ORIGINAL ARTICLE

Evaluation of the MAST indirect carbapenemase test and comparison with a modified carbapenem inactivation method for the detection of carbapenemase enzymes in Gram-negative bacteria

Julie Creighton and Clare Tibbs

Canterbury Health Laboratories, Christchurch

ABSTRACT

Introduction: Carbapenemase-producing Enterobacteriaceae (CPE) are no longer rarely encountered in New Zealand and worrying aspects of increasing prevalence include two hospital-associated outbreaks, infections in patients with no travel history, and a report of household transmission. It is critically important for both patient management and infection control purposes that carbapenemase-producing organisms (CPO) are rapidly and reliably detected and identified in clinical laboratories. However, this can be problematic due to the diversity of carbapenemase enzymes, the different genera they can reside in, and the difficulties of discriminating CPO from carbapenem-resistant-non-carbapenemase producers. Thus, the aim of this study was to evaluate and compare the recently released MAST indirect carbapenemase test (ICT) and a modified carbapenem inactivation method (mCIM) test, in order to determine their ability to detect carbapenemase production, and to reliably 'rule out' a non-carbapenemase producer.

Methods: A total of 100 non-duplicate isolates, consisting of 80 *Enterobacteriaceae*, 12 *Pseudomonas aeruginosa*, and 8 *Acinetobacter baumannii* were included in the study. The panel included 63 carbapenemase-producing strains and 37 non-carbapenemase producing multi-drug resistant strains. Each isolate was tested by the MAST ICT and a mCIM assay, with sensitivities and specificities determined.

Results: Both the MAST ICT and mCIM tests performed with 100% sensitivity, detecting all carbapenemase-producing strains. For the non-carbapenemase-producing strains, 3 false positive results were observed with the mCIM assay, giving a specificity of 91.9% and PPV of 95.5%. The MAST ICT was more subjective to interpret, with the assay initially producing 11 equivocal or false positive results (specificity 70.3%). Upon repeat testing, 4 strains were negative; giving a final specificity of 81.1% and PPV of 90.0%.

Conclusion: Our evaluation of the MAST ICT and a modified CIM assay found high sensitivity and specificity for both assays across a range of Gram negative bacteria. To reliably distinguish CPO from carbapenem-resistant-non-CPO, we would recommend that the mCIM is used in tandem with the MAST ICT, or with another high performing assay as such as Carba NP, rather than as stand-alone tests. Advantages of these tests include ease of use, simple to interpret, inexpensive and an ability to detect carbapenemase production, regardless of class type, in *Enterobacteriaceae* as well as Pseudomonas and Acinetobacter. An all-in-one test format rather than having to use multiple inhibitor-based tests, is favourable for laboratories with limited resources and experience.

Key words: indirect carbapenemase test, carbapenem inactivation method, carbapenemase, Gram- negative bacilli.

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INTRODUCTION

Carbapenemase-producing Enterobacteriaceae (CPE) are no longer rarely encountered in New Zealand (NZ) (1). Although most findings of CPE have been from patients who have recent overseas travel history, worrying aspects include infections in patients with no travel history, two hospital-associated outbreaks, two probable cross-transmissions, and a report of a household transmission (1-3). A worldwide increasing prevalence of these often extremely multi-drug resistant organisms is an immediate threat to global health systems (4). Furthermore, there is a very real concern that the spread of CPE might mimic that seen with extended-spectrum beta-lactamase (ESBL) spread, especially the globally successful bla_{CTX-M} epidemic (5). It is critically important for both patient management and infection control purposes that carbapenemase-producing organisms (CPO) are promptly and reliably detected and identified in clinical laboratories. However, this can be a challenge for many laboratories as the carbapenemase enzymes are a diverse group; being found in a variety of Gram-negative bacteria, they can sometimes express only low levels of carbapenem resistance and test susceptible to extended spectrum cephalosporins (e.g. OXA-48-like group), or conversely be transmitted on a plasmid which also harbours other resistant mechanisms such as ESBLs (6).

Complicating detection are organisms which are resistant to carbapenems due to other resistant mechanisms such as outer membrane porin mutations or up-regulated efflux pumps, often in combination with AmpC hyper production or ESBLs. Organisms that can sometimes possess these mechanisms, such as *Enterobacter cloacae* complex, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* complex, are more frequently encountered in most New Zealand clinical laboratories, making for problematic differentiation.

There are a range of phenotypic, enzymatic and molecular methods available for clinical laboratories to choose from, many of which are commercially available (7). Unfortunately, many of the phenotypic based tests are only recommended for *Enterobacteriaceae* and do not include Pseudomonas or Acinetobacter. For laboratories with limited expertise and resources it is important to be able to utilise methods which are accurate, simple, inexpensive, and can reliably discriminate non-carbapenemase resistance from the more concerning carbapenemase producers.

One method which is suitable for a variety of Gram negative bacteria is the recently released Indirect Carbapenemase Test (ICT) from MAST (Liverpool, UK). Using an indirect method to detect a β-lactamase is not new, being described by Moland *et al.* (8) as a method to detect Class A carbapenemases. The indirect method is a simple disc diffusion test whereby a reporter organism, such as *E.coli* ATCC 25922, is the lawn culture and the test organism is spread onto a bacterial permeabiliser-containing disc (e.g. EDTA), then the inverted disc is placed adjacent to an indicator disc (e.g. imipenem). The plate is incubated and any resulting distortion of the indicator disc zone is considered positive for carbapenemase production (8). Another simple phenotypic test, also suitable for Pseudomonas and Acinetobacter, is the carbapenem inactivation method (CIM), which was first described by van der Zwaluw *et al.* (9) in 2015. This method was subsequently modified by Tamma and colleagues (10), essentially replacing water with tryptic soy broth to suspend organisms, and incubating for four hours instead of two hours.

Thus, the aim of this study was to evaluate and compare the MAST ICT and a modified CIM (mCIM) test, in order to determine their ability to detect carbapenemase production, and to reliably 'rule out' a non-carbapenemase producer; against a panel of isolates, including 63 CPO and 37 non-CPO, constituting a diverse range of Gram-negative species and resistant markers.

MATERIALS AND METHODS

Bacterial isolates

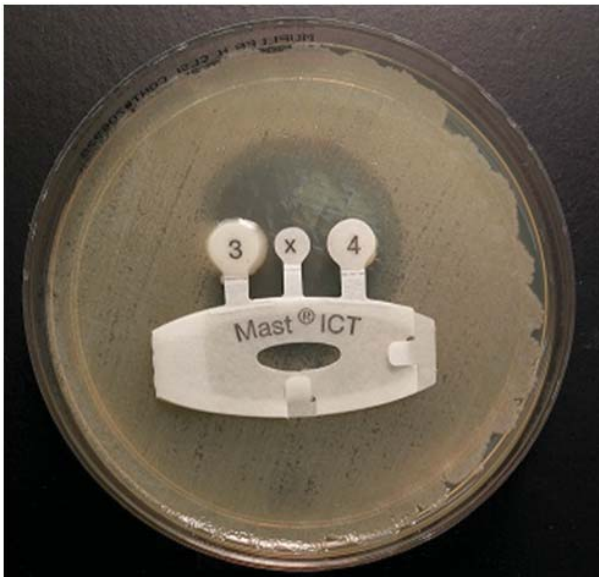
A total of 100 non-duplicate isolates, consisting of 80 *Enterobacteriaceae*, 12 *Pseudomonas aeruginosa*, and eight *Acinetobacter baumannii* were included in the study (Table 1). The study isolates were collected between 2008 and 2017, with the carbapenemase-producing isolates collected consecutively. The panel included 63 carbapenemase-producing strains: NDM (*n* = 18), OXA-48-like (*n* = 19), KPC (*n* = 6), IMP (*n* = 5), VIM (*n* = 4), OXA-23 (*n* = 3), and one each of OXA-24, OXA-25, OXA-27, OXA-58, GES-5, IMI-1, VIM/IMP, OXA-48/NDM; and 37 non-carbapenemase producing isolates that produced other resistant mechanisms such as ESBL, plasmid-mediated AmpC, and derepressed AmpC. Thirteen of the non-carbapenemase strains were non-susceptible to one or more carbapenem. All bacterial isolates used in the study were either isolates obtained from Canterbury Health Laboratories or reference strains provided by the Institute of Environmental Science and Research Limited (ESR). *Klebsiella pneumoniae* BAA1705 (KPC positive) and *K. pneumoniae* BAA1706 (KPC negative/ESBL positive) were used as positive and negative controls respectively. Isolates were stored at -80°C and subbed twice onto Columbia base blood agar before tests were performed.

MAST ICT

The MAST ICT consists of a paper device which has three disc-like tips; one for a negative control, one for the test organism, and a middle disc containing a carbapenem antibiotic. The organism tips contain ESBL and AmpC inhibitor products as well as a permeabilising agent. The test was performed according to the manufacturer's instructions. Briefly, 3-5µl of test organism was spread onto tip 2 and *E.coli* ATCC 25922 was similarly spread onto tip 1. The device was then inverted and placed firmly onto a Mueller Hinton Agar plate which had previously been seeded with a lawn of the reporter organism, *E.coli* ATCC 25922. The plate was then incubated at 35°C aerobically for 16 to 20 hours. For *Pseudomonas* spp., the lawn organism was *K. pneumoniae* ATCC 700603, and the plate was incubated anaerobically. A positive result was indicated by a distortion of the carbapenem zone approximate to the test organism tip (Figure 1). The negative control should show no zone distortion.

mCIM: The mCIM test was performed with a slight modification to previously published methods. Briefly, approximately 5µl of test organism was evenly suspended in 400µl of tryptic soy broth in an Eppendorf tube. A 10µg meropenem disc was added and the tube was vortexed for a few seconds before incubating for 4 hours ± 15 mins. The meropenem disc was carefully removed and placed onto a Mueller Hinton Agar plate which had previously been seeded with a lawn of the reporter organism, *E.coli* ATCC 25922. The plate was then incubated at 35°C aerobically for 16 to 20 hours. A zone of <20mm indicated a positive result. Each test was read by three scientists, at least two of whom were blinded to the organism's resistance mechanisms. All initial equivocal interpretations and any discrepant results were repeated.

(a)



(b)

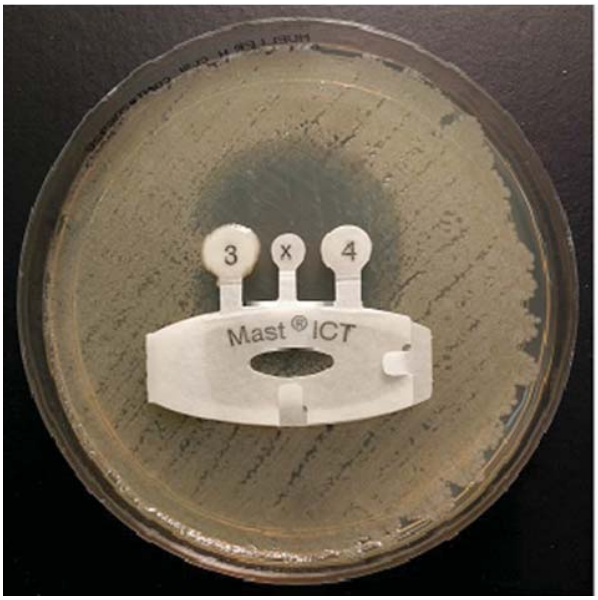


Figure 1.
(a) OXA-232 producing *K.pneumoniae* (weak positive)
(b) pAmpC producing *K. pneumoniae* (false positive)

Table 1. MAST ICT and mCIM results for carbapenemase and non carbapenemase-producing isolates

Resistance mechanism(s)	Species	n	Number of Positive Test Results	
			MAST ICT	mCIM
Carbapenemase				
NDM	*K.pneumoniae*	6	**6**	**6**
	K. oxytoca	2	**2**	**2**
	E.coli	4	**4**	**4**
	C. freundii	2	**2**	**2**
	P. mirabilis	1	**1**	**1**
	P. stuartii	1	**1**	**1**
	M. morganii	1	**1**	**1**
	P. aeruginosa	1	**1**	**1**
NDM/OXA-48-like	*K.pneumoniae*	1 1	**1**	**1**
OXA-48-like	*K.pneumoniae*	1	**11**[a]	**11**
	E.coli	8	**8**[b]	**8**
KPC	*K.pneumoniae*	6	**6**	**6**
IMP	*E.coli*	2	**2**	**2**
	K.pneumoniae	1	**1**	**1**
	K. oxytoca	1	**1**	**1**
	P. aeruginosa	1	**1**	**1**
VIM	*K. pneumoniae*	1	**1**	**1**
	P. aeruginosa	3	**3**	**3**
VIM/IMP	*P. aeruginosa*	1	**1**	**1**
GES-5	*P. aeruginosa*	1	**1**	**1**
IMI-1	*E. cloacae*	1	**1**	**1**
OXA-23	*A. baumannii*	3	**3**	**3**[c]
OXA-24	*A. baumannii*	1	**1**	**1**
OXA-25	*A. baumannii*	1	**1**	**1**
OXA-27	*A. baumannii*	1	**1**	**1**
OXA-58	*A. baumannii*	1	**1**	**1**[c]
Total (% sensitivity)		**63**	**63 (100%)**	**63 (100%)**
Non-carbapenemase				
ESBL	*E.coli*	4	**0**	**0**
	P. mirabilis	2	**0**	**0**
	P. vulgaris	1	**0**	**0**
	K. pneumoniae	1	**0**	**0**
	K. oxytoca	1	**0**	**0**
	C. koseri	1	**0**	**0**
ESBL/pAmpC	*E.coli*	2	**0**	**0**
	K.pneumoniae	1	**0**[d]	**0**
ESBL/AmpC	*E.coli*	2	**0**[d]	**0**
	C. freundii	1	**0**	**0**
	E. cloacae	1	**1**	**0**
	P. aeruginosa	1	**0**	**0**
pAmpC	*E. coli*	3	**0**	**0**
	P. mirabilis	3	**1**	**0**
	K. pneumoniae	1	**0**[d]	**0**
	K. oxytoca	1	**1**	**0**
	C. koseri	1	**0**	**0**
AmpC derepressed	*E. cloacae*	3	**1**	**1**
	E. aerogenes	1	**0**	**0**
	E.coli	1	**0**	**0**
AmpC/porin/efflux	*P. aeruginosa*	4	**2**[d]	**2**
	A. baumannii	1	**1**	**0**
Total (% specificity)		**37**	**7/37 (81.1%)**	**3/37 (91.9%)**

N=Number of strains tested; [a] Two isolates weak positive; [b] One isolate weak positive; [c] Zone sizes 19mm; [d] One isolate initially tested equivocal, repeat tested negative

RESULTS

Results of the trial are displayed in Table 1. Both the MAST ICT and mCIM tests performed with excellent sensitivity, detecting 63/63 (100%) of the carbapenemase-producing strains, giving a negative predictive value of 100%. Regarding the MAST ICT, three of the OXA-48-like producers (one *K. pneumoniae* carrying OXA-162, one *K. pneumoniae* carrying OXA-232 and one *E.coli* carrying OXA-181) were determined to have a weak positive result by at least one of the readers. Repeat testing produced a clear positive result for the *K. pneumoniae* carrying OXA-162, but the other two remained weak positive/equivocal. Carbapenem MIC values did not have any correlation to strength of zone distortion (data not shown). In comparison, reading and interpretation of the mCIM was less subjective, with the zone diameter reading being more clear-cut, yielding no equivocal results.

For the non-carbapenemase-producing strains, three false positive results were observed with the mCIM assay, including two strains of *P. aeruginosa*, which had high-level resistance to imipenem and meropenem, and a hyper-AmpC producing *E. cloacae*, which was non-susceptible to ertapenem. Repeat testing of these three strains reproduced identical results with no discernible zone size, giving final specificity and positive predictive value (PPV) of 91.9% and 95.5% respectively. For interest (results not shown), we also tested these three strains with Tamma's method (10) using 1 µl of organism suspended in 2 ml of TSB. The *E. cloacae* and one of the *P. aeruginosa* yielded zones of 19mm (negative by Tamma's criteria) and the other *P. aeruginosa* yielded a zone of 17mm (indeterminate by Tamma's criteria). However, it is unknown how this lower inoculum version may have affected the overall sensitivity of the assay for all of the test strains.

Compared with the mCIM, the initial specificity of the MAST ICT was lower at 70.3%, with the assay producing 11 equivocal or false positive results, including the same three isolates that were also mCIM false positive. Upon repeat testing, four strains which were initially interpreted as equivocal by some readers were negative on repeat testing, giving a final specificity of 81.1% and PPV of 90.0%. Of note is that the majority of strains that produced a false positive result in the MAST ICT assay were hyper-AmpC producing.

DISCUSSION

Clinical isolates possessing a carbapenemase is becoming more common in NZ. Worrying aspects of this increasing prevalence include hospital, healthcare and household transmission events (1). Therefore it is imperative that all NZ hospital and community laboratories should be adept at detecting CPO and have the ability to rapidly discriminate carbapenemase producers from non-carbapenemase producers. Indeed, there has been a recent call for the implementation of a nationally coordinated response plan, akin to the preparation that would be required for an Ebola outbreak (11). Included in the recommendations by Blakiston and colleagues is the development of minimum patient screening criteria and laboratory testing standards for CPE.

Due to the diverse nature of carbapenemase genes and the many Gram-negative species that can harbour them, there is no single 'gold standard' phenotypic test available, making for complicated laboratory identification and confirmation. Many phenotypic tests have sensitivity issues, especially with the detection of OXA-48-like enzymes, or tests have been designed for a geographical area with a predominating gene-type e.g. KPC producers in the USA, rather than for a diverse range of CPO or Gram-negative organisms (12).

Van der Zwaluw's (9) initial presentation and evaluation of the CIM test included a large panel of carbapenemase-producing *Enterobacteriaceae* as well as *P. aeruginosa* and *A. baumannii*. Their original method consisted of a full 10 µl loop of organisms suspended in 400 µl of water, the addition of a 10 µg meropenem disc and incubation for a minimum of two hours at 35°C. A positive result was classified as allowing uninhibited growth, whereas a negative result was classified as a clear inhibition zone. The authors reported sensitivities of 100% for *Enterobacteriaceae* and *P. aeruginosa*, but two OXA-23-producing *A. baumannii* were not detected.

Similarly Tijet *et al.* (13) evaluated the CIM and compared to Carba NP, against 182 *Enterobacteriaceae*; 100 of which were carbapenemase producers. They used a zone size of ≥20 mm to indicate a negative result. The authors found excellent sensitivity and specificity (99% and 100% respectively) with the CIM assay, which was superior to the performance of the Carba NP, the latter failing to detect 11/100 CPE (mainly OXA-48-like).

A modified version of the CIM (mCIM) was compared to 10 other phenotypic assays by Tamma *et al.* (10), including various modifications of the CarbaNP, Blue Carba and CIM assays. The mCIM substituted 400 µl of water for 2 ml of tryptic soy broth, adding just 1 µl of organism instead of 10 µl and extending the incubation period from 2 h to 4 h ± 15 min. In addition they incorporated zone diameter interpretations of 6 to 15 mm = positive, 16 to 18 mm = indeterminate and negative if ≥19 mm. This study determined that excellent sensitivity and specificity was achieved by the mCIM as well as Rapidec Carba NP, modified Carba NP and the manual Blue Carba. Extending the work by Tamma's group was a more comprehensive study by Pierce *et al.* (14), which involved a multi-laboratory evaluation on a variety of *Enterobacteriaceae*, as part of a CLSI working group. Overall, a high level of sensitivity, specificity and reproducibility was found by this group for the mCIM. In addition, laboratory staff found the mCIM simple to perform, with less subjective results compared to their experiences with Carba NP or MHT (14). This mCIM assay has subsequently been added to the 27th edition of the CLSI M100 supplement (15). A disadvantage of both studies is the exclusion of non-fermentative Gram-negative bacilli.

There are scant published studies evaluating the use of the ICT for carbapenemase detection. Mathers *et al.* (16) evaluated an in-house ICT method against 127 *Enterobacteriaceae* with ertapenem MICs ≥1 mg/L, 56 of which were bla_{KPC} positive. They found the overall sensitivity to be 90.0%, but ranged as low as 84.3% for *Klebsiella* spp. The authors concluded that this ICT method may not be adequate for KPC-producing *K. pneumoniae*. It is important to note that Mather's ICT version consisted of just EDTA as the bacterial cell lysing agent, whereas the cell permeabilising agent is not disclosed in the MAST ICT documentation.

Our evaluation of the MAST ICT and the mCIM would indicate that both methods have a high capacity to detect CPO, with a sensitivity and negative predictive value of (100%). An area of concern with the MAST ICT would be the number of equivocal reactions (false positives) and the weak positive results for some of the OXA-48-like group; which are one of the most common enzymes currently found in NZ (1,17). The weak positive results were indistinguishable from false positive/ equivocal results (direct communication from MAST), as shown in Figure 1. Problems with the detection of OXA-48-like genes are a well-recognised problem with many phenotypic assays. Any equivocal results should be regarded as initially positive until further confirmation is known. Most of the mCIM tests produced no discernible zone, but of note are the two *A. baumannii* strains, one with OXA-23 and one with OXA-58, which both gave zone diameters of 19 mm. While our study used a cut-off value of <20 mm for the mCIM, these isolates would have been considered negative by Tamma (10) and Pierce (14); although their studies both used a lower bug/broth ratio and described zone interpretations for *Enterobacteriaceae* only, so it is unclear how these method variations would relate

to Pseudomonas and Acinetobacter. In addition, Van der Zwaluw, using the original CIM assay, also reported two false negative results with OXA-23-producing *A. baumannii* (9). Further research is needed in order to set mCIM method variations and zone interpretations for all Gram-negative species.

In our study the specificity and PPV of the mCIM was high (91.9%, 95.5%), indicating an excellent ability to reliably 'rule out' a non-carbapenemase-producer. The positive false results can mostly likely be attributed to hyper-AmpC production in a high-inoculum test environment. On the other hand the MAST ICT produced appreciably more equivocal or false positive results, delivering a final specificity of 81.1%. While four false equivocal results were retested as negative, repeat testing involves another day waiting for results. To reliably distinguish CPO from carbapenem-resistant-non-CPO, we would recommend that the mCIM is used in tandem with the MAST ICT, or with another high performing assay as such as Carba NP, rather than stand-alone tests.

Limitations of this study include the inability of either method to differentiate carbapenemase class, requiring further phenotypic testing and molecular sequencing. The number of Class A carbapenemase genes in the study were small as were the number of unusual enzymes such as GES and IMI; however, the panel reflects the most common carbapenemase genes that are currently found in NZ (1,17).

To our knowledge, this is the first report on the performance of the recently released MAST ICT assay. Our findings would suggest that both the MAST ICT assay and modified CIM assay have a high level of sensitivity and specificity across a range of Gram-negative bacteria. Advantages of these tests include ease of use, simple to interpret, inexpensive, and an ability to detect carbapenemase production, regardless of class type, in *Enterobacteriaceae* as well as Pseudomonas and Acinetobacter. An all-in-one test format rather than having to use multiple inhibitor-based tests, is favourable for laboratories with limited resources and experience. All phenotypic testing should be tempered with the requirement for molecular confirmation.

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AUTHOR INFORMATION

Julie Creighton, DipMLT, Senior Medical Laboratory Scientist[1] and Clinical Lecturer[2]
Clare Tibbs, BMLSc, Medical Laboratory Scientist[1]

[1]Department of Microbiology, Canterbury Health Laboratories, Christchurch
[2]University of Otago, Christchurch

Corresponding author: Julie Creighton, Microbiology Laboratory, Canterbury Health Laboratories, P. O. Box 151, Christchurch, New Zealand.
Email: julie.creighton@cdhb.health.nz

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