Section: Microbiology

Original Research Article

PHENOTYPIC DETECTION OF METALLO-B-LACTAMASE PRODUCTION AND COLISTIN SUSCEPTIBILITY IN GRAM-NEGATIVE BACILLI: A TERTIARY CARE HOSPITAL STUDY

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ABSTRACT

Background: Metallo-β-lactamases (MBLs) belong to class B beta-lactamase of Ambler classification are enzymes that hydrolyze the β-lactam group of drugs and confer resistance to carbapenems, posing a significant threat in healthcare settings. The emergence of Carbapenem-resistant Gram-negative bacilli in both the community and hospital environments has been an alarming sign in the field of infectious disease management and control. Colistin, the last resort antibiotic, is increasingly encountering the resistance. So, it is important to evaluate the colistin resistance in these isolates. **Materials and Methods:** A cross-sectional descriptive study was conducted from November 2022 to February 2023 in the Department of Microbiology, NRI Institute of Medical Sciences, Visakhapatnam. A total of 452 Gram-negative bacilli isolates recovered from various clinical samples were included. All isolates were subjected to the Kirby–Bauer disc diffusion method for antimicrobial susceptibility testing per CLSI guidelines. Imipenem-resistant isolates (n=108) were subjected to Imipenem-EDTA combined disc test(CDT), Imipenem-EDTA double-disc synergy test (DDST), EDTA disc potentiation test using Ceftazidime, Cefepime and Cefotaxime for MBL confirmation. Minimum inhibitory concentration (MIC) of colistin was determined using Broth Micro Dilution according to NCDC guidelines India (August 2020). **Results:** Among 452 Gram-negative isolates, most common isolate was *Klebsiella pneumoniae* (31.4%). Imipenem resistance was detected in 23.8% (108 isolates), of which 102(94.4%) were confirmed as MBL producers. Colistin resistance was found in 5.5% (6/108) of Carbapenem Resistant Gram negative isolates(CRGNB) by Broth Microdilution(BMD). **Conclusion:** The Imipenem-EDTA Combined Disc Test(CDT) proved effective for routine MBL detection due to its simplicity and ease of integration into clinical laboratories. Determining the Minimum Inhibitory Concentration (MIC) for colistin is crucial for guiding its judicious use as a last-resort antibiotic, particularly in an era of escalating carbapenem resistance.

INTRODUCTION

Metallo-β-lactamases (MBLs), class B enzymes of the Ambler classification, utilize zinc ions to hydrolyze virtually all β-lactam antibiotics, including carbapenems — historically considered last-line agents for serious Gram-negative infections.[1,2] MBL-encoding genes (blaNDM, blaVIM, blaIMP) reside on mobile genetic elements such as integrons, transposons, and plasmids, enabling rapid horizontal gene transfer among *Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and other members of family Enterobacteriaceae, driving the epidemic dissemination of carbapenem-resistant organisms (CROs) with significant morbidity and mortality.[3,4,5]

Carbapenem resistance has become endemic in the India with resistance rates of 23–48% among Gram-negative isolates in tertiary care hospitals, predominantly involving *Acinetobacter baumannii* and *Klebsiella pneumoniae*.[6,7] The New Delhi Metallo-β-lactamase (NDM), first identified in

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India, has since disseminated globally and remains a major driver of carbapenem resistance.[8]

Colistin (polymyxin E) has consequently been revived as a last-resort agent, acting by binding lipid A of LPS, displacing divalent cations (Mg^{2+}, Ca^{2+}), and disrupting outer membrane integrity.[9] Co-occurrence of colistin and carbapenem resistance leaves clinicians without viable therapeutic options, underscoring the urgent need for surveillance and stewardship.

Phenotypic MBL detection exploits the zinc-chelating properties of EDTA, using methods including the Combined Disc Test (CDT), Double Disc Synergy Test (DDST), Imipenem-EDTA disc method enabling timely infection control and appropriate therapeutic decisions.[11]

The present study aimed to: (i) determine the prevalence of imipenem resistance among Gram-negative isolates in a tertiary care hospital in Visakhapatnam; (ii) compare three phenotypic methods for MBL detection among imipenem-resistant isolates; and (iii) assess colistin susceptibility using Broth Microdilution (BMD) in Carbapenem resistant Gram negative bacilli.

MATERIALS AND METHODS

A cross-sectional descriptive study was conducted from September 2022 to February 2023 in the Department of Microbiology, NRI Institute of Medical Sciences, Visakhapatnam, AndhraPradesh. All Gram negative isolates from various clinical specimens — including pus, sputum, ET aspirate, BAL, urine, blood, and body fluids of all age groups which were sent to routine microbiological evaluation were included in the study. Repeated isolates from same patient and organisms which are intrinsically resistant to colistin like *Proteus, Serratia, Providencia, Burkholderia species* were excluded from the study.

A total of 452 non-duplicate Gram-negative bacilli isolates were recovered from various clinical samples and subjected to the Kirby–Bauer disc diffusion method on Mueller–Hinton agar (MHA) to study antimicrobial susceptibility pattern with recommended drugs following Clinical and Laboratory Standards Institute (CLSI) guidelines (M100, 32nd Edition, 2022).

Carbapenem-resistant isolates (n=108) were subjected to three phenotypic tests for MBL confirmation:

1. Disc diffusion test: This is a screening test. Imipenem disc (10µg) was placed on a lawn culture of test bacteria and incubated overnight at 37°C. Zone diameter is read the next day. It is considered resistant if it is ≤19mm, and isolates were further tested.
2. Imipenem-EDTA combined disc test(CDT): Two Imipenem discs (10µg) were placed on a plate inoculated with the test organism, and 10 µl of 0.5 M EDTA solution was added one disc to obtain the desired concentration of 750 µg. The zone diameter difference between the Imipenem and the Imipenem + EDTA of ≥7mm was interpreted as positive for MBL production.
3. Imipenem-EDTA double-disc synergy test (DDST): An Imipenem disc was placed 20mm apart from a blank disc to which 10µl of (Ethylenediaminetetraacetic acid) 0.5 M EDTA(750µg) was added. Augmentation of the zone of inhibition in the area between Imipenem and the EDTA disc was interpreted as a positive result.
4. EDTA disc potentiation test using Ceftazidime, Cefepime and Cefotaxime: A blank disc was placed in the middle of the plate, and the following discs [Ceftazidime (30 µg), Cefepime (30 µg), Cefixime (5µg), cefotaxime (30 µg),] were placed 25mm center to center from the blank disc.10 µl of 0.5 M EDTA solution was added to the blank disc and incubated. Augmentation of the zone of inhibition in an area between any one of the four cephalosporin discs and the EDTA disc compared to the zone of inhibition on the far side of the drug was interpreted as a positive.

Colistin susceptibility was determined for all Carbapenem resistant Gram negative isolates using Broth Microdilution (BMD) as the reference method, following NCDC India guidelines (August 2020). Two-fold serial dilutions of colistin sulfate were prepared in cation-adjusted Mueller–Hinton broth (CAMHB). MIC breakpoints of ≤2 µg/mL (susceptible) and ≥4 µg/mL (resistant) were considered.

RESULTS

A total of 452 Gram-negative bacilli isolates were analyzed during the study period. 108 Carbapenem resistant Gram negative bacilli(CRGNB) were found on screening with Imipenem disc diffusion test with *Klebsiella pneumoniae* was the most frequent organism (31.4%), followed by *Acinetobacter species* (25.9%), *Escherichia coli* (20.4%), *Pseudomonas aeruginosa* (18.5%), *Klebsiella oxytoca* (3.7%).

Table 1: Distribution of CRGNB among various isolates

Organism	CRGNB	Percentage
Klebsiella pneumoniae	34	31.4%
Acinetobacter species	28	25.9%
Pseudomonas aeruginosa	20	18.5%
Escherichia coli	22	20.3%
Klebsiella oxytoca	4	3.7%
Total	**108**	**100%**

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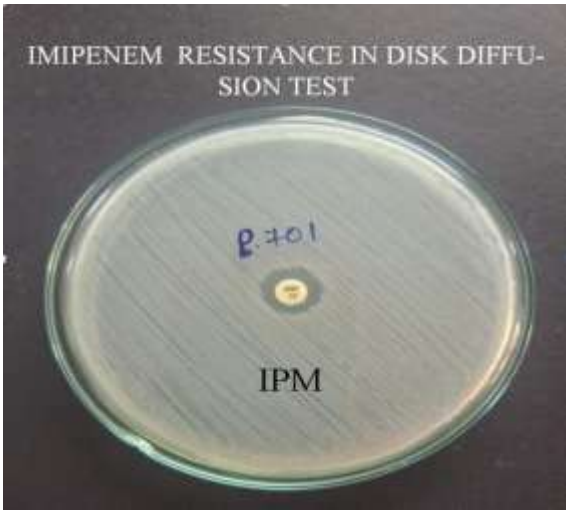
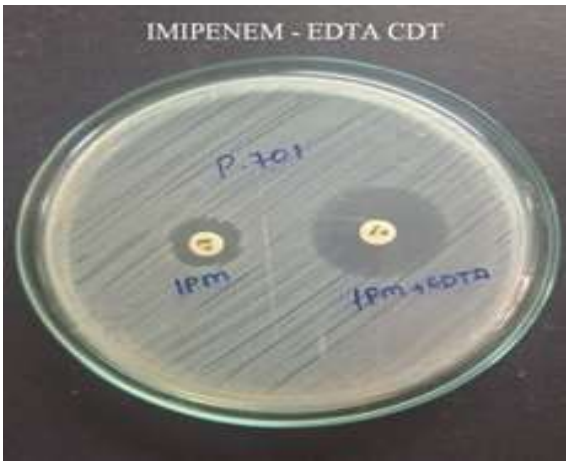
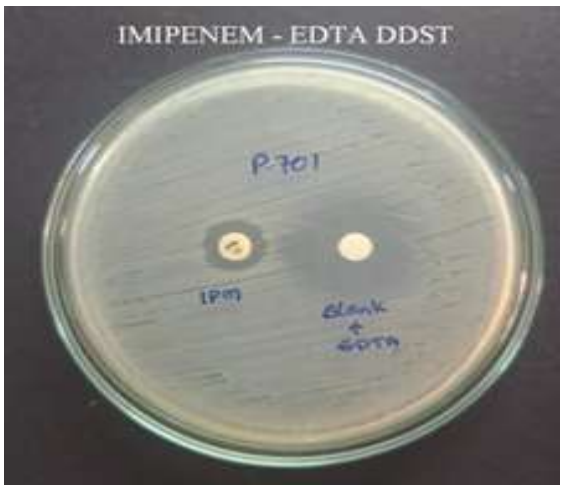
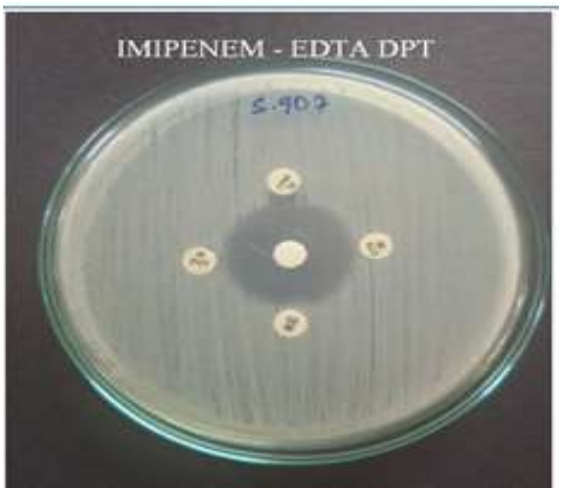
Of the 452 Gram-negative isolates, 108 (23.89%) demonstrated imipenem resistance on disc diffusion and all imipenem-resistant isolates were subjected to three phenotypic confirmatory tests. 102 isolates (94.4% of imipenem-resistant of total isolates) were confirmed as MBL producers by at least two concordant phenotypic methods.









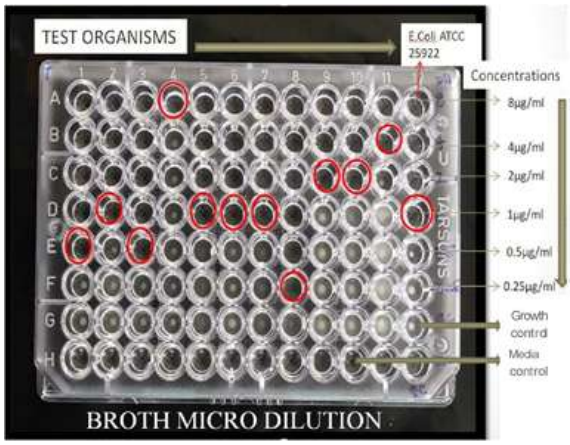


Table 2: Comparison of different tests for MBL detection

MBL Detection tests	Positives	Percentage
IMP-EDTA CDT	102	94.44%
IMP-EDTA DDST	94	87.03%
Disc potentiation test	47	43.51%

Imipenem-EDTA Combination disc test(CDT) was taken as the confirmatory test for MBL. Many studies considered it the most sensitive phenotypic test, which confirmed as 102 (94.44%) of the 108 screening positive isolates were MBL producers. 6 isolates that were imipenem-resistant but MBL-negative were presumed to harbour non-MBL carbapenemase mechanisms (KPC, OXA-type) or non-enzymatic resistance (porin loss, efflux pump upregulation).

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Colistin susceptibility was determined by BMD for all 108 Carbapenem Resistant Gram negative isolates. Colistin resistance (MIC ≥4 µg/mL) was identified in 6 isolates (5.5%): two Acinetobacter species and four *Klebsiella pneumoniae*. The remaining isolates (94.5%) were susceptible to colistin (MIC ≤2 µg/mL).

Table 3: Distribution of MIC in Broth micro dilution among MBL producers

	MIC OF BMD (µg/ml)						
Organism	**No.of isolates tested**	**0.25**	**0.5**	**1**	**2**	**4**	**8**
Klebsiella pneumoniae	**34**	0	4	16	10	**2**	**2**
Acinetobacter species	**28**	2	12	8	4	**2**	0
Escherichia coli	**22**	4	4	12	2	0	0
Pseudomonas aeruginosa	20	0	8	10	2	0	0
Klebsiella oxytoca	4	2	2	0	0	0	0

DISCUSSION

The present study documents a substantial burden of MBL-mediated carbapenem resistance among Gram-negative bacilli in a tertiary care hospital in Visakhapatnam. An imipenem resistance rate of 23.8% (108/452) was observed in the current study. This rate is comparable to the findings of Nordmann et al,[5] who reported carbapenem resistance rates ranging from 23% to 48% among Gram-negative isolates in Indian tertiary care hospitals. Sahoo et al,[7] documented a similar range of MBL prevalence in their study from Odisha. The highest imipenem resistance rate in comparable Indian studies was reported at 48% by Veeraraghavan et al,[6] among Klebsiella pneumoniae bloodstream isolates, whereas the lowest was reported at 20% by Kulkarni and Mulay,[13] in clinical isolates from a tertiary care setting.

Of the 108 imipenem-resistant isolates, 94.4% were confirmed as MBL producers by at least two concordant phenotypic methods. This high MBL confirmation rate is consistent with the study by Sahoo et al,[7] which reported MBL positivity in 91% of carbapenem-resistant Gram-negative isolates from a tertiary care hospital in eastern India. A comparable confirmation rate of 93.2% was documented by Kulkarni and Mulay,[13] among imipenem-resistant Escherichia coli and Klebsiella pneumoniae isolates. In contrast, Devarasetty et al,[17] reported a lower overall MBL confirmation rate of approximately 78% in their two-year retrospective analysis from South India, while Nordmann et al,[8] documented the highest MBL burden globally, with carbapenemase-producing Enterobacteriaceae confirmed in nearly 100% of NDM-positive isolates. These comparisons collectively affirm that the predominance of MBL as the mechanism of carbapenem resistance.

Klebsiella pneumoniae was the most prevalent overall isolate (31.4%) among the 452 Gram-negative bacilli, consistent with national epidemiological data identifying it as the leading Carbapenem-resistant Enterobacteriaceae. Veeraraghavan et.al[6] similarly identified *K. pneumoniae* as the predominant carbapenem-resistant Enterobacteriaceae in bloodstream infections across Indian hospitals, reporting it as the highest contributor among Gram-negative organisms.

Comparison of the three phenotypic MBL detection methods revealed that the Imipenem-EDTA Combined Disc Test (CDT) demonstrated the highest positivity rate (94.44%), followed by the Double Disc Synergy Test (DDST) at 87.03%, and the EDTA Disc Potentiation Test at 43.51%. Franklin et al,[12] similarly validated the superiority of the CDT over other phenotypic methods, reporting it as the most sensitive and specific phenotypic test for MBL detection in clinical laboratories. Kulkarni and Mulay,[13] reported CDT positivity rates of approximately 92% in their study of *E. coli* and *K. pneumoniae*, the closest result to the 94.44% observed in the current study, suggesting strong concordance between the two methods across different Indian centres. These comparative findings reinforce the recommendation that CDT should be the preferred phenotypic screening method in routine clinical microbiology laboratories due to its simplicity, cost-effectiveness, and superior diagnostic accuracy.[12]

Colistin resistance was detected in 5.5% (6/108) of Carbapenem resistant Gram negative isolates using Broth Microdilution (BMD) in the present study which correlates with Ayushi sharma et.al[9], who documented colistin resistance rate of 6.2% and Sujatha et.al[17] reported resistance rate of 11% which demands implementation of Antimicrobial Stewardship Programs (AMSP) and robust Infection Prevention and Control (IPC) measures.

CONCLUSION

This study establishes a significant prevalence of MBL-mediated carbapenem resistance (22.6%) among Gram-negative bacilli in a tertiary care hospital in Visakhapatnam. *Klebsiella pneumoniae* and *Acinetobacter species* are the principal MBL producers. Incorporating simple screening methods like the Combined Disk Test (CDT) represents a

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vital step towards widespread monitoring of these emerging resistance markers. Since CDT is easy to perform and interpret, it should be adopted as a routine screening tool for detecting MBL production. Despite being detected in only 5.5% of isolates, colistin resistance poses serious clinical consequences. Broth Microdilution remains the gold standard for colistin MIC determination. Effective containment of these multidrug-resistant organisms requires continuous surveillance of carbapenem and colistin resistance, alongside strict antimicrobial stewardship and infection control measures.

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