



DETECTION OF METALLO BETA LACTAMASE PRODUCTION AMONG ENTEROBACTERIACEAE ISOLATES RECOVERED FROM CLINICAL SPECIMENS IN A TERTIARY CARE IN CENTRAL INDIA

Shubham Shekhawat¹, Dr. Munesh Kumar Sharma²

¹*Ph.D. Scholar, Dept. of Medical Microbiology, Malwanchal University, Indore, MP
Email id:muneshmanipal@gmail.com

²Professor Dept. of Microbiology, Amaltas Institute Of Medical Sciences Dewas, MP

***Corresponding Author:** Shubham Shekhawat
*email id:shubhsmshekhawat06@gmail.com

ABSTRACT

Introduction: Recent observations have shown a rise in reports of metallo-beta-lactamase (MBL) producing isolates within the Enterobacteriaceae family. This alarming trend highlights a critical concern in the medical field: the diminishing number of effective therapeutic options for treating infections caused by these resistant organisms. As MBL enzymes enable bacteria to evade the effects of an array of beta-lactam antibiotics, healthcare providers face increasingly challenging scenarios in managing infections, underscoring the urgent need for novel treatment strategies and heightened surveillance.

Materials & Methods: This study was conducted in the Department of Microbiology at Index Medical College, Hospital & Research Centre in Indore, M.P. A total of 200 isolates of Enterobacteriaceae were included in this study. Resistance to imipenem was determined in isolates by disc diffusion and the minimum inhibitory concentration (MIC) method. Imipenem-resistant isolates were tested for MBL production by the Double Disk Synergy Test, MRP-EDTA IMP-EDTA combined disc test, and E-test.

Results: out of 200 isolates, 37(44.6%) were MBL-producing Enterobacteriaceae strains. Among which, *E. coli* (20, 24.1%) was the most prevalent organism, followed by *K. pneumoniae* (14, 16.9%), and *Citrobacter Spp.* (3, 3.6%).

Conclusion: This study demonstrated a high prevalence of MBL-producing *Enterobacterales* alongside widespread multidrug resistance, highlighting the need for regular surveillance and strict infection control measures to limit the dissemination of resistant strains.

KEY WORDS: Enterobacteriaceae, mCIM, Metallo-Beta-Lactamase Producer, Minimum Inhibitory Concentration, Double Disk Synergy Test, E-test, IPM-Double Disc test.

Key words: Metallo B-lactamase, carbapenem resistant, Antimicrobial resistant, Phenotypic detection

Introduction

Carbapenem-resistant Gram-negative bacteria (CR-GNB) have emerged as a major cause of both community-acquired and healthcare-associated infections (HCAIs), posing a significant threat to global public health. [1,2] Carbapenems are bactericidal β -lactam antibiotics with proven efficacy

against severe infections caused by extended-spectrum β -lactamase (ESBL)-producing organisms. [3]

India is considered one of the major epicentres for MBL-producing organisms, particularly New Delhi metallo-beta-lactamase (NDM). Indian studies have reported MBL prevalence rates ranging from **20% to 70%** among carbapenem-resistant Enterobacteriaceae, with higher rates observed in tertiary care hospitals and intensive care units.[3]

Commonly used carbapenems such as meropenem, ertapenem, and imipenem are often the drugs of choice for treating infections caused by multidrug-resistant bacteria. They are also employed in microbiology laboratories for screening carbapenem resistance. [4]

Carbapenem resistance in Gram-negative bacteria may be intrinsic or acquired, resulting from mutations or the acquisition of resistance genes through horizontal gene transfer mechanisms such as plasmids, transposons, or integrons. [5]

Both the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) annually establish susceptibility breakpoints for commercially available carbapenems—including doripenem, ertapenem, imipenem, and meropenem—for Gram-negative bacterial species, although EUCAST has discontinued doripenem breakpoints in recent updates [6,7]

When a bacterial isolate is found to be non-susceptible to carbapenems (i.e., exhibiting intermediate or resistant phenotypes), the underlying mechanism of resistance remains undetermined [8-10].

Therefore, confirmatory testing through biochemical assays and/or molecular (gene-based) methods is required to identify carbapenemase production or other resistance mechanisms [6,9,11]

The modified Carbapenem Inactivation Method (mCIM) is a reliable and straightforward phenotypic test designed to detect carbapenemase-producing Gram-negative bacteria. It has been validated primarily for use on bacterial colonies [12]

Gram-negative bacteria, particularly members of the *Enterobacteriaceae* family, are common etiological agents of both community-acquired and hospital-acquired infections, including urinary tract, bloodstream, and lower respiratory tract infections [13,14]. These organisms possess the ability to acquire genes encoding various antibiotic resistance mechanisms, such as extended-spectrum β -lactamases (ESBLs), AmpC β -lactamases, and carbapenemases [15-17]. β -lactam antibiotics remain the mainstay of therapy for severe infections; however, carbapenems are often regarded as the agents of last resort for treating infections caused by multidrug-resistant Gram-negative pathogens. [18,19].

Several phenotypic methods, such as the disc diffusion test and minimum inhibitory concentration (MIC) determination, are routinely employed in diagnostic microbiology laboratories to assess carbapenem susceptibility or resistance patterns among Gram-negative bacteria [20]. Among the available carbapenems, ertapenem is often preferred over imipenem or meropenem for *in vitro* susceptibility testing due to its superior ability to detect carbapenemase-producing organisms, particularly those producing KPC or OXA-type enzymes [21]. Consequently, meropenem serves as a helpful screening agent for identifying potential carbapenemase producers before confirmatory testing is performed [22].

This study aimed to determine the prevalence of carbapenem-resistant Enterobacteriaceae (CRE) among clinical isolates and to identify metallo-beta-lactamase-producing strains using phenotypic methods, including the modified carbapenem inactivation method (mCIM), the Double Disc Synergy Test, the combined disc test (IMP–EDTA), and the E test.

Materials & Methods

This study was conducted in the Department of Microbiology at Index Medical College, Hospital & Research Centre in Indore, M.P.

Following approval from the institutional ethics committee, a total of 200 Enterobacteriaceae isolates were identified for meropenem and imipenem resistance and they were tested for MBL production from various clinical specimens collected from hospitalised patients.

The Modified Carbapenem Inactivation Method (mCIM) was performed to detect carbapenemase production in all *Enterobacteriaceae* isolates. A 1 µL loopful of the test strain was suspended in 2 mL of tryptic soy broth (TSB) with a meropenem (MRP) disc and incubated at 37 °C for 4 ± 0.25 hours. After incubation, a meropenem-susceptible *E. coli* ATCC 25922 suspension (0.5 McFarland) was inoculated onto Mueller–Hinton agar, and the pre-incubated disc was placed on the plate. A reduced inhibition zone around the disc indicated carbapenemase production. Although mCIM detects carbapenemase activity, it does not differentiate between metallo-β-lactamases (MBLs) and serine carbapenemases; hence, the EDTA-modified version (eCIM) was used for MBL detection.[22]

Control strains for the detection of Metallo Beta Lactamase (MBL): We used the following control strains for MBL detection.

Positive control strain

- *Klebsiella pneumoniae* ATCC BAA -1705

Negative control strain

- *E. coli* ATCC 25922

MBL production was carried out in Meropenem-resistant isolates. MBL detection tests were performed using the following methods with *P. aeruginosa* ATCC 27853 (Hi-media) as a negative control strain.

1. **Double Disk Synergy Test (DDST):** This test was performed with an overnight broth culture of the test strain inoculated on the MHA plate and allowed to dry. 10 µl of the 0.5 M EDTA solution was added to a 6-mm blank filter paper disk (Whatman filter paper, no. 1), which contained approximately 750 µg of EDTA. A 10 µg imipenem disk was placed in the centre of the plate, flanked by an EDTA disk at a distance of 20 mm centre-to-centre from a blank disc containing 10 µl of 0.5 M EDTA (750 µg). After overnight incubation, the presence of an enlarged zone of inhibition towards the EDTA disk was interpreted as positive for an MBL producer [23].

2. The IMP-EDTA combined disc test was performed as described by Yong *et al.* Test organisms were inoculated onto Mueller–Hinton agar (CLSI guidelines). A 0.5 M EDTA solution (pH 8.0) was prepared by dissolving 18.61 g in 100 mL of distilled water and sterilised by autoclaving. Two 10 µg imipenem discs were placed on the agar, and 10 µL of EDTA (750 µg) was added to one disc. After incubation at 35 °C for 16–18 hours, an increase of ≥7 mm in the inhibition zone around the IMP–EDTA disc compared with the IMP disc alone indicated metallo-β-lactamase (MBL) production. [24]

3. **E-Test:** E-test Metallo-beta-lactamase strips consisted of a double-sided seven dilution range of imipenem IP (4 to 256µg/ml) and IP (1 to 64µg/ml) overlaid with a constant gradient of EDTA. Individual colonies were picked from overnight agar plates and suspended in 0.85% saline to a turbidity of 0.5 McFarland's standard. A sterile cotton swab was dipped into the inoculum's suspension, and a lawn culture of the inoculum was done on an MHA plate. The excess moisture was allowed to be absorbed for about 15 min before the E-test MBL (Hi-media) strip was applied. Plates were incubated for 16 to 18 h at 37°C. The MIC endpoints were read where the inhibition ellipses intersected the strip. A two-fold reduction in imipenem MIC to 3 in the presence of EDTA will be interpreted as suggesting MBL production. [25]

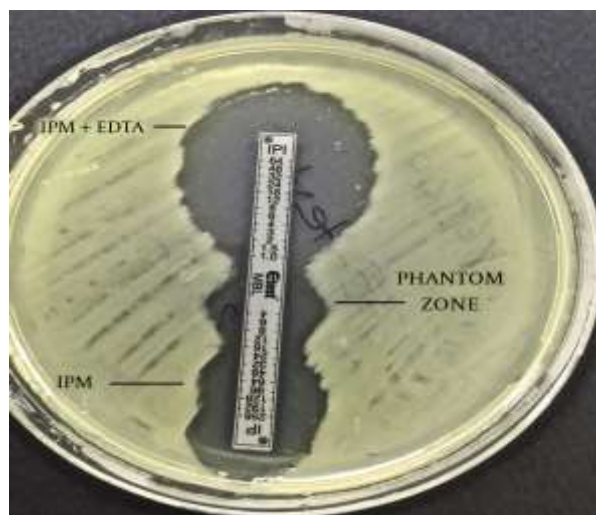
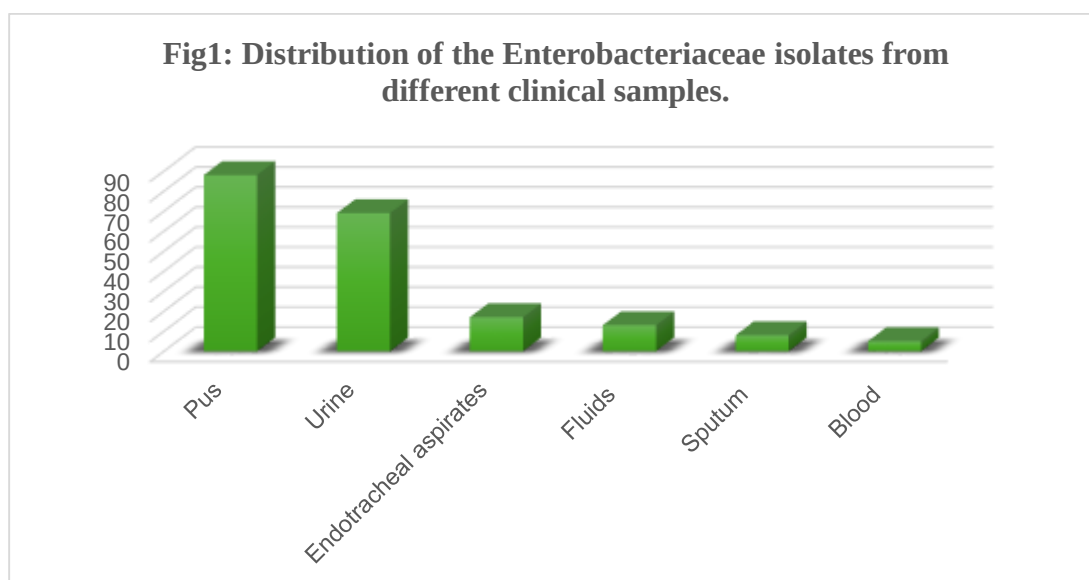


Fig.: Image of MBL E-test positive isolate

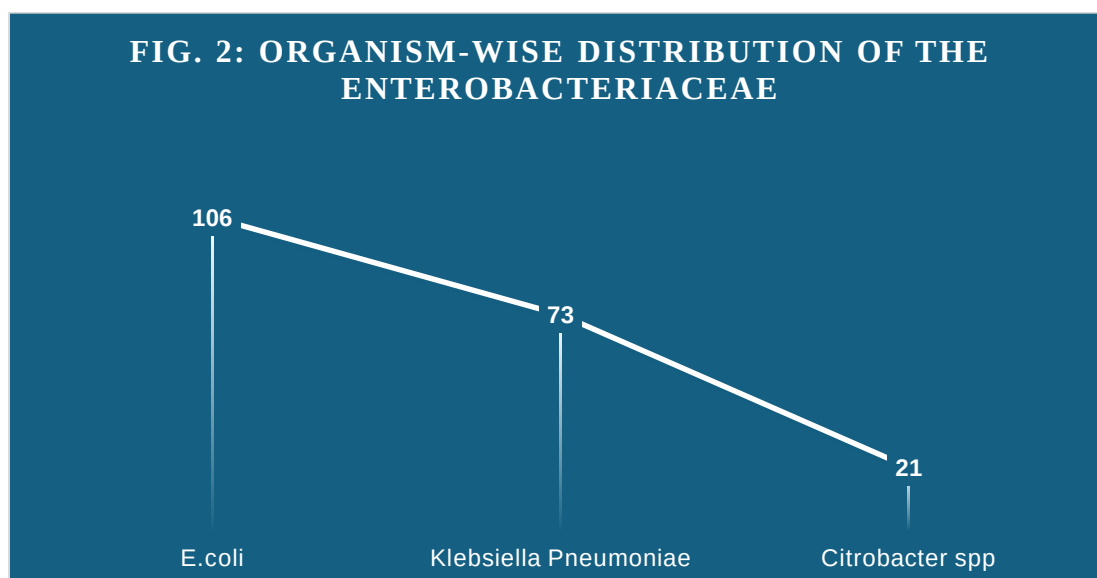
Result

Out of 200 isolates from various clinical specimens, the maximum was from pus, 88 (44%), followed by Urine, 69 (34.5%), endotracheal aspirates, 17 (8.5%), Sputum, 8 (4%), and Blood, 5 (2.5%). Distribution of *Enterobacteriaceae* isolates among specimen types. Most are found in pus and urine specimens. (Table 1 & Fig. 1)

Table 1: Distribution of the Enterobacteriaceae isolates from different clinical samples.		
Specimen	Total no. of Enterobacteriaceae isolates	Chi-square=158.6 p-value <0.0001
Pus	88(44%)	
Urine	69(34.5%)	
Endotracheal aspirates	17(8.5%)	
Fluids	13(6.5%)	
Sputum	8(4%)	
Blood	5(2.5%)	



Out of 200 isolates, 106(52%) were *E. coli* followed by 73(36.5%) were *Klebsiella pneumoniae* and 21(10.5%) were *Citrobacter* species. (Figure 2)



Screening test for metallo-beta-lactamases (MBL) production

Modified carbapenem inactivation methods (mCIM) were performed for all isolates using the disk diffusion test, which involved detecting resistance to Meropenem and Imipenem disks. of the isolates tested, 83 (41.5%) were found to be resistant. (Table 2)

Table 2: Modified carbapenem inactivation methods (mCIM)				
organism	MRP resistant	MRP sensitive	Imipenem resistant	Imipenem sensitive
<i>Enterobacteriaceae</i> isolates	83/200 (41.5%)	117/200 (58.5%)	83/200 (41.5%)	117/200 (58.5%)

Confirmation for MBL Production

Among the three tests, the overall percentage of MBL production was 37/83(44.6%). The **CDT, Double Disc Synergy test and MBL by E-test gave similar results, 37(44.6%)**. The table shows the compression of screening and confirmatory tests in phenotypic detection of MBL. Science **p-value <0.05**. There is no difference between the three phenotypic MBL detection methods — they gave identical results in this dataset. (Table 3)

Table 3: Comparison of screening and confirmatory tests in the phenotypic detection of MBL					
Total number of Enterobacteriaceae isolated	CRE	MBL by CDT	Double Disc Synergy test	MBL by E-test	p-value <0.05
200	83	37(44.6%)	37(44.6%)	37(44.6%)	

Metallo beta-lactamase distribution among Carbapenem-resistant Enterobacteriaceae (CRE)

Among 83 carbapenem-resistant Enterobacteriaceae, 37 (44.6%) were MBL producers, detected by different phenotypic methods. In total, out of 200 isolates, 37(44.6%) were MBL-producing Enterobacteriaceae strains. Among which, E. coli (20, 24.1%) was the most prevalent organism, followed by K. pneumoniae (14, 16.9%), and Citrobacter Spp. (3, 3.6%). Since there was no statistically significant difference in the detection of metallo beta lactamase producing isolates among CDT, Double Disc Synergy Test, and E test, as all three methods identified an identical number of MBL positive isolates ($\chi^2 = 0$, $p = 1.000$). This demonstrates complete concordance among the phenotypic methods evaluated. (Table 4)

Table 4: Metallo beta-lactamase distribution among Carbapenem-resistant Enterobacteriaceae				
Organisms name	MBL by CDT	Double Disc Synergy test	MBL by E-test	Chi square (χ^2) = 0 P-value=1.000
<i>E. coli</i>	20(24.1%)	20(24.1%)	20(24.1%)	
<i>Klebsiella pneumoniae</i>	14(16.9%)	14(16.9%)	14(16.9%)	
<i>Citrobacter species</i>	3(3.6%)	3(3.6%)	3(3.6%)	
Total	37(44.6%)	37(44.6%)	37(44.6%)	

Antibiotic susceptibility and resistance pattern of all 200 isolates of Enterobacteriaceae

The antibiotic sensitivity pattern amongst isolates. 152(76%) were resistant to ceftriaxone, followed by 147(73.5%) ceftazidime, 146(73%) Amikacin, 137(68.5%) cefepime, 127(63.5%) Piperacillin/tazobactam, 133(66.5%) ciprofloxacin, 83(41.5%) were to meropenem & imipenem. Colistin, Polymyxin B, and CAZ+AVI+AT were found to be 100% sensitive, followed by meropenem & Imipenem 117(58.5%), Piperacillin/tazobactam 73(36.5%), cefepime 63(31.5%), ciprofloxacin 67(33.5%), amikacin 58(27%), ceftazidime 53(26.5%), and aztreonam were 47(23.5%). There is a **highly significant difference** ($p < 0.0001$) in resistance patterns among antibiotics. Resistance is **much higher** for cephalosporins and aztreonam, **moderate** for carbapenems and piperacillin–tazobactam, and **absent** for colistin, polymyxin B, and CAZ+AVI+AT

Table 5: Antibiotic susceptibility and resistance pattern of all 200 isolates of Enterobacteriaceae			
Antibiotics	Resistant	Sensitive	Ch-square = 830 p-value <0.0001
Amikacin	146(73%)	58(27%)	
Ceftazidime	147(73.5%)	53(26.5%)	
Ceftriaxone	152(76%)	48(24%)	
Cefepime	137(68.5%)	63(31.5%)	
ciprofloxacin	133(66.5%)	67(33.5%)	
Aztreonam	153(67.5%)	47(23.5%)	
Meropenem	83(41.5%)	117(58.5%)	
Imipenem	83(41.5%)	117(58.5%)	
Piperacillin/Tazobactam	127(63.5%)	73(36.5%)	
Colistin	0	100	
Polymyxin B	0	100	
CAZ+AVI+AT	0	100	

Discussion

Carbapenem-resistant *Enterobacteriaceae* (CRE) represent a serious global public health concern due to their multidrug-resistant nature and limited treatment options, often leading to high morbidity and mortality, particularly among immunocompromised patients and those undergoing invasive procedures [26].

The present study aimed to detect metallo- β -lactamase (MBL) production among carbapenem-resistant clinical isolates of *Enterobacteriaceae* obtained from samples received in the hospital microbiology laboratory. A total of 200 *Enterobacteriaceae* isolates were included. Of these, 111 (55.5%) were collected from male patients and 89 (44.5%) from females. The highest number of isolates was obtained from patients aged 31–40 years (50; 25%), followed by 41–50 years (38; 19%), 21–30 years (37; 18.5%), 51–60 years (32; 16%), 60–70 years (18; 9%), 10–20 years (9; 4.5%), and >70 years (16; 8%).

Among the clinical specimens, pus accounted for the highest number of isolates (88; 44%), followed by urine (69; 34.5%), endotracheal aspirates (17; 8.5%), sputum (8; 4%), and blood (5; 2.5%). These findings are consistent with studies by Naim *et al.* and Balan *et al.*, who reported recovery

rates of 73.2% and 64.5% of *Enterobacteriaceae* isolates from pus samples, respectively, highlighting the predominance of these pathogens in wound infections [27,28].

Out of 200 *Enterobacteriaceae* isolates, 83 (41.5%) were identified as CRE. The majority were recovered from pus (57; 68.7%), followed by urine (17; 20.5%), endotracheal aspirates (5; 6%), body fluids (2; 2.4%), and sputum and blood (1 each; 1.2%). These results align with Zaidah *et al.* (2017), who found most isolates in pus samples (49.3%), followed by urine (15.9%), blood (9.6%), and sputum (4.6%), underscoring the clinical importance of purulent infections as reservoirs for resistant pathogens [29].

Similarly, Khare *et al.* (2017) observed the highest number of isolates from pus (42.8%), followed by sputum (35.3%), blood (32.3%), and endotracheal samples (31%) [17]. In the present study, most MBL-positive isolates were recovered from pus (78.6%), followed by urine (14.3%), endotracheal aspirates (4.8%), and sputum (2.4%). Comparable studies by Jagnath *et al.* (2014) and Deshmukh *et al.* (2011) reported lower MBL positivity rates in pus samples (40% and 36.8%, respectively) [30,31].

In our study, 152(76%) were resistant to ceftriaxone, followed by 147(73.5%) ceftazidime, 146(73%) Amikacin, 137(68.5%) cefepime, 127(63.5%) Piperacillin/tazobactam, 133(66.5%) ciprofloxacin, 83(41.5%) were to meropenem & imipenem. Colistin, Polymyxin B, and CAZ+AVI+AT were found to be 100% sensitive, followed by meropenem & Imipenem 117(58.5%), Piperacillin/tazobactam 73(36.5%), cefepime 63(31.5%), ciprofloxacin 67(33.5%), amikacin 58(27%), ceftazidime 53(26.5%), and aztreonam were 47(23.5%).

A study done by Saseedharan S *et al.* (2016) reported 96.15% resistance to amikacin and 92% resistance to Ceftazidime. [32]

Another study by Jain P *et al* in 2012 reported 90% resistance to amikacin and 100% resistance to ceftazidime, ceftriaxone and ampicillin. [33]

Iman F *et al* in 2016 also reported high resistance to drugs like amikacin (89.3%), ceftazidime (100%), ceftriaxone (100%), cefepime (98.2%), aztreonam (98.2%), ciprofloxacin (78.6%), norfloxacin (100%) and polymyxin B (19.6%). [34]

Conclusion

The high prevalence of multidrug-resistant (MDR) and metallo- β -lactamase (MBL)-producing *Enterobacteriales* in developing countries like India is a major concern. Early detection in clinical laboratories and strict infection control measures are essential to prevent their spread. When carbapenems fail in vivo or isolates show resistance to ceftazidime and/or meropenem, MBL production should be suspected. In the absence of molecular methods, the modified and extended carbapenem inactivation methods (mCIM and eCIM) serve as reliable phenotypic alternatives for routine detection. Further molecular studies are needed to characterize resistance genes and better understand the mechanisms underlying MDR patterns.

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