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The Modified Carbapenem Inactivation Method (mCIM): a highly sensitive and specific tool to assess carbapenemase-producing and non-producing in Gram-negative bacilli

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RESUMEN

Introducción: Los bacilos Gram negativos productores de carbapenemasas representan un problema de salud pública mundial, debido a que el mecanismo de resistencia, en su mayoría, se encuentra codificado en plásmidos, por lo que son fácilmente transmisibles en el ambiente hospitalario. Se han propuesto muchos métodos para detectar dicha resistencia, pero la detección sigue siendo un desafío. Recientemente, el método de inactivación de carbapenémicos modificado (mCIM) ha mostrado resultados prometedores; sin embargo, se necesitan realizar más estudios. Teniendo en cuenta lo anterior, en este estudio se propuso evaluar el rendimiento del mCIM en asilados de Enterobacterias y bacilos Gram negativos no fermentadores resistentes a carbapenémemicos. **Materiales y métodos**: A partir de una colección de aislados, previamente caracterizados para la detección molecular de genes que codificaban para carbapenemasas, se seleccionaron 100 aislados de bacilos Gram negativos, 52 no productores de carbapenemasas y 48 productores de carbapenemasas. El mCIM se realizó de acuerdo con las directrices del CLSI. Para evaluar el rendimiento del método se calcularon la sensibilidad y la especificidad. **Resultados:** La sensibilidad del mCIM observada en este estudio fue del 96 % (46/48) y la especificidad del 96,2 % (50/52). La mayoría de los bacilos Gram negativos productores de carbapenemasas fueron positivos para el mCIM; adicionalmente, en los aislados resistentes a carbapenémicos no productores de carbapenemasas (Enterobacterias y no fermentadores), los resultados de mCIM fueron negativos. **Conclusión:** En general, el mCIM proporciona una buena alternativa para la detección de bacilos Gram negativos productores de carbapenemasas. Nuestros hallazgos destacan que el mCIM es un método sensible y específico para evaluar la producción de carbapenemasas en bacilos Gram negativos no fermentadores y Enterobacterias como *Enterobacter cloacae*. **Palabras claves:** Resistencia a Carbapenémicos, Bacilos Gram negativos productores de carbapenemasas, método de inactivación de carbapenémicos modificado, Enterobacterias, no fermentadores productores de carbapenemasas, *E. cloacae, P. aeruginosa*

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ABSTRACT

Introduction: Carbapenemase-producing Gram-negative bacilli are a worldwide problem, which represent a health concern because most of their resistance mechanisms are encoded by plasmids and therefore easily transmissible in hospital settings. Many methods have been proposed to detect such resistance, but screening is still challenging. Recently, the modified carbapenem inactivation method has shown promising results; however, more studies need to be performed. Hence, this study aimed to evaluate the performance of the mCIM in carbapenem-resistant Enterobacteriaceae and nonfermenting Gram-negative bacilli isolates. **Materials and methods:** From a microbial collection with molecular characterization of carbapenemase genes previously conducted, 100 Gram-negative bacilli isolates were selected: fifty-two non-carbapenemase-producing and 48 carbapenemase-producing isolates. The mCIM was performed according to the CLSI guidelines, and to assess the performance of the method, sensitivity and specificity were calculated. **Results:** The sensitivity of the mCIM observed in this study was 96% (46/48), and the specificity was 96,2% (50/52). Most of the Gram-negative bacilli carrying a carbapenemase gene were mCIM-positive. Moreover, in carbapenem-resistant isolates that do not produce a carbapenemase (Enterobacteriaceae and non-fermenters), the results of the mCIM were negative. **Conclusion:** Overall, the mCIM provides a good alternative for the screening of carbapenemase-producing Gram-negative bacilli. Our findings highlight that mCIM is a sensitive and specific method to assess carbapenemase production in non-fermenting Gram-negative bacilli and Enterobacteriaceae such as *Enterobacter cloacae.* **Keywords:** Carbapenem-resistant, Gram-negative Bacilli. Carbapenemase-producing, Modified carbapenem inactivation method, Enterobacteriaceae, Carbapenemase-producing non-fermenters, *E. cloacae, P. aeruginosa.*

INTRODUCTION

Carbapenem-resistant bacteria have become a worldwide problem, representing a major public health concern due to their ability to inactivate antibiotics that are considered a last resort for treating infections caused by Gram-negative bacilli [1]. Carbapenem resistance may occur through different mechanisms: the production of hydrolytic enzymes such as carbapenemases, and others like the overexpression of efflux pumps, AmpC, and porin loss [2]. The carbapenemase-mediating resistance is a major problem because it is plasmid-encoded, and therefore easily transmissible among bacteria in hospital settings [3]. Thus, the detection of such pathogens and the distinction between carbapenemase-mediating resistance and resistance mediated by other mechanisms are critical for rapid infection prevention and control measures [4]. However, distinction and screening are still challenging.

Many phenotypic methods have been proposed for the detection of carbapenemase production, including the modified Hodge test (disused), disk diffusion methods such as the synergy test, biochemical tests like RAPIDEC® CARBA NP, and lateral flow immunoassays; however, each has its limitations and differences in performance related to sensitivity and specificity. RAPIDEC® CARBA NP has been recommended by the CLSI as a capture method; however, the preparation of the reagents required for this test is complicated, and the solutions cannot be stored for extended periods. On the other hand, although immunoassay is easy to perform and results are fast, it is expensive, and the detection of carbapenemases is limited to the variants specified by the manufacturer. One of the most commonly used methods is disk diffusion, which is easy to use in the clinical laboratory and does not present higher costs; however, it has limitations with the detection of carbapenemases in non-fermenting Gram-negative bacilli, due to the presence of false positives in non-carbapenemase producer resistant strains [5–6]. Finally, molecular methods like PCR, also employed to detect carbapenemases, face some problems because they can only detect known encoding genes, but these are increasing rapidly. Also, it could be more expensive as a daily clinical routine test, especially in low-income countries [7].

Based on the enzymatic hydrolysis of a meropenem disk after the exposure to a carbapenemase-producing stain and its consequent inactivation, the carbapenem inactivation method (CIM) described in 2015 [8] and its subsequent published reports have shown promising results detecting carbapenemase-mediated resistance [9,10]. On April 2017, the modified car-

bapenem inactivation method (mCIM) was published showing greater sensitivity compared with the CIM, and is now recommended by the CLSI for the detection of carbapenemase activity in *Enterobacteriaceae*. [11] However, more studies on non-fermenting bacteria such as *Acinetobacter spp.* and *Pseudomonas spp.*, and on carbapenemase non-producing isolates still need to be performed [12]. Therefore, the aim of this study was to evaluate the performance of the mCIM in *Enterobacteriaceae* and non-fermenters.

MATERIALS AND METHODS.

Bacterial isolates and study population: To assess the performance of the mCIM, bacterial isolates were selected from a microbial collection from a previous study conducted at several tertiary care hospitals located in Medellín from 2014 to 2016, in which bacterial identification and susceptibility testing were performed as well as carbapenemase detection by conventional and multiplex PCR using previously described protocols. [13,14]. A total of 100 isolates were tested in this study; 48 carbapenemase-producing isolates including *Acinetobacter baumannii* (*bla*-$_{\text{OXA-23-51}}$; n = 12), *Pseudomonas aeruginosa* (*bla*-$_{\text{KPC}}$ or *bla*-$_{\text{VIM}}$; n = 12), and Enterobacteriaceae (n = 24) such as *Klebsiella pneumoniae, Klebsiella oxytoca,* and *Enterobacter cloacae* producer of *bla*-$_{\text{KPC}}$, *bla*-$_{\text{OXA-48}}$, *bla*-$_{\text{NDM-1,}}$ and *bla*-$_{\text{VIM}}$ carbapenemases. Fifty-two non-carbapenemase producing isolates, including *P. aeruginosa* (n = 20), *A. baumannii* (n = 3), *E. cloacae* (n = 20; 16 of them HMT), *K. pneumoniae* (n = 4), *E. coli* ATCC25922 (n = 1), *E. coli-bla*-$_{\text{CTX-M-G1}}$ (n = 1), *K. pneumoniae-bla*-$_{\text{TEM}}$ (n = 1),and *E. coli bla*-$_{\text{AmpC}}$ (n = 2).

Modified Carbapenem Inhibition Method (mCIM): From frozen (-80°C) stock, each isolate was subcultured twice on Brain Heart Infusion Agar (BHI). Using a sterile inoculation loop, 1μL of the stain to be tested was added into a tube containing 2mL of tryptic soy broth (TSB; BD BBL™ Tryptic Soy Broth); the suspension was vortexed immediately for 15 seconds. After, a 10 μg Meropenem disk (MEM BD BBL™ Sensi-Disc™ Susceptibility Test Disc) was placed into the suspension. The tube was then incubated for four hours at 37°C. Fifteen minutes before the four-hour incubation, a suspension of the indicator organism (*E. coli* ATCC 25922, a carbapenem-susceptible strain) with turbidity equivalent to a 0.5 McFarland standard was prepared and the surface of an MHA plate (BD BBL™ Mueller-Hinton Agar or RemelTM Mueller-Hinton Agar) was inoculated using the procedure for standard disk diffusion susceptibility testing. The MEM disk was then removed from the TSB bacterial suspension using a 10μL inoculating loop. The loop was dragged along the edge of the tube during the procedure to remove excess liquid, and the disk was placed into the inoculated MHA plate, which was then incubated in an inverted position for 18–24 hours at 37°C in ambient air. Regarding carbapenemase positive, negative, or indeterminate mCIM results were interpreted according to the CLSI 2017 guides [15].

Controls: The positive and negative quality control (QC) strains used in this study were *K. pneumoniae* ATCC BAA-1705 (*bla*$_{\text{-KPC}}$-positive by PCR) and *K. pneumoniae* ATCC BAA-1706, respectively. The MEM disk control was also performed according to de CLSI guidelines.

Statistical analysis: Sensitivity, specificity, positive predictive value, negative predictive value, and accuracy were calculated to assess the performance of the mCIM results versus the gold standard PCR by medCalc available online (https://www.medcalc.org/calc/diagnostic_test.php).

RESULTS

Carbapenemase-producing isolates

Out of the 48 Carbapenemase-producing isolates, the mCIM showed positive results in 46 isolates that were previously characterized with carbapenemase encoding genes. The two mCIM-negative isolates were *K. pneumoniae* and *E. cloac*ae carrying *bla*-$_{\text{KPC-2/VIM}}$ and *bla*-$_{\text{KPC-3,}}$ respectively, for which the carbapenem MICs were low (IMP 2, MEM 4; IMP 2, MEM, 0,25 mg/mL). Moreover, all non-fermenters carrying a previously carbapenemase gene were mCIM-positive.

The *A. baumannii* isolates inhibition zone was up to 18 mm and almost all presented multiple small bacterial colonies growing within the zone of inhibition. *P. aeruginosa* was evaluated with inoculation loops of 1μL and 10μL but no significant difference was observed, overall non-fermenters showed an excellent performance (Table 1).

Table I. mCIM results for carbapenemase producing isolates.

Species Strain N°	PCR Resistance genes	MICS ug, mL		mCIM (diameter, mm)	mCIM
		IMP	MEM	Meropenem	
Non-fermenters (n=24)					
Acinetobacter baumannii					
AC1	OXA-23, OXA-51 (ISAba1)	16	16	15	+
AH1	OXA-23, OXA-51 (ISAba1)	16	16	16	+
AH4	OXA-23, OXA-51 (ISAba1)	16	16	18*	+
AP5	OXA-23, OXA-51 (ISAba1)	16	16	18*	+
AL1	OXA-23, OXA-51 (ISAba1)	16	16	16*	+
AL4	OXA-23, OXA-51 (ISAba1)	16	16	17*	+
AL9	OXA-23, OXA-51 (ISAba1)	16	16	6	+
AH10	OXA-23, OXA-51 (ISAba1)	16	16	15*	+
AH6	OXA-23, OXA-51 (ISAba1)	16	16	16*	+
AP2	OXA-23, OXA-51 (ISAba1)	16	16	16*	+
AR2	OXA-23, OXA-51 (ISAba1)	16	16	16*	+
AR3	OXA-23, OXA-51 (ISAba1)	16	16	16*	+
Pseudomonas aeruginosa					
PH90	VIM	16	16	6	+
PP9	VIM, KPC	16	16	6	+
PC8	VIM	16	16	6	+
PR35	VIM	16	16	16	+
PH15	VIM	16	4	10	+
PL35	VIM, KPC	16	16	10	+
PC16	KPC	16	16	6	+
PH29	KPC	16	16	6	+
PH67	KPC	16	16	6	+
PL5	KPC	16	16	6	+
PL25	KPC	16	16	10	+
PR2	KPC	16	16	8	+
Enterobacteriaceae (n=24)					
Klebsiella pneumoniae					
KL15	KPC-3	16	16	6	+
KP58	KPC-3	16	16	6	+
KH10	KPC-3	8	2	6	+
KP24	KPC-2	4	2	6	+
KH43	KPC-2	16	16	6	+
KL49	KPC-2	16	16	6	+
KP4	KPC-2	2	4	19	-
KC4	KPC-2	8	4	6	+
KC8	KPC-3	16	16	6	+
KH7	KPC-2	2	1	6	+
KH8	KPC-2	16	16	6	+
CAR119	NDM-1	16	16	6	+
Klebsiella oxytoca					
CAR118	NDM-1	8	16	6	+
CAR105	OXA-48	16	16	15	+
Enterobacter cloacae					
ER2	KPC-2	4	1	6	+
EH15	VIM, KPC-3	2	0.25	19	-
EP5	VIM, KPC-3	1	0.5	17*	+
EP8	VIM, KPC-3	8	16	6	+
EC3	KPC-2	16	16	6	+
EH19	KPC-2	16	16	6	+
EP9	KPC-2	16	16	6	+
EL10	KPC-2	16	16	6	+
EL38	KPC-2	16	16	6	+
ER14	KPC-2	16	8	6	+

(+) = positive; (-) = negative *presence of colonies within a 16–18 mm zone

Non- Carbapenemase-producing isolates

Of the 52 isolates characterized as not carrying a carbapenemase gene, 50 were mCIM-negative. Almost all non-fermenters that were non-carbapenemase producing isolates were mCIM negatives except for a false positive isolate belonging to *P. aeruginosa*. Regarding *E. cloacae*, 15 out of 16 previously HTM Positive isolates but non-carbapenemase producing yield an mCIM negative result. (Table 2)

Sensitivity and Specificity

The sensitivity of the mCIM observed in this study was 96% (46/48), and the specificity was 96,3% (50/52) for non-carbapenemase-producing isolates (Table 3).

Table II. mCIM results for non-carbapenemase producing isolates.

Non-Carbapenemase Producer (n= 52)	Molecular characteristic	Positive mCIM (%)	Negative mCIM (%)
P. aeruginosa (20)		1(5)	19 (95)
A. baumannii (3)		0 (0)	3 (100)
E. cloacae (4)		0 (0)	4 (100)
E. cloacae HMT positive (16)		1 (6.3)	15 (93.7)
K. pneumoniae (4)		0 (0)	4 (100)
E. coli (ATCC 25922)		0 (0)	1 (100)
E. coli (HD13)	CTX-M-G1	0 (0)	1 (100)
K. pneumoniae (HD13)	TEM	0 (0)	1 (100)
E. coli (BAE11)	AmpC LAT-1	0 (0)	1 (100)
E. coli (BEE25)	AmpC MOX-1	0 (0)	1 (100)

Table III. Performance of the mCIM results versus the gold standard PCR

Statitistic	value	95% CI
Sensitivity	96.00%	86.29% to 99.51%
Specificity	96.30%	87.25% to 99,55%
Positive Predictive Value	96.00%	86,02% to 98,94%
Negative Predictive Value	96.30%	86,98% to 99.20%
Accuracy	96.15	90.44% to 98.94%

*Confidence intervals for sensitivity, specificity and accuracy are "exact" Clopper-Pearson confidence intervals.

DISCUSSION

The results of the mCIM in this study show that it is capable of detecting carbapenemase production not only in *Enterobacteriaceae* but to a greater extent, it is an excellent tool to identify carbapenemase in non-fermenters showing high concordance 92% (22/24) for Enterobacteriaceae and 100% (24/24) for non-fermenters. Overall, the mCIM results display a sensitivity of 96%. These results are in accordance with other studies evaluating mCIM, which showed low false-negative results with a sensitivity up to 98.8% [11,12]. Additionally, our study reported two false-positive results, with a specificity of 96,3%.

Despite the fact that other articles focus on *Enterobacteriaceae* [23,24], the results of non-fermenting isolates in the present study showed them to be very efficient in the recognition of carbapenemase-producing isolates, regardless of the initial inoculum. Likewise, the present results also evidenced a good performance of the mCIM in isolates with different types of carbapenemases, which may differ in their hydrolytic profile. We suggest that mCIM can be used routinely in the laboratory for the detection of carbapenem-producing non-fermenter isolates.

Our study included isolates of *Enterobacter spp*. with MHT positive results and *P. aeruginosa,* both non-carbapenemase producers. Our findings highlight that mCIM is an excellent method with greater specificity compared to previously used methodologies due to its ability to differentiate true negative results. The use of more sensitive and specific methods in the clinical laboratory allows for a better selection of the antibiotic treatment since the presence of carbapenemases directly influences the administration of the type of antibiotic and dosage. In this sense, mCIM offers great advantages in microorganisms such as *P. aeruginosa*, which may present resistance to carbapenems due to the presence of carbapenemases or by non-enzymatic mechanisms that include decreased permeability [25].

Many methods to identify carbapenemase-producing bacteria have been proposed. The modified Hodge test was recommended by the CLSI in 2009 as a screening method for carbapenemases, but regardless of its advantages like being easy to perform and that it does not need special equipment [16], this procedure has false-negative results with mucoid colonies and problems detecting low carbapenemase expression, especially difficulties with oxacilinase and metalobetalactamase-producing isolates [17]. Furthermore, it has many detractors due to the disagreements observed with some stains showing false-positive results with hyperproduction of AmpC, porin loss, and*bla*-$_{\text{CTX-M}}$ producing bacteria, especially *Enterobacteriaceae* isolates (mostly *Enterobacter spp*) [18]. Some variations of the test have been proposed, especially for non-fermenters like *P. aeruginosa*, but none of them have shown promising results [19].

The Carba NP test was developed by Nordmann & Poirel [20]. This method was approved by CLSI in 2015 [21], given its high sensitivity and rapid detection (≤ 2 h). However, this method has a high cost in contrast to other phenotypic methods [9]. The same

year a new method called the carbapenem inactivation method—CIM—a low-cost and effective screening method that can reliably detect carbapenemase activity [8] was published. Despite its initial results, other papers did not find a 100% sensitivity as claimed by Van der Zwaluw et al [12, 22]. Therefore, a modification of the protocol was needed to improve the detection of some carbapenemases [11]. The CLSI Performance Standards for Antimicrobial Susceptibility Testing was charged with validating this modification, and in Supplement M100 (2017) they proposed the modified carbapenem inactivation method –mCIM- as a reliable standardized method available to laboratories that aim to identify carbapenem-resistant *Enterobacteriaceae* for epidemiologic or infection control purposes [15].

Conclusion: We have shown that the mCIM provides a user-friendly alternative for the screening of carbapenemase production. This provides a great tool for early detection not only in *Enterobacteriaceae* but also in non-fermenters, consequently in the implementation of control measures and infection prevention, avoiding the spread of such pathogens in health care settings, and finally in low-income countries.

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