Egyptian Journal of Medical Microbiology Volume 32 / No.1 / January 2023 37-46 Online ISSN: 2537-0979

ORIGINAL ARTICLE

Assessment of Phenotypic Testing by mCIM with eCIM for Determination of the type of Carbapenemase Produced by Carbapenem-resistant Enterobacterales

[1]Aliaa G. Aboulela*, [2]Mawj F. Jabbar, [2]Abdelfattah Hammouda, [2]Medhat S. Ashour
[1]Microbiology Department, Medical Research Institute, Alexandria University, Alexandria, Egypt
[2]Microbiology Department, High Institute of Public Health, Alexandria University, Alexandria, Egypt

Key words:
Carbapenem-resistant Enterobacterales, mCIM with eCIM, metallo-beta-lactamase detection, serine-carbapenemase detection, blaOXA-48 gene, blaNDM-1 gene

****Corresponding Author:***
Aliaa Gamaleldin Aboulela (PhD)
Lecturer, Department of Microbiology Medical Research Institute
Alexandria University, Egypt
aliaagamaleldin@alexu.edu.eg
ORCID: 0000-0002-2923-1027
Tel.: +201002104138

ABSTRACT

Background: *Carbapenemase-producing Enterobacterales are increasingly spreading in healthcare facilities. Identifying the type of carbapenemase can help epidemiologic surveillance and proper selection of antimicrobials.* ***Objective:*** *This study assessed the sensitivity and specificity of carbapenem inactivation method (mCIM with eCIM) for identification of carbapenemase-production.* ***Methodology:*** *The study involved 150 isolates of Enterobacterales. Carbapenem-resistant isolates by Kirby Bauer method were further tested for carbapenemase production phenotypically by mCIM with eCIM, and genotypically by multiplex PCR using specific primers for bla_{KPC}, bla_{OXA-48}, bla_{NDM-1}, bla_{VIM}, and bla_{IMP}.* ***Results:*** *Resistance to carbapenem was observed in 53/150 isolates. Phenotypically, 28/53 isolates produced metallo-β-lactamase, 16/53 produced serine carbapenemase, 5/53 isolates gave inconclusive results, and 4/53 were negative by mCIM with eCIM test. Genotypically, 30 isolates carried bla_{NDM-1}, and 41 isolates carried bla_{OXA-48}. Both genes co-existed in 18 Metallo-β-lactamase producers. The 9 isolates with negative or inconclusive results carried carbapenemase-encoding genes. For mCIM with eCIM test the sensitivity and specificity of detecting Metallo-β-lactamase production were higher (87% and 91%) than for serine carbapenemase detection (34% and 83%), respectively.* ***Conclusion:*** *It was concluded that the mCIM with eCIM test does not always lead to true conclusions about the existence and the type of carbapenemase produced by Enterobacterales.*

INTRODUCTION

Carbapenems are highly potent β-lactam antimicrobial agents. They exert their broad-spectrum activity by interfering with the process of cell wall synthesis in bacteria via inhibition of transpeptidase enzyme, also known as Penicillin-Binding Protein (PBP), which is the main target of these drugs. This results in autolysis and eventually death of bacterial cells [1]. Carbapenems are of high antimicrobial efficacy as they can bind to various types of PBPs, mainly PBP 1a, PBP 1b, PBP 2 and PBP 3 [1, 2].

Variation in the chemical structure of different carbapenem antimicrobials results causes variation in their efficacy. For instance, the anti-Gram-negative activity of Meropenem and Ertapenem is slightly higher than Imipenem. However, the anti-pseudomonal activity of Ertapenem is less than that of other carbapenems. On the other hand, Imipenem affects *Acinetobacter baumannii* more than Meropenem. Meanwhile, Doripenem, exerts better antimicrobial effect against *Acinetobacter baumannii and Pseudomonas* spp., being less affected by the hydrolytic activity of β-lactamase enzymes[3, 4].

Carbapenem-resistant Enterobacterales (CRE) are of growing importance as pathogens of major morbidity, for their resistance to one of the safest last-line drugs available against infections by multidrug-resistant bacteria, either as single agents or in combination with other drugs [5]. Enterobacterales can resist the action of carbapenems by various mechanisms, the most common among which is carbapenem-hydrolyzing enzymes. Other mechanisms include alteration of porin channels and efflux pumps that extrude the drug [6].

Carbapenem-hydrolyzing enzymes are broadly classified into 2 main categories; Serine- ß-Lactamases, which use serine at the enzyme's active motif (belonging to Ambler's classes A, and D) and Metallo-ß-Lactamases which use Zinc at the enzyme's active motif (belonging to Ambler's class B) [7].

Carbapenemase-encoding enzymes are rapidly disseminated among Enterobacterales, as most of which undergo horizontal gene transfer [8]. In the US, the plasmid-mediated Ambler's classes A serine ß-Lactamase KPC is the most predominant. Variants of this kind of ß-Lactamases are increasingly spreading worldwide. The OXA-48 serine ß-Lactamase is another common plasmid-mediated enzyme belonging to

Ambler's classes D. Since its discovery in 2003, it has been widely identified in countries from all over the world particularly in the Mediterranean region and Europe[9,10].

EDTA inhibits the activity of Ambler's class B Metallo-ß-Lactamases by chelation of zinc ions at their active site, thereby depriving the enzyme from its beta-lactamase hydrolyzing activity. The most predominant among this category are the IMP, the NDM-1, and the VIM. The genes encoding for these beta-lactamases are often carried on integrons within gene cassettes [11, 12].

Although AmpC β-lactamases exert minimal anti-carbapenem activity, yet exposure to Imipenem is a potent inducer to the production of AmpC in bacteria that carry chromosomal *amp*C genes [13, 14].

With the availableness of antibiotics effective against bacteria producing carbapenemase of distinct Ambler classes, it is of particular importance to inform the clinicians about carbapenemase-production by the bacteria isolated from clinical specimens, and their Ambler class. In addition, this information is necessary for surveillance purposes [15].

Different phenotypic tests have been suggested to test Enterobacterales for carbapenemase activity. Among these tests, the CLSI has adopted the modified Hodge test which was included in the M100 Performance standards for antimicrobial susceptibility testing from 2009 to 2017. Later, this test has been omitted, due to its low sensitivity and specificity, leaving only CarbaNP test and mCIM with eCIM tests (modified carbapenem inactivation method with EDTA-modified carbapenem inactivation method) starting from the 28th edition of the M100 CLSI guidelines till the latest edition to date (32nd edition). The guidelines stated some limitations for the CarbaNP test, including the invalid results with some isolates, and its being unable to consistently detect certain carbapenemase types like the chromosomally encoded OXA-type carbapenemase. Meanwhile, the only limitation stated for mCIM with eCIM tests was that they require overnight incubation [16, 17].

This study assessed the reliability of phenotypic detection and determination of the category of carbapenemase by the CLSI-recommended phenotypic tests (mCIM with eCIM) in correlation with the results of molecular detection of the 5 commonly encountered carbapenemase -encoding genes; bla_{KPC}, bla_{OXA-48}, bla_{NDM-1}, bla_{VIM}, and bla_{IMP}.

METHODOLOGY

The study involved 150 isolates of Enterobacterales, collected at the microbiology department of the Medical Research Institute from clinical samples over the period From July 2019 to March 2020. The isolates were mostly from urine samples (76, 50.7%), followed by sputum (42, 28%), pus (29, 19.3%), and blood (3, 2%).

Isolates identification was accomplished by conventional biochemical reactions and was confirmed by VITEK2 system. Susceptibility to antimicrobials was tested by Kirby Bauer method, as per the CLSI M100 guidelines, 31st edition, using Oxoid™ disks (Thermo Scientific™). Carbapenem-resistant isolates were screened out, based on displaying resistance to Meropenem, Imipenem and Ertapenem disks. The screened-out isolates were tested by the mCIM with eCIM tests as described in the CLSI M100 guidelines [17]. In brief, a loopful of each test bacteria from blood agar plate was emulsified in 2 tubes each containing two milliliters of TSB (tryptic soy broth). A tube of the two was labelled as eCIM, and 20 µL of 0.5 M EDTA was added to it. A meropenem disk (10-µg) was fully immersed in each the 2 tubes of bacterial emulsion, followed by aerobic incubation for 4 hours at 37 °C. After the end of incubation, the disks were placed over a plate of Muller Hinton agar inoculated by a 0.5 McFarland saline suspension of *E. coli* ATCC® 25922. The plates were overnight incubated aerobically at 37 °C, followed by measurement of the inhibition zone diameters. Results were interpreted as per the CLSI-M100 guidelines.

Regarding molecular analysis, DNA was extracted by boiling method [18]. Briefly, 2 colonies of identical morphology were suspended in 200 µl of sterile distilled water and put in a boiling water bath for 15 minutes, then immediately chilled on ice, followed by centrifugation for 10 minutes at 13,000 RPM. The supernatant served as a template for multiplex PCR reactions.

For PCR, specific primers (Biosearch Technologies, California., USA) were employed to amplify bla_{NDM-1}, bla_{VIM}, and bla_{IMP}, which encode for metallo-ß-Lactamase, in addition to bla_{KPC} and bla_{OXA-48} which encode for serine carbapenemase [19, 20] (**Table S1, supplementary material**). Three multiplex PCR reactions were caried out; the first one employed the primers for the 3 metallo-ß-lactamase genes, the second employed the primers for the 2 serine carbapenemase genes, and the third one was a confirmatory reaction that employed primers of genes from the two groups to confirm their co-existence.

Each 15 µl PCR reaction-mixture contained 7.5 µl of HotStarTaq DNA Polymerase (Qiagen, Valencia, CA, USA), 0.5 µl of each primer (10 picomole), and 0.5 µl of extracted DNA. The thermal cycling conditions for all multiplex PCR reactions were 15 minutes of initial denaturation at 95 °C, 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 40 seconds and extension at 72 °C for 50 seconds, followed by 1 cycle of final extension at 72 °C for 10 minutes.

Agarose gel electrophoresis using 1.5% (w/v) agarose gel in TAE buffer (Tris-Acetate-EDTA) stained with 0.5 µg/ml ethidium bromide was employed to detect the amplified PCR products. Gene-specific bands were identified by comparison against a 100 bp DNA

ladder (ThermoScientific, California., USA). The bands were visualized on a 302 nm UV-transilluminator.

Phenotypic tests (mCIM with eCIM) results were correlated with PCR results, to evaluate their sensitivity and specificity, and the added value of performing them compared to mere screening for carbapenem resistance by Kirby Bauer method followed by PCR-screening for common carbapenemase-production genes.

RESULTS

Isolates identification and antimicrobial susceptibility testing results

The 150 screened isolates of Enterobacterales included *K. pneumoniae* 81/150 (54 %), *E. coli* 49/150 (32.7 %), *Klebsiella oxytoca* 9/150 (6%), *Citrobacter* spp. 8/150 (5.3%), and *Enterobacter* spp. 3/150 (2%).

The highest resistance within the 150 isolates was to penicillins, cephalosporins, and trimethoprim-sulfamethoxazole, followed by fluroquinolones. The numbers of isolates with resistance to aminoglycosides and to carbapenems were similar (**Table S2, supplementary material**).

Fifty-three isolates displayed reduced susceptibility to carbapenems by Kirby Bauer method, and accordingly were screened out for subsequent testing. Most CRE were from urine (47.2%), then sputum (30.2%), pus (9.4%), and blood (3.8%).

The 53 CRE displayed marked resistance to most classes of antibiotics. Apart from resistance to β-lactam drugs, resistance to the fluroquinolones was the highest, followed by resistance to trimethoprim-sulfamethoxazole, tetracyclines, and aminoglycosides (**Table S3, supplementary material**).

K. pneumoniae was the commonest among CRE 37/53 (70%) mainly preceding *E. coli* 15/53 (28%). The remaining isolate of CRE was identified as *K. oxytoca* 1/53 (2%).

Results of phenotypic testing by mCIM with eCIM

Detection of carbapenemase and determination of the category of carbapenemase produced by mCIM with eCIM tests could be achieved for only 44/53 CRE (83%).

Carbapenemase production tests (mCIM with eCIM) results revealed that 28 (52.8%) isolates were metallo-β-lactamase producers **(Fig. 1)**, including 19 isolates of *K. pneumoniae* and 9 isolates of *E. coli*. On the other hand, 16 (30.2%) isolates were producers of serine carbapenemase **(Fig. 2)**, including 15 isolates of *K. pneumoniae* and 1 isolate of *K. oxytoca*. Five isolates (9.4%) gave inconclusive results, and 4 isolates (7.5%) were carbapenemase negative by phenotypic tests **(Fig. 2)** despite being unsusceptible to carbapenems by disk diffusion method. A statistically significant association between bacterial species and carbapenemase production was detected, $p < .001$ (**Table 1**).

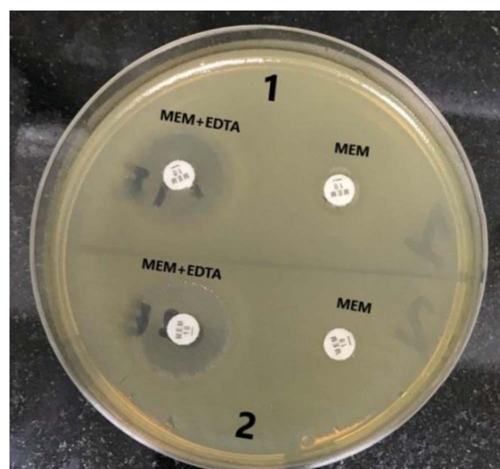

Fig. 1: Metallo-β-lactamase production by the mCIM with eCIM test. Muller Hinton agar plate is inoculated by E. coli ATCC® 25922. Meropenem disks immersed and 4 hours incubated (37 °C) with isolate 1 (upper part) and 2 (Lower part) show inactivation of the disk by carbapenemase (right), and inhibition of carbapenemase by EDTA (left).

Fig. 2: Serine carbapenemase production mCIM with eCIM test (lower part). Muller Hinton agar plate is inoculated by E. coli ATCC® 25922. Meropenem disks were immersed and 4 hours incubated (37 °C) with isolate 72 (lower part) and isolate 89 (upper part). Meropenem disk was inactivated by carbapenemase production by isolate 72 (right) with failure of carbapenemase inhibition by EDTA (left). Isolate 89 (upper part) was negative for carbapenemase production with failure of inactivation of meropenem disk as indicated by the zone diameter (≥19mm).

Table 1: Relationship between the species of Enterobacterales and the result of phenotypic detection of carbapenemase production by mCIM with eCIM tests

Species of Enterobacterales	Carbapenemase production by mCIM with eCIM test								$\chi^{2\ a}$	^{MC}p [b]
	Serine Carbapenemase (n = 16)		Metallo-β-lactamase (n = 28)		Negative (n = 4)		Inconclusive (n = 5)			
	No.	%	No.	%	No.	%	No.	%		
***K. pneumoniae* (n=37)**	15	93.6	19	67.9	3	75.0	0	0.0	22.576*	< .001*
***E. coli* (n=15)**	0	0.0	9	32.14	1	25.0	5	100.0		
***K. oxytoca* (n=1)**	1	6.3	0	0.0	0	0.0	0	0.0		
Total No. (%)	53 (100.0)									

[a] χ^2: Chi square test
[b] MC: Monte Carlo, *p*: *p* value for comparing between different categories
*: Statistically significant at $p \leq .05$

Results of PCR detection of carbapenemase genes

All 53 carbapenem-resistant isolates by disk diffusion method harbored at least one of the 5 common carbapenemase genes.

Amplification of the 3 Metallo-β-lactamases genes (bla_{NDM-1}, bla_{VIM}, and bla_{IMP}) revealed the existence of bla_{NDM-1} gene in 30/53 isolates (56.6%), whereas bla_{VIM}, and bla_{IMP} were undetected in all isolates. As for the serine β-lactamase genes, bla_{OXA-48} existed in 41/53 isolates (77.4%), while bla_{KPC} was not amplified in any of the tested CRE. Both of bla_{NDM-1} and bla_{OXA-48} genes co-existed in 18/53 isolates (34 %).

The bla_{OXA-48} gene was amplified in 29/37 (78%) of the carbapenem resistant *K. pneumoniae*, either alone in 15/37 (40.5%), or together with the bla_{NDM-1} in 14/37 (37.8%). The 8 remaining isolates of carbapenem resistant *K. pneumoniae* (21.6%) carried only the bla_{NDM-1} gene.

The gene bla_{OXA-48} was found in 11/15 (73.3%) carbapenem resistant *E. coli.* It was detected alone in 7/15 (46.7%), and alongside bla_{NDM-1} gene in 4/15 (26.7%). The bla_{NDM-1} gene was solely found in the remaining 4 isolates of *E. coli.* The only isolate of carbapenem resistant *Klebsiella oxytoca* carried bla_{OXA-48} gene (**Table 2**).

Table 2: Carbapenemase-encoding genes among different species of CRE

	bla_{OXA-48} (n = 23)		bla_{KPC} (n = 0)		bla_{NDM-1} (n = 12)		bla_{VIM} (n = 0)		bla_{IMP} (n = 0)		bla_{OXA-48} bla_{NDM} (n = 18)	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
***E. coli* (n=15)**	7	30.4	0	0.0	4	33.3	0	0.0	0	0.0	4	22.2
***K. pneumoniae* (n=37)**	15	65.2	0	0.0	8	66.7	0	0.0	0	0.0	14	77.8
***K. oxytoca* (n=1)**	1	4.4	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
Total No. (%)	53 (100.0)											

Correlation between phenotypic and genotypic results

bla_{NDM-1} gene alone was detected in 8 isolates phenotypically identified as metallo-beta-lactamase producers, 2 isolates phenotypically identified as serine carbapenemase producers, 1 isolate negative for carbapenemase production phenotypically by mCIM with eCIM tests and 1 isolate with inconclusive result by these phenotypic tests.

bla_{OXA-48} gene alone was found in 14 isolates phenotypically identified as serine beta-lactamase producers. It was also detected in 2 metallo-beta-lactamase producers, 3 isolates negative for β-lactamase production and 4 isolates with inconclusive results by mCIM and eCIM.

Both bla_{NDM-1} and bla_{OXA-48} were found in 18 Metallo-β-lactamase producers (**Fig. 3**), but the 2 genes did not co-exist in any of the serine beta-lactamase producers or any of the CRE with negative or inconclusive results.

The existence of bla_{OXA-48} gene was statistically significantly higher among isolates displaying the serine carbapenemase ($P < .001$). The co-existence of the two genes bla_{NDM-1} and bla_{OXA-48} together was statistically significantly higher among isolates displaying metallo-β-lactamase production phenotypically ($P < .001$). Regarding the genotypic findings in the isolates with negative and inconclusive phenotypic test results, we found that among the 4 isolates which were negative, 1 (25.0%) was carrying bla_{NDM-1} gene, and 3 (75.0%) were carrying bla_{OXA-48} gene. As for the 5 isolates with inconclusive phenotypic results; 1 (20.0%) was carrying bla_{NDM-1} gene, and 4 (80.0%) were carrying bla_{OXA-48} gene (**Table 3**).

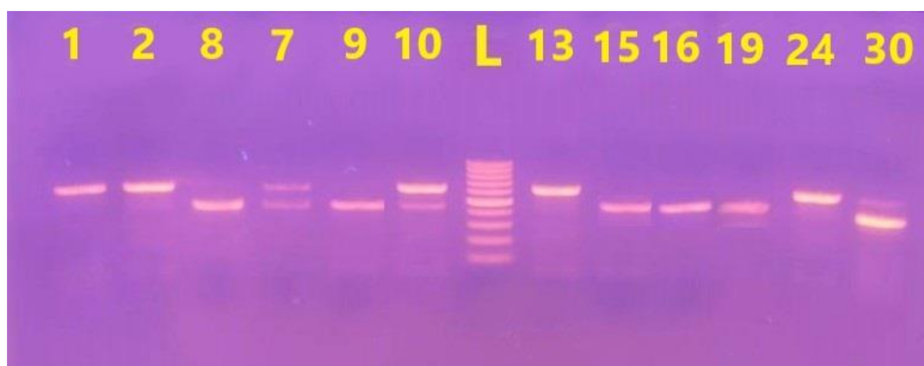

Fig. 3: Ethidium bromide-stained agarose gel showing amplicons from multiplex PCR of metallo-β-lactamase and serine carbapenemase genes and the 100bp DNA ladder (L) (ThermoScientific, California., USA). Bands of bla_{NDM-1} (621 bp) are detected alone in isolates (1,2,13, and 24). Bands of bla_{OXA-48} (438 bp) are detected alone in isolates (8,9,15,16, and 19). Both bands are detected in isolates (7,10, and 30).

Table 3: The Relationship between Phenotypic test (mCIM with eCIM) and Genotypic test results among carbapenem-resistant Enterobacterales

Multiplex PCR results	Phenotypic mCIM with eCIM test result								$\chi^{2\ a}$	^{MC}p [b]
	Serine carbapenemase (n = 16)		Metallo-β-lactamase (n = 28)		Negative (n = 4)		Inconclusive (n = 5)			
	No.	%	No.	%	No.	%	No.	%		
***bla**$_{NDM-1}$* (n=12)	2	12.5	8	28.6	1	25.0	1	20.0	1.736	0.708
***bla**$_{VIM}$* (n= 0)	0	0.0	0	0.0	0	0.0	0	0.0	–	–
***bla**$_{IMP}$* (n= 0)	0	0.0	0	0.0	0	0.0	0	0.0	–	–
***bla**$_{OXA-48}$* (n=23)	14	87.5	2	7.14	3	75.0	4	80.0	34.264*	< .001*
***bla**$_{KPC}$* (n= 0)	0	0.0	0	0.0	0	0.0	0	0.0	–	–
***bla**$_{OXA-48}$* **and** ***bla**$_{NDM-1}$* (n=18)	0	0.0	18	64.3	0	0.0	0	0.0	24.660*	< .001*
Total No. (%)	53 (100.0)									

[a] χ^2: Chi square test
[b] MC: Monte Carlo, *p*: *p* value for comparing between different categories
*: Statistically significant at $p \leq .05$

Calculation of the sensitivity and specificity of mCIM with eCIM phenotypic tests

The sensitivity of mCIM with eCIM tests for detecting serine carbapenemase potential producers was calculated to be 34.14 %, whereas their specificity was 83.33%. The positive predictive value (PPV) was 87.5%, and the negative predictive value (NPV) was 27.03 %. On the other hand, the sensitivity of these tests for detection of Metallo-β-lactamase production was 86.67%, and their specificity was 91.3 %. Their PPV was 92.86 %, and the NPV was 84% **(Table 4)**.

Table 4: Sensitivity and specificity of mCIM with eCIM for detecting serine carbapenemase and metallo-β-lactamase potential producers

	TP [a]	FP [b]	FN [c]	TN [d]	Sensitivity (%)	Specificity (%)	PPV [e] (%)	NPV [f] (%)
Serine carbapenemase production by mCIM with eCIM	14	2	27	10	34.14 %	83.33%	87.5%	27.03 %
Metallo-β-lactamase production by mCIM with eCIM	26	2	4	21	86.67%	91.3 %	92.86 %	84%

[a] TP: true positive, [b] FP: false positive, [c] FN: false negative, [d] TN: true negative
[e] PPV: positive predictive value, [f] NPV: negative predictive value

DISCUSSION

Despite their documented broad-spectrum antimicrobial effect against Enterobacterales and many other species of medically important bacteria, carbapenems are increasingly becoming ineffective antibacterial drugs against clinical isolates of Enterobacterales, particularly in geographic regions where the prescription of antimicrobials is unrestricted. This is appertaining to the rapidly rising incidence of resistance to these drugs, with an associated increase in morbidity and mortality [21]. CRE are commonly isolated from healthcare-associated infections and also community-acquired infections. Horizontal transfer of carbapenemase genes widely occurs whilst CRE clonally spread from an infected patient to another [22].

Timely and reliable identification of CRE in the microbiology laboratory is indispensable for limiting their spread, by initiation of an alternative effective therapeutic plan [23].

Historically, the CLSI recommended the "modified Hodge test" as confirmatory tests for Carbapenemase-production [24]. However, starting from 2009, it has been declined. Meanwhile, the CLSI still recommend phenotypic tests such as mCIM with eCIM for epidemiologic surveillance of isolates with low susceptibility to carbapenems [17].

With carbapenemase production being the most wide-spread resistance mechanism to carbapenems in Enterobacterales, and considering the sufficient sensitivity of Kirby Bauer method to detect insusceptibility to carbapenems [6]; this study aimed to assess the sensitivity, specificity, and added value of performing mCIM with eCIM in identifying carbapenemase production and differentiation between serine-carbapenemase and Metallo-β-lactamase enzymes produced by clinical isolates of Enterobacterales.

Relying on the updated CLSI breakpoints for testing the susceptibility of bacteria to carbapenems by Kirby Bauer method has been reported to be of a satisfactory level of sensitivity and specificity. In this aspect, meropenem is preferred to imipenem and ertapenem as an indicator drug. This is owing to Imipenem's liability to resistance by various mechanisms, while Ertapenem being less liable to hydrolysis by non-KPC carbapenemase [23, 24]. Consistently, our results revealed a slightly higher degree of resistance to Imipenem and Ertapenem (35.3%) compared with Meropenem (34%) by Kirby Bauer method. Owing to the negligible difference, all isolates displaying resistance to any of the 3 carbapenem drugs were screened out as CRE, for subsequent phenotypic and genotypic analysis.

The marked resistance to antimicrobials among Enterobacterales, and the relatively high abundance of carbapenem resistance is a common problem in Egypt. Two recent Egyptian studies in Suez and Cairo governorates reported that carbapenems' resistance was 34% in the former, which is like our results, and it ranged from 57% to 66.8% in the latter [25, 26].

Egypt may be considered as the African country with the highest spread of CRE. Several studies from other African countries reported the prevalence of CRE to be 28.6% in Uganda [27], 15.2% in Nigeria [28], 12.12% in Ethiopia [29].

Meanwhile, aminoglycosides and fluroquinolones cannot be used as a frontline therapy for CRE, a fact that further limits the available treatment choices for infection by these multidrug resistant organisms. The marked resistance to aminoglycosides and fluroquinolones encountered with our 53 CRE was similarly reported in other Egyptian studies and in studies from several regions around the world. In Cairo, it was reported that 93.3% of their CRE resisted aminoglycosides, and 80% were resistant to fluroquinolones [30]. In addition, a study in Italy reported elevated resistance to aminoglycosides among CRE, that was approaching 80% [31]. Moreover, a study in Shanghai reported that the susceptibility of their CRE isolates to aminoglycosides did not exceed 13 % [32].

The prevalence of serine and metallo β-lactamase production in CRE is highly subject to regional variation. The phenotypic subcategorization of our isolates based on the mCIM with eCIM test results revealed that the production of Metallo-β-lactamase (52.8%) was higher than serine β-lactamase (30.2%). Meanwhile, another Egyptian study stated that Serine-carbapenemase was produced by 37.2 % of their CRE examined isolates, while 30.2% produced Metallo-β-lactamase [25]. Meanwhile, in Taiwan, Wang et al. reported 61.35% of their CRE isolates were Serine-carbapenemase producers, while only 12.9% were metallo-β-lactamase producers by mCIM with eCIM tests [33]. Similarly, in Austria, Segagni Lusignani reported that among their CRE isolates, 63.8% produced serine carbapenemase and only 27.6% produced Metallo-β-lactamase [34].

Among the 53 CRE in our study, *Klebsiella pneumoniae* was the most predominant species, representing 69.8%, followed by *E. coli* (28.3%) and *Klebsiella oxytoca* (1.9%). Similarly, in Turkey, Baran and Aksu reported that the most common CRE species was *Klebsiella pneumoniae* (38.12 %), followed by *E. coli* (7.18 %) and *Klebsiella oxytoca* (0.55 %) [35]. On the contrary, a study in Nigeria reported that the most common CRE species were *K. oxytoca* 41%, then *K. pneumoniae* 32% and *E. coli* 27% [36].

At the genome level, PCR detection of the 5 common carbapenemase-encoding genes revealed that 43.4% of our CRE carried the bla_{OXA-48} gene solely, 34% carried both bla_{NDM-1} and bla_{OXA-48} genes, and 22.6% carried bla_{NDM-1} gene. The bla_{VIM}, bla_{IMP} and bla_{KPC}

genes were not amplified in any of the CRE under study. Similarly, a study in Cairo reported that the genes *bla*$_{NDM-1}$ and *bla*$_{OXA-48}$ were the most predominant, and none of their isolates carried *bla*$_{IMP}$ nor *bla*$_{KPC}$ genes. However, *bla*$_{VIM}$ was amplified at a low incidence (2.3%) among their isolates [37]. Similar results were encountered with other studies in Egypt, Saudi Arabia, and Turkey [25, 26, 35, 38-41].

In Uganda, however, the 5 genes under study were detected in CRE. The most prevailing gene was *bla*$_{VIM}$ (10.7%), followed by *bla*$_{OXA-48}$ (9.7%), *bla*$_{IMP}$ (6.1%), *bla*$_{KPC}$ (5.1%), and *bla*$_{NDM-1}$ (2.6%) [27]. Meanwhile, it was reported in China that *bla*$_{KPC}$ was the most amplified carbapenemase gene in *K. pneumoniae* (77%), while *bla*$_{NDM-1}$ was predominant in *E. cloacae* (53%) and *E. coli* (75%) [42].

Correlation between phenotypic and genotypic test results of our 53 CRE revealed that out of the 16 isolates displaying serine carbapenemase phenotypically, 14 (87.5%) were carrying *bla*$_{OXA-48}$ gene, and 2 (12.5%) were solely carrying the metallo-β-lactamase encoding gene *bla*$_{NDM-1}$. The phenotypic expression of serine carbapenemase by the latter 2 isolates might be explained by the existence of other genes that encode for serine carbapenemase which were preferentially expressed over the *bla*$_{NDM-1}$ gene. In this case the existence of *bla*$_{NDM-1}$ without being preferentially expressed has resulted in false negative phenotypic result for metallo-β-lactamase enzyme production by mCIM with eCIM test.

Out of the 28 metallo-β-lactamase producing isolates phenotypically, only 8 (28.6%) were solely carrying the *bla*$_{NDM-1}$ gene, while 2 (7.14%) were carrying solely the serine carbapenemase encoding *bla*$_{OXA-48}$ gene. This might also be explained by the existence of other genes that encode for metallo-β-lactamase in these 2 isolates which were preferentially expressed over the *bla*$_{OXA-48}$ gene. In this case the presence of *bla*$_{OXA-48}$ gene without being expressed has resulted in false negative phenotypic result for serine carbapenemase enzyme by mCIM with eCIM test.

Most of CRE identified as metallo-β-lactamase producers phenotypically (18/28, 64.3%) were carrying the 2 genes *bla*$_{NDM-1}$ and *bla*$_{OXA-48}$ together. This indicated that when a bacterial isolate carries both genes, it usually tests positive for metallo-β-lactamase enzyme phenotypically by mCIM with eCIM tests, and this was statistically significant ($P < .001$). Thus, with the co-production of a serine carbapenemase and a metallo-β-lactamase by the same organism, differentiation between enzymes is not achievable by this phenotypic method. The limited sensitivity of the CIM in detecting both *bla*$_{NDM-1}$ and *bla*$_{OXA-48}$ type producers was previously reported [43, 44].

Regarding PCR deciphering of the determinants behind the insusceptibility to carbapenems in isolates with negative and inconclusive phenotypic test results; among the 4 isolates that tested negative phenotypically, 1 (25.0%) was carrying *bla*$_{NDM-1}$ gene, and 3 (75.0%) were carrying *bla*$_{OXA-48}$ gene, inferring that this was a false negative result. PCR assessment of the 5 isolates with inconclusive phenotypic test results also revealed that 1 isolate (20.0%) was carrying *bla*$_{NDM-1}$ gene, and 4 (80.0%) were carrying *bla*$_{OXA-48}$ gene. This reflects the relatively limited capability of the mCIM with eCIM method in detecting carbapenemase enzyme production.

Our calculated sensitivity and specificity values for serine-carbapenemase detection by mCIM were 34.14% and 83.33% respectively. The percentages were higher for metallo-β-lactamase detection by mCIM with eCIM, as they were 86.67% and 91.3 %, respectively. Overall, these values are less than those reported by previous studies. For instance, Tsai et al. [44] reported sensitivity and specificity values of 100% for serine-carbapenemase detection by mCIM, and a sensitivity of 89.3% for metallo-β-lactamase detection by mCIM with eCIM, with 98.7% specificity. Similarly, Hu et al. [45] reported results of 100% for sensitivity and specificity of mCIM in detecting serine carbapenemase, and the same value for the specificity of metallo-β-lactamase detection by mCIM with eCIM tests. Their reported sensitivity for the latter test was 92.9%.

CONCLUSION

Accordingly, the negative and inconclusive results of carbapenemase phenotypic detection tests (mCIM with eCIM) in some isolates (9/53, 17%) cannot be guaranteed as true negative and cannot be ignored, as the isolates at the genome level did carry the genes of carbapenemase enzymes and were not susceptible to inhibition by carbapenems by Kirby Bauer method.

Molecular amplification of the common carbapenemase-encoding genes is much more reliable in detecting the categories of carbapenemases produced by bacteria that display insusceptibility to carbapenems. This might lead to the conclusion that carbapenem susceptibility testing by Kirby Bauer method might be sufficient to point to the possibility of production of carbapenemase enzymes by Enterobacterales, and the phenotypic tests mCIM with eCIM could lead to confounding results and false conclusions about the category of carbapenemase produced by CRE.

This manuscript has not been previously published and is not under consideration in the same or substantially similar form in any other reviewed media. I have contributed sufficiently to the project to be included as author. To the best of my knowledge, no conflict of interest, financial or others exist. All authors have participated in the concept and design, analysis, and interpretation of data, drafting and revising of the manuscript, and that they have approved the manuscript as submitted.

Acknowledgment

The authors acknowledge Dr. Asmaa Abdel Hameed Ahmed, Lecturer of Biomedical Informatics and Medical statistics for performing statistical analysis of data using the Statistical Package for Social Sciences (SPSS ver.20 Chicago, Il, USA).

Statements and Declarations

Funding

The authors did not receive any funds, grants, or other support for conducting this study.

Conflict of Interest

The authors declare that there is no conflict of interest. All authors revised and approved the submission of the manuscript. The article is not published and not under consideration for publication elsewhere.

Author Contributions:

Aliaa Gamaleldin Aboulela: Conceived the original idea, planned and supervised the experiments, worked out almost all of the technical details, performed the analysis, discussed the results, and wrote the final manuscript with input from all authors. **Mawj Fahad Jabbar:** Carried out the experiments, discussed the results, drafted the work, and approved the final version to be published. **Abdelfattah Hammouda:** Supervised the findings of this work, revised the work for important intellectual content, and approved the final version to be published

Medhat Saber Ashour: Supervised the findings of this work, revised the work critically for important intellectual content, discussed the results, and approved the final version to be published. All authors agree to be accountable for all aspects of the work.

Ethics approval

The research design has been approved by the Ethics Committee of the High Institute of Public Health, and the Ethics Committee of the Medical Research Institute, Alexandria University in March 2020 (IORG0008812). All experiments were performed in accordance with relevant guidelines and regulations. The study does not involve any humans or animals. The study was performed on clinical isolates obtained from samples that were submitted to the Microbiology laboratory of the Medical Research Institute for routine analysis, with anonymization of patients' data.

REFERENCES

1. Codjoe FS, Donkor ES (2017) Carbapenem resistance: a review. Med. Sci. 6(1):1. https://doi.org/10.3390/medsci6010001
2. Papp-Wallace KM, Endimiani A, Taracila MA, Bonomo RA (2011) Carbapenems: past, present, and future. Antimicrob Agents Chemother 55(11):4943-60. https://doi.org/10.1128/AAC.00296-11
3. Kattan J, Villegas M, Quinn J. (2008) New developments in carbapenems. Clin Microbiol Infect 14(12):1102-11. https://doi.org/10.1111/j.1469-0691.2008.02101.x
4. Chahine EB, Ferrill MJ, Poulakos MN (2010) Doripenem: a new carbapenem antibiotic. Am J Health Syst Pharm 67(23):2015-24. https://doi.org/ 10.2146/ajhp090672
5. Hansen GT (2021) Continuous evolution: perspective on the epidemiology of carbapenemase resistance among enterobacterales and other gram-negative bacteria. Infect Dis Ther 10(1):75-92. https://doi.org/10.1007/s40121-020-00395-2
6. Suay-García B, Pérez-Gracia MT (2021) Present and Future of Carbapenem-Resistant Enterobacteriaceae Infections. In: Advances in Clinical Immunology, Medical Microbiology, COVID-19, and Big Data, 1st ed, Jenny Stanford Publishing, pp 435-56. eBook ISBN 9781003180432.
7. Sawa T, Kooguchi K, Moriyama K (2020) Molecular diversity of extended-spectrum β-lactamases and carbapenemases, and antimicrobial resistance. j intensive care 8(1):1-13. https://doi.org/10.1186/s40560-020-0429-6
8. Rada AM, De La Cadena E, Agudelo C, Capataz C, Orozco N, Pallares C, Dinh AQ, Panesso D, Ríos R, Diaz L, Correa A, Hanson BM, Villegas MV, Arias CA, Restrepo E (2020) Dynamics of blaKPC-2 dissemination from non-CG258 *Klebsiella pneumoniae* to other Enterobacterales via IncN plasmids in an area of high endemicity. Antimicrob Agents Chemother 64:e01743-20. https://doi.org/10.1128/AAC.01743-20.
9. Nordmann P, Naas T, Poirel L (2011) Global spread of carbapenemase-producing Enterobacteriaceae. Emerg Infect Dis. 17(10):1791. http://dx.doi.org/ 10.3201/eid1710.110655
10. Nockelmann L, Cremanns M, Gatermann SG, Pfennigwerth N (2021) Evaluation of the rCIM for carbapenemase detection in Enterobacterales and Pseudomonas aeruginosa and description of the TSBrCIM, an optimized variant. J Microbiol Methods 190:106345. https://doi.org/10.1016/j.mimet.2021.106345
11. Queenan AM, Bush K (2007) Carbapenemases: the versatile β-lactamases. Clin. Microbiol. Rev. 20(3):440-58. https://doi.org/10.1128/CMR.00001-07
12. Nordmann P, Poirel L, Toleman MA, Walsh TR (2011) Does broad-spectrum β-lactam resistance due to NDM-1 herald the end of the antibiotic era for treatment of infections caused by Gram-negative

bacteria? J Antimicrob Chemother. 66(4):689-92. https://doi.org/10.1093/jac/dkq520

13. Tamma PD, Doi Y, Bonomo RA, Johnson JK, Simner PJ (2019) A primer on AmpC β-lactamases: necessary knowledge for an increasingly multidrug-resistant world. Clin Infect Dis. 69(8):1446-55. https://doi.org/10.1093/cid/ciz173.
14. Marsik FJ, Nambiar S (2011) Review of carbapenemases and AmpC-beta lactamases. Pediatr Infect Dis J. 30(12):1094-5. https://doi.org/10.1097/INF.0b013e31823c0e47
15. Mitteregger D, Wessely J, Barišić I, Bedenić B, Kosak D, Kundi M (2022) A Variant Carbapenem Inactivation Method (CIM) for Acinetobacter baumannii Group with Shortened Time-to-Result: rCIM-A. Pathogens 11(4):482. https://doi.org/ 10.3390/ pathogens11040482.
16. Al-Zahrani IA (2018) Routine detection of carbapenem-resistant gram-negative bacilli in clinical laboratories: A review of current challenges. Saudi Med J. 39(9):861. https://doi.org/10.15537/ smj.2018.9.22840
17. CLSI. Performance Standards for Antimicrobial Susceptibility Testing. 31st ed. CLSI supplement M100 ISBN 978-1-68440-105-5 [Electronic] Clinical and Laboratory Standards Institute, USA2021.
18. Purighalla S, Esakimuthu S, Reddy M, Varghese GK, Richard VS, Sambandamurthy VK (2017) Discriminatory power of three typing techniques in determining relatedness of nosocomial Klebsiella pneumoniae isolates from a tertiary hospital in India. Indian J Med Microbiol. 35(3):361-8. https://doi.org/10.4103/ijmm.IJMM_16_308
19. Hatrongjit R, Kerdsin A, Akeda Y, Hamada S. (2018) Detection of plasmid-mediated colistin-resistant and carbapenem-resistant genes by multiplex PCR. MethodsX. 5:532-6. https://doi.org/10.1016/j.mex.2018.05.016
20. Poirel L, Hombrouck-Alet C, Freneaux C, Bernabeu S, Nordmann P. (2010) Global spread of New Delhi metallo-β-lactamase 1. Lancet Infect Dis. 10(12):832. https://doi.org/10.1016/S1473-3099(10)70279-6
21. Xie S, Fu S, Li M, Guo Z, Zhu X, Ren J, Hu F (2020) Microbiological characteristics of carbapenem-resistant Enterobacteriaceae clinical isolates collected from county hospitals. Infect Drug Resist. 13:1163. https://doi.org/10.2147/IDR. S248147
22. Pollett S, Miller S, Hindler J, Uslan D, Carvalho M, Humphries RM (2014) Phenotypic and molecular characteristics of carbapenem-resistant Enterobacteriaceae in a health care system in Los Angeles, California, from 2011 to 2013. J Clin Microbiol. 52(11):4003-9. https://doi.org/10.1128/ JCM.01397-14
23. Maurer FP, Castelberg C, Quiblier C, Bloemberg GV, Hombach M (2015) Evaluation of carbapenemase screening and confirmation tests with Enterobacteriaceae and development of a practical diagnostic algorithm. J Clin Microbiol. 53(1):95-104. https://doi.org/10.1128/JCM.01692-14
24. Willems E, Verhaegen J, Magerman K, Nys S, Cartuyvels R (2013) Towards a phenotypic screening strategy for emerging β-lactamases in Gram-negative bacilli. Int J Antimicrob Agents 41(2):99-109. https://doi.org/10.1016/j.ijantimicag.2012.07.006
25. Raheel A, Azab H, Hessam W, Abbadi S, Ezzat A (2020) Detection of carbapenemase enzymes and genes among carbapenem-resistant Enterobacteriaceae isolates in Suez Canal University Hospitals in Ismailia, Egypt. Microbes and Infectious Diseases. 1(1):24-33. https://doi.org/ 10.21608/mid.2020.25702.1007
26. Tawfick MM, Alshareef WA, Bendary HA, Elmahalawy H, Abdulall AK (2020) The emergence of carbapenemase blaNDM genotype among carbapenem-resistant Enterobacteriaceae isolates from Egyptian cancer patients. Eur J Clin Microbiol Infect Dis. 39(7):1251-9. https://doi.org/10.1007/s10096-020-03839-2
27. Okoche D, Asiimwe BB, Katabazi FA, Kato L, Najjuka CF (2015) Prevalence and characterization of carbapenem-resistant Enterobacteriaceae isolated from Mulago National Referral Hospital, Uganda. PloS one. 10(8):e0135745. https://doi.org/10.1371/journal.pone.0135745
28. Oduyebo O, Falayi O, Oshun P, Ettu A (2015) Phenotypic determination of carbapenemase producing Enterobacteriaceae isolates from clinical specimens at a tertiary hospital in Lagos, Nigeria. Niger Postgrad Med J. 22(4):223. https://doi.org/10.4103/1117-1936.173973
29. Legese MH, Weldearegay GM, Asrat D (2017) Extended-spectrum beta-lactamase-and carbapenemase-producing Enterobacteriaceae among Ethiopian children. Infect Drug Resist. 10:27-34. https://doi.org/10:27. 10.2147/IDR. S127177
30. Hager R, Ahmed S, Salam A, Abo N, Magd E. (2019) Emergence of carbapenem resistant Enterobacteriaceae coharboring New Delhi metallo-β-lactamase (NDM-1) and Klebsiella pneumoniae carbapenemase (KPC) isolated from a tertiary hospital in Egypt. Int. J. Appl. Microbiol. Biotechnol. Res. 7:86-94. https://doi.org/10.33500/ ijambr.2019.07.011

31. Capone A, Giannella M, Fortini D, Giordano A, Meledandri M, Ballardini M, et al. (2013) High rate of colistin resistance among patients with carbapenem-resistant Klebsiella pneumoniae infection accounts for an excess of mortality. Clin Microbiol Infect. 19(1):E23-E30. https://doi.org/10.1111/1469-0691.12070
32. Hu F, Chen S, Xu X, Guo Y, Liu Y, Zhu D, et al. (2012) Emergence of carbapenem-resistant clinical Enterobacteriaceae isolates from a teaching hospital in Shanghai, China. J Med Microbiol. 61(1):132-6. https://doi.org/10.1099/jmm.0.036483-0
33. Wang J-T, Wu U-I, Lauderdale T-LY, Chen M-C, Li S-Y, Hsu L-Y, et al. (2015) Carbapenem-nonsusceptible enterobacteriaceae in Taiwan. PloS one. 10(3):e0121668. https://doi.org/10.1371/journal.pone.0121668
34. Segagni Lusignani L, Presterl E, Zatorska B, Van den Nest M, Diab-Elschahawi M (2020) Infection control and risk factors for acquisition of carbapenemase-producing enterobacteriaceae. A 5 year (2011–2016) case-control study. Antimicrob Resist Infect Control 9(1):1-10. . https://doi.org/10.1186/s13756-019-0668-2
35. Baran I, Aksu N (2016) Phenotypic and genotypic characteristics of carbapenem-resistant Enterobacteriaceae in a tertiary-level reference hospital in Turkey. Ann Clin Microbiol Antimicrob.15(1):1-11. https://doi.org/10.1186/s12941-016-0136-2
36. Oli AN, Itumo CJ, Okam PC, Ezebialu IU, Okeke KN, Ifezulike CC, et al. (2019) Carbapenem-resistant Enterobacteriaceae posing a dilemma in effective healthcare delivery. Antibiotics (Basel). 8(4):156. https://doi.org/10.3390/antibiotics8040156
37. Shawky AM, Tolba S, Hamouda HM (2019) Emergence of New Delhi Metallo Beta Lactamase blaNDM-1 and Oxacillinases blaOXA-48 Producing *Klebsiella pneumoniae* in an Egyptian Hospital. Egyptian Journal of Microbiology. 54(1):25-37. https://doi.org/10.21608/EJM.2019.12395.1096
38. ElMahallawy HA, Zafer MM, Amin MA, Ragab MM, Al-Agamy MH (2018) Spread of carbapenem resistant Enterobacteriaceae at tertiary care cancer hospital in Egypt. Infect Dis (Lond) 50(7):560-4. https://doi.org/10.1080/23744235.2018.1428824
39. Memish ZA, Assiri A, Almasri M, Roshdy H, Hathout H, Kaase M, et al. (2015) Molecular characterization of carbapenemase production among gram-negative bacteria in Saudi Arabia. Microb Drug Resist. 21(3):307-14. https://doi.org/10.1089/mdr.2014.0121
40. Kilic A, Aktas Z, Bedir O, Gumral R, Bulut Y, Stratton C, et al. (2011) Identification and characterization of OXA-48 producing, carbapenem-resistant Enterobacteriaceae isolates in Turkey. Ann Clin Lab Sci. 41(2):161-6. PMID: 21844575.
41. Zowawi HM, Sartor AL, Balkhy HH, Walsh TR, Al Johani SM, AlJindan RY, et al. (2014) Molecular characterization of carbapenemase-producing Escherichia coli and Klebsiella pneumoniae in the countries of the Gulf cooperation council: dominance of OXA-48 and NDM producers. Antimicrob Agents Chemother. 58(6):3085-90. https://doi.org/10.1128/AAC.02050-13
42. Wang Q, Wang X, Wang J, Ouyang P, Jin C, Wang R, et al. (2018) Phenotypic and genotypic characterization of carbapenem-resistant Enterobacteriaceae: data from a longitudinal large-scale CRE study in China (2012–2016). Clin Infect Dis. 67(suppl_2):S196-S205. https://doi.org/10.1093/ cid/ciy660
43. Tamma PD, Opene BN, Gluck A, Chambers KK, Carroll KC, Simner PJ (2017) Comparison of 11 phenotypic assays for accurate detection of carbapenemase-producing Enterobacteriaceae. J Clin Microbiol. 55(4):1046-55. https://doi.org/10.1128/JCM.02338-16
44. Tsai Y-M, Wang S, Chiu H-C, Kao C-Y, Wen L-L (2020) Combination of modified carbapenem inactivation method (mCIM) and EDTA-CIM (eCIM) for phenotypic detection of carbapenemase-producing Enterobacteriaceae. BMC microbiol. 20(1):1-7. https://doi.org/10.1186/s12866-020-02010-3
45. Hu Y, Li J (2021) Clinical application of mCIM and eCIM combined detection of carbapenemase-producing Enterobacteriaceae. Zhonghua Yu Fang Yi Xue Za Zhi 55(4):506-11. https://doi.org/10.3760/cma.j.cn112150-20200821-01142