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Original Research Article

Characterizing carbapenemase production in *Enterobacterales* through combined disk test and genetic profilingShuchita Gaur^{1*}, Rupam Gahlot¹, Nikita Sherwani¹, Suchita Netam¹, Sneha Dadarya¹¹Dept. of Microbiology, Pt. JNM Medical College, Raipur, Chhattisgarh, India

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ABSTRACT

Background: Carbapenem-resistant *Enterobacterales* (CRE) are major organisms amongst the critical group of drug-resistant bacteria and are associated with high morbidity and mortality. With limited treatment options, the detection and characterization of carbapenemase is important for appropriate management. This study aims to characterize carbapenemase produced by *Enterobacterales* using combined disk test and their molecular profiling.

Materials and Methods: All carbapenem-resistant *Enterobacterales* isolated from various clinical samples were included in the study. Carbapenemase production was characterized by observing synergy on combining meropenem disk with beta-lactamase inhibitors like phenylboronic acid, EDTA, and cloxacillin, following which genetic profiling was done using multiplex PCR.

Results: Out of 445 *Enterobacterales* isolated, 104 (23.4%) were carbapenem-resistant. The most common CRE isolated was *Klebsiella pneumoniae* (62 out of 104) followed by *Escherichia coli* (40 out of 104) and 2 out of 104 CRE isolates were *Enterobacter* species. Co-production of NDM and OXA-48-like enzymes (39.4%) was the most common mechanism followed by NDM alone (19.2%) and OXA-48 alone (16.3%). NDM was the most common gene detected overall with 72 out of 104 CRE (69.2%) isolates showing its presence, followed by OXA-48 present in 63 of 104 (60.6%) isolates.

Conclusion: Metallo-beta-lactamases (NDM) was the predominant type of carbapenemase gene detected among the *Enterobacterales* isolates, with the co-production of NDM and OXA-48 enzymes being the most common mechanism of resistance.

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1. Introduction

Antimicrobial resistance (AMR) represents a well-documented global threat, significantly undermining the efficacy of commonly used antibiotics. This resistance leads not only to increased mortality rates but also escalates overall healthcare costs due to prolonged illness and the need for more complex treatments.¹ The decline in the development of new antibiotics, coupled with the growing ineffectiveness of existing ones, portends a grim scenario

where healthcare systems could revert to conditions reminiscent of the pre-antibiotic era.²

The World Health Organization (WHO) has highlighted the critical urgency of addressing antimicrobial resistance, particularly within the *Enterobacterales* order, which includes bacteria that are increasingly resistant to carbapenems, a class of last-resort antibiotics.³ The Centers for Disease Control and Prevention (CDC) has identified carbapenem-resistant *Enterobacterales* (CRE) — such as *Klebsiella* species, *Escherichia coli*, and *Enterobacter* species — as significant emerging threats to global health.⁴

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In the last decade, the misuse and overuse of carbapenems have precipitated an alarming rise in resistance among bacterial pathogens, especially those within the *Enterobacterales* family.⁵ This escalating trend presents a formidable challenge to global public health.⁶ The genes responsible for carbapenemase production, which confer resistance to carbapenems, are often located on mobile genetic elements such as plasmids and transposons.⁷ The presence of these genes on mobile elements facilitates their rapid transfer between bacteria through horizontal gene transfer mechanisms, thereby accelerating the spread of carbapenem resistance within bacterial communities.⁸

This study was conducted to identify the most prevalent genes responsible for carbapenemase production. By pinpointing these genes, we aim to implement effective infection control measures and enhance antimicrobial stewardship practices to combat the spread of carbapenem-resistant pathogens.

2. Materials and Methods

2.1. Study design and setting

This study was a prospective observational study conducted over one year, from October 2022 to September 2023, at the Department of Microbiology, Pt. Jawaharlal Nehru Memorial Medical College (Pt. JNMMC), is a tertiary care center located in Raipur, Chhattisgarh, India. The primary objective was to characterize carbapenemase production in *Enterobacterales* isolates through phenotypic methods and perform their molecular characterization.

2.2. Sample collection

A total of 445 *Enterobacterales* organisms were isolated from various clinical specimens, which included pus, sputum, other lower respiratory tract specimens, blood, other sterile body fluids, and urine. These specimens were received in the Microbiology laboratory from different clinical departments of Dr. BR Ambedkar Memorial Hospital Raipur, with cases of suspected or clinically diagnosed bacterial infections.

2.3. Microbiological processing and identification

Upon receipt, each specimen was processed according to standard microbiological practices. Identification and antimicrobial susceptibility testing (AST) of each *Enterobacterales* isolate were performed using the VITEK-2 automated system. Isolates showing resistance to meropenem were selected for further study.⁹

2.4. Phenotypic detection of carbapenemase production

The phenotypic detection of carbapenemase production was conducted using an inhibitor-based combined disk test. This involved the use of disks containing a combination

of meropenem with various beta-lactam inhibitors such as phenylboronic acid (PBA), ethylenediaminetetraacetic acid (EDTA), and cloxacillin (CLX). The detection of carbapenemase classes was determined based on the inhibition pattern observed, which manifested as a synergistic effect, indicated by an increase in the zone diameter of ≥ 5 mm with the combination of meropenem and inhibitor disks compared to the meropenem disk alone.

2.5. Genetic profiling

Genetic profiling was undertaken to identify the genes responsible for carbapenemase production. DNA purification for each carbapenem-resistant *Enterobacterales* (CRE) isolate was performed using the HiPurA Bacterial Genomic DNA Purification Kit (MB505). The detection of carbapenemase genes was achieved through multiplex PCR, utilizing the Hi-PCR® Carbapenemase Gene (Multiplex) Probe PCR Kit (MBPCR 132).

2.6. Ethical consideration

This study was approved by the Institutional Ethics Committee of Pt. J.N.M. Medical College, Raipur on 06-09-2022 (Approval No. /MC/Ethics/2024/374). All procedures followed the ethical standards set forth in the 2008 Declaration of Helsinki. Informed consent was obtained from all participants, and their confidentiality was rigorously maintained throughout the study.

2.7. Statistical analysis

Statistical analyses were carried out using SPSS Statistics version 27.0 (Statistical Package for the Social Sciences, IBM Corp., Armonk, NY, USA) and Microsoft Excel. The data were presented in the form of tables, charts, and graphs to elucidate the findings comprehensively.

3. Results

Among the 445 *Enterobacterales* isolates, 104 (23.4%) were identified as carbapenem-resistant *Enterobacterales* (CRE). *Klebsiella pneumoniae* was the most commonly isolated CRE, constituting 62 (59.6%) of the total CRE organisms. *Escherichia coli* accounted for 40 (38.5%), while *Enterobacter* species made up 2 (1.9%) of the CRE isolates (Figure 1).

The majority of CRE isolates (42.3%) were obtained from pus samples (44 out of 104), followed by urine samples and tracheal aspirates, each contributing 21.2% (22 out of 104). Blood samples accounted for 9.6% (10 out of 104), and pleural fluid samples made up 5.8% (6 out of 104). A significant proportion of CRE isolates (69%) were from patients admitted to intensive care units (ICUs) (72 out of 104), while 26.9% were from wards (28 out of 104), and 3.8% were from outpatient departments (OPD) (4 out of

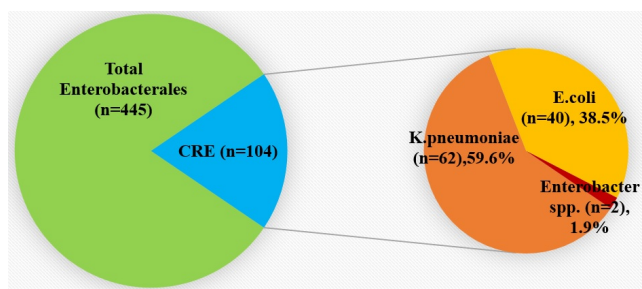


Figure 1: Distribution of isolates

104).

The phenotypic detection of carbapenemase production was performed using combined disk testing of meropenem with various carbapenemase inhibitors. The type of carbapenemase produced was identified based on the inhibition pattern observed. Synergy with meropenem combined with EDTA indicated the production of metallo-beta-lactamase. (Figure 2)

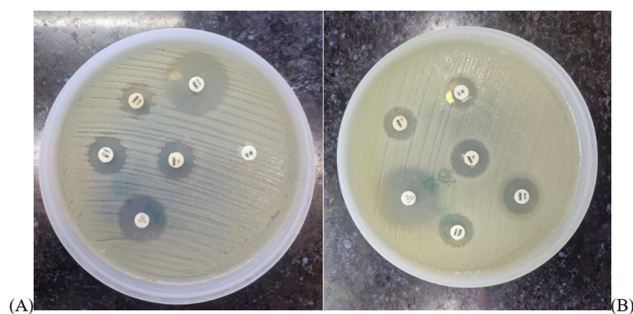


Figure 2: Combined disk test: (A) Synergy seen with Meropenem+EDTA disk showing production of metallo-beta-lactamase (B) No synergy seen with any of the combined disk

Out of the 104 meropenem-resistant isolates tested, 77.9% (81 of 104) showed synergy with the meropenem+EDTA disk, indicating the production of metallo-beta-lactamase (class B carbapenemase). Only 1.9% (2 of 104) showed synergy with cloxacillin, suggestive of AmpC beta-lactamase hyperproduction with porin loss. The remaining 20.2% showed no synergy with any of the drugs, indicating the potential presence of OXA-like enzymes (class D carbapenemase) or non-carbapenemase mechanisms responsible for resistance, such as ESBL with porin loss. No isolates showed synergy with the meropenem+phenyl boronic acid disk, thus no class A carbapenemase was detected (Table 1).

The concordance, also known as the percentage of agreement (POA), is the percentage of chance that an identical sample analysed by two different methodologies will yield the same result. The concordance of combined disk testing with molecular test in detection of metallo-

beta-lactamase was found to be 91.3% in this study; and in detection of OXA-like enzymes production alone was found to be 95.1%. Combined disk test could not detect presence of OXA-like enzymes production in combination with other carbapenemase production.

Molecular testing revealed that the presence of both NDM and OXA-48 genes was the main mechanism responsible for carbapenemase production among CRE isolates. Specifically, 41 (39.4%) isolates carried both NDM and OXA-48, followed by 20 (19.2%) isolates with NDM alone. OXA-48 alone was present in 17 (16.3%) isolates, and OXA-23 alone was detected in 10 (9.6%) isolates. Overall, NDM was the most commonly detected gene, found in 72 out of 104 CRE isolates (69.2%), followed by OXA-48 in 63 out of 104 (60.6%) isolates. Some CRE isolates showed the presence of more than one gene, and no carbapenemase gene was detected in 3 (2.9%) isolates, suggesting other mechanisms of carbapenem resistance (Table 2).

The most common resistance gene combination in carbapenem-resistant *Klebsiella pneumoniae* (CRKpn), the predominant isolate, was NDM and OXA-48 in 31 (29.8%) isolates, followed by NDM alone in 9 (8.7%), OXA-48 alone and OXA-23 alone in 8 (7.7%) isolates each. In carbapenem-resistant *E. coli*, the most common gene was NDM alone in 11 (10.6%) isolates, followed by the combination of NDM and OXA-48 in 9 (8.7%) isolates, and OXA-48 alone in 8 (7.7%) isolates. Among the carbapenem-resistant *Enterobacter* species, 1 isolate had a combination of NDM and OXA-48, and 1 had the OXA-48 gene alone (Table 3).

4. Discussion

This study aimed to detect carbapenemase production in *Enterobacteriales* through phenotypic methods and molecular characterization, with the objective of identifying the most prevalent genes responsible for carbapenemase production. Given the rising prevalence of carbapenem-resistant *Enterobacteriales* (CRE) globally, understanding the predominant genes responsible for carbapenemase production in specific regions is crucial. Such knowledge assists healthcare professionals in selecting optimal empirical treatments for infections caused by CRE.^{10,11}

The prevalence of CRE in our study was notably high at 23.4%. This finding is consistent with studies conducted in various regions of India, which report similarly high prevalence rates: 29% in Gujarat,¹² 27.18% in Bengaluru,¹³ and 29.4% in Bhubaneswar.¹⁴ These consistent findings across different parts of the country highlight the widespread issue of CRE and underscore the urgent need for robust infection control measures and antibiotic stewardship programs nationwide (Figure 1).

In our study, *Klebsiella pneumoniae* was the most commonly isolated CRE, accounting for 59.6% of the total

Table 1: Distribution of Carbapenem-resistant *Enterobacterales* (CRE) isolates according to combined disk test

Class of Carbapenemase	Number of CRE isolates showing synergy	Concordance
Class B Carbapenemase / Metallo-betalactamase (synergy with EDTA)	81 (77.9%)	91.3%
Class C Carbapenemase / AmpC (with porin loss) (synergy with CLX)	2 (1.9%)	
Class A Carbapenemase / KPC (synergy with PBA)	0 (0%)	
Class D carbapenemase / ESBL with porin loss (no synergy with any disk)	21 (20.2%)	
Total CRE isolates	104 (100%)	

Table 2: Genes detected in Carbapenem-resistant *Enterobacterales* (CRE) isolates.

Gene Detected	Number (%)
NDM + OXA 48	41 (39.4%)
NDM	20 (19.2%)
OXA 48	17 (16.3%)
OXA 23	9 (8.7%)
NDM + OXA 23	8 (8.7%)
OXA 48 + OXA 23	3 (2.9%)
NDM + OXA 48 + OXA 23	2 (1.9%)
NDM + OXA 51	1 (0.96%)
No gene isolated	3 (2.9%)
Total CRE isolates	104 (100%)

Table 3: Carbapenem-resistant *Enterobacterales* (CRE) organisms and identified genes responsible for carbapenem resistance

Organism	Carbapenemase gene detected	Number (%) of isolates
Carbapenem resistant <i>Klebsiella pneumoniae</i> (CRKpn) 62 (59.6%)	NDM + OXA 48	31 (29.8%)
	NDM	9 (8.7%)
	OXA 48	8 (7.7%)
	OXA 23	8 (7.7%)
	NDM + OXA 23	1 (0.96%)
	NDM + OXA 48 + OXA 23	1 (0.96%)
	OXA 48 + OXA 23	2 (1.9%)
	NDM + OXA 51	1 (0.96%)
	No gene detected	1 (0.96%)
	Carbapenem resistant <i>Escherichia coli</i> (CREco) 40 (38.5%)	NDM
NDM + OXA 48		9 (8.7%)
OXA 48		8 (7.7%)
NDM + OXA 23		7 (6.7%)
OXA 23		1 (0.96%)
OXA 48 + OXA 23		1 (0.96%)
NDM + OXA 48 + OXA 23		1 (0.96%)
No gene detected		2 (1.9%)
Carbapenem resistant <i>Enterobacter</i> species (CREbc) 2 (1.9%)	NDM + OXA 48	1 (0.96%)
	OXA 48	1 (0.96%)
Total CRE isolates		104 (100%)

CRE organisms, followed by *Escherichia coli* at 38.5%. This is in alignment with most other studies,^{12,13} which also identified *Klebsiella pneumoniae* as the predominant species, followed by *Escherichia coli*. Although some studies have reported high carbapenem resistance rates in *Enterobacter species*,¹⁵ our findings indicated that *Enterobacter species* were relatively uncommon. The consistent identification of *Klebsiella pneumoniae* as the most prevalent CRE across various studies, including ours, underscores its significant role in CRE infections.

Phenotypic detection of carbapenemase production using inhibitor-based combination disk testing revealed that 77.9% of the isolates produced metallo-beta-lactamase, while 1.9% exhibited AmpC carbapenemase activity. The remaining 20.2% of isolates did not show inhibition by any of the combination disks used in the test, suggesting the presence of OXA-like enzymes or non-carbapenemase mechanisms such as ESBL with porin loss. Notably, no isolates showed synergy with phenylboronic acid, indicating the absence of class A carbapenemase genes (Table 1).

Our findings are consistent with other studies. For instance, one study found metallo-beta-lactamase production in 56% of isolates and KPC production in 18%.¹⁶ Another study reported metallo-beta-lactamase production in 59.09% and KPC production in 13.63% of isolates.¹⁷ These findings, in conjunction with ours, highlight the critical need for comprehensive surveillance and molecular diagnostics to inform targeted treatment strategies for CRE infections.

The main mechanism responsible for carbapenemase production among the CRE isolates in our study was the presence of NDM and OXA-48 together. A significant proportion (39.4%) of the isolates exhibited both NDM and OXA-48, followed by 19.2% with NDM alone and 16.3% with OXA-48 alone. Overall, NDM was the most frequently detected gene, present in 69.2% of the CRE isolates, followed closely by OXA-48 in 60.6%. These findings are consistent with other studies, although the prevalence of specific carbapenemase genes can vary regionally.^{13,15,18–20} This variability underscores the importance of localized surveillance and tailored treatment strategies to manage CRE infections effectively. The differences in carbapenemase types suggest the need for region-specific antimicrobial policies and diagnostic approaches to ensure appropriate and effective therapeutic interventions (Table 2).

The concordance of the combined disk test with the molecular test in the detection of metallo-beta-lactamase was 91.3% in this study, and in the detection of OXA-like enzymes production alone was 95.1%. Thus combined disk test is a good alternative for the detection of carbapenemase where molecular tests are not available.

No variability was seen in the organism-wise characterization of carbapenemase genes (Table 3). In

other studies also, the same genes were identified in all CRE isolates.^{13,15,19} This shows that these genes have become widespread amongst *Enterobacterales*. These genes via their capability of horizontal transfer can spread both inter-species and intra-species. Therefore, surveillance of these genes is a must to monitor both their spread and the effectiveness of control measures

This study has several limitations. Conducted in a single center with a relatively small sample size, the findings may lack generalizability. The phenotypic method used could not detect certain carbapenemases, such as OXA-48. Additionally, the molecular methods employed did not identify non-carbapenemase mechanisms of resistance. Furthermore, the study did not examine risk factors and outcomes, which are crucial for a comprehensive understanding of CRE infections. Further research is required to know the outcomes in organisms according to their molecular pattern.

The landscape of CRE indeed varies significantly across regions, influencing treatment choices.²¹ The treatment of CRE infections depends on the infection site, the isolated pathogen, and the resistance profile.¹ Preventing the spread of these organisms is as important as their prompt detection and treatment. This necessitates strict infection control practices, including standard and transmission-based precautions. Robust antimicrobial stewardship practices are also crucial in preventing the overuse and misuse of antibiotics, which are major risk factors for the emergence of resistant strains.^{22,23}

5. Conclusion

Our study identified Metallo-beta-lactamases (NDM) as the predominant type of carbapenemase gene among the *Enterobacterales* isolates, with the co-production of NDM and OXA-48 enzymes being the most common mechanism of resistance. OXA-48 enzymes, which were the second most frequently produced, often exhibit weaker activity and can be easily missed by standard detection methods.

The treatment options for infections caused by metallo-beta-lactamase producers are extremely limited. Current therapeutic strategies typically involve the use of drugs such as ceftazidime-avibactam in combination with aztreonam and colistin. However, these treatment options are associated with significant limitations, including high costs and considerable toxicity profiles, which further complicate clinical management.

Given the increasing prevalence of carbapenem-resistant *Enterobacterales* (CRE), there is an urgent need for the development and implementation of newer point-of-care diagnostic tests. These tests should possess high sensitivity and specificity to facilitate early screening and detection of CRE. Prompt and accurate identification of these resistant organisms will enable timely and appropriate treatment interventions, as well as the effective implementation of

infection control measures to curb the spread of these pathogens.

To address the rising threat of antimicrobial resistance, it is imperative that healthcare systems invest in advanced diagnostic technologies and foster robust antimicrobial stewardship programs. These efforts are crucial to mitigating the impact of CRE on public health and ensuring the availability of effective treatment options for future generations.

6. Sources of Funding

None.


7. Conflict of Interest

None.

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