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A Modified Carbapenem Inactivation Method, CIMTris, for Carbapenemase Production in *Acinetobacter* and *Pseudomonas* Species

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ABSTRACT The carbapenem inactivation method (CIM) and modified CIM (mCIM) are simple and economical phenotypic screening methods for detecting carbapenemase production in Gram-negative bacteria. Although the mCIM has been recommended by the Clinical and Laboratory Standards Institute, both the CIM and mCIM have limitations. This study describes another modified CIM, called CIMTris, in which carbapenemase was extracted from bacteria with 0.5 M Tris-HCl (pH 7.6) buffer. The ability of the CIMTris to detect carbapenemase production was examined in *Acinetobacter* and *Pseudomonas* species. The CIMTris had an overall sensitivity of 97.6% and an overall specificity of 92.6%, whereas the mCIM had a sensitivity of 45.1% and a specificity of 100% for the isolates tested. These findings indicate that the CIMTris is useful for detecting carbapenemase production in *Acinetobacter* and *Pseudomonas* species.

KEYWORDS *Acinetobacter* species, *Pseudomonas* species, carbapenem inactivation method, carbapenem resistance

The emergence and dissemination of carbapenem-resistant *Acinetobacter* and *Pseudomonas* species have been a serious health concern worldwide, because infection with these bacteria is associated with high mortality rates and limited medical treatment options (1, 2). The high carbapenem resistance of these bacteria is due to their production of acquired carbapenemases, including metallo-β-lactamases, such as IMP-, NDM-, and VIM-type enzymes, and KPC-type β-lactamases (3). In addition, some variants of OXA-type and extended-spectrum β-lactamases, including GES-5, and OXA-23, OXA-48, and OXA-72, have shown carbapenemase activity (3).

Carbapenemase activity in Gram-negative pathogens can be detected using the carbapenem inactivation method (CIM) (4). Its characteristics have been closely examined, and the CIM has been compared with the Rapidec Carba NP (bioMerieux, France), a commercially available modified version of the Carba NP test, as well as the modified Hodge and Carba NP tests (5, 6). The Clinical and Laboratory Standards Institute (CLSI) now recommends the mCIM, a modified carbapenem inactivation method, for the detection of carbapenemase activity in *Enterobacteriaceae* (7). The mCIM has greater sensitivity than the CIM in detecting carbapenemase production in *Enterobacteriaceae* (8) and in glucose-nonfermenting Gram-negative bacteria (9). However, the ability of the mCIM to detect carbapenemase production in certain Gram-negative bacteria can

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TABLE 1 Time dependence of incubation with MEM disks in the CIMTris

Bacterial isolates[a]	Presence of acquired carbapenemase-encoding genes[b]	No. of isolates tested	No. (%) of CIMTris-positive isolates after incubating with MEM disks for:			
			0.5 h	1 h	2 h	4 h
Acinetobacter species	Positive	29	3 (10.3[c])	8 (27.6)	27 (93.1)	29 (100)
	Negative	15	0 (0)	0 (0)	2 (13.3)	4 (26.7)
Pseudomonas species	Positive	53	35 (66.0)	48 (90.6)	53 (100)	53 (100)
	Negative	12	0 (0)	0 (0)	0 (0)	12 (100)

[a]All bacterial isolates used are listed in Table 2.
[b]Acquired carbapenemase-encoding genes are shown in Table 2.
[c]Percentage of isolates tested that were CIMTris-positive (3/29).

be improved. This study describes a further modified CIM, called CIMTris, which uses 0.5 M Tris-HCl buffer instead of Trypticase soy broth (TSB) to extract carbapenemase. This study also tested the ability of the CIMTris to detect carbapenemase production in *Acinetobacter* and *Pseudomonas* species.

RESULTS AND DISCUSSION

To optimize the incubation time of bacterial suspensions with meropenem (MEM) disks in CIMTris, the suspensions were incubated with MEM disks for 0.5, 1, 2 and 4 h (Table 1). The results of the CIMTris improved with incubation time up to 2 h. Twenty-seven of the 29 (93.1%) *Acinetobacter* isolates and 53 of the 53 (100%) *Pseudomonas* isolates harboring acquired genes encoding carbapenemases were positive on the CIMTris after 2 h, whereas all these isolates harboring acquired carbapenemase genes were positive after 4 h; four (26.7%) *Acinetobacter* and 12 (100%) *Pseudomonas* isolates not harboring acquired carbapenemase genes were also positive on the CIMTris after incubating for 4 h (Table 1). Therefore, the 4-h incubation was not suitable to detect carbapenemase production, especially in *Pseudomonas* species. Incubation for 2 h was defined as optimal and used in subsequent experiments.

Of the 44 *Acinetobacter* isolates tested, 29 (65.9%) harbored acquired genes encoding carbapenemases, including IMP-14, NDM-1, OXA-23, OXA-58, and OXA-72 (Table 2), and 27 of the 29 (93.1%) were positive on the CIMTris after incubating for 2 h (Tables 1 and 2). The remaining two isolates, *A. calcoaceticus* and *A. radioresistens*, were negative on the CIMTris but harbored genes encoding the OXA-58 and OXA-23-like carbapenemases, respectively. These isolates were susceptible to MEM, suggesting they produced insufficient amounts of carbapenemases. Of the 15 isolates not harboring acquired carbapenemase genes, nine were *A. baumannii*, which harbors intrinsic carbapenemase genes (1). Two of these nine isolates had different intrinsic carbapenemase genes, OXA-69 and OXA-82, and they showed MICs to MEM of 64 and 16 μg/ml, respectively (Table 2). Both isolates were positive on the CIMTris, with sequencing showing the insertion of an IS*Aba1* element upstream of these intrinsic genes. This upstream element harbors a promoter sequence, resulting in the overproduction of carbapenemases (1). The remaining seven isolates were negative on the CIMTris and did not have an IS*Aba1* element upstream of the intrinsic genes. Six of the 15 isolates that did not harbor acquired carbapenemase gene were found to consist of species other than *A. baumannii*, with all six being negative on the CIMTris. Thus, after incubating for 2 h, the CIMTris showed a sensitivity of 93.1% and a specificity of 86.7% for detecting carbapenemase activities in *Acinetobacter* species ($P < 0.01$).

Although the mCIM was originally designed to detect carbapenemase production in *Enterobacteriaceae*, we tested its ability to detect carbapenemase production in *Acinetobacter* species isolates. Eight of 29 (27.6%) isolates harboring acquired carbapenemase genes were positive on the mCIM, and 15 of 15 (100%) isolates not harboring acquired carbapenemase genes were negative on the mCIM. Of the eight mCIM-positive isolates, two, one, one, and four harbored acquired genes encoding IMP-10, IMP-14, NDM-1, and OXA-72, respectively. The remaining 21 isolates harboring acquired

TABLE 2 List of clinical isolates of *Acinetobacter* and *Pseudomonas* species and their MICs (IPM and MEM), CIMTris, and mCIM results

Species	No. of isolates	Carbapenemase-encoding gene(s)	MICs (μg/ml)		CIMTris			mCIM (no. positive/ no. of isolates tested)	Reference or source
			IPM	MEM	No. positive/ no. of isolates tested	Inhibited by SMA	Inhibited by CVA		
Acinetobacter spp. harboring acquired carbapenemase-encoding genes									
A. baumannii	1	NDM-1, OXA-69[a]	128	256	1/1	+	−	0/1	This study
	1	OXA-23, OXA-104[a]	32	64	1/1	−	−	0/1	13
	1	OXA-23, OXA-371[a]	8	16	1/1	−	−	0/1	13
	2	OXA-23, OXA-402[a]	16–32	16–32	2/2	−[b]	−[b]	0/2	This study
	1	OXA-23, OXA-51-like[a]	32	16	1/1	−	−	0/1	This study
	1	OXA-23, OXA-58, OXA-66[a]	8	8	1/1	−	−	0/1	This study
	1	OXA-23, OXA-64[a]	16	32	1/1	−	−	0/1	This study
	4	OXA-23, OXA-66[a]	16–32	16–64	4/4	−[b]	−[b]	0/4	This study
	1	OXA-23-like, OXA-91[a]	32	64	1/1	−	−	0/1	This study
	5	OXA-72, OXA-66[a]	64–128	64–256	5/5	−[b]	−[b]	4/5	12
A. bereziniae	1	NDM-1	8	128	1/1	+	−	1/1	This study
A. calcoaceticus	1	NDM-1	128	128	1/1	+	−	0/1	This study
	1	OXA-58	16	4	0/1	ND[c]	ND	0/1	This study
A. indicus	1	NDM-1	32	32	1/1	+	−	0/1	This study
A. nosocomialis	1	IMP-14	128	128	1/1	+	−	1/1	This study
	2	OXA-23	16–32	32	2/2	−[b]	−[b]	0/2	This study
A. pittii	1	OXA-23	32	32	1/1	ND	ND	0/1	This study
A. radioresistens	1	OXA-23-like	<0.5	<0.5	0/1	ND	ND	0/1	This study
A. ursingii	2	IMP-10	64–128	256	2/2	+[b]	−[b]	2/2	This study
Acinetobacter spp. not harboring acquired carbapenemase-encoding genes									
A. baumannii	1	OXA-69[a] (IS*Aba1* positive)[d]	32	64	1/1	−	−	0/1	This study
	1	OXA-70[a] (IS*Aba1* negative)	<0.5	2	0/1	ND	ND	0/1	13
	1	OXA-82[a] (IS*Aba1* positive)	8	16	1/1	−	−	0/1	13
	1	OXA-98[a] (IS*Aba1* negative)	1	2	0/1	ND	ND	0/1	13
	5	OXA-51-like[a] (IS*Aba1* negative)	<0.5–2	<0.5–2	0/5	ND	ND	0/5	This study
A. nosocomialis	4		<0.5	<0.5	0/4	ND	ND	0/4	This study
A. pittii	1		<0.5	<0.5	0/1	ND	ND	0/1	This study
A. ursingii	1		<0.5	<0.5	0/1	ND	ND	0/1	This study
Pseudomonas spp. harboring acquired carbapenemase-encoding genes									
P. aeruginosa	1	DIM-1	2	16	1/1	+	−	1/1	17
	23	GES-5	32–64	64–512	23/23	−[b]	+[b]	2/23	This study
	1	GES-6	32	64	1/1	−	+	0/1	This study
	1	IMP-1	64	512	1/1	+	−	1/1	10
	1	IMP-6	64	256	1/1	+	−	1/1	15
	1	IMP-7	64	128	1/1	+	−	1/1	11
	1	IMP-10	64	128	1/1	+	−	1/1	15
	1	IMP-15	64	32	1/1	+	−	0/1	16
	1	IMP-26	256	512	1/1	+	−	1/1	16
	2	IMP-34	16–512	32–1,024	2/2	+[b]	−[b]	2/2	15
	1	IMP-43	512	512	1/1	+	−	1/1	11
	1	IMP-44	512	512	1/1	+	−	1/1	11
	1	IMP-51	32	512	1/1	+	−	1/1	14
	1	NDM-1	128	128	1/1	+	−	1/1	This study
	2	VIM-1	256	256–512	2/2	+[b]	−[b]	2/2	This study
	3	VIM-2	16–256	8–128	3/3	+[b]	−[b]	2/3	This study
P. fulva	1	NDM-1	128	1,024	1/1	+	−	1/1	This study
P. mendocina	2	DIM-1	<0.5–1	8	2/2	+[b]	−[b]	2/2	This study
P. monteilii	1	NDM-1	512	>1,024	1/1	−	−	1/1	This study
P. putida	2	NDM-1	32–64	128–512	2/2	+[b]	−[b]	2/2	This study
	5	VIM-2	1–512	8–512	5/5	+[b]	−[b]	5/5	This study
Pseudomonas spp. not harboring acquired carbapenemase-encoding genes									
P. aeruginosa	12[e]		4–16	4–16	0/12	ND	ND	0/12	This study

[a]OXA-type β-lactamase encoding genes that are intrinsic genes in *Acinetobacter baumannii*.

[b]All isolates tested showed the same results.

[c]ND, not determined.

[d]IS*Aba1* positive/negative indicates the presence/absence of this gene.

[e]Of 12 *P. aeruginosa* isolates, 5 harbored a GES-1-encoding gene, 1 harbored GES-7, and 2 harbored GES-9, whereas 4 did not harbor any of these β-lactamase genes. GES-1, GES-7, and GES-9 encode proteins that do not have carbapenemase activity.

carbapenemase genes and the 15 not harboring these acquired genes were negative on the mCIM (Table 2).

Of the 65 *Pseudomonas* isolates tested, 53 (81.5%) harbored acquired genes encoding carbapenemases, including DIM-1, GES-5, GES-6, IMP-1, IMP-6, IMP-7, IMP-10, IMP-15, IMP-26, IMP-34, IMP-43, IMP-44, IMP-51, NDM-1, VIM-1, and VIM-2 (Table 2), and all 53 isolates were positive on the CIMTris. The remaining 12 isolates did not harbor these acquired genes and were negative on the CIMTris. Thus, after 2 h of incubation, the CIMTris showed 100% sensitivity and 100% specificity for detecting carbapenemase production in *Pseudomonas* species ($P < 0.01$) (Tables 1 and 2).

Testing of the mCIM in *Pseudomonas* isolates showed that 29 of 53 (54.7%) isolates harboring acquired carbapenemase genes were positive on the mCIM. Of the 24 mCIM-negative isolates, 21 harbored an acquired carbapenemase gene encoding GES-5, and one each harbored acquired genes encoding GES-6, IMP-15, and VIM-2 (Table 2). That is, 22 of the 24 isolates harboring acquired genes encoding GES-type β-lactamases were negative on the mCIM. Twelve of the 12 (100%) isolates not harboring acquired carbapenemase genes were negative on the mCIM (Table 2).

Collectively, the CIMTris showed 97.6% sensitivity, with 80 of 82 *Acinetobacter* and *Pseudomonas* isolates harboring acquired carbapenemase genes being positive on the CIMTris, and 92.6% specificity, with 25 of 27 isolates not harboring acquired carbapenemase genes being negative on the CIMTris. In contrast, the mCIM showed 45.1% sensitivity, with 37 of 82 isolates harboring acquired carbapenemase genes being positive, and 100% specificity, with 27 of 27 isolates not harboring acquired carbapenemase genes being negative. Most isolates of *A. baumannii* harboring OXA-type carbapenemase genes and *P. aeruginosa* harboring GES-type carbapenemase genes were negative on the mCIM, reducing the sensitivity of this method in detecting carbapenemase activities in *Acinetobacter* and *Pseudomonas* species.

Of 36 isolates of *Acinetobacter* and *Pseudomonas* species harboring metallo-β-lactamase encoding genes, 35 (97.2%) were positive on the CIMTris, and their carbapenemase activities were inhibited by sodium mercaptoacetic acid (SMA) (Table 2). The remaining isolate, a *Pseudomonas monteilii* harboring an NDM-1-encoding gene, was positive on the CIMTris, but its carbapenemase activity was not inhibited by SMA. This isolate probably produced an extremely large amount of NDM-1, as its MIC to MEM was higher than 1,024 μg/ml. All 24 *P. aeruginosa* isolates harboring GES-type carbapenemase genes were positive on the CIMTris, and their carbapenemase activities were inhibited by clavulanic acid (CVA). These results indicate that most of the activities detected by the CIMTris are carbapenemase activities.

The CIMTris differed in only one aspect from the CIM (4), in that Tris-HCl buffer was used instead of water during the MEM inactivation step. The CIMTris showed markedly higher sensitivity than the mCIM (97.6% versus 45.1%, respectively) in *Acinetobacter* and *Pseudomonas* species. The Tris-HCl buffer used in the CIMTris seemed to effectively extract carbapenemases without inactivating them. In contrast, the specificity of the CIMTris was lower than that of the mCIM (92.6% versus 100%, respectively), likely because the CIMTris detected overproduction of carbapenemase by intrinsic carbapenemase genes. In comparing the performance of the CIMTris and mCIM, we used the presence or absence of acquired carbapenemase genes as a standard definition but not the presence or absence of intrinsic carbapenemase genes. The carbapenemase activities of the two *A. baumannii* isolates harboring OXA-69 and OXA-82 genes were probably due to overexpression of intrinsic genes (Table 2), indicating that CIMTris was able to detect carbapenemases produced by intrinsic as well as acquired carbapenemase genes.

This study had several limitations. The isolates tested were not completely comprehensive and balanced, and all were from Asia. We were not able to test important carbapenem-producing isolates, including VIM-producing *Acinetobacter* and KPC-producing *Pseudomonas* species, because these isolates are currently very rare in Japan, Nepal, and Vietnam. Therefore, the CIMTris requires further testing with a more diverse group of Gram-negative bacteria, including *Enterobacteriaceae*. Our preliminary experiment showed that in *Enterobacteriaceae*, the CIMTris showed 84.2% sensitivity, with 16

of 19 isolates harboring carbapenemase-encoding genes being positive, and 100% specificity, with all 10 isolates lacking carbapenemase-encoding genes being negative (data not shown).

In conclusion, the CIMTris is a promising approach for detecting carbapenemase activity in *Acinetobacter* and *Pseudomonas* species and merits further testing.

MATERIALS AND METHODS

The selected isolates, consisting of Gram-negative bacteria producing a variety of carbapenemases, were readily available in the two participating laboratories (10–17). These isolates included 23 GES-5-producing isolates obtained throughout Japan and not epidemiologically related; GES-5 producers were included because they have spread and are causing serious problems in medical settings throughout Japan (unpublished data). Forty-four clinical isolates of *Acinetobacter* species and 65 isolates of *Pseudomonas* species were obtained from hospitalized patients in Japan (70 isolates), Nepal (36 isolates), and Vietnam (3 isolates). *Acinetobacter* species isolates were from the respiratory tracts of 20 patients, wounds of 11, urinary tracts of 8, gastrointestinal tracts of 2, and blood of 2; 1 isolate was from an unknown source. *Pseudomonas* species isolates were from the urinary tracts of 27 patients, respiratory tracts of 16, wounds of 12, and gastrointestinal tracts of 8; 2 isolates were from unknown sources. The carbapenem-susceptible reference strain was *Escherichia coli* ATCC 25922.

Bacterial species were determined based on genome sequences, as assessed by MiSeq (Illumina, San Diego, CA). The MICs of imipenem (IPM) and MEM were determined using a microdilution method, as recommended by the CLSI (18). The entire genome of each isolate was extracted using DNeasy blood and tissue kits (Qiagen, Tokyo, Japan) and sequenced through MiSeq. These sequences were compared with the sequences of drug resistance genes available at the National Center for Biotechnology Information (NCBI) website (https://www.ncbi.nlm.nih.gov/pathogens/beta-lactamase-data-resources/), and the drug resistance genes were determined using CLC genomics workbench version 9.0.1.

The mCIM for the detection of suspected carbapenemase production, originally developed for *Enterobacteriaceae*, was used in this study for *Acinetobacter* and *Pseudomonas* species. Briefly, each isolate was cultured overnight on a sheep blood agar plate, and a 10-μl loopful of bacteria was emulsified in 2 ml TSB and vortexed for 10 to 15 s. A 10-μg MEM disk (Becton, Dickinson and Company, Tokyo, Japan) was added to each tube, and the suspension was incubated for 4 h at 35°C. The MEM disks were subsequently removed from each suspension with 10-μl loops and then placed on Mueller-Hinton II agar plates with freshly inoculated MEM-susceptible *E. coli* ATCC 25922 on the surfaces.

In the CIMTris method, a 10-μl loopful of bacteria from each isolate was emulsified in 400 μl of 0.5 M Tris-HCl buffer (pH 7.6) (Tris-aminomethane; Sigma-Aldrich Co., Tokyo, Japan) and vortexed for 10 to 15 s. A 10-μg MEM disk was added to each of three tubes. Tube 1 contained only a MEM disk, tube 2 contained a MEM disk and a 3-mg SMA disk (Eiken Chemical Co., Ltd., Tokyo, Japan), and tube 3 contained a MEM disk and 1 mg of CVA made with 10 μl of a 100-mg/ml solution of CVA (Sigma-Aldrich). The tubes were incubated at 35°C for 2 h, unless otherwise indicated, and the MEM disks were removed and placed onto Mueller-Hinton II agar plates inoculated with MEM-susceptible *E. coli* ATCC 25922. The plates were incubated for 18 h at 35°C, and the diameter (mm) of each inhibition zone was measured. Carbapenemase activity was regarded as positive if the inhibition zone measured 6 to 15 mm or measured 16 to 18 mm with satellite colonies. Carbapenemase activity was regarded as negative if the inhibition zone measured $\geq$19 mm and as indeterminate if the inhibition zone measured 16 to 18 mm without satellite colonies. SMA and CVA inhibition of the CIMTris-positive isolates was assessed by incubating each isolate on plates containing MEM disks and SMA or CVA. The results were compared with those of a reference plate containing a MEM disk with no additional compound. Differences of $\geq$5 mm and $<$5 mm in the diameters of the inhibition zone were regarded as positive and negative, respectively. Chi-square tests were performed for statistical analysis.

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The authors declare that there are no conflicts of interest.

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