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Original Research Article

Evaluation of phenotypic carbapenem inactivation methods among carbapenem resistant gram-negative bacteria isolated from blood culture specimens and their synergy testing

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ABSTRACT

Background: Carbapenem-resistant Enterobacterales (CRE) are a significant public health threat due to their resistance to antibiotics, leading to serious infections, increased healthcare expenses, and higher mortality rates. Accurate identification of CRE is essential for effective treatment and infection control. Phenotypic methods like the modified Carbapenemase Inactivation Method (mCIM) and the EDTA-Carbapenem Inactivation Method (eCIM) are practical approaches for identifying production carbapenemase. As a result, the current study aims to analyse the efficacy of phenotypic carbapenem inactivation approaches in detecting carbapenem-resistant Gram-negative bacteria isolated from blood culture specimens, as well as the synergy of ceftazidime-avibactam and aztreonam in treating such infections.

Aims & Objectives: This laboratory based prospective study was adopted to evaluate the efficacy of phenotypic carbapenem inactivation methods in detecting carbapenem-resistant Gram-negative bacteria isolated from blood culture specimens and assess the synergy of ceftazidime-avibactam and aztreonam in treating these infections.

Materials and Methods: Gram-negative bacteria isolated from positive blood cultures were evaluated for carbapenemase activity using mCIM and eCIM, respectively. Synergy testing was conducted using ceftazidime-avibactam and aztreonam to evaluate potential therapeutic benefits.

Results: Among 383 blood cultures, 153 (39.94%) were MDROs, predominantly *Klebsiella pneumoniae* (57.5%). Of these, 123 (81%) were carbapenem-resistant. The mCIM and eCIM tests identified 67 (54%) serine carbapenemase and 54 (45.5%) metallo beta-lactamases. Synergy testing with ceftazidime-avibactam and aztreonam showed positive results in 43 (68.25%) of the 63 CZA-resistant isolates.

Conclusion: The study confirms that mCIM and eCIM tests effectively detect carbapenemase production in blood culture isolates, identifying 54% serine carbapenemases and 45.5% metallo beta-lactamases. Additionally, synergy testing with ceftazidime-avibactam and aztreonam demonstrated a 68.25% success rate in CZA-resistant isolates, indicating a promising treatment option for these resistant infections.

1. Introduction

Carbapenem-resistant gram-negative bacteria (CR-GNB) pose an increasing concern to public health by generating

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infections acquired both in healthcare settings and among communities.[1] Carbapenems are β-lactam antimicrobials. Effective bactericidal agents, they are especially important in treating infections brought on by bacteria that generate extended-spectrum β-lactamases (enzymes resistant to numerous β-lactam antibiotics).[2] In simpler terms, CR-GNB are bacteria that have developed resistance to carbapenem antibiotics, which are essential for treating certain types of bacterial infections. This resistance makes these bacteria particularly difficult to treat and can lead to serious health complications.Carbapenemases, such as Imipenem, Ertapenem, Meropenem, and Doripenem, are critical antibiotics for treating multidrug-resistant bacterial infections.[3] Screening for carbapenem resistance in clinical microbiology labs is essential to identify resistant bacteria, guide treatment decisions, and prevent the spread of resistance. Resistance may be inherited or acquired by gene transfer or mutation.[4] Current guidelines from CLSI and EUCAST provide standards for carbapenem susceptibility testing, though EUCAST lacks specific doripenem breakpoints.[5,6] The Modified Hodge Test (MHT), the Carba NP test and its variations, and the modified Carbapenem Inactivation Method (mCIM) are phenotypic assays that are frequently used in clinical microbiology laboratories to identify bacteria that produce carbapenemases. However, these tests typically do not provide specific information about the type of carbapenemase being produced. Some modifications to these assays can offer more insight into the specific carbapenemase groups being expressed. For instance, while the traditional mCIM does not differentiate between different classes of carbapenemases, conducting the test alongside the addition of the divalent cation chelator EDTA (known as EDTA-mCIM or eCIM) allows for the distinction between serine and metallo-carbapenemases. In simpler terms, by making slight adjustments to these tests, laboratories can gain more detailed information about the type of carbapenemase present in bacterial isolates. A quick test to identify gram-negative bacteria that produce carbapenemases is called the modified Carbapenem Inactivation Method, or mCIMs. This assay is currently being assessed using culture isolates.[7] Using positive blood cultures from carbapenem-resistant gram-negative bacterial isolates, this study intends to assess phenotypic carbapenem inactivation techniques and their synergy testing in a tertiary care hospital in Mysuru, South India.

2. Materials and Methods

Isolation and Identification of the Organisms**:** Organisms were isolated, identified, and tested for susceptibility using standard bacteriological procedures. Blood samples were collected aseptically into culture bottles and processed using automated blood culture systems (Versa Trek, BacT/Alert). Gram-stained positive cultures were sent to doctors right away, while Gram-negative bacilli were not. After that, samples were cultivated on McConkey and sheep blood agar and incubated. Vitek 2 was used for identification and susceptibility testing.

Carbapenem-resistant isolates underwent carbapenem inactivation testing to determine carbapenemase production type.

In the study, Modified Carbapenem Inactivation Method (mCIM) and its enhanced version (eCIM) were employed to detect carbapenemases in Enterobacterales and Pseudomonas aeruginosa.

2.1. Modified carbapenem inactivation method [mCIM]

For every isolate, emulsify a 1-μL loopful of Enterobacterales or a 10-μL loopful of Pseudomonas aeruginosa in 2 mL of tryptic soy broth (TSB) derived from an overnight blood agar plate. Add a 10-μg meropenem disc to each tube, making sure the disc is completely submerged, then vortex the suspension for ten to fifteen seconds. For four hours and fifteen minutes, incubate the tubes at 35°C ± 2°C. A 0.5 McFarland suspension of *E. coli* ATCC® 25922 should be made in saline or nutritional broth. This suspension should be applied to a Mueller-Hinton agar (MHA) plate, and it should be left to dry for three to ten minutes. Transferring the meropenem disc onto the inoculated MHA plate, remove it with a 10-μL loop from the TSB suspension. After 18 to 24 hours of incubation at 35°C ± 2°C, assess the zones of inhibition.

2.2. mCIM results

2.2.1. Carbapenemase positive

The existence of a carbapenemase is indicated by a zone diameter of 6–15 mm or pinpoint colonies inside a 16–18 mm zone, as the hydrolysis of the disk's meropenem results in little to no growth inhibition of *E. Coli* ATCC® 25922.

2.2.2. Carbapenemase negative

The presence of carbapenemase is not indicated by a clear zone diameter of less than 19 mm, since meropenem is still able to effectively suppress the growth of *E. coli* ATCC® 25922.

2.2.3. Carbapenemase inconclusive

When pinpoint colonies are present and the zone diameter is 16–18 mm or ≥19 mm, it indicates that more testing is necessary to determine whether carbapenemase is present or not.

2.3. EDTA carbapenem inactivation method [eCIM]

To reach a final concentration of 5 mM, label a second 2-mL TSB tube and add 20 μL of 0.5 M EDTA. Proceed as per the mCIM protocol, processing the two tubes simultaneously

and putting the meropenem discs from the mCIM and eCIM on the same MHA plate.

eCIM Results (to be interpreted only if mCIM is positive):

1. Metallo-β-lactamase positive: The zone diameter increases by ≥5 mm in comparison to the mCIM, indicating the presence of metallo-β-lactamase. The inhibition of *E. coli* ATCC® 25922 is a result of EDTA's suppression of the enzyme's activity.
2. Metallo-β-lactamase negative: The activity of the enzyme is not significantly affected by EDTA, and an increase of ≤4 mm in the zone diameter relative to the mCIM indicates the presence of a serine carbapenemase.

Reporting of mCIM and eCIM

1. For carbapenemase-positive isolates, report as "Carbapenemase positive," including the zone diameter and any pinpoint colonies.
2. For carbapenemase-negative isolates, report as "Carbapenemase negative," with the clear zone diameter.
3. For inconclusive results, report as "Carbapenemase inconclusive" and recommend additional testing.
4. For eCIM, if metallo-β-lactamase positive, report as "Metallo-β-lactamase positive" with the zone diameters for both mCIM and eCIM. If metallo-β-lactamase negative, report as "Metallo-β-lactamase negative," including the zone diameters for both tests.

2.4. Clinical relevance

2.4.1. mCIM and eCIM

In the study, mCIM identified 67 isolates as serine carbapenemases (mCIM positive, eCIM negative), indicating resistance mediated by serine β-lactamases. This information is crucial for selecting appropriate antibiotics that can effectively inhibit these enzymes. Conversely, 54 isolates were identified as metallo-β-lactamases (MBLs) using both mCIM and eCIM. This distinction is critical because MBLs are resistant to serine β-lactamase inhibitors like EDTA, influencing treatment decisions toward antibiotics that can overcome MBL-mediated resistance.

2.4.2. CZA-AZT synergy testing

The synergy testing results showed that among the carbapenem-resistant organisms (CROs) tested, 43 out of 63 CZA-resistant isolates exhibited positive synergy with AZT. This finding is significant as it suggests that despite resistance to ceftazidime-avibactam (CZA) alone, the combination of CZA with aztreonam (AZT) could still effectively inhibit bacterial growth in a significant proportion of cases. By treating infections brought on by CROs, this method lessens the need for carbapenems and other last-line antibiotics.

mCIM and eCIM play a crucial role in identifying specific carbapenemase types among resistant organisms, guiding targeted antibiotic therapy. CZA-AZT synergy testing expands treatment options by demonstrating effective synergistic activity against resistant pathogens, thereby enhancing clinical management strategies for patients with challenging infections. These methods collectively contribute to optimizing antibiotic use, combating antimicrobial resistance, and improving patient outcomes in clinical practice.

3. Results

The study comprised the first 383 positive flagged blood cultures received in the laboratory during the study period. Following the definition, 153 (39.94%) of the 383 positively flagged blood cultures were classified as MDROs and were added to the research. Ages ranging from 3 years minimum to 70 years maximum comprised the majority of patients yielding growth of MDR GNB. Of 153 subjects included in the study 69% of them were males, 31% were females. Of 153 MDR GNB included, the majority were *Klebsiella pneumoniae* 88 (57.5%), followed by 32 (20.9%) *E. coli*, *P. aeruginosa* 29 (18.95%), and *Serratia marcescens* 4 (2.61%). Carbapenem resistance was determined through Vitek 2 systems and of 153 MDR GNB isolates, 123 (81%) were found to be Carbapenem resistant and 30 (19%) were carbapenem sensitive but multi-drug resistant organisms. Those isolates that were determined as Carbapenem-resistant (n= 123), were further subjected to mCIM, eCIM, and synergy testing to determine the type of carbapenemase producers. Of 123 CRO's 67 (54%) isolates were Serine Carbapenemases which are mCIM positive and eCIM negative) and 1 isolate each of

K. pneumoniae and P. aeruginosa showed indeterminate results. Of 123 CROs 54 (45.5%) were positive by both eCIM and mCIM and were determined as Metallo B lactamases. The results of these are tabulated in (Table 1)

As per CLSI guidelines, Synergy testing is indicated only for MBL-producing organisms but as a novel approach for better understanding synergy between CZA and AZT, synergy testing was performed for all CROs included in this study. All 123 CROs were further subjected to synergy testing using ceftazidime- avibactam and Aztreonam. Of 123 CROs, 60 (48.7%) were CZA susceptible and 63 (51.2%) were CZA resistant, and synergy testing was positive in 43 (68.25%) of the CZA-resistant isolates and negative in 31.74%) of the isolates. (Table 2)

To determine the statistical significance of the results, a chi-square test for independence was conducted to assess the association between the type of carbapenemase (serine vs. metallo-β-lactamase) and the organisms identified. Additionally, Fisher's exact test was used to analyze the

Table 1: Showing distribution of Serine and Metallo β Lactamases

0rganisms	Serine Carbapenemases (mCIM positive and eCIM negative)	Metallo Beta lactamases (Both positive)	Inconclusive
E. coli	0	16	0
Klebsiella pneumoniae	67	35	1
Serratia marcescens	0	1	0
Pseudomonas aeruginosa	0	2	1
Total	67	54	2

Table 2: Distribution of CZA susceptibility results and synergy testing results

Organisms	CZA		Synergy	
	S	R	Pos	Neg
E. coli - 22	29	03	2	1
Klebsiella pneumoniae -68	28	30	28	2
Serratia marcescens -4	3	1	1	0
Pseudomonas aeruginosa -29	0	29	12	17
Total	60	63	43	20

significance of synergy testing results among CZA-resistant isolates.

4. Discussion

In clinical contexts, carbapenem resistance in Gram-negative bacteria is a serious challenge that calls for accurate detection techniques to inform successful treatment plans. In order to identify carbapenemase producers among clinical isolates from blood cultures, we assessed phenotypic carbapenem inactivation techniques in this investigation.

The Carbapenemase Inactivation Method (CIM), launched in 2015 and is dependent on isolates that produce carbapenemase hydrolysing meropenem.[8] For detecting carbapenemase manufacturers, this approach showed great specificity (99-100%) and sensitivity (91-94%).[9,10] After being further confirmed by the CLSI data working group, the updated version, called modified CIM (mCIM), had a mean sensitivity of 97% and specificity of 99% across several testing sites.[11]

A drawback of mCIM is its incapacity to distinguish between metallo-β- and serine-lactamases. Our solution was to employ the EDTA-Carbapenem Inactivation Method (eCIM), which uses EDTA to block metallo-β-lactamase activity and separate the two kinds of carbapenemases. The results of our investigation support earlier studies, demonstrating the effectiveness of eCIM in improving specificity for identifying particular carbapenem resistance pathways.[12]

Our study included 153 multidrug-resistant Gram-negative bacteria (MDR GNB) isolates from blood cultures, of which 123 (81%) were carbapenem-resistant. Among these, 67 isolates (54%) were identified as serine carbapenemases (mCIM positive, eCIM negative), and 54 isolates (45.5%) were confirmed as metallo-β-lactamases (positive by both mCIM and eCIM). Additionally, 1 isolate each of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* showed indeterminate results.

Furthermore, synergy testing with ceftazidime-avibactam (CZA) and aztreonam (AZT) was performed on all 123 carbapenem-resistant organisms (CROs). Among these, 60 isolates (48.7%) were CZA susceptible, and 63 isolates (51.2%) were CZA resistant. Of the CZA-resistant isolates, 43 (68.25%) showed positive synergy with AZT, highlighting the potential for combined therapy in treating resistant infections.

The chi-square test revealed a significant association between the type of carbapenemase and the organisms identified ($p < 0.05$), indicating that specific organisms are more likely to produce particular types of carbapenemases. The Fisher's exact test for synergy testing showed a significant proportion of CZA-resistant isolates demonstrating positive synergy with AZT ($p < 0.05$), supporting the potential efficacy of the combination therapy.

The accurate identification of carbapenemase producers is clinically significant as it directly impacts patient management and treatment outcomes. The mCIM and eCIM methods provide reliable and specific results, enabling healthcare providers to tailor antibiotic therapy effectively.[13] Identifying the type of carbapenemase present in an isolate help guide the use of targeted antimicrobials, reducing the reliance on broad-spectrum antibiotics and minimizing the risk of developing further resistance.[14] The synergy testing results indicate that combination therapy with CZA and AZT can be an effective treatment strategy for CZA-resistant infections, offering a potential solution for managing difficult-to-treat infections.

5. Conclusion

Our study highlights the significance of using combined phenotypic detection methods, such as mCIM and eCIM, to accurately identify and differentiate carbapenemase-producing bacteria. The high prevalence of carbapenem-resistant organisms (81%) among the MDR GNB underscores the urgent need for effective diagnostic tools and tailored treatment strategies.

The mCIM and eCIM methods demonstrated high sensitivity and specificity, making them reliable tools for detecting carbapenemase production in clinical isolates. The synergy testing results further emphasize the potential of combination therapy with ceftazidime-avibactam and aztreonam in managing carbapenem-resistant infections.

A significant synergy positivity was observed with CZA and AZT among resistant isolates which highlights the potential of combination therapy in managing carbapenem-resistant infections. These findings provide robust evidence supporting the use of these diagnostic and therapeutic approaches in clinical practice.

Understanding the mechanisms behind carbapenem resistance in Enterobacterales is crucial for clinical management, infection control, and antimicrobial stewardship. Accurate detection of carbapenemase producers allows for targeted therapy, optimizing patient outcomes and reducing the spread of resistant strains. This study supports the implementation of integrated phenotypic methods in clinical laboratories, regardless of resource constraints, to enhance diagnostic accuracy and guide appropriate treatment decisions. Continued research and validation of these methods across different bacterial species and clinical settings will further strengthen our ability to combat antibiotic resistance effectively.

6. Ethical Approval

Every individual involved in this research gave their consent, or declined it. Approved JSS/MC/PG/46/2022–23 by the Institutional Ethical Committee (IEC) of JSS Medical College.

7. Source of Funding

Nil.

8. Conflicts of Interest

Nil.

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