DETECTION OF CARBAPENEMASE PRODUCING CARBAPENEM RESISTANT ENTEROBACTERALES IN BLOOD CULTURE ISOLATES BY MCIM AND ECIM AND ITS SUSCEPTIBILITY TO TIGECYCLINE AND MINOCYCLINE

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ABSTRACT

Objective: In this study, we aimed to detect different Carbapenemase-producing carbapenem-resistant Enterobacterales (CREs) in blood isolates by phenotypic, modified carbapenem inactivation methods (MCIM) and amp; EDTA-carbapenem inactivation methods (ECIM), and also to study the susceptibility of these CREs toward Tigecycline and Minocycline.

Methods: This prospective study included 100 non-duplicate Enterobacterales organisms isolated from 250 blood samples positive for Enterobacterales that showed resistance to carbapenem (Imipenem). The isolates were identified by conventional routine biochemical tests. CRE isolates were screened for Carbapenemase production by the Clinical and Laboratory Standards Institute (CLSI)-recommended, MCIM and ECIM for evidence of the production of matello-beta-lactamase. Antimicrobial susceptibility for Tigecycline and Minocycline drugs was tested by the disk diffusion method on Mueller–Hinton agar according to CLSI guidelines, and susceptibility patterns were recorded. Clinical diagnosis data were collected from the requisition forms sent to our laboratory during test procedures.

Results: Out of 100 (40%) CRE isolates tested for MCIM, 34 samples showed positive results for the Carbapenemase enzyme. Among the MCIM-positive isolates, Klebsiella spp. showed the highest prevalence of 58.8% (20/34). While among MCIM-positive isolates (22/34), 64.7% were positive for eCIM (Matello beta-lactamase producer). Maximum samples had been received from the NICU ward from patients diagnosed with early-onset sepsis; 41.2% of these were Carbapenemase-producing Enterobacterales. Among CRE isolates, 86 isolates were susceptible to Tigecycline and 24 isolates were susceptible to Minocycline. 70 CRE isolates were susceptible to Tigecycline but resistant to minocycline, and all CRE isolates resistant to Tigecycline were not susceptible to Minocycline.

Keywords: Carbapenem resistant Enterobacterales, Modified carbapenem inactivation method, EDTA-carbapenem inactivation method.

INTRODUCTION

Enterobacterales cause community-acquired as well as health-care-associated infections with a broad clinical and epidemiological spectrum [1]. The major challenge the world has faced with these groups of organisms is the emergence of multidrug-resistance strains causing high mortality and morbidity burden [1-3]. Among various therapeutic antibiotic options, Carbapenems are considered the last resort of choice to treat this infection [4]. However, Enterobacterales has started developing resistance to this antibiotic through various modes of acquisition of resistance mechanisms. This resistance pattern usually creates challenges in diagnosis, and proper antibiotic selection for treatment ultimately results in clinical failure. The Centre for Disease Control and Prevention has termed this group of organisms carbapenem-resistant Enterobacterales (CRE) and defined them as Enterobacterales that are resistant to at least one of the carbapenem antibiotics (Meropenem, Imipenem, Ertapenem, and Doripenem, approved by the FDA for clinical use) or produce a carbapenemase, or carbapenem hydrolyzing enzyme [3]. CREs are responsible for causing pneumonia, mainly associated with ventilator-associated pneumonia, bloodstream infections, Urinary tract infections, wound infections, meningitis, and intra-abdominal abscesses. In 2017, CRE caused an estimated 13,100 infections in hospitalized patients and 1,100 deaths in the USA [3,4].

CRE confers resistance to Carbapenem either because of the production of the hydrolytic enzyme Carbapenemase (CP-CRE) or by other mechanisms like porin channel mutation or reduced expression of the same (Non-CP-CRE) [5]. While the presence of carbapenemase-producing genes on plasmids results in the emergence of high-rate CRE [6,7]. According to a CDC report, 30% of CRE carry the carbapenemase-producing gene [2]. Carbapenemases are members of Class A, B, and D beta-lactamases [5,6]. These are mainly classified into Matello-beta-lactamases (MBLs, Zinc dependent class B) and Serine Carbapenemases (Zinc independent A and D classes) [6]. Therefore, known Chelators like EDTA or dipicolinic acid can block carbapenemase activity by binding the activator ion ZINC, which can be assessed to detect and differentiate both groups of carbapenemase. Various MBL genes such as blaNDM, blaIMP, and blaVIM and serine Carbapenemases like blaOXA (class D) and blaKPC (class A) have been reported frequently in different CREs [8].

Although the characterization of the involved mechanisms leading to carbapenem resistance is not routinely detected in clinical laboratories for therapeutic choice selection, understanding the presence of an inhibiting enzyme in an isolate has significant epidemiological implications for monitoring the resistant isolates and selecting effective antibiotics to treat the infections [9].

There are different phenotypic tests that are deployed to detect the presence of carbapenemase. Most of the phenotypic tests not only determine the mere presence of resistance but also the nature of resistance patterns [10,11]. A previously modified Hodge test was recommended by the Clinical and Laboratory Standards Institute (CLSI) in 2009 to detect carbapenemase. However, because of its limitations and lack of sensitivity, the test has been discarded from the recommended profile [12]. Currently, CLSI has introduced the modified carbapenem inactivation method (MCIM) to identify carbapenemases,
while a further modification to mCIM with the addition of the EDTA carbapenem inactivation method (eCIM) has been recommended to specifically identify MBLs [12,13].

While considering the treatment of CRE infection, various old, new, and combined antibiotic therapy combinations have been recommended. Among these, the tetracycline group of antibiotics is commonly prescribed in clinical settings. Minocycline and its semisynthetic derivative, Tigecycline, are the most commonly used drugs. Tigecycline is glycycline, which can overcome the common resistance seen in other tetracycline drugs. Therefore, Tigecycline is one of the few remaining old therapeutic options for the treatment of multi-drug-resistant Gram-negative organisms, including CRE. However, as the use of Tigecycline increases, an increasing number of tetracycline-resistant strains have emerged, especially among the CREs. In this context, accurate detection of Tigecycline susceptibility in comparison to other tetracyclines is important to correctly choose the appropriate antibiotic. At present, the CLSI offers no break points or recommended methods for tigecycline susceptibility testing for Enterobacteriaceae [12], while studies have shown that the modified Kirby-Bauer disk diffusion method does a simple, accurate, and inexpensive method for testing Tigecycline susceptibility in CRE [14].

Our study aimed to detect
• Carbapenemase production in Enterobacteriaceae isolated from blood samples by mCIM and eCIM
• The susceptibility patterns of CRE strains to Minocycline and Tigecycline.

METHODS

Isolates from blood cultures were identified as Enterobacteriaceae by standard identification methods. Susceptibility to Carbapenem (Imipenem 10 μg/disc) for Enterobacteriaceae isolates was determined by the Kirby Bauer disk diffusion method on Mueller-Hinton (MH) agar plates based on the CLSI guidelines (M100-S30).

CRE isolates were further tested by phenotypic methods (mCIM and eCIM) to detect the mechanism and type of carbapenemase production.

mCIM and eCIM were performed on CRE isolates according to the CLSI guidelines to detect the presence of carbapenemase [15]. In brief, a 1-μL loopful of bacterial inoculum was resuspended in a 2-mL tube of TSB. Another 1-μL loopful of bacteria was resuspended in a 2-mL tube of TSB supplemented with EDTA (Thermo Fisher Scientific, Carlsbad, CA, USA) at a final concentration of 5 mM (addition of 20 μL of 0.5 M EDTA to 2 mL of TSB). A meropepenem disk was placed in each tube, and the tubes were incubated at 35°C for 4 h. Subsequently, the disks were removed and applied to MH agar plates, which were freshly plated with a 0.5 McFarland suspension of a carbapenem-susceptible Escherichia coli ATCC 25922 strain. Then the plates were incubated at 35°C for 16-20 h, and the mCIM and eCIM results were interpreted as described by CLSI21. The mCIM is considered negative if the zone size is ≥19 mm, positive if the zone size is 6-15 mm, or intermediate (defined as positive) if pinpoint colonies are present within a 16-18-mm zone [2]. An isolate is positive for metallo-beta lactamase production when the eCIM zone size increases by ≥5 mm compared to the zone size observed for the mCIM and is considered negative for a metallo-beta lactamase if the increase in size is <4 mm [2].

Susceptibility of CRE to minocycline and tigecycline

Susceptibility testing to Minocycline and Tigecycline was performed on CRE isolates by the Kirby-Bauer disk diffusion method on MH agar and interpreted as per the CLSI zone diameter corresponding categorical break points. Minocycline (30 μg) >16 mm and Tigecycline (30 μg) >18 mm diameter were considered susceptible zones of inhibition.

RESULTS

During the study period in our laboratory, a total of 250 Enterobacteriaceae were isolated from blood cultures. 100 of these isolates were found to be resistant to Carbapenem and diagnosed as CRE.

CRE and ward-wise distribution

CRE blood isolates were reported from different wards of the attached hospitals. Among the 100 tested CRE isolates, 40 were from the NICU, which represented the highest distribution of CRE among all clinical setting in our hospital. This was followed by 20 isolates recovered from the PICU, 19 from the MICU, 13 from the SICU, and 8 from the medical emergency ward. The highest number of CRE isolates in our study were documented in pediatric setting.

CRE and organism-wise distribution

Among the CRE isolates, four major Enterobacteriaceae were identified. Among the isolates, 58 were Klebsiella spp., 30 belonged to Enterobacter spp., 7 were E. coli spp., and 5 were Citrobacter spp. Klebsiella spp. (50%) had the highest proportion of CRE isolates in our study.

Carbapenemase producer (positive mCIM and eCIM)

Among 100 isolates, 34 isolates of CRE were positive for carbapenemase, which showed mCIM positivity. Among these mCIM-positive isolates, 64.7% (22/34) were positive for eCIM, representing the MBL producers. Among these MBL producers, 77% were Klebsiella spp., 9% were Enterobacter and E. coli spp., and 5% were Citrobacter spp.

Susceptibility to minocycline and tigecycline

Carbapenem-resistance organisms were tested for Susceptibility to Minocycline and Tigecycline. Among 100 CRE isolates, 86 were Susceptible to tigecycline while only 24 were susceptible to Minocycline. 70 CRE isolates were susceptible to Tigecycline but resistant to Minocycline. However, all CRE isolates resistant to Tigecycline were not...
Among the CREs, the highest isolates were from the NICU (40%), and early-onset neonatal sepsis was the most common clinical cause among these groups. A study by Thomas et al. showed that, along with other multi-drug-resistant strains, CRE isolates have about 18% incidence in neonates admitted to intensive care units [19]. Another study in Shanghai, China, by Yin et al. found CRE incidence in the NICU to be 11.2% [20]. Both our studies showed that receiving mechanical ventilation, malnutrition, and critical conditions with high SNAP-II scores were independent risk factors for acquiring the infection. In comparison to these studies, our study found a higher incidence of CRE infection, which may be because of the higher number of sample received from the NICU. However, the high incidence of CRE indicates the urge to screen the respective samples for Carbapenem resistance and the mode of their resistant patterns.

Our study showed that *Klebsiella* spp. (47%) of the isolates was the highest isolate among all CREs. Most of these were isolated from the NICU (50%, 29/58) and associated with Neonatal sepsis. In the study by Mukherjee et al. in *Neonatal Sepsis: The Impact of Carbapenem-Resistant and Hypervirulent Klebsiella pneumonia*, found *Klebsiella* spp. as the highest prevalent CRE responsible for early-onset sepsis [15]. In a nationwide database of reports from 300 microbiology laboratories, the cultures obtained from children in the U.S. from 1999 to 2012 showed that the rate of infection had increased significantly among children of all ages and settings, from 0% in 1999–2000 to 0.47% in 2011–2012. The greatest increase, from 0% to 4.5%, was found in cultures from children between the ages of 1 and 5 years in intensive care units. In addition, CRE isolates found in the bloodstream increased from zero to 3.2% during the study period. This is important because, according to the CDC, up to half (50%) of people who develop CRE bloodstream infections die from the infection [2].

Our study found that 34% of CRE isolates possess the carbapenemase enzyme. The study by Petit et al. [7] showed a concordant result with our findings. While a study by Bushnell et al. showed the prevalence of beta-lactamase enzyme producer CRE (CP-CRE) in India was 31–92% [21]. Our study showed similar findings to these studies in detecting the carbapenemase enzyme in CRE by phenotypic mCIM testing.

A study by Han et al. showed 97.4% of the CRE strains possessing the carbapenemase enzyme overall [22]. This high incidence is definitely a concern for the dissemination of the multi-drug-resistant strain among the population. In contrast to our study, this study was a genotypic screening of carbapenemase-producing CRE.

Among the carbapenemase-producing CRE (CP-CRE) isolates, 64.7% were found to be eCIM-positive, representing the MBL producers. This is a relatively higher incidence of CRE harbouring Metallo-beta lactamase. The CDC has reported that among CP-CRE, around 10% are Metallo-beta lactamases [2]. The high incidence of MBL in our study might be due to the limited number of study samples as well as the fact that most of the CRE samples were from children. In this regard, a study by Han et al. showed that, in comparison to adult patients, pediatric population had a higher rate of harbouring Metallo-beta lactamases [22]. Among the CRE strains isolated from children, 49.0% were MBL-producers, mostly NDM. In our study, the highest number of MBL producer isolates were from the NICU, which represented 50% of all eCIM-positive isolates.

Our study found that CRE harboring different beta-lactamase enzymes are better susceptible to Tigecycline (86%) than to Minocycline. In a study by Bajaj et al. overall Tigecycline resistance among CRE isolates was also found to be 15.40% [61/396], which was highly concordant with our study finding of 14% resistant CRE to Tigecycline [17].

In the study "Global Assessment of the Activity of Tigecycline Against MDR-GN Pathogens Between 2004 and 2014 as Part of the Tigecycline
Evaluation and Surveillance Trial,” showed Resistance to Tigecycline in *E. coli* (0.2%), *Klebsiella* spp. (6%) and *Enterobacter* spp. (12%), while resistance was shown to Minocycline in *E. coli* (92.8%), *Klebsiella* spp. (75.2%), and *Enterobacter* spp. (82.8%) [23]. Our study also found similar results.

In the study by Garrison et al, overall tigecycline susceptibility in CRE was 84% [24], which was found to be relatively higher (91.6%) in the present study.

Interestingly, our study found that all CRE isolates resistant to Tigecycline were also resistant to Minocycline. Among these *Klebsiella* spp. isolates recovered from MBW and PICU, 100% incidence rate of the specific resistance pattern. In this regard, *in vitro* resistance to the antibiotic Tigecycline indicates a high probability of resistance to Minocycline. For a better understanding of these resistance patterns, further study is required. Tigecycline could definitely be a better option in the treatment of CRE infection, though further therapeutic efficacy should be evaluated for effective treatment.

**CONCLUSION**

CRE infection is an emerging public threat. Carbapenemase-producing CREs are responsible not only for therapeutic failure but are also a major cause of concern in disseminating multidrug resistance gene. Routine screening for CP-CRE strains by simple, inexpensive, and highly specific mCIM and eCIM methods will be helpful in mitigating the multi-drug resistance CRE infection. In a resource-limiting set up, the availability of the next and novel modalities to treat the CRE infection is a dire challenge. This could be mitigated by evaluating old groups of drugs like Tigecycline in the treatment of the same.

**AUTHORS’ CONTRIBUTION**

All the mentioned authors contributed to the design and implementation of the study and to the writing of the manuscript.

**CONFLICT OF INTEREST**

None.

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**REFERENCES**