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Original Article

Evaluation of phenotypic tests for detection of carbapenemases: New modifications with new interpretation

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ABSTRACT

Introduction: The emergence and spread of carbapenemase-producing *Enterobacterales* (CPE) is a worldwide public health threat. Rapid and accurate detection of CPE is essential to prevent their dissemination within health care settings. The aim of this study was to evaluate the performance of CIM, mCIM and mCIM with ammonium bicarbonate (mCIM-A) methods by using different interpretation criteria for detection of carbapenemases.

Methods: One hundred and fifty-three *Klebsiella pneumoniae* isolates previously characterized by molecular tests, including 133 carbapenemase producers and 20 non-carbapenemase producers, were collected in this study. CIM and mCIM tests were performed as described previously. mCIM-A by adding 50 mM ammonium bicarbonate to the bacterial suspension prepared in tryptic soy broth. The inhibition zone diameter of around meropenem disc was measured and interpreted as positive according to i) Pierce and colleagues (<19 mm), ii) EUCAST meropenem susceptibility breakpoint (<22).

Results: CIM, although seems to be good for carbapenemases other than OXA-48-like and NDM, is not satisfactory (42.3% and 83.4%, respectively) for those enzymes with any of the interpretation criteria. OXA-48-like and NDM were detected with a better performance (88.7% and 92.8, respectively) with mCIM when results were interpreted according to <22 mm zone diameter for OXA-48-like and NDM. The best results were obtained with mCIM-A using <22 mm criteria without any difference in the results of other enzymes and negative strains.

Conclusions: mCIM-A method interpreted with <22 mm meropenem zone diameter seems to be preferable compared to CIM and mCIM. mCIM-A is simple and useful tool for identification of CPEs in clinical microbiology laboratories.

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1. Introduction

Over the last decade, carbapenemase-producing *Enterobacterales* (CPEs), especially *Klebsiella pneumoniae*, are become major public health concern in all over the world. Rapid and reliable detection of CPEs which are easily carried from person to person through mobile genetic elements is more important compared to other carbapenem resistance mechanisms, especially the production of AmpC beta-lactamases or extended-spectrum beta-lactamases (ESBLs) combined with porin deficiency. Because the detection of their presence can rapidly initiate infection control procedures to avoid potential outbreaks and spread of the resistance [1–4].

The most common types of carbapenemases in *Enterobacterales* are Ambler class A (i.e. KPC and GES), class B (i.e. VIM, IMP, and NDM), and class D (i.e. OXA-48-like). But epidemiological distribution of carbapenemases varies between countries and geographic regions [1–4]. OXA-48 which was firstly described in 2001 is endemic in Turkey. The prevalence of this enzyme is alarmingly increased in many European (France, Belgium, Spain) the Middle East, and North African countries (Morocco, Tunisia, Egypt, and Libya). In recent years, NDM type of carbapenemase is also increasingly encountered in Turkey [5–7].

Although antibiotic susceptibility testing is determined which antibiotic will be used in treatment of infections, it is not sufficient

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alone. The development of highly sensitive and simple detection methods for CPE carriers or infections is important for antibiotic stewardship and infection control measures to be effective at an early stage. Molecular tests which have high sensitivity and specificity are considered the gold standard. But it cannot be performed in many clinical microbiology laboratories, because of the expensive equipment and trained personnel are required [8,9]. On the other hand, phenotypic detection of carbapenemases in clinical microbiology laboratories is still the most challenging issue. So far, different phenotypic methods have been recommended by antibiotic susceptibility guidelines (EUCAST, CLSI) to the detection of carbapenemases for epidemiological or infection control purposes [10,11]. The performance of these methods varies due to diversity of the carbapenem hydrolysis activity of enzymes and there is still no a phenotypic method which can detect 100% of carbapenemases.

The carbapenem inactivation method (CIM) described by van der Zwaluw et al. is based on the enzymatic hydrolysis of a meropenem disc after its exposure to a carbapenemase-producing strain and its consequent inactivation which allows uninhibited growth of a full susceptible indicator strain [12]. While it is easy to interpret and inexpensive test for the detection of CPEs, it has low sensitivity for the detection of OXA-48 and NDM type carbapenemases [13–15]. The modified carbapenem inactivation (mCIM) method was described by Pierce and colleagues, using less microorganisms (1 μl versus 10 μl loopful), longer incubation time (4 h versus 2 h) and a different suspension medium (tryptic soy broth versus water) to increase the sensitivity [16]. Studentova et al. were found that the activity of the carboxylated lysine residues in the active region of OXA-48 can be increased with bicarbonate compounds. Researchers were pointed out that bicarbonates in media or reaction buffers can enhance the sensitivity of screening methods and diagnostic tests for carbapenemase producers [17]. However, there are limited studies about the effect of bicarbonate compounds on the performance of phenotypic tests used to detect carbapenemases [18–20].

The aim of this study was to evaluate the performance of CIM, mCIM, and mCIM with ammonium bicarbonate (mCIM-A) methods by using different interpretation criteria.

2. Materials and methods

2.1. Bacterial isolates

A total of 153 non-duplicate *Klebsiella pneumoniae* isolates were used in this study. The strains were recovered from various clinical samples (abscess, bronchoalveolar lavage, sputum, blood, deep tracheal aspirate, urine, wound) at Marmara University Hospital, Istanbul, Turkey. All strains have previously been characterized at the molecular level for their carbapenemase content by in-house polymerase chain reaction (PCR). PCR was performed using specific primers and reaction conditions for genes encoding bla$_{VIM}$, bla$_{IMP}$, bla$_{NDM}$, bla$_{KPC}$, and bla$_{OXA\text{-}48\text{-}like}$ [21–24]. Among them, 133 isolates were carbapenemase producers which expressed OXA-48-like (n = 71), NDM (n = 42) IMP(n = 11), VIM (n = 5) and KPC (n = 4); and remaining 20 isolates were susceptible to all carbapenems and did not have any carbapenemase genes. All of the isolates were identified to the species level by matrix-assisted laser desorption ionization-time of flight mass spectrometry (VITEK-MS, bioMerieux, Marcy l'Etoile, France).

2.2. Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed using an automated VITEK2 system (bioMerieux, Marcy-l'E'toile, France). Minimum inhibitory concentrations (MICs) of imipenem, meropenem, and ertapenem were determined by the gradient diffusion strip test (Etest®, bioMerieux, Marcy l'Etoile, France) and results were interpreted according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines [10].

2.3. Carbapenem inactivation method (CIM)

The bacterial colonies grown on 5% sheep blood agar were picked up using 10 μl inoculation loop and suspended in 400 μl water. Subsequently, a 10 μg meropenem disc (Oxoid, Thermo Scientific, USA) was added to the suspension and incubated at 35 °C for 2 h. After incubation, the meropenem disc was removed from the suspension using an inoculation loop and then placed on a Mueller-Hinton agar plate (bioMerieux, Marcy l'Etoile, France) inoculated with *Escherichia coli* ATCC 25922 equivalent to a 0.5 McFarland standard. The plate was incubated at 35 °C for 6 h and the diameter of the inhibition zone around the meropenem disc was measured [12].

2.4. Modified carbapenem inactivation method (mCIM)

The bacterial colonies grown on 5% sheep blood agar were picked up using 1 μl inoculation loop and suspended in 2 ml of tryptic soy broth (Oxoid, Thermo Scientific, USA). Subsequently, a 10 μg meropenem disc was added to the suspension and incubated at 35 °C for 4 h. After incubation, the meropenem disc was removed from the suspension using an inoculation loop and then placed on a Mueller-Hinton agar plate which was inoculated with *E. coli* ATCC 25922 equivalent to a 0.5 McFarland standard. The plate was incubated at 35 °C for overnight and the diameter of the inhibition zone around the meropenem disc was measured [16].

2.5. Modified carbapenem inactivation method with ammonium bicarbonate (mCIM-A)

The bacterial colonies grown on 5% sheep blood agar were picked up using 1 μl inoculation loop and suspended in 2 ml of tryptic soy broth. Ammonium bicarbonate (NH_4HCO_3) (pH was adjusted to 7.0) (Sigma-Aldrich, St. Louis, Missouri, USA) at the final concentration of 50 mM was added to the bacterial suspension. The next steps of this method were applied like mCIM.

Additionally, we investigated that whether NH_4HCO_3 has an inhibitory effect or not. The meropenem susceptibility of *Escherichia coli* ATCC 25922 was performed by disc diffusion method with and without NH_4HCO_3. The method was repeated 20 times and the meropenem zone diameters were recorded.

2.6. Interpretation of the results

The results of three methods were separately interpreted according to following two criteria;

i) The results were considered as positive if the diameter of the inhibition zone around the meropenem disc was <19 mm which recommended by Pierce et al. [16].
ii) The results were considered as positive if the diameter of the inhibition zone around the meropenem disc was <22 mm which was EUCAST meropenem susceptibility breakpoint [10].

2.7. Statistics

All statistical analyses were performed with the SPSS 22.0. The sensitivity and specificity were calculated, with their respective 95% confidence interval (CI). McNemar test was used to compare

the sensitivity and specificity of three methods. The significance was set at $p < 0.05$.

Cohen's kappa (k) coefficient was calculated to indicate the degree of consistency among CIM, mCIM, mCIM-A and PCR. Interpretation of Cohen's k coefficient was as follows: $k > 0.75$, excellent; $k = 0.4–0.75$, fair to good; and $k < 0.4$, poor.

3. Results

The minimum inhibitory concentration ranges of imipenem, meropenem, and ertapenem against CPEs were 0.125 to >32 mg/L, 0.25 to >32 mg/L, and 0.75 to >32 mg/L, respectively (Table 1). All KPC, NDM, IMP, and VIM producers were nonsusceptible to imipenem, meropenem, and ertapenem. While all OXA-48-like producers were resistant to ertapenem, 50.7% and 25.3% of them were susceptible to imipenem and meropenem, respectively.

The results of three methods by using different interpretation criteria are shown in Table 2. Meropenem zone diameter was observed 6 mm in all KPC, IMP, and VIM producers, and these isolates were successfully detected with all phenotypic methods according to two interpretation criteria. But, the performance of these methods differed according to two interpretation criteria for the detection of OXA-48-like and NDM producers. The meropenem zone diameters were determined between 28 and 30 mm in non-carbapenemase producers and no false-positive result was observed according to all interpretation criteria.

CIM method, although seems to be good for carbapenemases other than OXA-48-like and NDM, is not satisfactory (42.3% and 83.4%, respectively) for those enzymes with any of the interpretation criteria. When the interpretation criteria were considered at <19 mm, OXA-48-like and NDM producers were detected with mCIM-A 84.5% and 92.8%, while they were detected with mCIM 71.9% and 90.5%, respectively. OXA-48-like and NDM were detected with better performance (88.7% and 92.8, respectively) with mCIM when results were interpreted according to <22 mm zone diameter. Best results were obtained with mCIM-A using <22 mm criteria without any difference in the results of other enzymes and negative strains.

The overall sensitivity and specificity of the three methods are shown in Table 3. The sensitivity was significantly higher (89.47%) for mCIM-A compared to CIM (63.9%) and mCIM (81.95%) when the interpretation criteria were considered at <19 mm ($p=<0.00001$ and $p = 0.004$, respectively). There was no statistical difference between the sensitivity of mCIM (91.73%) and mCIM-A (95.49%) ($p = 0.0736$), but the sensitivity of CIM was statistically different ($p=<0.00001$) when the interpretation criteria were considered at <22 mm. All methods displayed 100% specificity.

The meropenem zone diameters obtained by mCIM and mCIM-A in OXA-48-like producers are compared in Fig. 1. According to our results, there was change in the meropenem zone diameter of the 29 OXA-48-like producers (40.8%). The decrease of meropenem zone diameters with mCIM-A was 1–4 mm in 15 OXA-48-like producers (21.1%) and ≥5 mm in 14 OXA-48-like producers (19.7%).

Additionally, the meropenem zone diameter of *E. coli* ATCC 25922 was determined as 33 mm with and without of NH_4HCO3. Thus, it has been determined that NH_4HCO_3 has no inhibitory effect.

Table 1
The minimum inhibitory concentration (MIC) of imipenem, meropenem, and ertapenem in *K. pneumoniae*.

Carbapenemase	MIC (mg/L)		
	Imipenem	Meropenem	Ertapenem
OXA-48-like (n = 71)	0.125- >32	0,25- >32	0,75- >32
NDM (n = 42)	3- >32	3- >32	3- >32
IMP (n = 11)	>32	>32	>32
VIM (n = 5)	>32	>32	>32
KPC (n = 4)	4- >32	12- >32	12- >32
Negative (n = 20)	0.016–0.06	0.016–0.03	0.004–0.03
Total (n = 153)	0.016- >32	0.016- >32	0.016- >32

4. Discussion

Over the last decade, the emergence of carbapenemases in *Enterobacterales* has become a major challenge for health care settings. The rapid and accurate detection of CPEs is essential for rapid implementation of infection prevention and control measures.

Several phenotypic and genotypic methods are available for detection of CPEs. While genotypic methods are considered as a gold standard for detection of CPEs, they are of limited practical use in most microbiology laboratories due to disadvantages such as the need for expensive equipment and trained personnel. Over the last decade, many phenotypic methods [Modified Hodge test (MHA), carbapenem inactivation method (CIM), modified carbapenem inactivation method (mCIM) inhibitor-based methods, chromogenic medium, matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS), etc.] have been developed for the detection of CPEs. The performance of these tests varies considerably, depending on hydrolysis activity of the enzyme. The most challenging enzymes to be detected by phenotypic methods are OXA-48 and NDM [25].

Although CIM and its modified version (mCIM) require an extra overnight incubation of the plates to obtain results, both methods have several advantages over phenotypic detection methods. They are simple to perform and interpretation. They require inexpensive consumables that are easily available in most microbiology laboratories. Additionally, six different isolates can be tested simultaneously on a single plate inoculated with *E. coli* ATCC 25922. However, some studies have shown that these tests have a low sensitivity in the detection of OXA-48 and NDM [13–15,26–28]. We thought that the sensitivity of these tests could be improved by modifications on method and interpretation criteria. Therefore, these tests can play important role for accurate detection of CPEs in microbiology laboratories where molecular tests cannot be performed.

The first modification was to add ammonium bicarbonate to the mCIM method for better detection of OXA-48-like producers. Previous studies have shown that the most important factor of the activity of OXA-type β-lactamases is the carboxylated lysine residue in the active site. Studentova et al. were found that the activity of the carboxylated lysine residues in the active region of OXA-48 can be increased with bicarbonate compounds. However, there are limited studies investigating the effect of bicarbonate compounds on the performance of phenotypic tests used to detect carbapenemases [17–20]. In this study, the meropenem zone diameter significantly decreased in 40.8% of OXA-48-like producers with mCIM-A method. Also, we have confirmed that the ammonium bicarbonate does not have an inhibitory effect in carbapenem-susceptible *K. pneumoniae* isolates.

The second modification was the change in the interpretation criteria. Two different interpretation criteria (<19 mm recommended by Pierce and colleagues and <22 mm recommended by EUCAST as meropenem susceptibility breakpoint) were used in our study. The CIM yielded the worst performance according to both interpretation criteria. The CIM was unable to detect more than 40% of the OXA-48-like producers even if the interpretation criteria were taken <22 mm differently than defined by Zwaluw et al. The sensitivity of the CIM in studies performed in our country were

Table 2
Results of three phenotypic methods according to different interpretation criteria.

Carbapenemase	CIM		m CIM		m CIM-A	
	Meropenem zone diameter (mm)		Meropenem zone diameter (mm)		Meropenem zone diameter (mm)	
	<19[a]	<22[b]	<19[a]	<22[b]	<19[a]	<22[b]
OXA-48-like (n = 71)	30 (42.3)	41 (57.7)	51 (71.9)	63 (88.7)	60 (84,5)	66 (92,9)
NDM (n = 42)	35 (83.4)	38 (90.4)	38 (90.5)	39 (92.8)	39 (92.8)	41 (97.6)
IMP (n = 11)	11 (100)	11 (100)	11 (100)	11 (100)	11 (100)	11 (100)
VIM (n = 5)	5 (100)	5 (100)	5 (100)	5 (100)	5 (100)	5 (100)
KPC (n = 4)	4 (100)	4 (100)	4 (100)	4 (100)	4 (100)	4 (100)
Negative[c] (n = 20)	–	–	–	–	–	–

[a] Test results were interpreted as positive according to Pierce and colleagues (<19).
[b] Test results were interpreted as positive according to EUCAST meropenem susceptibility breakpoint (<22).
[c] Meropenem zone diameters were detected 28–30 mm in non-carbapenemase producers.

Table 3
Performance of the three phenotypic methods for detection of carbapenemase production.

Method and interpretation criteria	Sensitivity % (Cl)	Specificity % (Cl)	Kappa
CIM			
19 mm	63.91% (55.13%–72.05%)	100 (83.16%–100%)	0.316
22 mm	74.44% (66.15%–81.60%)	100 (83.16%–100%)	0.432
mCIM			
19 mm	81.95% (74.35%–88.08%)	100 (83.16%–100%)	0.543
22 mm	91.73% (85.68%–95.80%)	100 (83.16%–100%)	0.744
mCIM-A			
19 mm	89.47% (82.97%–94.12%)	100 (83.16%–100%)	0.69
22 mm	95.49% (90.44%–98.33%)	100 (83.16%–100%)	0.847

Cl: Confidence interval.

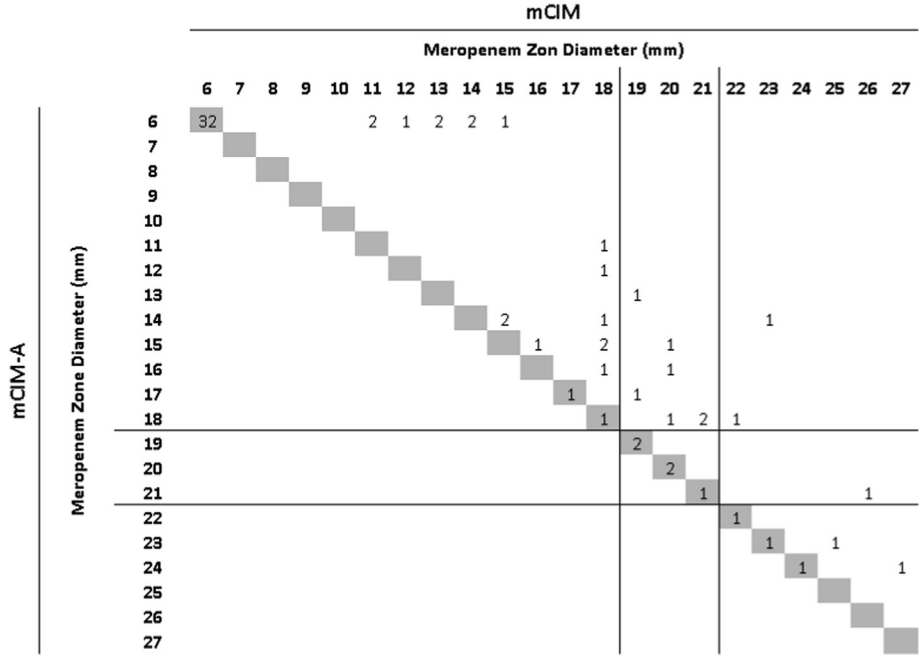


Fig. 1. Comparison of meropenem zone diameter obtained by mCIM and mCIM-A in OXA-48-like producers.

significantly found lower unlike the studies reported from countries other than Turkey [14,27,28]. As a result, CIM were found to be unsuccessful in detecting especially OXA-48-like producers. When the interpretation criteria were considered at <19 mm, better results were obtained with the mCIM for OXA-48-like and NDM producers (71,9% and 90,5% respectively). In this study, the mCIM test displayed a lower sensitivity than was initially reported studies (98–100%), mainly due to false-negative results obtained with OXA-48-like and NDM producers [29–33]. This may be related to the high prevalence of OXA-48-like which have low hydrolysis activity in our country. Fifty-three percent of our study population was OXA-48-like and 31% were NDM. The detection rate of OXA-48-like and NDM producers has increased to 92,9% and 97,6%, respectively, by mCIM-A using <22 mm criteria. Although the best results were obtained with the mCIM-A, it has missed 5 OXA-48-like and 1 NDM producers. All of the KPC, IMP and VIM producers

were detected by all method and interpretation criteria. The major limitations of our study is that it contains a small number of KPC, IMP, and VIM producing isolates and includes only *K. pneumoniae* isolates. The mCIM were not evaluated in carbapenemase producers other than *K. pneumoniae*. It will be useful to determine of effectiveness of mCIM with additionally studies using carbapenemase producers other than *K. pneumoniae*.

So far, some modifications have been made by the researchers to both increase the sensitivity of the CIM and reduce the detection time. Muntean et al. was developed another method as called rCIM. The most important advantage of this method is less than 3 h of the detection time [34]. Uechi et al. recently developed a further modified CIM, called CIMTris, which uses 0.5 M Tris-HCl buffer for improve the sensitivity of test in *Acinetobacter* and *Pseudomonas* species [35]. Baeza et al. have developed the zCIM method to better detect metallo-beta lactamases in a shorter time. They found the sensitivity of zCIM higher than CIM using a new cut-off value (97.4% versus 69.6%) [36]. Gauthier et al. reported that the best substrate was imipenem for the evaluation of the CIM (100% sensitivity) [37]. On the other hand, according to some study results, meropenem disk was found to be more suitable than ertapenem for CIM and mCIM [28,37,38]. However, the number of OXA-48 producers used in these studies is very low. All OXA-48-like producers used in our study were resistant to ertapenem. Therefore, ertapenem seems to be a better marker for OXA-48-like producers. Therefore, in countries where OXA-48 is common, it may be more ideal to use ertapenem instead of meropenem. This issue should be supported by further studies which including more OXA-48 producers.

Although antibiotic susceptibility guidelines (EUCAST, CLSI) suggest various phenotypic methods for the detection of carbapenemases, each country should determine the most appropriate method and algorithm to detect the most common carbapenemases in accordance with their epidemiological data. OXA-48-like and NDM type of carbapenemases are predominant in Turkey and are represented largely (53% and 31%, respectively) in our study. But, it is still a major problem to detection of OXA-48-like producers by several phenotypic tests due to weak hydrolysis activity. Therefore, the development of effective detection methods for these enzymes is very important. Although the mCIM-A is found to be more sensitive compare to mCIM, false-negative results have been observed, especially in OXA-48-like and NDM producers. Our results which were obtained with ammonium bicarbonate are promising for the detection of OXA-48-like producers. It may be facilitate for the better detection of OXA-48-like which is the most problematic carbapenemase in phenotypic methods. However, it requires further studies with increased numbers of strains and different concentrations of ammonium bicarbonate Thus, the performance of the phenotypic tests used to detect carbapenemases can be increased.

In summary, the mCIM-A can be an ideal method for the reliable detection of carbapenemases as it is a simple, inexpensive, and useful method in clinical microbiological laboratories with limited resources and no molecular equipment.

ICMJE statement

G. Altinkanat Gelmez and G. Soyletir developed the concept for the study and, together with U. Hasdemir, were responsible for the study design and its conduct. Data acquisition was performed by G. Altinkanat Gelmez and B. Can. The data were analyzed and interpreted by G. Altinkanat Gelmez and B. Can. G. Altinkanat Gelmez were involved in drafting the article. All authors critically reviewed the manuscript and approved the final version.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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