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ORIGINAL ARTICLE

Phenotypic Detection of Carbapenem Resistant Enterobacteriaceae Isolates and Assessment of Their Susceptibility to the Novel Ceftazidime-Avibactam Combination

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Key words:
Carbapenem Resistant Enterobacteriaceae (CRE), Phenotypic Detection of Carbapenemase-producing CRE, modified Carbapenem Inactivation Method (mCIM), ceftazidime-avibactam susceptibility

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ABSTRACT

Background: *Carbapenems have become one of the last lines of antimicrobials against Gram negative resistant microorganisms. But in last few years, Carbapenem Resistant Enterobacteriaceae (CRE) have been reported all over the world. Numerous phenotypic tests have been planned for detection of carbapenemase activity including the newer modified Carbapenem Inactivation Method (mCIM). There is a strong need for new antibiotics to mitigate the growing threat of antimicrobial resistance (AMR).* ***Objectives:*** *Evaluation of the mCIM as a new method for phenotypic detection of carbapenemase-producing CRE and to test the in vitro susceptibility of the isolates to ceftazidime-avibactam.* ***Methodology:*** *Total of 25 CRE, isolated from 120 clinical specimens then identified by conventional methods over a 6 months period from Intensive Care Units of Surgery Department of Ain Shams University Hospitals. Phenotypic detection of carbapenemase-producing CRE was done by mCIM method compared to meropenem E-test as well as testing the susceptibility of isolates to ceftazidime- avibactam by E test.* ***Results:*** *Twenty-five CRE isolates were detected by meropenem disk diffusion method [Klebsiella pneumoniae (n=19) and E.coli (n=6)]. mCIM was positive for 23 out of 25 isolates (92%) showing 100% sensitivity, 66.6% specificity and 96% accuracy. There is an excellent agreement between meropenem E-test and modified CIM (kappa =0.77; P value <0.001). Twenty-two of CRE isolates (88%) were sensitive to ceftazidime-avibactam.* ***Conclusion:*** *The mCIM method is simple, less subjective, cost effective, sensitive method and plays an important role in detection of CRE. Also, Ceftazidime-avibactam appears to be a promising agent for the treatment of serious CRE infections.*

INTRODUCTION

The emergence and spread of carbapenemase-producing Gram-negative rods are a worldwide emerging public health threat. Particularly in health care centers, this may pose a major problem as carbapenems are becoming more frequently needed to treat infections caused by Enterobacteriaceae producing extended-spectrum β-lactamases (ESBL) and AmpC β lactamases[1].

Prevention of spread of carbapenemase producers requiring rapid detection of these bacteria[2]. Identification is of primary importance for the choice of appropriate therapeutic schemes and the implementation of proper infection control measures[3].

A new straight forward inexpensive phenotypic test called mCIM, was developed to detect carbapenemase production in Enterobacteriaceae[4]. It is currently recommended by Clinical and Laboratory Standards Institute (CLSI) for detection of carbapenemase among Enterobacteriaceae clinical isolates[5]. This technique showed high concordance with results obtained by PCR to detect genes coding for the carbapenemases KPC, NDM, OXA-48, VIM, IMP and OXA-23[6].

Enterobacteriaceae producing carbapenemases have conferred broad resistance to most beta lactam antibiotics including last line carbapenems[7]. So, new antibiotic options are urgently needed for the treatment of carbapenem-resistant Enterobacteriaceae infections. Ceftazidime-avibactam is a combination of the established third-generation cephalosporin ceftazidime, with the novel non-β-lactam β- lactamase inhibitor avibactam[8].

It has an activity against many carbapenem-resistant Enterobacteriaceae (CRE) as avibactam inhibits a broad range of serine β-lactamases including Ambler class A (ESBL and KPC), class C (AmpC) and some class D (OXA-48) enzymes[9]. In combination with ceftazidime, avibactam restores activity of ceftazidime against a number of clinically relevant β-lactamase-producing Gram-negative pathogens causing serious infections[10].

METHODOLOGY

This study was conducted on 120 patients admitted to different Intensive Care units of Surgery Department of Ain Shams University Hospitals during the period from June 2018 to November 2018. The study was approved by the ethics committee and informed consent was obtained from the patients or from their relatives after explaining the study and its goals to them.

Thorough history and examination were performed with emphasis on received antibiotics.

Isolation of Enterobacteriaceae strains:

Out of 120 patients, 80 mid-stream urine samples (MSU), were collected from non-catheterized patients, and catheter-stream urine (CSU) from catheterized patients, from the catheter tube using a sterile syringe, 30 respiratory specimens [18 sputum and 12 endotracheal aspirate (ETA)], 5 pus specimens were collected from open wounds using a sterile swab, and 5 blood samples were collected under complete aseptic conditions. Samples were collected in sterile containers to be examined bacteriologically.

Sputum specimens were mechanically liquefied and homogenized. Blood samples were inoculated into diphasic culture medium bottle (Castenada medium) (Himedia, India) and incubated for at least 7 days aerobically at 37°C. All samples (urine, respiratory, pus and from blood culture bottles) were plated on MacConkey's agar and blood agar. Urine & respiratory specimens were inoculated using 1 µl calibrated loop to detect colony forming unit (CFU) per milliliter. Urine samples were also inoculated on Cystine-Lactose-Electrolyte-Deficient (CLED) Agar. All the inoculated plates were incubated aerobically at 37°C for 24 h.

Urine culture & respiratory specimens' culture were expressed as colony-forming units (CFU) per milliliter. For urine specimens: a threshold concentration of 10^5 cfu/ml MSU, while threshold concentration of 10^4 cfu/ml of catheter specimen was used to define urinary tract infections. For respiratory specimens: a threshold concentration of 10^6 cfu/ml sputum and 10^5 cfu/ml ETA was used to define lower respiratory tract infections.

The isolated organisms were worked up microbiologically and identified by conventional methods according to Cheesbrough[11] based on colonial morphology, microscopic examination of Gram stained films and biological activity of the isolated organisms.

Detection of carbapenem resistance in Enterobacteriaceae strains:

All *Enterobacteriaceae* isolates were tested for carbapenem resistance by disc diffusion method using commercially prepared meropenem (MEM) (10µg) disks *(Oxoid, England)* and results were interpreted according to the recommendations of the CLSI[12] until obtaining 25 CRE isolates.

Detection of Antimicrobial susceptibility pattern in CRE strains:

Twenty-five CRE isolates were tested by disk diffusion test for different antibiotics (Oxoid, England): Aztreonam 30µg (ATM), Cefepime 30µg (FEP), Ceftazidime 30µg (CAZ), Cefotaxime 30µg (CTX), Ampicillin 10µg (AM), Piperacillin /Tazobactam 110µg (TPZ), Amoxicillin/clavulanic acid 20/10µg (AMC), Sulphamethoxazole/Trimethoprim 25µg (SXT), Ciprofloxacin 5µg (CIP), Gentamycin 10µg (GN), Amikacin 30µg (AK), and Cefoxitine 30µg (FOX). Figure (1) showed multidrug resistant *klebsiella pneumoniae*.

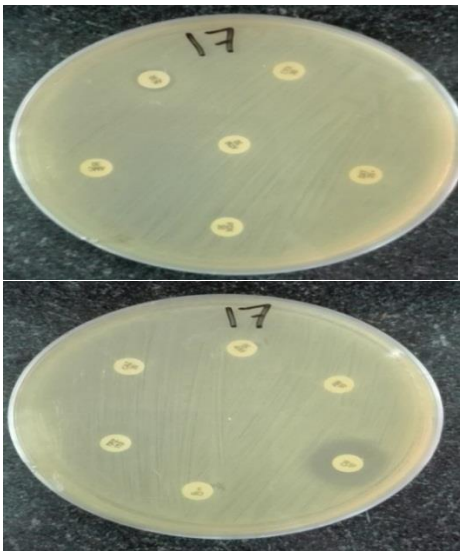

Fig. 1: A plate of Muller-Hinton agar inoculated by *klebsiella pneumoniae* showing multidrug resistance.

Phenotypic detection of carbapenemase production in CRE isolates by using modified carbapenem inactivation method:

1 µL loopful of CRE isolate from blood agar plates was emulsified in 2 mL trypticase soy broth (TSB) (Sigma-Aldrich). A meropenem disk was then immersed in the suspension and incubated for a minimum of 4 h at 35°C. A 0.5 McFarland suspension of carbapenem susceptible strain of *E. coli* (ATCC 25922) (Central Laboratories of Egypt) and of carbapenem resistant strain (obtained from previous work and confirmed by PCR, used as a control for meropenem disc) was prepared in saline using the direct colony suspension method. A Mueller–Hinton agar (MHA) plate (Oxoid, England) was inoculated with *E. coli* ATCC 25922 using the routine disk diffusion procedure. The meropenem disk was removed from the TSB and placed on an MHA plate previously inoculated with the *E. coli* ATCC 25922 indicator strain. Plates were incubated at 35°C in ambient air for 18−24 h. An inhibition zone diameter of 6–15 mm or colonies within

a 16–18 mm zone was considered to be a positive result, and a zone of inhibition ≥19 mm was considered to be a negative result[12]. Figure (2) showed Carbapenemase negative and positive results.

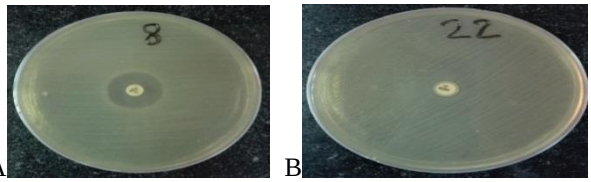

Fig. 2: Plates of Muller-Hinton agar inoculated by *E.coli* ATCC® 25922 A: showing Carbapenemase negative result (zone of inhibition =19 mm), B: showing Carbapenemase positive result (no zone of inhibition)

Minimal Inhibitory Concentration (MIC) of meropenem and ceftazidime- avibactam using E test:

E test strips containing range of antibiotic concentrations (0.016-256 ug/ml) for ceftazidime-avibactam and (0.002-32 ug/ml) for meropenem (Biomerieux, France). A suspension equivalent in density to a McFarland 0.5 opacity standard was prepared and spread uniformly across the surface of Mueller-Hinton agar plate. E-Test strips were placed in the center of the plates ensuring that they were evenly placed with the MIC scale facing upwards. The agar plate was then incubated at 37˚C and results were read after 24 hours of incubation. The values of the MIC of each antibiotic were interpreted according to CLSI[12] as presented in table (1).

Table 1: MIC values of antibiotics

Antibiotic	MIC (ug/ml)		
	Sensitive	Intermediate	Resistant
Meropenem	≤ 1 ug/ml	2	≥4 ug/ml
Ceftazidime-avibactam	≤ 8 ug/ml	-	≥ 16 ug/ml

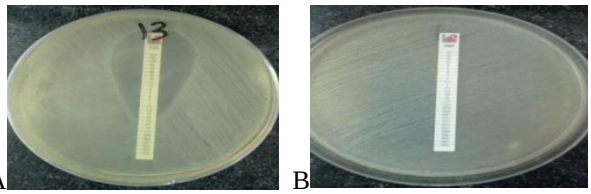

Fig. 3: Plate of MHA inoculated with CRE; A: ceftazidime- avibactam MIC (0.38) µg/ml (susceptible to CZA). B: meropenem no zone of inhibition (resistant to meropenem)

Statistical Analysis:

Data were analyzed using a personal computer Statistical package for Social Science (SPSS 20) (Stat Soft Inc. USA). Quantitative data were statistically represented in terms of mean, standard deviation (SD) and range for parametric numerical data, while Median for non-parametric numerical data. Qualitative data were statistically represented in terms of numbers and percentages. Kappa statistics used to compute the measure of agreement between two investigational methods Kappa's over 0.75 is excellent, 0.40 to 0.75 is fair to good, and below 0.40 is poor. The sensitivities, specificities, positive predictive values (PPV) and negative predictive values (NPV) of the phenotypic methods for BF production were calculated as described by Ilstrup[13]. A p value <0.05 was considered statistically significant and p> 0.05 was considered non-significant.

RESULTS

Out of 120 clinical, 45 Enterobacteriaceae isolates were obtained (25 *K. pneumoniae*, 17 *E.coli and* 3 Proteus isolates).Twenty-five CRE isolates were detected by meropenem disk diffusion method (19 *K. pneumoniae* and 6 *E.coli*). They were isolated from 15 males (60%) and 10 females (40%). Their mean age was 54.9± 16.2 ranging from 22 years to 82 years. The distribution of the Enterobacteriaceae isolates in different clinical samples was demonstrated in figure (4). Figure (5) showed that the prevalence rate of CRE infections 25/45 (55.6%) among ICU patients infected with Enterobacteriaceae.

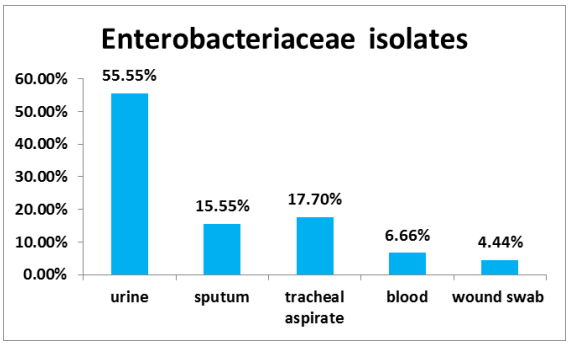

Fig. 4: The distribution of the Enterobacteriaceae isolates in different clinical samples

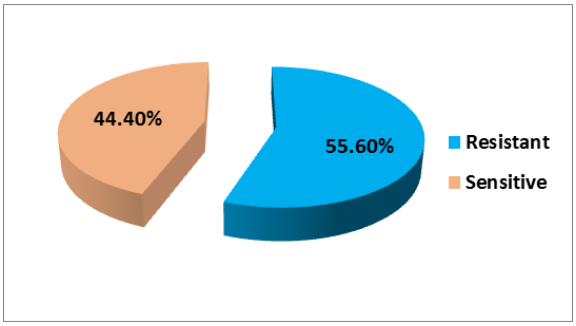

Fig. 5: The percentage of CRE infections among the Enterobacteriaceae isolates

Table (2) showed that 19/25 (76%) of isolated *K. pneumoniae* and 6 /17 (35.3%) of isolated *E.coli* were resistant to carbapenems while no resistant strains were detected among Proteus isolates. The distribution of CRE isolates in different clinical samples was illustrated in table (3). Table (4) showed the in-vitro susceptibility pattern of CRE isolates to different tested antimicrobials. It was noticed that 40% of CRE isolates (10 of 25) were susceptible to gentamycin followed by amikacin and sulphamethoxazole-trimethoprim (2; 8%) then Piperacillin/ Tazobactam, Cefoxitine, Cefepime and Aztreonam (1; 4%).

Table 2: The distribution of susceptible and resistant strains of Enterobacteriaceae isolates to carbapenems

Enterobacteriaceae	Sensitive	Resistant	Total
K. pneumoniae	6 (24%)	19 (76%)	25 (100%)
E. coli	11 (64.7%)	6 (35.3%)	17 (100%)
Proteus	3 (100%)	0 (0%)	3 (100%)
Total	20 (44.4%)	25 (55.6%)	45 (100%)

Table 3: The distribution of CRE isolates in different clinical samples

Sample type	Number	Percentage
Sputum	4	16%
Tracheal aspirate	5	20%
Urine	12	48%
blood	2	8%
Wound swab	2	8%

Table 4: In-vitro susceptibility pattern of CRE isolates to different antimicrobials.

Antimicrobial	Susceptible	Intermediate	Resistant
AM	0 (0%)	0 (0%)	25 (100%)
AMC	0 (0%)	0 (0%)	25 (100%)
Tpz	1 (4%)	0 (0%)	24 (96%)
CTX	0 (0%)	0 (0%)	25 (100%)
CAZ	0 (0%)	0 (0%)	25 (100%)
FOX	1 (4%)	0 (0%)	24 (96%)
FEP	1 (4%)	0 (0%)	24 (96%)
MEM	0 (0%)	0 (0%)	25 (100%)
AK	2 (8%)	2 (8%)	21 (84%)
GN	10 (40%)	0 (0%)	15 (60%)
SXT	2 (8%)	0 (0%)	23 (92%)
ATM	1 (4%)	1 (4%)	23 (92%)
CIP	0 (0%)	0 (0%)	25 (100%)

(AK: Amikacin, AMC: Amoxicillin / calvulinic acid, AM: Ampicillin, ATM: Aztreonam, FEP: Cefepime, CTX: Cefotaxime, FOX: Cefoxitine, CAZ: Ceftazidime, CIP: Ciprofloxacin, GN: Gentamycin, CIP: Ciprofloxacin, MEM: meropenem, SXT: Sulphamethoxazole / Trimethoprim, TPZ: Piperacillin / Tazobactam)

Carbapenemase-production in CRE isolates by mCIM result was illustrated in figure (6). It was noticed that that 23 (92%) of CRE were positive and only 2 isolates were negative. Figure (7) showed that 22 (88%) of CRE isolates were resistant to meropenem E-test and 3 (12%) of isolates were sensitive. The co-ordnance between the result of meropenem E-test and modified CIM was demonstrated in table (5). There is an excellent agreement between two investigational methods (kappa =0.77; p value <0.001). The performance of mCIM test was demonstrated in table (6), it was found that sensitivity of mCIM is 100%, specificity is 66.6% and accuracy is 96%.

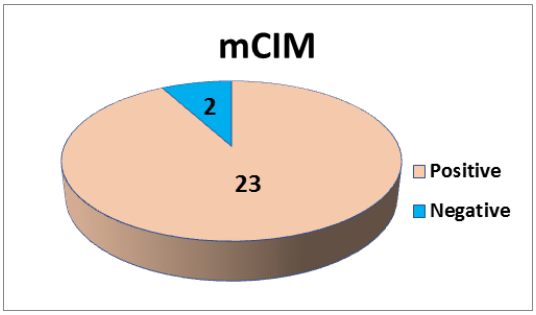

Fig. 6: The number of Carbapenemase-producing CRE isolates using mCIM

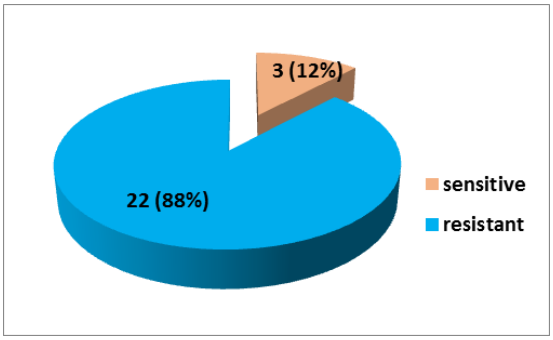

Fig 7: Antimicrobial susceptibility of CRE isolates to Meropenem E-test

Table 5: Co-ordnance between result of meropenem E-test and modified CIM.

Test		Meropenem E-test		Total	Kappa agreement		
		Resistant	Sensitive		Kappa	p value	sig.
modified CIM	Positive	22	1	23	0.779	<0.001	HS
	Negative	0	2	2			
Total		22	3	25			

Table 6: Sensitivity and Specificity of modified CIM.

Sensitivity	Specificity	PPV	NPV	Accuracy
100%	66.60%	95.60%	100%	96%

Table (7) showed that 5 clinical isolates (22.7%) were inhibited at concentration of 0.023 µg/ml of ceftazidime- avibactam , 4 isolates were inhibited at concentration of 0.016 µg/ml , 3 isolates were inhibited at concentration of 0.064 µg/ml, 2 isolates were inhibited at each of these concentrations of 0.38 µg/ml and 0.75µg/ml, one isolate was inhibited at each of these concentrations: 0.032, 0.047, 0.125, 0.25, 0.5, 2 µg/ml. Figure (8) showed that 22 (88%) of CRE isolates were sensitive to Ceftazidime- avibactam E-test and 3 (12%) of isolates were resistant.

Table 7: Number of CRE isolates inhibited by different concentrations of ceftazidime- avibactam.

Antibiotic conc.	MIC (µg/ml)										
	0.016	0.023	0.032	0.047	0.064	0.125	0.25	0.38	0.5	0.75	2
Number of isolates	4	5	1	1	3	1	1	2	1	2	1
Percentage	18.2%	22.7%	4.5%	4.5%	13.6%	4.5%	4.5%	9.1%	4.5%	9.1%	4.5%

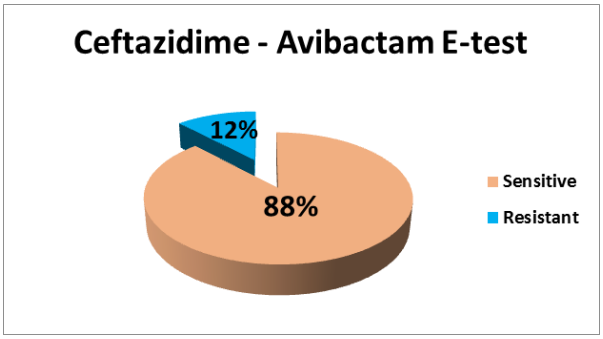

Fig. 8: Antimicrobial susceptibility of CRE isolates to Ceftazidime- avibactam E-test.

DISCUSSION

Rapid and effective detection of carbapenemase-producers is important for clinicians treating patients and for infection preventionists to limit the spread of carbapenem-resistant organisms[14]. mCIM suggested by CLSI in 2017 is a simple and cheap method to perform and is well established in many clinical microbiology laboratories supported by its high sensitivity and specificity to detect carbapenemase-producing *Enterobacteriaceae* isolates compared with the current published or available phenotype method such as modified hodge testing and Craba-NP method[15]. The increased prevalence of isolates carrying carbapenemases in recent years constitutes a greater challenge, leading to multidrug-resistant (MDR), extensively-drug resistant (XDR), and pandrug-resistant (PDR) bacteria[16]. Developing new antibiotics to combat these resistant strains is an urgent need[17]. Ceftazidime-avibactam is a novel β-lactam/β-lactamase inhibitor with activity against carbapenem-resistant Enterobacteriaceae[18].

The current study revealed that the prevalence rate of CRE was 25/45 (55.6%) of Enterobacteriaceae infected patients. This finding goes in accordance with the results of studies carried out in Egypt by Amer et al.[19] and Mahmoud et al.[20] who found that (47/75) 62.7% and (79/121) 65.29% respectively of isolated Enterobacteriaceae were CR. On the other hand, Baran and Aksu[1] in Turkey, Li and Ye[21] in China, and Mohamed et al.[22] in Egypt found that (181/6426) 2.8%, (26/148) 17.6%, and (35/176) 19.9% respectively of isolated Enterobacteriaceae were CR.

All cases infected with CRE in this study were previously exposed to empirical misuse of antibiotic intake prior to result of culture and sensitivity and this could explain the high prevalence rate of CRE. These results are supported by a study done by Teo et al.[23] in Singapore and found that 73% of cases receiving previous empirical antibiotics were infected with CRE. Also, Amer et al.[19] in Egypt reported that the high level of resistance in his study can be attributed to the unrestricted use of antibiotics which plays an important role in increasing carbapenem resistance. However, Mouloudi et al.[24] found that previous exposure to antibiotics is not a risk factor for CRE infection.

The current study showed that most of CRE were isolated from urine samples. Similar finding was found by Mohamed et al.[22] in Egypt. However, Amer et al.[19] in Egypt; Pang et al.[25] in China reported that most of CRE were isolated from blood, tracheal aspirate samples respectively. This difference could be explained by large number of urine samples tested in this study.

Nineteen out of 25 CRE isolates were *K. pneumoniae* (76%), so it was the most common CRE isolated from ICU. Other studies performed by Xu et al.[26], Baran and Aksu[1], Pang et al.[25], and Mohamed et al.[22] also reported that *K. pneumoniae* was the most common CRE isolated. On the other hand, Amjad et al.[27] reported that *E-coli* was the most common CRE.

In present study, 40% of CRE isolates (10 of 25) were susceptible to gentamycin, followed by amikacin and sulphamethoxazole trimethoprim (2 of 25). This is similar to results of Pollett et al.[28] in USA, Wang et al.[29] in china, and Baran and Aksu[1] in Turkey. On the other hand, Pang et al.[25] found that quinolone was the most effective antibiotics. In this study about 50% of CRE were multi-drug-resistant (MDR); being resistant to all antibiotics tested. These results go in accordance with studies performed by El-Sweify et al.[30] and Mahmoud et al.[20] who found that 45.7% and 52.89% respectively of CRE isolates were MDR.

In this study, CRE isolates were tested for carbapenemases production using the mCIM where this test showed 100% sensitivity in comparison with meropenem E.test. Similarly, Yu et al.[5] reported 100% sensitivity of mCIM when compared with PCR (117 out of 117 isolates). Also, Pawar et al.[31] reported 98.48% sensitivity of mCIM when compared with Modified Hodge test (MHT) and Combined Disc Test (CDT) methods (65 out of 66 isolates). The mCIM in the current study showed specificity 66.6%, in contrast to the result reported by Creighton and Tibbs[32], Tamma et al.[33], and Yu et al.[5] who showed specificity 91.9%, 100%, 100% respectively. This may be due to difference in sample size between studies.

The results of meropenem E.test showed that 88% of CRE isolates were resistant to meropenem, on the other hand Yu et al. [5] reported that 96.6% of CRE isolates were resistant to meropenem E.test. This study showed that there is an excellent agreement between mCIM and meropenem E.test (kappa =0.77; p value <0.001). These results were similar to Yu et al.[5] and Pierce et al.[4].

In this study 88% (22 of 25) of CRE isolates were sensitive to Ceftazidime- avibactam E-test. However, Karlowsky et al.[34] and Sader et al.[35]; found that 99.5% (33,877 of 34,062 isolates) and 98% (185 of 189 isolates) respectively of Enterobacteriaceae were susceptible to ceftazidime avibactam. In a study done by Kazmierczak and coworkers[36], they reported that Ceftazidime-avibactam showed potent activity against carbapenemase-positive MBL negative isolates (100% susceptible) and lower sensitivity in carbapenemase-negative isolates (87.5-100% susceptible) and they concluded that Ceftazidime-avibactam was not active against MBL-positive isolates (<5% susceptible). As a result, ceftazidime-avibactam showed diminished activity in regions where MBLs were more frequently encountered in CRE, so regional differences in the incidence of MBL-mediated resistance are important to consider when assessing the value of ceftazidime-avibactam.

In the current study, MIC range of ceftazidime-avibactam was ranged from (0.016 - 2 µg/ml) which is similar to the results of Urban et al.[37]. However, a higher MIC range (0.06 - 8 µg/ml) was reported by Sader et al.[35]. In this study, the highest percentage of inhibition (22.7% of CRE isolates) was at concentration (0.023 µg/ml) of ceftazidime- avibactam. However, Sader et al.[35] found that the highest percentage of inhibition (30.6% of the CRE isolates) was at a higher concentration (1 µg/ml) of ceftazidime- avibactam. Sader et al.[35] and Karlowsky et al.[34] also reported that ceftazidime-avibactam had MIC 90 of (2 µg/ml) and (0.5 µg/ml) respectively, while only one of our isolates was inhibited at these concentrations.

CONCLUSION

The mCIM method is simple, less subjective, cost effective, sensitive method and plays an important role in detection of CRE. Also, Ceftazidime-avibactam appears to be a promising agent for the treatment of serious CRE infections.

Recommendation:

Continuous searching for other highly effective and low-cost alternative antimicrobials to combat CRE. Regular monitoring of local antimicrobial resistance to guide the prescription of effective antimicrobials and for early initiation of control measures to stop the spread of highly resistant CRE.

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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