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Original Research Article

Phenotypic Detection of Carbapenemase-Producing Gram-Negative Bacteria Using Modified and EDTA-Carbapenem Inactivation Methods: A Prospective Study from a Tertiary Care Center in India

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Abstract:

Introduction: Carbapenem-resistant (CR) gram-negative organisms present a critical global threat. Phenotypic tests such as modified Carbapenem Inactivation method (mCIM) and EDTA carbapenem inactivation method (eCIM) provide affordable options for detecting carbapenemases in resource-limited settings. This study aimed to identify CROs, differentiate resistance mechanisms using mCIM/eCIM, and evaluate effective strategies for treatment and control of carbapenem-resistant organisms (CROs) infections.

Methods: A prospective observational study was conducted in the department of Microbiology, Kakatiya Medical College, from January 2023 to May 2024. A total of 210 non-duplicate Gram-negative isolates resistant to one or more carbapenems were tested using mCIM and eCIM as per CLSI guidelines.

Results: Of 210 isolates, 108 (51.4%) were carbapenemase producers: 67 (32%) were metallo-β-lactamases (MBLs) and 41 (20%) were serine carbapenemases. *Klebsiella pneumoniae* was the predominant isolate. The highest proportion of MBLs was found in urine samples, while serine carbapenemases were more common in blood cultures.

Conclusion: The mCIM and eCIM tests provide effective phenotypic screening for carbapenemases. These methods are essential in guiding empirical therapy, especially in settings lacking molecular diagnostics.

Keywords: Antimicrobial Resistance, Carbapenem Resistant Organisms, Modified Carbapenem Inactivation method, EDTA Carbapenem inactivation method, Metalloβlactamases, Serine Carbapenemases.

Introduction

Excessive and or unrestricted usage of antimicrobials is the biggest driver of antimicrobial resistance [1]; by 2050, these will kill 10 million per year which is 700,000 currently. Carbapenem-resistant (CR) Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacterales listed as priority pathogens by the WHO [2]. Carbapenemase enzyme production is the most common mechanism of CR by the gram-negative bacilli (GNB).

Carbapenemases, according to the Ambler molecular classification, are broadly divided into serine and metallo-β-lactamases. The serine group includes Class A enzymes such as KPC, SHV, IMI, SME, and CTX-M; Class C enzymes such as OXA-48, OXA-181, OXA-40, and OXA-58; and Class D enzymes like AmpC. The metallo-β-lactamases (MBLs), on the other hand, belong to Class B and include NDM, VIM, and IMP [3]. Isolates that produce KPC enzymes may remain susceptible to newer β-lactam/β-lactamase inhibitor (BL/BLI) combinations, whereas MBL-producing isolates are generally not inhibited by these agents. Despite the availability of novel BL/BLI therapies for CR GNB infections, emerging resistance has been reported, highlighting the urgent need for robust antimicrobial and diagnostic stewardship [4, 5].

The Clinical Laboratory Standards Institute (CLSI) recommends that an isolate be considered carbapenemase-producing if it is intermediate or resistant to more than one carbapenem. This identification is crucial for tailoring therapy and implementing institutional infection control protocols [6]. Early and accurate recognition of carbapenemase-producing organisms (CROs) plays a key role in preventing their spread and guiding rational antibiotic use. Therefore, diagnostic microbiology must integrate phenotypic methods to differentiate the resistance mechanisms for effective patient management.

In this context, the present study was undertaken to identify carbapenem-resistant organisms from clinical isolates and interpret their antimicrobial susceptibility test (AST) reports. The mechanisms of resistance were further differentiated using the modified carbapenem inactivation method (mCIM) combined with the EDTA-carbapenem inactivation method (eCIM). The results were communicated to clinicians during rounds, enabling culture-guided escalation or de-escalation of therapy based on the type of carbapenemase produced [7]. This approach supports timely diagnosis, rational therapeutic decisions, and development of effective strategies to control CRO infections [4].

Methods:

It was a laboratory based prospective observational study, conducted in the department of Microbiology, Kakatiya Medical College. Study was conducted from January 2023 to May 2024. Written informed consent was collected and study protocol was approved by the Institutional Ethical Committee (ECR/840/Inst/TG/2016/RR/20/40). Isolates identified to be resistant to $\geq$1 Carbapenems were included and repeat specimen was rejected.

Various clinical samples were obtained from the symptomatic patients from Intensive Care Units (ICU) / wards were considered within 2 hours of collection. All samples were processed in the Biosafety Cabinet. Blood and body fluids were inoculated in blood culture bottles and incubated in BacT alert automated instrument. Bottles flagged positive were sub cultured on Blood agar (BA), MacConkey agar (MA). Other samples such as pus, swab, urine, tissue were inoculated on BA and MA and incubated at 37^0C for 18 – 24 hours. Isolates were identified by conventional biochemical reactions. Antibiotic susceptibility was done using Kirby Bauer Disc diffusion method as per the recent CLSI guidelines. After successful processing, the samples were stored in the refrigerator, blood culture bottles were discarded after reporting.

Isolates showed resistance to one or more carbapenems on Mueller Hinton agar plate were further tested by mCIM with eCIM tests as described in the CLSI M100 guidelines. A 1µl loopful of bacteria from Enterobacterales and 10 µl from Non fermentative GNB from overnight incubated blood agar plate was emulsified in 1 tube containing 2ml of Tryptic soy broth. Another 1µl loopful of bacteria from Enterobacterales and 10 µl from Non fermentative Gram negative Bacilli was suspended in another tube containing 2ml of Tryptic soy broth with 20 µL of 0.5 M Ethylene Diamine Tetra acetic Acid (EDTA) added to it. A Meropenem disk (10-µg) was placed in each tube, followed by aerobic incubation for 4 hours at 37 °C. Subsequently the disks were removed and applied over a plate of Muller Hinton agar inoculated by a 0.5 McFarland saline suspension of E. coli ATCC 25922. The plates were incubated for 16 – 20 hours at 37^0C. The mCIM and eCIM were used in combination for differentiation of carbapenemases. An mCIM zone size ≥19 mm was considered negative, while 6–15 mm or pinpoint colonies within 16–18 mm indicated positivity. An increase ≥5 mm with eCIM confirmed MBL, whereas <4 mm supported serine carbapenemase (Figure 1). Reporting was done according to CLSI-M100 guidelines [8, 9].

Results

Total 210 CR strains were isolated; majority from blood culture (63: 30.1%) followed by wound swab (60; 29%), urine (47; 22.4%) pus (22; 10.5%), sputum (11; 5.2%) and the remaining 7 from bronchial washing, ascitic fluid, endotracheal aspiration and tissue sample. Pathogen wise, Klebsiella pneumoniae (KP) was the leading isolate (69; 33%) followed by Escherichia coli (45; 21.4%), Acinetobacter baumanii (39; 18.5%), Klebsiella oxytoca (27; 13%), Pseudomonas aeruginosa (22; 10.4%), Citrobacter freundii (4; 2%), Proteus vulgaris and Proteus mirabilis, 2 (1%) each, respectively (Table 1). Carbapenemases were detected in 108 (51.5%) isolates; among these 67 (32%) were Metalloβlactamases (MBLs) and the rest (41; 20%) were Serine Carbapenemases (Table 2).

Majority (21) of MBLs were isolated from urine followed by blood (16), swab (15), pus (7) and 4 each from sputum, rest of the isolates, respectively (table 3). Out of 41 Serine carbapenemases, 14 were from Blood 14/41, 10 from Swab, 7 from Pus, 6 from Urine, 4 from Sputum Table 3). Further organism-specific distribution revealed that in urine samples with MBLs, E. coli predominated, followed by KP and K. oxytoca. Blood samples with MBLs were dominated by KP along with K. oxytoca, A. baumannii, and C. freundii (Table 4). For serine carbapenemases in blood, A. baumannii was the leading isolate, followed by KP, K. oxytoca, and E. coli. In swab samples, serine carbapenemases were most commonly associated with KP, K. oxytoca, E. coli, and a smaller proportion of A. baumannii (Table 5).

Table 1: Sample wise distribution of CROs

Sample	KP	*E. coli*	*K. oxytoca*	*A. baumanii*	*P. aeruginosa*	*C. freundii*	*P. species*
Blood	26	3	6	28	-	-	-
Urine	12	21	3	3	4	2	2
Swab	18	9	12	6	12	1	2
Pus	7	7	3	1	3	1	-
Sputum	4	3	2	1	1	-	-
Rest of samples	2	2	1	-	2	-	-
Total	69	45	27	39	22	4	4

E. coli: Escherichia coli, K. oxytoca: Klebsiella oxytoca, A.baumanii: Acinetobacter baumanii, P. aeruginosa: Pseudomonas aeruginosa, C. freundii: Citrobacter freundii, P.species: Proteus species.

Table 2: Isolation of MBL, Serine Carbapenemase and Non- Carbapenemases; n (%)

mCIM	eCIM	Interpretation	Isolates
+	+	MBL	67 (32)
+	-	Serine Carbapenemase	41 (20)
-	-	Carbapenemase not detected	102 (48)

Table 3: Specimen wise distribution of Carbapenemase

Type of Carbapenemase	Blood	Swab	Urine	Pus	Sputum	Rest
MBL	16	15	21	7	4	4
Serine Carbapenemases	14	10	6	7	4	4
Non-Carbapenemases	33	35	20	8	3	3

Table 4: Pathogen wise distribution of MBLs among the clinical sample

Specimen	KP	*E. coli*	*K. oxytoca*	*A. baumannii*	*Ps. aeruginosa*	*C. freundii*	Total
Blood	13	-	2	-	1	-	16
Urine	6	11	3	-	1	-	21
Swab	4	4	5	-	1	1	15
Pus	2	2	-	-	3	-	7
Sputum	2	1	-	-	1	-	4
Rest	2	2	-	-	-	-	4

Table 5: Distribution of Serine Carbapenemases in isolates among the clinical samples

Sample	KP	E. coli	K. oxytoca	A. baumannii	Total
Blood	4	1	3	6	14
Urine	3	2	1	-	6
Swab	3	3	3	1	10
Pus	1	3	2	1	7
Sputum	2	1	1	-	4

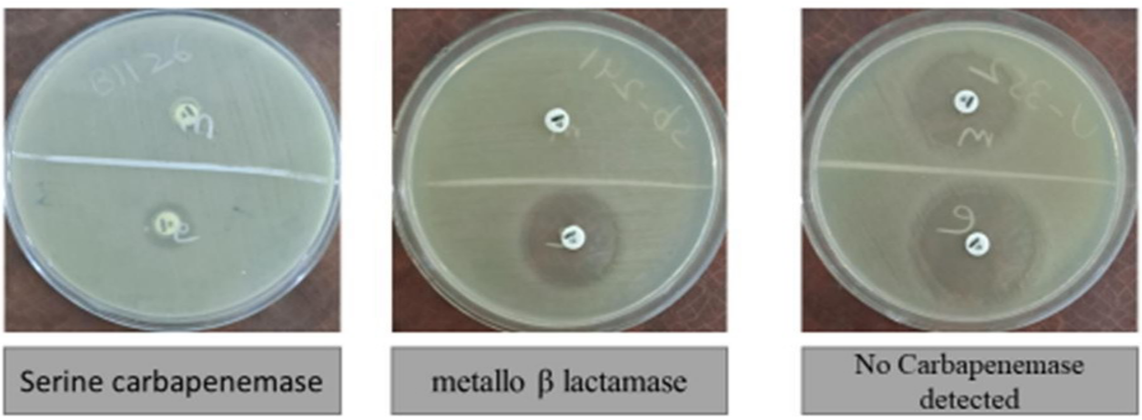

Figure 1: Interpretation of Carbapenemase producing organisms

Discussion

A total of 210 CR strains were isolated during the study period, with the highest proportion derived from blood cultures (63; 30.1%), followed closely by wound swabs (60; 29%), and urine samples (47; 22.4%). Pus and sputum samples accounted for 10.5% and 5.2% respectively, while the remaining seven isolates originated from bronchial washings, ascitic fluid, endotracheal aspirates, and tissue samples. Pathogen-wise, KP was the predominant isolate (69; 33%), followed by Escherichia coli (45; 21.4%), Acinetobacter baumannii (39; 18.5%), Klebsiella oxytoca (27; 13%), Pseudomonas aeruginosa (22; 10.4%), with Citrobacter freundii (4; 2%), Proteus vulgaris (2; 1%), and Proteus mirabilis (2; 1%) comprising the remainder. These distributions align with global trends showing that K. pneumoniae and E. coli are leading causes of carbapenem resistance in both clinical and bloodstream infections. Sisay et al. reported similar prominence of these pathogens among carbapenem-resistant and cephalosporin-resistant isolates, underlining their clinical significance in the spread of multidrug-resistant infections [10].

Carbapenemase production was confirmed in 108 of the isolates (51.5%), of which 67 (32% of total isolates) produced MBLs and 41 (20%) produced serine carbapenemases. This nearly equal distribution underscores the dual threat of MBLs such as NDM, IMP, and VIM which hydrolyze carbapenems via zinc-dependent mechanisms, and serine carbapenamases such as KPC, OXA variants, and others which employ a serine active site. Codjoe and Donkor detail the epidemiology and clinical importance of these Ambler classes in Enterobacteriaceae, emphasizing that both mechanisms are widespread and pose substantial challenges to antimicrobial therapy [11]. Moreover, Wu et al. highlighted the frequent co-occurrence of MBLs like NDM-1 and serine carbapenemases such as OXA-48 in K. pneumoniae, suggesting that multiple resistance mechanisms may coexist within single strains, exacerbating treatment complexity [12].

The detection of carbapenemase producers in $\geq$50% of the CR isolates has critical clinical and epidemiological implications. As Yahav et al. discuss, the increasing availability of new β-lactam/β-lactamase inhibitor combinations offers therapeutic options for KPC or other serine carbapenemase producers; however, MBL producers remain largely resistant to such inhibitors, reinforcing the need for precise phenotypic or molecular differentiation of resistance mechanisms [13]. Additionally, the dissemination of MBL-encoding genes like bla_NDM-1 across globally circulating pathogenic strains including E. coli and KP has been well documented, with Newman et al. and others stressing the threat posed by horizontal gene transfer driving the spread of these resistance determinants [14]. In this regard, efforts such as the development of broad-spectrum inhibitors targeting both serine and metallo-carbapenemases like taniborbactam are encouraging, though they remain in early stages of translational research [15].

Together, these findings underline the urgent need for integrated diagnostic stewardship including phenotypic assays like mCIM/eCIM and molecular testing to accurately phenotype carbapenemases, inform tailored therapy, and guide infection control measures. Moreover, they reinforce the imperative for ongoing surveillance and research into novel therapeutics and inhibitors capable of tackling both serine and MBL resistance mechanisms.

The analysis of CROs revealed a noteworthy distribution of resistance mechanisms across sample types. A total of 67 isolates were identified as MBLs, the majority of which were recovered from urine (21), followed by blood (16) and swabs (15). Smaller numbers originated from pus (7), sputum (4), and miscellaneous samples (4). Serine carbapenemases were detected in 41 isolates, with blood accounting for the highest proportion (14), followed by swabs (10), pus (7), urine (6), and sputum (4). The predominance of MBLs in urinary isolates, particularly Escherichia coli and KP, is significant as these pathogens are key agents of community- and hospital-acquired urinary tract infections. Similar observations were highlighted by Wu et al., who described NDM-1 producing E. coli as a major contributor to multidrug resistance in urinary infections worldwide [12]. The presence of MBLs in bloodstream infections, largely dominated by KP, emphasizes their potential to cause severe invasive disease with limited therapeutic options [16].

Organism-specific analysis highlighted distinct patterns of resistance within sample categories. In urine samples, E. coli was the dominant MBL producer, followed by KP and K. oxytoca. In blood, KP accounted for the majority of MBLs, with additional contributions from K. oxytoca, Acinetobacter baumannii, and Citrobacter freundii. Serine carbapenemases in blood were led by A. baumannii, followed by KP, K. oxytoca, and E. coli. In swab isolates, both KP and K. oxytoca were frequent producers, along with E. coli and a smaller contribution from A. baumannii. This distribution supports existing literature, where Enterobacterales, particularly KP and E. coli, remain the leading hosts of carbapenemases, while non-fermenters such as A. baumannii contribute significantly to nosocomial infections [11]. The high representation of serine carbapenemases in A. baumannii isolates underscores its role as an opportunistic pathogen with remarkable ability to acquire diverse β-lactamase genes [17].

The clinical implications of these findings are profound. MBL producers are notoriously resistant to nearly all β-lactam/β-lactamase inhibitor combinations, necessitating reliance on agents such as colistin, tigecycline, or cefiderocol, all of which present limitations in toxicity or availability. Conversely, serine carbapenemase producers, such as KPC-producing KP, may remain susceptible to newer inhibitors including ceftazidime-avibactam, meropenem-vaborbactam, or imipenem-relebactam [13]. Differentiating resistance mechanisms, therefore, is not merely an academic exercise but an essential step in guiding tailored therapy and institutional infection control practices. The predominance of MBLs in urinary and bloodstream isolates and serine carbapenemases in wound and respiratory pathogens highlights the heterogeneity of resistance and the importance of comprehensive diagnostic stewardship. Together, these data emphasize the necessity for ongoing surveillance, molecular confirmation of resistance genes, and antimicrobial policy reinforcement to curb the escalating threat of CROs.

This study has certain limitations, including its single-centre design and reliance on mCIM and eCIM methods, which require overnight incubation. These procedures cannot distinguish when MBLs and serine carbapenemases are co-produced, nor can they differentiate individual enzyme subtypes. Despite these constraints, the findings highlight the crucial role of clinical microbiologists in combating antimicrobial resistance. Their contributions include strengthening laboratories with rapid diagnostics, ensuring accurate resistance detection, monitoring CRO prevalence across time and facilities, and providing clinicians with antibiogram data for informed decisions. For clinicians, appropriate culture and sensitivity testing before antibiotic initiation, reviewing therapy within 48–72 hours, culture-guided de-escalation, discontinuing antibiotics in non-infectious cases, and optimizing dosage and duration remain pivotal in preserving antibiotic efficacy.

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