Performance of the Carbapenemase Inhibition Method (CIM) to Accurately Identify Carbapenemase-Producing Organisms (CPO) and the Impact of Decreasing Carbapenem Inactivation Times (CIT)

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ABSTRACT

Background: Reliable cost-effective phenotypic methods for detecting carbapenemase activity in low-resource settings are lacking. The CIM is recommended as an accurate low-cost way to detect CPO within 24h that utilizes supplies available in most laboratories. This study assessed CIM performance and impact of decreasing CIT to make it more amenable to clinical laboratory workflow.

Methods: For CIM, >2 MacFarland STD equivalent bacterial suspensions are prepared in 400uL sdH2O, then 10µg meropenem discs are added to the micro-tubes. The suspended discs are incubated at 35°C. After 2h-CIT, discs are removed and placed onto Mueller-Hinton agar pre-seeded with reporter organism *Escherichia coli* ATCC 29566 (0.5 MacFarland). After overnight at 37°C, inhibition zones of <21mm are considered evidence of carbapenemase activity (meropenem inactivation), while zones >20mm are considered negative (Tijet JAC 2015). In this study, varied inactivation times were compared in parallel using the following blinded species-diverse highly-characterized clinical isolates, respectively: 2h-CIT: n=258 [221 CPO of classes: A (108: 99 KPC, 4 SME, 2 GES5, 2 NMCA, 1 IMI1), B (80: 73 NDM, 6 VIM, 1 IMP7), D (26: 15 OXA48, 6 OXA181, 4 OXA232, 1 OXA244), B+D (7: 4 NDM+OXA181, 3 NDM+OXA232); non-CPO (37)]; 1h-CIT: n=211 [174 CPO: A (69: 62 KPC, 2 SME, 2 GES, 2 IMI, 1 NMC), B (72: 65 NDM, 6 VIM, 1 IMP7), D (26), B+D (7); non-CPO (37)]; and 30min-CIT: n=219 [182 CPO: A (69), B (80: 73 NDM, 6 VIM, 1 IMP7), D (26), B+D (7); non-CPO (37)]. Zones were measured independently by 5 readers. Sensitivities/specificities per CIT were determined from consensus measurements; 95% confidence intervals were calculated in www.graphpad.com.

Results: CPO detected/CPO tested (% sensitivity) by CIM overall [and by class] using the above criteria were as follows: 2h-CIT – 204/221 (92.3%) [A: 107/108 (99.1%); B: 77/80 (96.3%); D 13/26 (50%); B+D: 7/7 (100%)]; 1h-CIT – 149/174 (85.6%) [A: 67/69 (97.1%); B: 67/72 (95.8%); D: 7/26 (26.9%), B+D: 6/7 (85.7%)]; and 30min-CIT – 155/182 (85.2%) [A: 65/69 (94.2%); B: 77/80 (96.3%); D: 7/26 (85.7%), B+D: 6/7 (85.7%)]. If pin-point colonies inside zones >20mm were interpreted as evidence of carbapenemase activity, overall sensitivities would increase slightly to 95.5% for 2h-CIT, 89.1% for 1h-CIT and 83.1% for 30min-CIT, respectively. Specificities for all CIT were 97.3% as the only CIM-positive non-CPO was a meropenem-intrinsically resistant *Aeromonas hydrophila* (chromosomal *cphA*).

Conclusions: These data show CIM with 2h-CIT may offer a relatively accurate means to separate CPO from non-CPO in 24h if no other tests are available. However, it should be implemented with caution recognizing that 7.7% CPO (mostly OXA48-type) were missed in this evaluation. If pinpoint colonies inside zones are taken as clues to indicate CPO, the proportion missed may be reduced to 4.5%.

INTRODUCTION

Rapid and accurate detection of pan-resistant carbapenemase-producing organisms (CPO) is crucial for risk-reduction in patient management and to prevent outbreaks. In the clinical microbiology laboratory setting, in the absence of direct-from-specimen PCR tests, CPO are typically first detected after routine antimicrobial susceptibilities (in clinical isolates) or routine disc screen tests (in surveillance isolates) indicate resistance to ≥1 carbapenems may be present.

Reliable cost-effective phenotypic methods for detecting carbapenemase activity in low-resource settings are lacking. Molecular tests are costly and do not target all CPO. Currently available pH indicator-based phenotypic tests designed to detect imipenem hydrolysis appear not to be uniformly sensitive for class D OXA48-like CPO in all laboratory settings.

The carbapenem inactivation method (CIM) has been recommended as an accurate low-cost alternative to detect CPO in <24h that utilizes supplies available in most laboratories, requiring only an incubator, micro-tubes, water, meropenem discs, Mueller-Hinton plates and ubiquitous laboratory tools such as loops, swabs, toothpicks, etc. It is based on the simple principle that if a suspect CPO is incubated with a carbapenem disc, it will inactivate the carbapenem in the disc, if no CPO is present, its activity will remain intact. After the "carbapenem inactivation time" (CIT) has lapsed, the disc is placed onto a plate with a carbapenem-susceptible reporter organism, and if after overnight incubation there is no zone, a CPO is present, and if there is a >20mm zone, it was not a CPO.

This retrospective study assessed CIM accuracy and determined the impact of shorter CIT (from 2h to 1h or 30min) in an attempt to make CIM more amenable to clinical laboratory workflow.

Figure 1. The "CIM" or Carbapenem Inactivation Method - An extremely cheap screening method for detecting CPO

- **A meropenem disc is added to a heavy suspension prepared from a suspect CPO after which tubes are placed at 37°C for a 2h Carbapenem Inactivation Time or 2h-CIT**
- **A Mueller-Hinton agar plate is swabbed as for disc diffusion with the reporter strain *E. coli* ATCC 29566**
- **After time for enzyme inactivation of the meropenem, the disc is removed from the tube and placed on a designated spot on the surface of Mueller-Hinton agar**
- **After overnight incubation, inhibition zones are measured**
- **Zones of <21mm are considered to indicate CPO**
- **Zones of >20mm indicate no carbapenem inactivation has taken place and thus the organism is a non-CPO (8 of 8 tests)**
- **Negative Control (bottom left); Positive control (bottom right)**

METHODS

Table 1 (below) describes 258 species-diverse clinical isolates selected for study. Isolates were characterized by conventional PCR for genes encoding *ampC*/ESBL/CPO, with sequencing as needed. Isolates were identified to species-level by MALDI-TOF (bioMérieux's VITEK MS Plus). Phenotypic CPO expression had been measured by meropenem disc diffusion using the screen breakpoint of ≤25mm. Reactions to boronic and dipicolinic acid inhibitors and susceptibility to temocillin were completed using ROSCO's KPC+MBL+OXA48 Confirm kit. Only 1 isolate/patient was included, unless ≥1 genotype or ≥1 genus/species was confirmed as CPO from a patient. Isolate identities were blinded to prevent bias.

For CIM, >2 MacFarland STD equivalent bacterial suspensions were prepared in micro-tubes containing 400uL sdH2O, after which a 10µg meropenem disc (Oxoid) was added to each tube. Tubes with discs were incubated at 35°C for predetermined carbapenem inactivation times (CIT). After 2h-CIT, discs were removed and placed onto Oxoid Mueller-Hinton agar that had been evenly swabbed as for disc diffusion with reporter organism *Escherichia coli* ATCC 29566. After overnight at 37°C, inhibition zones <21mm were considered as evidence of carbapenemase activity, and ergo, meropenem inactivation; zones >20mm were considered to indicate no carbapenemase activity and were called CPO-negative. The use of the latter breakpoint was as per the modification of the original method by Tijet JAC 2015.

This study also evaluated CIM outcomes in a subset of study isolates after reducing CIT to 1h-CIT and 30min-CIT. Results from these were compared to the 2h-CIT tested in parallel (isolate subset, see Table 1). Zones were measured independently by 5 readers blinded to each others results. Sensitivities/specificities per CIT were determined from consensus measurements; 95% confidence intervals were calculated in www.graphpad.com.

Table 1. Characteristics of 258 isolates tested by the Carbapenem Inactivation Method (CIM 2h-CIT) to detect Carbapenemase-Producing Organisms (CPO) showing numbers used to assess reduced Carbapenem Inactivation Times (CIT)

Ambler class (No. per CIT)	CPO Genotypes (No. per CIT)	Species identification	CIM 2h-CIT (n=258)	CIM 1h-CIT (n=211)	CIM 30min-CIT (n=219)
A CPO 2h: 108 1h: 69 30 min: 69	*blaKPC* (2h: 99; 1h+30min: 69)	*Citrobacter freundii*	2	2	2
		Enterobacter aerogenes	3	1	1
		Enterobacter cloacae	35	14	14
		Escherichia coli	12	6	6
		Klebsiella oxytoca	1	0	0
		Klebsiella pneumonia	46	39	39
	blaGES5 (2)	*Klebsiella oxytoca*	2	2	2
	blaIMI1 (1)	*Enterobacter cloacae*	1	1	1
	blaNMCA (2)	*Enterobacter cloacae*	2	2	2
	blaSME (4)	*Serratia marcescens*	4	2	2
B CPO 2h: 80 1h: 72 30 min: 80	*blaIMP7 (1)*	*Pseudomonas aeruginosa*	1	1	1
	blaNDM (73)	*Acinetobacter baumannii*	1	1	1
		Citrobacter freundii	1	1	1
		Enterobacter cloacae	3	2	3
		Escherichia coli	30	25	30
		Klebsiella pneumoniae	33	31	33
		Morganella morganii	4	4	4
		Proteus mirabilis	1	1	1
	blaVIM (6)	*Citrobacter freundii*	1	1	1
		Enterobacter cloacae	4	4	4
		Pseudomonas putida	1	1	1
B + D CPO 2h: 7 1h: 7 30 min: 7	*blaNDM+blaOXA181 (1)*	*Escherichia coli*	1	1	1
	blaNDM+blaOXA181 (3)	*Klebsiella pneumoniae*	3	3	3
	blaNDM+blaOXA232 (1)	*Escherichia coli*	1	1	1
	blaNDM+blaOXA232 (2)	*Klebsiella pneumoniae*	2	1	2
D CPO 2h: 26 1h: 26 30 min: 26	*blaOXA48 (15)*	*Escherichia coli*	8	8	8
		Klebsiella pneumoniae	7	7	7
	blaOXA181 (6)	*Escherichia coli*	2	2	2
		Klebsiella pneumoniae	4	4	4
	blaOXA232 (4)	*Escherichia coli*	1	1	1
		Klebsiella pneumoniae	3	3	3
	blaOXA244 (1)	*Escherichia coli*	1	1	1
Non-CPO 2h: 37 1h: 37 30 min: 37	*ompC-ompF* mutants (2)	*Enterobacter cloacae*	1	37	37
		Escherichia coli	2	2	2
	ompK35-ompK36 (6)	*Klebsiella pneumoniae*	6	6	6
	Weak OXY promoter(1)	*Klebsiella oxytoca*	1	1	1
	Other mechanisms (26)	*Enterobacteriaceae*	26	26	26
	blaOXA252 (1)	*Shewanella putrifaciens*	1	1	1
	cphA	*Aeromonas hydrophila*	1	1	1

RESULTS

CIM performance for detecting CPO by varied CIT (meropenem inactivation times), is presented in Table 2. Even after the most favorable CIT (2h-CIT), CIM missed 50% OXA48 CPO. Of note, micro-colonies (MC, see Figure 2 below) were present within the >20mm zones in 16 false-negatives (13 OXA48, 2 NDM, 1 GES5) but not in zones of true-negatives. If micro-colonies were interpreted as evidence of carbapenemase activity, CIM sensitivities for each CIT increased slightly with no drop in specificity (Table 2), elevating CIM's 2h-CIT sensitivity to 95.5%.

CIM specificities for all CIT were 97.3%; the only CIM-positive non-CPO was intrinsically meropenem-resistant due to chromosomal *cphA in A. hydrophila*, which could be excluded.

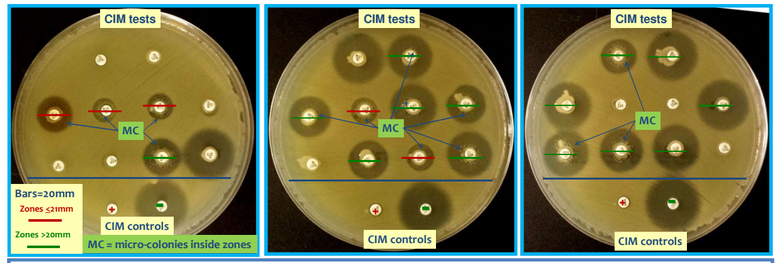

Figure 2. Examples of 3 test batches showing wide variations in CIM results obtained after 2h-CIT (Each plate shows 10 tests (top 3 rows), CPO-POS (bottom left) and CPO-NEG (bottom right) controls.
1) Meropenem in discs with growth right to the discs' edge has clearly been inactivated by CPO; 2) Meropenem in discs with large inhibition zones and no micro-colonies (MC, see above) in zones are clearly non-CPO as the meropenem is active; 3) Zones >6mm but <21mm are CPO as per Tijet *et al* JAC 2105; 4) Zones >20mm should be non-CPO but zone ranges overlapped 50% of OXA48 CPO; 6) If >20mm zones with micro-colonies within the zone were considered CPO-POS, CIM sensitivity for all CIT increased (Table 2).

Table 2. Performance of the overnight Carbapenem Inactivation Method (CIM) for phenotypic detection of Carbapenemase-Producing Organisms (CPO) showing the impact of reduced Carbapenem Inactivation Times (CIT)

Ambler class; Meropenem zones of <21mm interpreted as CIM-POS (Tijet *et al* JAC 2015)	No. CIM positive/No. CPO tested (Sensitivity; 95% CI)		
	2h	1h	30 min
Class A	107/108 (99.1%; 94.4->99.9)	67/69 (97.1%; 89.4-99.8)	65/69 (94.2%; 85.6-98.2)
Class B	77/80 (96.3%; 89.1-99.2)	69/72 (95.8%; 88-99.1)	77/80 (96.3%; 89.1-99.2)
Class B+D	7/7 (100%; 59.6-100)	6/7 (85.7%; 46.7-99.5)	6/7 (85.7%; 46.7-99.5)
Class D	13/26 (50%; 32.1-67.9)	7/26 (26.9%; 13.5-46.3)	7/26 (26.9%; 13.5-46.3)
Any CPO	204/221 (92.3%; 88-95.2)	149/174 (85.6%; 79.6-90.1)	155/182 (85.2%; 79.2-89.7)
Any CPO using <21mm and/or micro-colonies in zone as CIM-POS	211/221 (95.5%; 91.8-97.6)	155/174 (89.1%; 83.5-93)	157/182 (86.3%; 80.5-90.6)
CIM by non-CPO	**No. CIM negative/No. non-CPO tested (Specificity; 95% CI)**		
	2h	1h	30 min
Non-CPO using >20mm zone breakpoint as CIM-negative	36/37 (97.3%; 85->99.9)	36/37 (97.3%; 85->99.9)	36/37 (97.3%; 85->99.9)
Total isolates tested	258	211	219

CONCLUSIONS & DISCUSSION

Use of the Carbapenem Inactivation Method (CIM) as an overnight CPO detection test
These data show CIM with a 2h-CIT may offer a relatively accurate (92.3-95.5% sensitive; 97.3-100% specific) means to separate CPO from non-CPO within 24h if no other tests are available.

However, it must be implemented with caution recognizing 7.7% CPO, representing 50% OXA48 *E. coli/K. pneumoniae,* were missed as their CIM zone ranges overlapped those of the non-CPO.

If pinpoint or micro-colonies inside zones of the false-negatives were taken as clues to indicate the presence of CPO, the proportion missed was able to be reduced to 4.5% very major errors.

From a practical perspective, CIM is extremely cheap and all materials are already available in a clinical microbiology laboratory. But it takes overnight to produce a result, is time consuming if large numbers need testing, and has a high potential for contaminating the laboratory environment, as the possibility of incurring micro-splashes is inherent in the awkward procedure of transferring discs (dripping with CPO) from tubes to plates .

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