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Original Research Article

DETERMINATION OF METALLO-B-LACTAMASES BY PHENOTYPIC METHODS IN GRAM NEGATIVE BACILLI ISOLATED IN A TERTIARY CARE HOSPITAL

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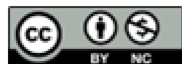

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Abstract

Background: Emergence of carbapenem-resistant Gram-negative bacteria in both the community and hospital environments constitutes an alarming development in the field of infectious disease management and control. Metallo-β-lactamases (MBLs) which belong to class B beta-lactamase of Ambler classification are enzymes that hydrolyze and confer on bacteria the exceptional ability to resist the antimicrobial action of the carbapenems such as imipenem and meropenem. **Objectives:** This study aims to know prevalence of MBL production in various gram negative bacilli, to evaluate different phenotypic methods to detect MBL production and to find out antibiotic susceptibility profile of MBL producing gram negative bacilli. **Materials and Methods:** A cross-sectional descriptive study was conducted in the Department of Microbiology, NRI Institute of Medical Sciences, and Visakhapatnam. A total of 100 Gram negative bacilli isolates were recovered from various clinical specimens like urine, sputum, pus, fluids & blood are included in this study. Specimen processing was done according to standard protocols. Kirby–Bauer disc diffusion technique was used to study antimicrobial susceptibility pattern with recommended drugs, whereas the Imipenem resistant isolates were subjected to 4 phenotypic tests for confirmation of MBL production. **Result:** Out of the 100 isolates majority were males (54%) and belong to the age group of 31-40 years (21%). The highest frequency of isolates were from pus and sputum (29%) with Klebsiella pneumoniae (33%) being the most common isolate. Varied antimicrobial susceptibility pattern has been noted among these different Gram-negative bacteria isolated. 34 Imipenem resistant strains were found on MBL screening with Imipenem disc diffusion tests of which 32 were confirmed by the other phenotypic tests. Major MBL production was seen among *Acinetobacter* species (60%) followed *Klebsiella pneumoniae* (36.3%) and *Pseudomonas aeruginosa* (35%). **Conclusion:** All the isolates should be routinely screened for MBL production by Imipenem-EDTA combined disc test (CDT) since this test is simple to perform and interpret. It can be performed as a routine antimicrobial susceptibility method as it can be easily introduced into the workflow of a clinical laboratory.

INTRODUCTION

The emergence of carbapenem-resistant Gram-negative bacteria in both the community and hospital environments constitutes an alarming development in infectious disease management and control. This menace has significant public health implications since they jeopardize the clinical significance of potent antibiotics used to treat serious infections. Metallo-β-lactamases (MBLs) belong to class B beta-lactamase of Ambler classification. These enzymes hydrolyze and confer on bacteria the exceptional ability to resist the antimicrobial action of the carbapenems such as Imipenem and Meropenem.[1] They require divalent cations of zinc as cofactors for enzyme activity, and thus the activity of these enzymes are usually

inhibited in vitro by chelating agents such as ethylenediamine tetra-acetic acid (EDTA)

The other mechanisms of carbapenem resistance are decreased permeability of the outer membrane and increased efflux pump.[2,3] There are many MBLs genes such as imipenemase (IMP), Verona integron-encoded Metallo-beta-lactamases (VIM), Sno Paolo Metallo (SPM), New-Delhi Metallo-β-lactamase (NDM), German imipenemase (GIM), Kyorin University Hospital imipenemase (KHM), and Australian imipenemase (AIM).[4,5] Genes encoding for MBL were shown to be carried on large transferable plasmids or were associated with transposons, allowing horizontal transfer of these MBL genes among different bacterial genera and species

Although PCR-based genotyping remains the golden standard for MBL detection and classification, its use is mainly restricted to research purposes, so diagnostic centers and laboratories still rely mostly on culture-based phenotypic tests to rapidly detect MBL activity. So far, many variations of phenotypic assays for MBLs detection have been reported, and these assays are not standardized. Early detection of MBL-producing organisms is critical as it allows for the prompt use of appropriate antibiotics to control infection effectively. It has been well documented that the activity of MBLs is dependent on zinc or cadmium.[6–11] Several screening methods incorporating the use of metal chelating agents, such as ethylenediaminetetraacetic acid (EDTA) and thiol-based compounds like 2-mercaptopropionic acid (2- MPA), which are capable of blocking MBL activity, have been developed to detect MBL-producing organisms.[12–16]

Rapid detection of Metallo-β-lactamase (MBL)-producing gram-negative pathogens is critical to prevent widespread dissemination. The clinical utility of carbapenems is under threat with the emergence of acquired carbapenemases, particularly Ambler class B Metallo-β-lactamases (MBLs).[6] Hence, the following study was conducted to know the prevalence MBL and communicate the same to the clinician to prevent the spread of these strains.

Aims & Objectives

This study aimed to know the prevalence of MBL production in various gram negative bacilli, evaluate different phenotypic methods to detect MBL production and determine the antibiotic susceptibility profile of MBL producing gram-negative bacteria bacilli.

MATERIALS AND METHODS

A cross-sectional descriptive study was conducted in the Department of Microbiology, NRI Institute of Medical Sciences, Sangivalasa, Visakhapatnam for a period of 6 months, from May to October.

The study included all the In-Patients in whom Gram-negative bacteria were isolated from various clinical specimens (urine, sputum, pus, fluids, blood).

Patients on systemic or topical antibiotics were excluded from the study.

All the specimens were inoculated onto Blood and MacConkey agar. Incubation of culture plates was done at 35°C for 18-24 hours. Organisms were identified by colony morphology, Gram staining, and various biochemical reactions according to the department's standard operating procedures.[17

Antimicrobial susceptibility testing was done using Disc diff usion technique with reference to Clinical and Laboratory Standards Institute (CLSI) guidelines against Amikacin (30µg), Gentamicin (10µg), Amoxicillin-clavulanate (20/10µg), Cotrimoxazole (25µg), Ampicillin (10µg), Ciprofloxacin (5µg), Cefazoline (30µg), Cefoxitin (30µg), Ceftriaxone (30µg), Cefotaxime (30µg), Ceftazidime (30µg), Cefepime (30µg), Piperacillin-Tazobactam (100/10µg), Norfloxacin (10µg) and Colistin (10µg).[18]

All Imipenem (IPM) resistant isolates were taken as positive for MBL screening. Isolates that gave MBL screening test positive were subjected to confirmation by four other phenotypic tests.

Phenotypic tests for MBL detection:

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. [Figure 1[18]
2) **Imipenem-EDTA combined disc test:** 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. [Figure 2][19]
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. [Figure 3][19]
4) **EDTA disc potentiation 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. [Figure 4][20]

5) **Modified Hodge test:** A 0.5 Mac Farland suspension of ATCC Escherichia coli 25922 was diluted 1 in 10 in sterile saline. It was inoculated on a Mueller Hinton agar plate, and after drying for 5 minutes, a disc of Imipenem 10µg was placed in the center of the plate. Colonies of test organism were picked and inoculated in a straight line, from the edge of the disc up to a distance of at least 20mm similarly Quality control strains like Modified Hodge test positive Klebsiella pneumonia ATCC BAA 1705 and Modified Hodge test negative Klebsiella pneumonia ATCC BAA 1706 were also streaked, they were incubated overnight. They were checked for enhanced growth around the test organism. The presence of cloverleaf zone or distortion of inhibition around the Imipenem disc was interpreted as positive for ESBL production. [Figure 5][18]

RESULTS

In the present study, 100 Gram-negative isolates from clinical specimens of patients were studied for MBL production. Out of 100 isolates, most of the isolates were in the age group of 31-40 years, i.e., 21%, followed by 41-50 years, i.e., 20% [Table 1]. Out of them, males accounted for 54% and females for 46%.

The highest frequency of isolates was pus and sputum (29%), followed by urine (28%). Among these isolates, Klebsiella pneumoniae (33%) was the commonest, followed by Escherichia coli (31%) and Pseudomonas aeruginosa (20%) [Figure 6]. A varied antimicrobial susceptibility pattern has been noted among these different Gram-negative bacteria isolated. Among these, Klebsiella pneumoniae isolates showed 75% susceptibility to Amikacin and 86% susceptibility to Imipenem, and 74% susceptibility to Piperacillin/Tazobactam. Only 32% and 40% susceptibility were seen in Cefazoline and Cefotaxime, respectively. All the Klebsiella pneumoniae isolates (100%) were resistant to Ampicillin.

Escherichia coli showed 89% susceptibility to Amikacin and 79% susceptibility to Imipenem, and 75% and 68% susceptibility to Piperacillin/Tazobactam and Gentamicin, respectively. 29% and 19% susceptibility was seen in Cefazoline and Cefotaxime, respectively. And only 16% susceptibility was seen in Ampicillin.

Pseudomonas aeruginosa showed 82% susceptibility to Imipenem and 74% susceptibility to Piperacillin/Tazobactam, and 67% susceptibility to Amikacin. Susceptibility to Ceftazidime and Ampicillin was 46% and 43%, respectively.

Among the Proteus species isolates, 100% susceptibility was seen in Imipenem and Piperacillin/Tazobactam, while 79% susceptibility was seen in Amikacin and Cotrimoxazole.74% susceptibility was seen in Ceftazidime, Norfloxacin, and Ceftriaxone. Only 21% susceptibility was seen in Cefazoline.

Acinetobacter species showed 80% susceptibility to Amikacin, 60% susceptibility to Imipenem, Piperacillin Tazobactam, Cotrimoxazole, Gentamicin, and 30% susceptibility to Cefoxitin, Ciprofloxacin, Cefepime, and Ceftriaxone. Only 10% susceptibility was seen in Cefazoline.

Citrobacter species showed 100% susceptibility to Imipenem and 80% susceptibility to Amikacin, Piperacillin/Tazobactam, Ciprofloxacin, Cefepime, Amoxicillin-clavulanate, and only 20% susceptibility to Cefazoline.

Detection of MBL

34 Imipenem resistant strains were found on MBL screening with Imipenem disc diffusion tests. These isolates were subjected to the other four phenotypic detection tests [Table 3].

Imipenem-EDTA Combination disc test was taken as the confirmatory test for MBL. Many studies considered it the most sensitive phenotypic test, which confirmed as 32 (94.11%) of the 34 screening positive isolates were MBL producers.[20–24] There was a significant statistical correlation between the Imipenem EDTA double-disc synergy test (p=0.0001) & EDTA disc potentiation test (p=0.035) with the Imipenem-EDTA Combination disc test. In contrast, the Modified Hodge test (p=0.223) doesn't correlate with it. Major MBL production was seen among Acinetobacter species (60%), followed by Klebsiella pneumoniae (36.3%) and Pseudomonas aeruginosa (35%) [Table 4].

Table 1: Age wise distribution (n=100)

Age (in years)	Percentage
0-10	4%
11-20	10%
21-30	13%
31-40	21%
41-50	20%
51-60	15%
>61	17%

Table 2: Sample wise distribution (n=100)

Sample	Percentage
Pus	29%
Urine	28%

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Sputum	29%
High vaginal swab	2%
Ascitic fluid	3%
Pleural fluid	1%
BAL	1%
Blood	7%

Table 3: Comparison of different tests for MBL detection (n=34, Imipenem resistant isolates)

MBL detection tests	Positive	Percentage
IMP-EDTA CDT	32	94.11%
IMP-EDTA DDST	30	88.23%
Modified Hodge test	23	67.64%
Disc potentiation test	14	41.17%

Table 4: Distribution of MBL producers among various isolates

Organism	Number of isolates	MBL producers	Percentage
Klebsiella pneumoniae	33	12	36.3%
Escherichia coli	31	10	32.2%
Pseudomonas aeruginosa	20	7	35%
Proteus species	9	0	0%
Acinetobacter species	5	3	60%
Citrobacter species	2	0	0%
Total	100	32	32%

Table 5: Comparison of the distribution of samples in various studies

Study series	Year	Urine	Pus	Blood	Sputum	High vaginal swab	Fluids
Present study	2022	28%	29%	7%	29%	2%	5%
Iswarya, M. et al.[27]	2019	30%	27%	18%	25%		
Narinder Kaur et al.[28]	2017	58%	31.8%		2.89%	4.3%	
Pathak et al.[25]	2017	36.22%	5.11%	8.66%	14.56%	-	0.78%
Kolhapure RM et al.[29]	2015	42.23%	2.12%	3.40%	28.61%		4.25%
Mita D.Wadekar et al.[26]	2013	26%	37%	30%	7%		

Table 6: Comparison of various tests for MBL detection with various studies

Study series	Year	IMP EDTA CDT	IMP EDTA DDST	Modified Hodge test	Disc potentiation test
Present study	2022	94.11%	88.23%	82.35%	41.17%
Munesh et al.[32]	2019	88.89%	94.44%	--	--
Sachdeva et al.[21]	2017	97.9%	82.3%	62.5%	--
Panchal et al.[19]	2016	63.33%	53.33%	--	--
Chauhan et al.[22]	2015	59.01%	--	48.08%	--
Ranjan et.al.[34]	2015	79.1%	70.8%	87.5%	54.1%
Haider et al.[39]	2014	--	40%	62.22%	--
Pandya et al.[23]	2011	96.30%	81.48%	--	--
Rawat V.[40]	2011	83.33%	50%	--	--

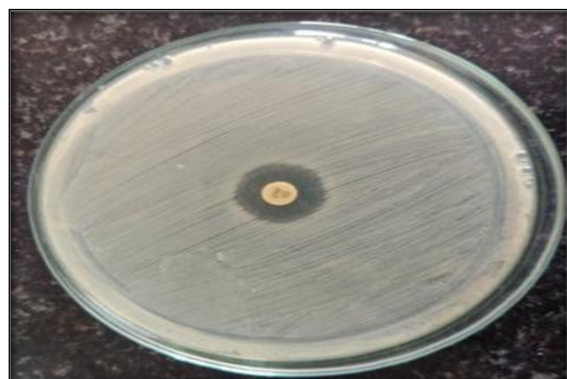
Figure 1: Disc diffusion test

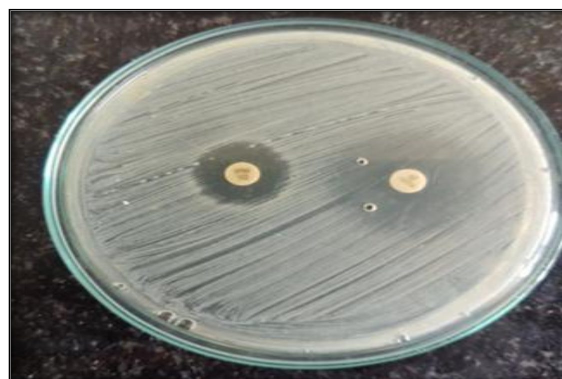
Figure 2: Imipenem-EDTA CDT

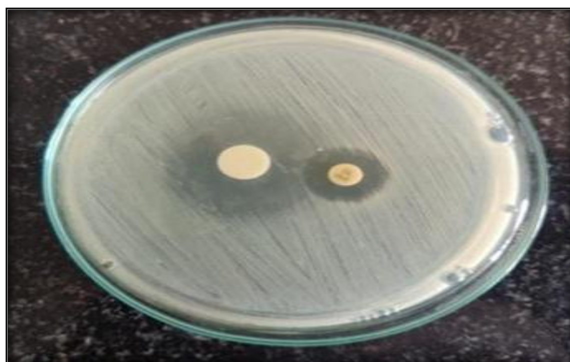

Figure 3: IMP-EDTA DDST

Figure 4: IMP-EDTA DPT

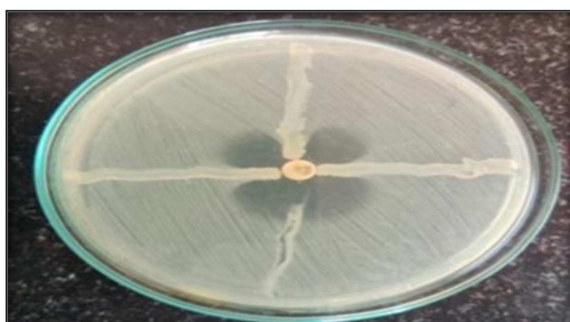

Figure 5: Modified Hodge test

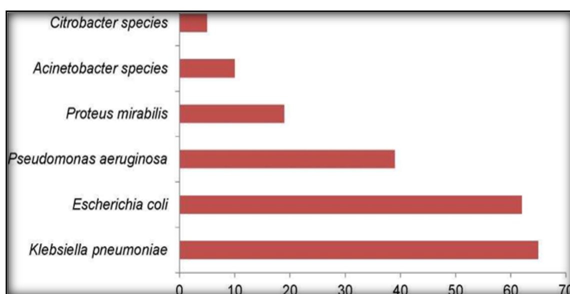

Figure 6: Organism wise distribution

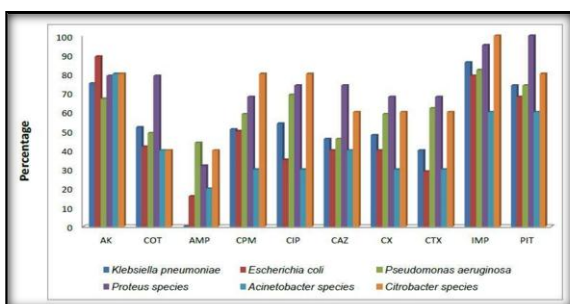

Figure 7: Antibiotic susceptibility pattern of isolates

DISCUSSION

The most common cause of resistance of bacteria to beta-lactam antibiotics is the production of beta-lactamases. MBL genes, in recent years, have spread from Pseudomonas aeruginosa to various other members of Enterobacteriaceae. These enzymes are plasmid-mediated, and multidrug resistance is characteristic of strains producing these enzymes.

The overall prevalence of MBL producers varies significantly in different geographical areas and different institutes.

In the present study, an attempt was made to know the prevalence of MBL in the Gram-negative bacterial isolates and their antibacterial susceptibility pattern. Out of 100 isolates screened, 32% were MBL producers.

This study mainly focused on in-patient samples because most of the risk factors are associated with infections which are present inside the hospital premises like cross-transmission, immune-compromised patients, patients with indwelling devices, heavy use of broad-spectrum antibiotics, and frequent contamination of the hands of health care workers while taking care of patients.

In the present study, 54% of the patients were male, and 46% were female. This finding correlates with the results of Pathak et al., where 51.57% were sampled from males, and 48.42% were female patient samples.[25] Among the 100 clinical samples, pus samples were 29%. The number of pus samples was more than the other samples in the study. This finding correlated with the study by Mita D Wadekar et al. (37%).[26] The percentage of urine samples were more in the studies conducted by Iswarya, M. et al. (30%), Narinder Kaur et al. (58%), Pathak et al. (36.22%), and Kolhapure RM et al., (42.23%) when compared to the present study.[27–29]

The most common isolate was Klebsiella pneumoniae (32.5%); similar to studies of Narinder Kaur et al. (70) (32.8%) and Pathak et al. (32.7%), whereas, in the study conducted by Iswarya, M. et al. (41%) & Vedavati B et al., (37.34%) Escherichia coli was the most common isolate.[25,27,28]

Klebsiella pneumoniae was the highest MBL producer, which correlates with the study conducted by Jena et al.[30] In contrast to this study, the highest MBL production was seen in Klebsiella pneumoniae in studies by Iswarya, M. et al., Binita Bhuyan et al., and Mita D Wadekar et al.[26,27,31] Pseudomonas aeruginosa was the highest MBL producer in a study conducted by Pathak et al.[25]

In the present study, IMP-EDTA CDT (94.11%) was more sensitive compared to IMP-EDTA DDST (88.23%); this is similar to the studies conducted by Sachdeva et al., who reported 97.9%(IMP-EDTA CDT) and 82.3%(IMP-EDTA DDST) and Pandya et al., 2011 who reported 96.30%(IMP-EDTA CDT) and 81.48% (IMP-EDTA DDST).[21,23] In the IMP-EDTA combined disc test with a cut-off >7 mm, the

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positive and negative results were more clearly discriminated.

Contrary to these findings, Munesh et al. reported a higher detection rate in IMP-EDTA DDST (94.44%) than in CDT (88.89%).[32] This contrast in findings may be due to differences in the population structure of MBL genes between different geographical areas. One of the main disadvantages of DDST was the subjective interpretation of the result.[21]

In the present study, MHT detected 82.35% of the isolates compared to CDT (94.11%). Modified Hodge Test often lacks specificity (false-positive results for high-level Amp C producers) and sensitivity (weak screening of NDM producers).[33] Contrary to these findings, Ranjan et al. reported 87.5% in MHT and 79.1% in CDT.[34]

EDTA disc Potentiation test was not a practical test for MBL detection, as in our study, its sensitivity was significantly low (41.17%). Behera et al., and Ranjan et al., observed similar findings in their study.[20,34]

The MBL E- test is very sensitive for the detection of MBL; however, it may not be practically possible for all laboratories to perform the E- test due to cost constraints and availability.

Molecular methods like Polymerase chain reaction (PCR), DNA hybridization, and sequencing are considered the gold standard for detecting carbapenemase production, which will help us know the actual prevalence of these enzymes and characterize them for epidemiological purposes. But these methods have limited practical use for daily application in clinical laboratories because of the cost restraints and are usually used in research settings.[35,36]

Thus, a simple and inexpensive testing method for detecting MBL producers is necessary. The use of simple screening tests like CDT will be a crucial step toward large-scale monitoring of these emerging resistant determinants. All the isolates should be routinely screened for MBL production by CDT since this test is simple to perform and interpret. It can be performed as a routine antimicrobial susceptibility method as it can be easily introduced into the workflow of a clinical laboratory. Many studies have reported the same.[20,37,38]

CONCLUSION

In recent years, the incidence of infections due to the organisms resistant to beta-lactam agents due to various enzymes' production has increased. Detection of MBL production is of paramount importance both in hospital and community isolates. These strains are probably more prevalent than currently recognized. Hospital outbreaks are increasing because of selective pressure due to the heavy use of expanded spectrum cephalosporins and lapses ineffective control measures.

So judicious use of antibiotics and evidence-based medicine is the need of the hour to stop the rise of these resistant strains. Stringent precautions are to be taken to avoid the availability of over-the-counter antibiotics, and their indiscriminate use and strict antibiotic policies are to be implemented.

Hospital infection control committees should meet regularly and make recommendations at all levels to prevent these drug-resistant strains. Strict adherence to standardized infection control policies and antibiotic policy will decrease hospital-acquired infection incidence due to multidrug-resistant organisms. Simple measures like Hand hygiene recommendations are essential to prevent cross-infection.

For the final confirmation of resistant strains, detection of molecular markers by gene demonstration is required, but this study was limited due to the constraint of resources. Simple disc methods can be used to detect resistant strains and have been proved to be rapid and convenient for detection in the clinical laboratory.

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