tris-HCl should not be used as the buffer when performing mCIM to assess MEM hydrolysis.

Building on the principles learned from this first set of experiments, we then moved forward with TSB as the incubation matrix of choice and performed several variations of the mCIM. Inoculum size and matrix volume were important parameters for adjustment of mCIM.

Our results showed an improvement with increasing inoculum size and reduced volume of the liquid base (Tables 1 and 2). No false-positives were detected for any modification of these two parameters; mCIM with 400 μL TSB had higher sensitivity than mCIM with 2 mL TSB.

The present study had several limitations. The isolates were not comprehensive and were mainly from a single medical center. In addition, the genotypes of the isolates were not determined. Therefore, further testing of various *A. baumannii* genotypes is required to evaluate the performance of the mCIM. Despite these limitations, the optimal mCIM is an easy-to-perform, inexpensive, and reproducible method using readily available materials and reagents for detecting carbapenemase-producing *A. baumannii* in clinical microbiology laboratories.

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Author Contributions

YC and DY designed the study and secured the funding. TNV, RD, NP, and LPN performed the experiments. TNV, J-HB, and DY analyzed and interpreted the data and wrote the manuscript.

Conflicts of Interest

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

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REFERENCES

1. Dijkshoorn L, Nemec A, Seifert H. An increasing threat in hospitals: multidrug-resistant *Acinetobacter baumannii*. Nat Rev Microbiol 2007;5:939-51.
2. Peleg AY, Seifert H, Paterson DL. *Acinetobacter baumannii*: Emergence of a successful pathogen. Clin Microbiol Rev 2008;21:538-82.
3. Poirel L and Nordmann P. Carbapenem resistance in *Acinetobacter baumannii*: mechanisms and epidemiology. Clin Microbiol Infect 2006;12:826-36.
4. Sunenshine RH, Wright MO, Maragakis LL, Harris AD, Song X, Hebden J, et al. Multidrug-resistant *Acinetobacter* infection mortality rate and length of hospitalization. Emerg Infect Dis 2007;13:97-103.
5. Queenan AM and Bush K. Carbapenemases: the versatile β-lactamases. Clin Microbiol Rev 2007;20:440-58.
6. Pierce VM, Simner PJ, Lonsway DR, Roe-Carpenter DE, Johnson JK, Brasso WB, et al. Modified carbapenem inactivation method for phenotypic detection of carbapenemase production among *Enterobacteriaceae*. J Clin Microbiol 2017;55:2321-33.
7. van der Zwaluw K, de Haan A, Pluister GN, Bootsma HJ, de Neeling AJ, Schouls LM. The carbapenem inactivation method (CIM), a simple and low-cost alternative for the Carba NP test to assess phenotypic carbapenemase activity in Gram-negative rods. PLoS One 2015;10:e0123690.
8. Walther-Rasmussen J and Høiby N. OXA-type carbapenemases. J Antimicrob Chemother 2006;57:373-83.
9. Simner PJ, Opene BN, Chambers KK, Naumann ME, Carroll KC, Tamma PD. Carbapenemase detection among carbapenem-resistant glucose-nonfermenting Gram-negative bacilli. J Clin Microbiol 2017;55:2858-64.
10. CLSI. Performance standards for antimicrobial susceptibility testing. 26th ed. CLSI supplement M100-S26. Wayne, PA, USA: Clinical and Laboratory Standards Institute, 2016.
11. CLSI. Performance standards for antimicrobial susceptibility testing. 27th ed. CLSI supplement M100-S27. Wayne, PA, USA: Clinical and Laboratory Standards Institute, 2017.
12. CLSI. Performance standards for antimicrobial susceptibility testing. 28th ed. CLSI supplement M100-S28. Wayne, PA, USA: Clinical and Laboratory Standards Institute, 2018.
13. CLSI. Test for carbapenemases in *Enterobacteriaceae* and *Pseudomonas aeruginosa*. 28th ed. CLSI supplement M100. Clinical and Laboratory Standards Institute, Wayne, PA 2018.
14. Uechi K, Tada T, Shimada K, Kuwahara-Arai K, Arakaki M, Tome T, et al. A modified carbapenem inactivation method, CIMTris, for carbapenemase production in *Acinetobacter* and *Pseudomonas* species. J Clin Microbiol 2017;55:3405-10.
15. CLSI. Performance standards for antimicrobial susceptibility testing. 20th ed. CLSI supplement M100-S20. Wayne, PA, USA: Clinical and Laboratory Standards Institute, 2010.
16. Woodford N, Ellington MJ, Coelho JM, Turton JF, Ward ME, Brown S, et al. Multiple PCR for genes encoding prevalent OXA carbapenemases in *Acinetobacter* spp. Int J Antimicrob Agents 2006;27:351-3.
17. Bahador A, Raoofian R, Farshadzadeh Z, Beitollahi L, Khaledi A, Rahimi S, et al. The prevalence of $IS_{Aba}1$ and $IS_{Aba}4$ in *Acinetobacter baumannii* species of different international clone lineages among patients with burning in Tehran, Iran. Jundishapur J Microbiol 2015;8:e17167.