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ORIGINAL ARTICLE

# Detection of Carbapenemase Producing *Enterobacteriaceae* using the Modified Carbapenem Inactivation Method

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***Key words:***
***Carbapenemases producing Enterobacteriaceae, broth microdilution method, modified carbapenem inactivation method***

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## ABSTRACT

***Background:*** *Carbapenem resistant Enterobacteriaceae (CRE) have been reported worldwide. Resistance to carbapenems in Enterobacteriaceae is caused mainly by carbapenemase production or by porin loss combined with the expression of beta (β) - lactamases like extended-spectrum β-lactamases (ESBL) or ampicillin class C (AmpC).* ***Objectives*** *are to determine the prevalence of carbapenemase-producing Enterobacteriaceae (CPE) among 202 clinical isolates of Enterobacteriaceae by the phenotypic test the modified carbapenem inactivation method (mCIM).* ***Methodology:*** *Initial screening for carbapenemase-producing isolates among the 202 Enterobacteriaceae isolates was done by minimum inhibitory concentration (MIC) determination for ertapenem by broth microdilution method. Confirmation of carbapenemase production among ertapenem-resistant isolates was done by the phenotypic test mCIM.* ***Results****: The prevalence of CRE by broth microdilution method was 36.1% and the prevalence of CPE among resistant isolates was 80.8% by mCIM.* ***Conclusion:*** *The mCIM is inexpensive, easy to perform, requires no specific reagents or media. It could be performed to detect CPE in Enterobacteriaceae that are non-susceptible to one or more carbapenems.*

## INTRODUCTION

*Enterobacteriaceae* are a common cause of both community-acquired and hospital-acquired infections; including urinary tract, blood stream and lower respiratory tract infections. There is a dramatic increase in the rate of antibiotic resistance among these pathogens that has reached a pandemic scale. [1]

Carbapenems served as the last line of defense against multidrug-resistant Gram-negative organisms since their introduction in the early 1980s[2]. They are the most broad-spectrum β-lactams active against Gram-negative organisms and very slowly hydrolyzed by most β-lactamases[2].

The reporting of carbapenem resistance among *Enterobacteriaceae* is increasing throughout the world, due to the wide spread of bacterial carbapenemases. The carbapenemases observed among *Enterobacteriaceae* have a broad spectrum of hydrolytic activity, including activity against almost all β-lactam antibiotics. Infections caused by CRE are accompanied with high deaths due to narrow treatment choices; treatment options involve antibiotics that are both less effective and more toxic than β-lactams.[3]

Detection of carbapenemases in microbiology laboratories is a challenge; accurate and fast detection methods are importantly needed. The detection of carbapenemases in *Enterobacteriaceae* consists of a screening step followed by a confirmatory step.[4]

The mCIM is a new phenotypic method recommended by the Clinical and Laboratory Standards Institute (CLSI) for detecting carbapenemase activity in *Enterobacteriaceae.*[5]

## METHODOLOGY

**Clinical isolates:**

This study was carried out at the Department of Medical Microbiology and Immunology, Faculty of Medicine, Cairo University during the period from January to May 2018. The study was approved by the Research and Ethical committee of Medical Microbiology and Immunology Department, Faculty of Medicine, Cairo University. The study included 202 different clinical isolates of *Enterobacteriaceae* obtained by cultivation of different clinical specimens: urine, sputum and pus. Specimens were cultivated on MacConkey's agar plates (Oxoid, UK) and incubated aerobically at 37°C for 24-48 hours. Identification of isolates was done according to the conventional microbiological standard tests (Gram's stain, glucose fermentation test and oxidase test). Isolates identified as Gram negative bacilli, glucose fermenters and oxidase negative were considered as *Enterobacteriaceae.* Further identification of *Enterobacteriaceae* genera was done using the following biochemical reactions (Triple sugar iron (TSI), urease test, citrate test, motility indole ornithine (MIO) and lysine iron agar (LIA).[6,7] Isolates

were stored at -80°C by emulsifying a loopful of bacteria in 500 µL of 50% glycerol broth in a 2 mL screw top tube[8]

**Carbapenemase screening test:**

Isolates were screened for carbapenemase production by MIC determination using broth microdilution method for ertapenem. Results were interpreted according to the standard guidelines in (table 1)[9]

**Table 1: The CLSI 2017 ertapenem breakpoints for MIC for *Enterobacteriaceae***

| Ertapenem MIC interpretive standards (µg/ml) | | |
|---|---|---|
| **Susceptible** | **Intermediate Resistant** | **Resistant** |
| **≤ 0.5** | **1** | **≥ 2** |

**Phenotypic confirmatory test for carbapenemase production:**

Isolates that were intermediate or resistant to ertapenem were subjected to the confirmatory mCIM[9]

**The modified carbapenem inactivation method:**

***Test procedure:***

From frozen (-80ºC) stock, each tested isolate was subcultured on Tryptic soy agar (TSA) plate (HiMedia, India) with 5% human blood incubating each subculture in ambient air at 35ºC ± 2ºC for 18-24 hours.[10] For each isolate to be tested, a 1µL loopful of bacteria was emulsified in 2 ml Tryptic soy broth (TSB) in a sterile test tube (HiMedia, India) and the bacterial suspension was vortexed for 10–15 seconds. A 10 µg Meropenem (MEM) disc (Oxoid, UK) was aseptically added to each tube using sterile forceps, the entire disc was fully immersed in the suspension and the tube was then incubated for 4 hours ± 15 minutes at 35ºC ± 2ºC in ambient air. Just prior to completion of the 4 hours carbapenem inactivation step, a suspension of the mCIM 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 a Mueller-Hinton agar (MHA) plate (Oxoid, UK) was inoculated using the procedure for standard disc diffusion susceptibility testing. A sterile cotton swab was dipped into the suspension, rotated several times and pressed tightly on the inside of the wall of the tube to remove excess inoculum from the swab. The swab was then streaked over the entire surface of MHA three times with the plate rotated 60° each time and left to dry. 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 removal to remove excess liquid, and the disc was placed onto the inoculated MHA plate, which was then incubated in an inverted position for 18-24 hours at 35ºC ± 2ºC in ambient air.[9]

***Reading and interpretation:***

The diameter of the zone of inhibition around each MEM disc was measured and interpreted according to the CLSI, 2017 as follows:

- The test was considered positive for carbapenemase production when the diameter of the indicator strain (*E. coli* ATCC 25922) growth-inhibitory zone around MEM disc was 6–15 mm or when colonies of growth were present within a 16–18 mm zone.
- The test was considered negative for carbapenemase production when the diameter of the growth-inhibitory zone around MEM disc was ≥19mm.
- The test was considered indeterminate for carbapenemase production when the diameter of the growth-inhibitory zone around MEM disc was 16-18 mm. [9]

***Statistical analysis:***

Data were coded and entered using computer program SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 25 for Microsoft Windows. Data was summarized using frequencies (number of cases) and relative frequencies (percentages) for categorical variables. For comparing categorical data, Chi square ($\chi 2$) test was performed. Exact test was used instead when the expected frequency is less than 5. P-values less than 0.05 were considered as statistically significant**.**

## RESULTS

**Analysis of clinical isolates:**

Out of the 202 isolated *Enterobacteriaceae*; 104 isolates were obtained from urine specimens, 67 isolates from pus specimens and 31 isolates from sputum specimens .They were identified by the standard biochemical reactions to be 112 *Klebsiella species*, 81 *E. coli*, 5 *Proteus species* and 4 *Enterobacter species* (Figure 1).

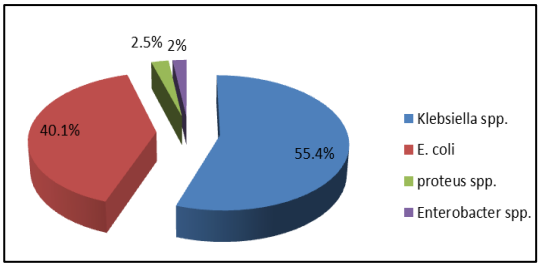


**Fig. 1:** Identification of the 202 *Enterobacteriaceae* isolates

**Carbapenemase screening:**

- ***MIC determination by broth microdilution method:***

By the broth microdilution method ertapenem MIC differed among the 202 *Enterobacteriaceae* isolates; 73

isolates (36.1 %) were resistant to ertapenem; while 129 isolates (63.9%) were sensitive (Figure 2).

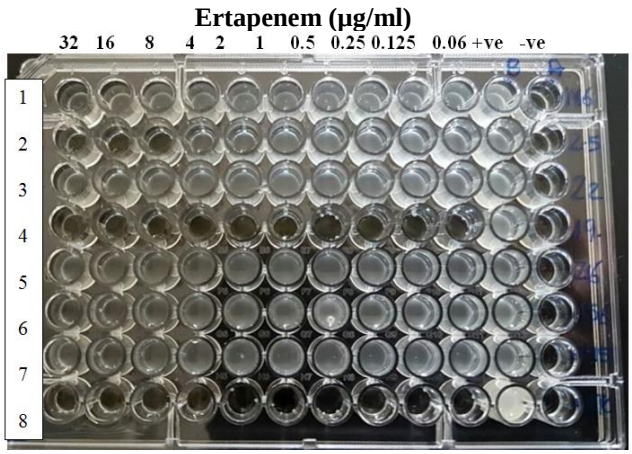


**Fig. 2:** Microtitre plate showing the MICs for tested isolates 1-8 for ertapenem

- Isolates 4 &8 MIC; <0.06 µg/ml (Susceptible)
- Isolates 1, 3 & 5-7 MIC; >32 µg/ml (Resistant)
- Isolate 2 MIC; 8 µg/ml (Resistant)

The prevalence of carbapenem resistance showed a statistically significant difference among different members of the 202 *Enterobacteriaceae* isolates; 57.1% among *Klebsiella species* Isolates (64/112), 25% among *Enterobacter species* isolates (1/4), 9.9% among *E. coli* isolates (8/81) and 0% among *proteus species* isolates (0/5) (Table 2).

**Table 2: Comparison of the prevalence of carbapenem resistance among different members of the 202 *Enterobacteriaceae* isolates**

| Organism | No. | Ertapenem MIC | | P value |
|---|---|---|---|---|
| | | Resistant | Sensitive | |
| ***Klebsiella species*** | **112** | 64 (57.1%) | 48 (42.9%) | **<0.001** |
| ***E. coli*** | **81** | 8 (9.9%) | 73 (90.1%) | |
| ***Enterobacter species*** | **4** | 1 (25%) | 3 (75%) | |
| ***Proteus species*** | **5** | 0 (0%) | 5 (100%) | |
| **Total** | **202** | 73 (36.1%) | 129 (63.9%) | |

*(P value < 0 .05 is considered statistically significant)*

The frequency of CRE showed a statistically significant difference among different clinical specimens from which the 202 *Enterobacteriaceae* isolates were retrieved; 67.7% among sputum specimens (21/31), 46.3% among pus specimens (31/67), and 20.2% among urine specimens (21/104) (Table 3).

**Table 3: Comparison of the frequency of CRE among different clinical specimens**

| Type of specimen | No. | Ertapenem MIC | | P value |
|---|---|---|---|---|
| | | Resistant | Sensitive | |
| **Urine** | **104** | 21(20.2%) | 83 (79.8%) | **<0.001** |
| **Pus** | **67** | 31 (46.3%) | 36 (53.7%) | |
| **Sputum** | **31** | 21 (67.7%) | 10 (32.3%) | |
| **Total** | **202** | 73 (36.1%) | 129(63.9%) | |

*(P value < 0 .05 is considered statistically significant)*

**Phenotypic confirmatory test for carbapenemase production:**

- ***Modified carbapenem inactivation method***

The prevalence of carbapenemase producers according to this test was as follows; out of the 73 suspected CPE isolates, 59 isolates (80.8%) were found to be mCIM positive as the diameter of the indicator strain (*E.coli* ATCC 25922) growth-inhibitory zone around the MEM disc was 6–15 mm, while 14 isolates (19.2%) were found to be mCIM negative as the diameter of the growth-inhibitory zone was ≥19mm (Figure 3).

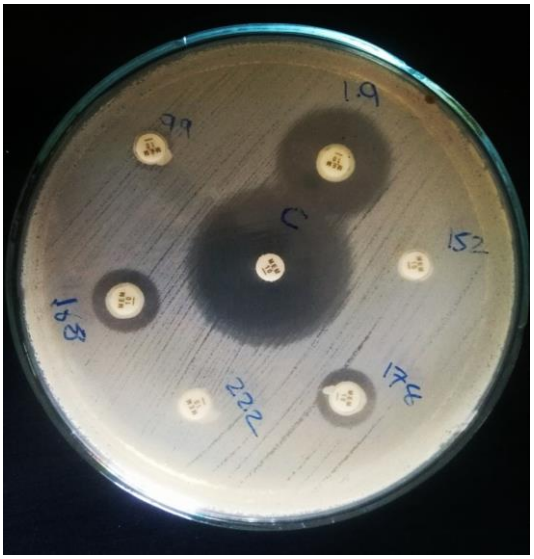


**Fig. 3:** mCIM test

- Isolates number 99, 152, 168, 178 and 222 showed positive results.
- Isolate number 109 was negative.
- C is the negative control *E. Coli ATCC 25922.*

- ***Prevalence of Carbapenemase producing Enterobacteriaceae:***

The prevalence of CPE among the 202 *Enterobacteriaceae* isolates was 29.2% (59/202) while that of non CP-CRE was 6.9% (14/202) (Figure 4).

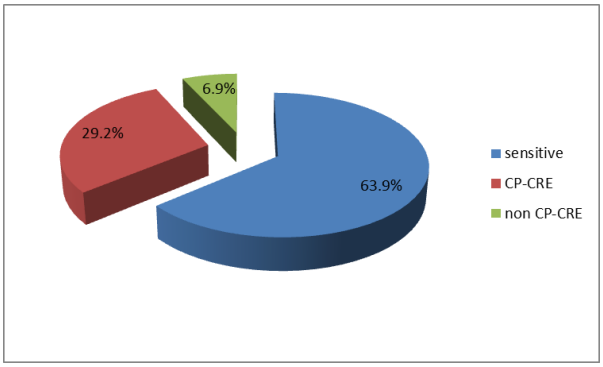


**Fig. 4: Prevalence of CPE and non CP-CRE among the 202 *Enterobacteriaceae* isolates.**

## DISCUSSION

The worldwide appearance of CPE constitutes a threat to the success of current medicine. CPE is lately classified as one of the most serious antimicrobial-resistance threats by the Centers for Disease Control and Prevention (CDC) and the World Health Organization (WHO). [11]

Resistance to carbapenem in *Enterbacteriaceae* may be due to several mechanisms, one of which is the production of carbapenemases. Although other mechanisms contribute to carbapenem resistance such as overexpression of AmpC or ESBLs combined with porin loss, CPE attract much concern. CPE are often resistant to all β-lactam drugs and frequently carry mechanisms conferring resistance to other antimicrobial classes, further limiting treatment options. Additionally, the plasmids harboring resistance genes could spread across other bacterial populations.[12, 13]

Definitive detection of CPE is of great value to guide infection control measures; identifying the presence of CPE is an essential element of outbreak investigations and in the assessment of possible colonization.[14]

The CLSI guidelines for the phenotypic detection of a carbapenemase-producing member of the *Enterobacteriaceae* is based on an initial screening test for ertapenem resistance by MIC determination, followed by the mCIM with or without EDTA carbapenem inactivation method (eCIM) or the Carba NP test, for confirmation.[5]

In the present study, we attempted to determine the presence and the prevalence of carbapenemases among 202 clinical isolates of *Enterobacteriaceae* by the phenotypic confirmatory test mCIM.

In our study, antimicrobial susceptibility testing was done for all of the 202 isolates of *Enterobacteriaceae* by ertapenem MIC determination by broth microdilution method as an initial screening test. By broth microdilution method; Seventy three isolates (36.1%) showed resistance to ertapenem, while 129 isolates (63.9%) were sensitive. The high prevalence of carbapenem resistance in the current study could be explained by the fact that our specimens were collected from patients hospitalized in different departments of a tertiary care hospital. Possibilities for acquiring of CRE include: Prolonged hospital stay, critical illness, surgery, the presence of wound and the use of invasive devices.[15]

This result was in line with another Egyptian study conducted at Alexandria Main University Hospital by El-Ghazzawy et al. who stated that 240 out of 706 (33.9%) *Enterobacteriaceae* isolates were ertapenem resistant.[16] Another study conducted at Mansoura University hospitals by Moemen and Masallat reported that 42 out of 125 (33.6%) *K. pneumoniae* isolates were ertapenem resistant.[17] Similar rates was reported by Metwally et al. who stated that out of 45 *K. pneumoniae* isolates, the resistance to ertapenem were found to be 44.4% (20/45). [18] Another study conducted by AlTamimi et al. reported 45% ertapenem resistance among *Enterobacteriaceae* isolates collected from patients admitted to Prince Sultan Military Medical City in Riyadh, Saudi Arabia.[19] Also, Lee and Chung reported that among the 72 isolates collected from a clinical microbiology laboratory of a tertiary university hospital, Seoul, Korea, 20 isolates (27.8%) were resistant to ertapenem.[20]

In disagreement with our study, lower resistance rates was reported by Huang et al. who stated that ertapenem resistance rate was only 2.2 %; (99 out of 4564) among *Enterobacteriaceae* isolates collected from 24 hospitals in Belgium.[21] Another surveillance study was conducted in four major teaching public hospitals in Kuwait by Jamal et al. and reported that only 8% (61/764) of *Enterobacteriaceae* isolates collected were ertapenem- resistant.[22] Similar rates were reported by Lee et al. who reported 1.6% CRE prevalence rate among 2,510 *Enterobacteriaceae* isolates.[23] These results agreed with the study done by Hayajneh et al. in Jordan who reported that CRE prevalence rate was 1.6%.[24]. Difference in the sample size, geographical distribution, antibiotics policies and application of infection control measures may explain the variations in the prevalence of CRE detected[25]

In our study, highest ertapenem resistance rate was detected among *klebsiella species* 57.1% (64/112) followed by *Enterobacter species* 25% (1/4) and *E.coli* 9.9% (8/81) while no resistance was detected among *proteus species* (0/5).

In agreement with our study, Faidah et al. reported a higher rate of carbapenem resistance among *K.pneumoniae* 459/1158 (38%) compared to *E. coli* 56/1001 (5.59%).[26] Similarly, AlTamimi et al. reported a higher rate of resistance among *K.pneumoniae* 40% (24/34) compared to *E.coli* 5% (3/22) isolates.[19] Also, Ibrahim et al. at Ain Shams University Hospitals in Egypt reported a higher carbapenem resistance among

*klebsiella pneumoniae* 76% (19/25) compared to *E.coli* 35.3% (6/17) and no resistant strains among *proteus species* [27]. Another study done by Oduyebo et al. at a Tertiary Hospital in Lagos, Nigeria showed that carbapenem resistance was higher among *klebsiella species* 19.5% (16/82) followed by *Enterobacter species* 16.7% (1/6) and lower resistance among *E.coli* 7.8% (5/64).[28]

The high rate of carbapenem resistance among *klebsiella species* may be due to that *K. pneumoniae* is likely to be the source of many hospital-acquired infections in critically ill patients and it is considered the most common enterobacterial species for spreading ESBL genes in health care institutions during the past 30 years. Additionally, it is recognized for its ability to accumulate and transfer resistance determinants. It may play the same role for spreading carbapenemase producers in health care facilites.[29, 30] .In disagreement with our study; Parimala reported a higher rate of carbapenem resistance among *E.coli* isolates (63%) compared to *klebsiella species* (42.8%).[31] similarly, Dahab et al. stated that the most resistant organism was *E. coli*, which constituted 45 out of 75 resistant bacteria (30.2%), followed by *Klebsiella spp.*15.4% (23 isolates).[32]

In the current study, we reported higher prevalence of CRE among sputum and pus specimens (67.7%, 46.3%) respectively compared to urine specimens (20.2%).

Our findings are in accordance with a study conducted at Large Teaching Hospital in Makah City, Saudi Arabia by Faidah et al. who stated that CRE prevalence was higher among sputum specimens 46.2% (186/403) followed by wound swabs 20.3% (124/611) and pus 18.2% (6/33) while lower CRE prevalence was detected among urine specimens 11.2% (53/474).[26] Similarly, Lee et al. reported that prevalence of CRE was higher among sputum samples (41.5%) compared to urine samples (29.3%).[23] Also El-Ghazzawy et al. stated that the highest number CRE isolates was isolated from respiratory cultures (25/80; 31.2%) followed by urine cultures (11/80; 13.8%).[16] Another study conducted by Zheng et al. reported that the respiratory tract was the most common infection site of CRE (32/51, 62.7%) followed by urinary tract (5/51, 9.8%).[33]

In disagreement with our study; Parimala. reported higher CRE rates among urine specimens (55.26%) compared to sputum specimens (18.18%).[31] Also, Teo et al. reported that urine (12/29, 41.4%) was the most common site of CRE isolation, followed by, respiratory secretions (3/29, 10.3%), skin and soft tissue wounds (2/29, 6.9%).[34]

The CLSI recommend the mCIM for phenotypic confirmation of the presence of carbapenemases as it is practical, can be easily performed and all necessary supplies are readily available. Also, interpreting the results of this test is not subjective as many other phenotypic assays as it is based on a defined zone diameter. The only disadvantage is the time needed; almost 24 hours are needed for results to be available.[35]

In the present study, 73 isolates (36.1%) fulfilled the CLSI criterion for performing carbapenemase detection by the mCIM (they were ertapenem resistant). These isolates were tested for the presence of carbapenemases by the mCIM using substrates recommended by the CLSI (2017). Out of 73 isolates, 59 (80.8%) proved to be carbapenemase-producers (positive results by mCIM) while 14 (19.2%) isolates tested negative for carbapenemases.

This result was in line with a study done by Li et al. who reported that 29 out of screened 29 carbapenem-resistant *k. pneumoniae* isolates (100%) tested positive for carbapenemase by mCIM.[36] Similarly, Engels-Schwarzlose et al. reported that 75 out of 120 (62.5%) CRE were positive by mCIM.[37]

CPE isolates are considered more serious than CRE by other mechanisms; as CPE strains usually carry carbapenemase genes on mobile genetic elements and can transfer it horizontally to other naïve, thus contributing to the reservoir of resistance in both environmental and clinical *Enterobacteriaceae* .[14, 38] On the other hand, a study conducted at Emergency Hospital Baia Mare, Romania by Földes et al. reported that only 19 out of 43(44.2%) CRE isolates were mCIM positive.[39] Similarly, Senchyna et al. stated that 24 isolate out of 62 (38.7%) CRE were mCIM positive.[40]

## CONCLUSION

- The current worldwide emergence of resistance to the powerful antibiotic carbapenem in *Enterobacteriaceae* constitutes an important growing public health threat.
- Determination of ertapenem MIC by broth microdilution method is the most sensitive indicator of carbapenemase production as ertapenem is the least active carbapenem against carbapenemase-producing organisms
- Detection of CPE is important to improve clinical management of infected patients and to initiate an appropriate infection control measures
- Rapid development of novel therapeutic agents is needed to face the rapidly emerging multidrug resistant pathogens

**Conflicts of interest:**

- The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.
- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.

- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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