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Original Article

Microbiology Section

Detection of Carbapenemase Producing Gram-negative Bacteria at a Tertiary Care Hospital: A Cross-sectional Study

RAJESWARI PILLI[1], ARAVA DURGA RANI[2], BARRE SUNITHA[3], SATYA CHANDRIKA VENNA[4]

ABSTRACT

Introduction: Gram-negative bacteria have developed a broad spectrum of resistance due to antibiotic degradation enzymes, including Extended-spectrum Beta-lactamases (ESBL), AmpC β-lactamases, and carbapenemases. Carbapenemase-producing organisms, such as *Escherichia coli, Klebsiella* species, *Acinetobacter* species, and *Pseudomonas* species, have become one of the most important causes of nosocomial and community-acquired infections.

Aim: To identify carbapenemase-producing organisms among Multidrug-resistant (MDR) clinical isolates and detect Metallo β-Lactam Carbapenemases (MBL).

Materials and Methods: The present study is a cross-sectional study that included 1266 clinical samples from patients visiting the Department of Microbiology, Ranagaraya Medical College, Kakinada, Andhra Pradesh, India. from July 2022 to December 2022. The samples were processed using the standard culture and antimicrobial susceptibility test, employing the Kirby-Bauer Disk Diffusion method. The present study included organisms that were resistant to either imipenem or meropenem. Carbapenemases were detected using the Modified Hodge test (MHT), and MBL carbapenemases were detected using the Imipenem Ethylene Diamine Tetraacetic acid (EDTA) Double Disk Test (DDT) following the guidelines of the Clinical and Laboratory Standards Institute (CLSI). Descriptive statistics were performed on the collected data.

Results: The study samples comprised 842 (66.5%) Gram-negative isolates and 424 (33.5%) Gram-positive isolates. Among the Gram-negative isolates, 80 (9.5%) were carbapenem-resistant, of which MHT showed 56 (70%) positive isolates (carbapenemase-producing) and 24 (30%) negative isolates. Imipenem-EDTA DDT showed 50 (62.5%) positive isolates (MBL-producing) and 30 (37.5%) negative isolates.

Conclusion: Carbapenemase production in Gram-negative organisms has reduced the clinical utility of the carbapenem group of drugs, posing a major challenge in the treatment of severe infections. In summary, the majority of carbapenem-resistant isolates in the present study were from the Enterobacteriaceae family. These isolates were subjected carbapenem-resistant isolates to MHT and DDT tests to identify carbapenemase and metallo-beta-lactamase-producing strains that were resistant to a wide spectrum of antibiotics. The present study enables the early detection of these MDR strains, ensuring appropriate management.

Keywords: Carbapenem-resistant, Double disk test, Meropenem, Modified Hodge test

INTRODUCTION

Multiple Drug Resistant (MDR) bacteria, causing high mortality and morbidity, have become a major public health problem in the country. Gram-negative bacteria have developed a broad spectrum of resistance due to antibiotic degradation enzymes like ESBL, AmpC β-lactamases, and carbapenemases. Carbapenems are β-lactam antimicrobials with demonstrated efficacy in severe infections caused by ESBL-producing bacteria [1]. Due to the increasing incidence of infections caused by MDR bacteria, there are very few options to treat such infections [2].

Carbapenem antimicrobial drugs, including imipenem, meropenem, ertapenem, and doripenem, are useful in MDR bacterial infections. Carbapenemase-producing organisms, such as *Escherichia coli, Klebsiella* species, *Acinetobacter* species, and *Pseudomonas* species, have become one of the most important causes of nosocomial and community-acquired infections [3].

The mechanisms of carbapenem resistance in Enterobacteriaceae are carbapenemase production or the loss of porins in combination with the expression of AmpC enzymes or ESBLs [4].

Carbapenemases are divided into three classes (Ambler):

- Class-A, which includes Klebsiella Pneumoniae Carbapenemase (KPC), Serratia Marcescens Enzyme (SME), Non Metallocarbapenemase-A (NMC-A), imipenemase (IMI), and others.
- Class B, which includes MBL like IMP, New Delhi metallo-β-lactamase (NDM-1), Verona integron-encoded MBL (VIM), etc.
- Class D, which includes Oxacillinase (OXA) and *Pseudomonas*-Specific Enzymes (PSE) [4].

Both MBL and KPC carbapenemases are known to co-exist, leading to difficulty in differentiating and identifying these enzymes through phenotypic testing. These enzymes hydrolyse almost all β-lactam antibiotics, making the detection of each one of them masked by the expression of the other. MBL-producing genes can be horizontally transferable via plasmids, resulting in rapid spread to other bacteria. MBL and KPC carbapenemases are mostly encoded by mobile transposons, facilitating faster spread to other Gram-negative bacteria [5].

Carbapenemases can recognise almost all hydrolysable β-lactams, and most of them are resistant to inhibition by β-lactamase inhibitors [2]. MBLs are encoded either by genes that are part of the bacterial chromosome in some bacteria or by heterologous genes acquired through the transfer of mobile genetic elements. Hence, acquired MBLs can be spread among various strains of bacteria [6]. MBLs can be inhibited by metal chelators like Ethylene Diamine Tetra Acetic Acid (EDTA), which contain zinc in their active sites. Confirming carbapenemase production is crucial to controlling the spread of carbapenemase-producing bacteria [5].

Molecular methods are the gold standard for detecting carbapenemase production. However, due to their time consumption

and cost-effectiveness, phenotypic tests have been developed [7]. The aim of the present study was to identify carbapenemase-producing organisms among MDR clinical isolates and detect MBL Carbapenemases. The primary objective of present study is the phenotypical detection of carbapenemases using the MHT. The secondary objective is the detection of MBLs using the DDT with imipenem and imipenem with EDTA.

Early phenotypic detection of carbapenem-resistant Gram-negative bacteria is of great importance as it guides clinicians and helps control the spread of carbapenemase-producing infections.

MATERIALS AND METHODS

The present is a cross-sectional study conducted in the Department of Microbiology, Rangaraya Medical College, Kakinada, Andhra Pradesh, India, for a period of six months from July 2022 to December 2022. Ethics committee approval (IEC/RMC/2022/754) for the present study was obtained before initiating the study. Informed consent was obtained from each patient before sample collection.

Inclusion criteria: Patients who were already admitted to the hospitals (with long hospital stays, any underlying diseases, or immunocompromised conditions).

Exclusion criteria: Patients who were on antibiotics.

Study Procedure

A total of 1266 clinical samples were analysed in the present study, collected from different clinical specimens such as pus, urine, sputum, endotracheal tube aspirate, blood, tissue, and pleural fluid from patients visiting the microbiological laboratory. A total of 842 Gram-negative bacteria, including Klebsiella, *Pseudomonas*, E.coli, and Proteus, from various samples were tested for carbapenemase production. All the samples available during the study duration were included in the analysis.

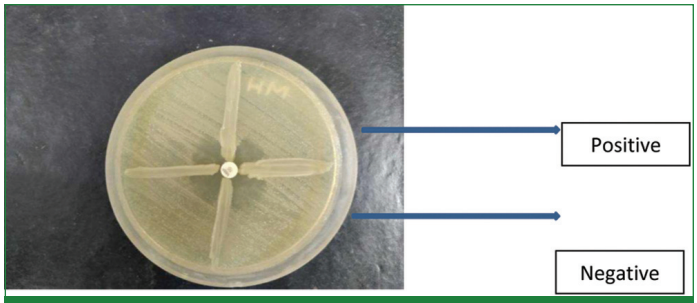
Samples inoculated on blood agar and MacConkey agar were processed using the standard conventional culture method, and identified by conventional biochemical methods. Antimicrobial susceptibility testing of isolates was performed using the standard Kirby-Bauer Disk Diffusion method on Mueller-Hinton Agar (CLSI).

The following antimicrobials were tested: Ampicillin (10 mcg), Ceftriaxone (30 mcg), Cefotaxime (30 mcg), Cefepime (30 mcg), Cotrimoxazole (25 mcg), Piperacillin-Tazobactam (100/10 mcg), Ciprofloxacin (5 mcg), Gentamycin (10 mcg), Amikacin (30 mcg), Meropenem (10 mcg), Imipenem (10 mcg), Cefoperazone-sulbactam (75/30 mcg). The CLSI 2021 standards were used to quantify and interpret zone diameters [8]. Organisms that are resistant to either imipenem or meropenem by the disk diffusion method were included in present study.

Carbapenemase detection methods:

Modified Hodge Test (MHT) for phenotypic detection of carbapenemase production:

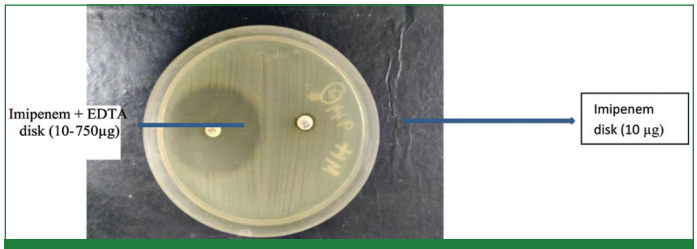
All carbapenem-resistant isolates were subjected to the MHT. A lawn culture of a 1:10 dilution of Escherichia coli ATCC 25922 was prepared on a Muller-Hinton agar plate, and a 10 µg meropenem disk was placed at the center of the plate. A straight line was drawn from the edge of the disk to the edge of the plate using the test organism. Four strains were tested on the same plate with one disk, and the plate was then incubated overnight at 35±2°C in an aerobic atmosphere. After 16-24 hours of incubation, the test result is interpreted as positive if there is a clover leaf-like indentation of the Escherichia coli ATCC 25922 strain growing along the test organism's streak within the disk diffusion zone, indicating the production of carbapenemase. A negative result indicates no growth of Escherichia coli ATCC 25922 along the test organism's streak within the disk diffusion zone [8,9]. [Table/Fig 1] shows the MHT.



[Table/Fig-1]: Modified Hodge Test (MHT) showing clover leaf like indentation for positive samples.

Phenotypic detection of Metallo β-lactamases (MBL) by Imipenem-EDTA Double Disk Test (DDT):

All the carbapenem-resistant isolates were subjected to the double disk synergy test using Imipenem and EDTA. To prepare a 0.5 McFarland standard of the test isolate, 2 to 3 colonies of the test isolate were inoculated into peptone water and incubated for 2-3 hours at 37°C. After incubation, a lawn culture of the organism was inoculated onto MHA following the CLSI guidelines. A sterile cotton swab was then dipped into the 0.5 McFarland standard inoculum and streaked across the entire MHA plate. After drying, a 10 µg imipenem disc was placed on the lawn culture, maintaining a distance of 24 mm center to center from the imipenem-EDTA (10-750 µg) disc. The plate was then incubated at 35±2°C for 16 to 18 hours. The zone diameter was measured using a calibrated zone scale. A MBL positive strain was considered when the increase in the inhibition zone with the imipenem-EDTA disk was ≥7 mm compared to the imipenem disk alone [10]. [Table/Fig 2] shows the DDT.



[Table/Fig-2]: Imipenem EDTA Double Disc Test showing increased zone of inhibition for imipenem- EDTA disk.

STATISTICAL ANALYSIS

Descriptive statistics were performed on the data collected in the study.

RESULTS

The [Table/Fig-3] shows a total of 1266 clinical samples that were analysed in present study from different clinical specimens: pus 573 (45%), sputum 305 (24%), urine 283 (22%), endotracheal tube aspirates 64 (5%), and others such as blood, tissue, pleural fluid, etc., 41 (3%).

Clinical sample	n	Percentage
Pus	573	45%
Sputum	305	24%
Urine	283	22%
Endotracheal tube aspirates	64	5%
Others	41	3%

[Table/Fig-3]: Total Distribution of clinical samples (N=1266).

The [Table/Fig-4] shows that out of all clinical samples, 842 (66.5%) were Gram-negative isolates, and 424 (33.5%) were Gram-positive isolates.

Isolates identified	n	Percentage
Gram-negative organisms	842	66.5%
Gram-positive organisms	424	33.5%

[Table/Fig-4]: Distribution of the clinical isolates (N=1266).

Out of all the Gram-negative isolates, the majority 395 (47%) were isolated from pus samples, 153 (18%) from urine samples, 257 (30.5%) from sputum samples, 32 (4%) from endotracheal tube aspirates, and the least 5 (0.5%) from other samples such as blood, tissue, or pleural fluid.

In the [Table/Fig-5] below, among all Gram-negative isolates, there were 296 (35.1%) Klebsiella species, 175 (21%) *Pseudomonas* aeruginosa, 164 (19.4%) Escherichia coli, 87 (10.3%) *Acinetobacter* species, 86 (10.2%) Proteus species, and 34 (4.03%) Citrobacter species.

Gram-negative isolates	n	Percentage
Klebsiella species	296	35.1%
Pseudomonas aeruginosa	175	21%
Escherichia coli	164	19.4%
Acinetobacter species	87	10.3%
Proteus species	86	10.2%
Citrobacter species	34	4.03%

[Table/Fig-5]: Organism wise distribution of Gram-negative isolates (n=842).

In [Table/Fig-6] below, among all the Gram-negative bacteria, the most resistant antibiotic was ampicillin 801 (95.2%), followed by ceftriaxone, cefotaxime, cefipime, piperacillin-tazobactam, cefoperazone-sulbactam, ciprofloxacin, cotrimoxazole, meropenem, and imipenem 80 (9.5%). The lowest resistance was seen in amikacin 67 (8%) and gentamycin 63 (7.5%).

Antibiotic	n	Resistance percentage
Ampicillin	801	95.2%
Ceftriaxone	657	78%
Cefotaxime	631	75%
Cefipime	623	74%
Piperacillin tazobactum	505	60%
Cefaperazone sulbactum	379	45%
Ciprofloxacin	354	42%
Cotrimoxazole	269	32%
Meropenem	84	10%
Imipenem	80	9.5%
Amikacin	67	8%
Gentamycin	63	7.5%

[Table/Fig-6]: Antibiotic resistance of Gram negative bacteria (n=842).

[Table/Fig-7] below shows that among all the Gram-negative Bacilli (GNB) isolate 80 (9.5%) were found to be Carbapenem-resistant according to the routine antibiotic susceptibility test.

	n	Percentage
Sensitive isolates	762	90.5%
Resistant isolates	80	9.5%

[Table/Fig-7]: Carbapenem-resistant isolates among GNBs isolated (n=842).

In [Table/Fig-8] below, the majority of Carbapenem-resistant isolates 41 (51%) were isolated from pus samples.

Sample	n	Percentage
Pus	41	51%
Urine	18	23%
Sputum	11	14%
Endotracheal tube aspirate	5	6%
Others (blood, pleural fluid, tissue)	5	6%

[Table/Fig-8]: Distribution of Carbapenem-resistant isolates (n=80) among clinical specimens.

[Table/Fig-9] below shows that among all the carbapenem-resistant isolates, the majority 37 (46%) were Klebsiella species, followed by 23 (29%) *Pseudomonas* aeruginosa, 12 (15%) Escherichia coli, 7 (9%) *Acinetobacter* species and 1 (1%) proteus species.

Organism	n	Carbapenem-resistant percentage
Klebsiella species	37	46%
Pseudomonas aeruginosa	23	29%
Escherichia coli	12	15%
Acinetobacter	7	9%
Proteus species	1	1%
Citrobacter species	0	0%

[Table/Fig-9]: Distribution of Carbapenem-resistant isolates (n=80) among organisms.

In [Table/Fig-10] above, among all Carbapenem-resistant isolates, 56 (70%) were MHT positive isolates, indicating carbapenemase-producing isolates, whereas 24 (30%) were MHT negative isolates.

Result	n	Percentage
Positive	56	70%
Negative	24	30%

[Table/Fig-10]: Distribution of Modified Hodge test (MHT) results among Carbapenem-resistant isolates (n=80).

In [Table/Fig-11] above, among all carbapenem-resistant isolates, 50 (62.5%) were MBL-producing isolates, whereas 30 (37.5%) were negative.

Result	n	Percentage
Positive	50	62.5%
Negative	30	37.5%

[Table/Fig-11]: Distribution of Imipenem-EDTA Double Disk test (DDT) results among Carbapenem-resistant isolates (n=80).

In [Table/Fig-12] below, among all carbapenem-resistant isolates, 42 (52.5%) were positive for both the MHT and DDT tests, whereas 16 (20%) were negative for both tests.

Carbapenem-resistant isolates	n	Percentage
Both MHT and DDT positive	42	52.5%
Both MHT and DDT negative	16	20%

[Table/Fig-12]: Distribution of both MHT and Imipenem-EDTA Double Disk Test (DDT) among Carbapenem-resistant isolates.
MHT: Modified hodge test; DDT: Double disk test

DISCUSSION

Carbapenems are considered the treatment of last resort for MDR infections. Carbapenem Resistant Enterobacterales (CRE) has emerged due to the production of carbapenem hydrolysing enzymes, also known as carbapenemases, by the Enterobacteriaceae family of bacteria. The clinical utility of carbapenem drugs has been reduced because of carbapenemase production, posing a significant challenge in the management of severe infections. Therefore, there is a need to detect carbapenemase production [11,12].

Carbapenem-resistant strains are characterised by their resistance to all β-lactam antibiotics, including third and fourth-generation cephalosporins, aminoglycosides, and fluoroquinolones [4]. The rapid emergence of Gram-negative bacteria that are resistant to carbapenems, such as imipenem and meropenem, poses a threat to public health.

In the present study, a total of 1266 clinical samples were tested, of which 842 (66.5%) were identified as Gram-negative bacteria. Among all the tested Gram-negative Bacteria (GNB) isolates, 9.5% were found to be carbapenem-resistant, which aligns with the findings of Alemayehu T et al., (9%) and Abdeta A et al., (10.7%) [1,3]. However, Kumari N et al., reported a higher rate of carbapenem-resistant GNB at 16.8% [9].

The average antibiotic susceptibility pattern observed in the present study showed higher resistance to ampicillin, followed by ceftriaxone, cefotaxime, cefepime, piperacillin-tazobactam, cefoperazone-sulbactam, ciprofloxacin, cotrimoxazole, meropenem, and imipenem. The lowest resistance was seen in amikacin and gentamycin for all the isolates. Similarly, Alemayehu T et al., reported higher resistance to ampicillin, followed by cefuroxime, cefotaxime, cotrimoxazole, ciprofloxacin, gentamycin, and the lowest resistance to piperacillin-tazobactam, meropenem, and amikacin for all isolates [1].

In the present study, the highest number of carbapenemase-producing isolates was recovered from 51% of pus samples, compared to Abdeta A et al., where urine samples showed the highest number (56.5%) of carbapenemase-producing isolates [3]. In the present study, carbapenemase production was found at a higher rate in Klebsiella species (46%), which aligns with Abdeta A et al., who reported a higher rate (73.4%) in Klebsiella species [3]. However, Pandurangan S et al., showed a higher rate (83%) in Enterobacter cloacae and Providencia rettgeri [10]. Klebsiella pneumoniae can easily survive in the hospital environment and is transmitted through admitted patients and hospital staff. The irrational use of antibiotics and frequent exposure of various Klebsiella pneumoniae isolates to antibiotics contribute to the development of multidrug resistance among the strains.

The present study showed 70% of the isolates as MHT positive, which aligns with Amjad A et al., who reported a similar percentage of 69% [13]. However, Pandurangan S et al., reported a lower percentage of MHT positive isolates at 30.5% [10]. The MHT is used for the detection of carbapenemase production and can identify different classes of carbapenemases on a single plate. However, it cannot differentiate between various classes of carbapenemases, leading to false-positive results for ESBL and AmpC producing isolates with reduced or absent porin expression [14]. False-positive results may occur in certain geographical areas where ESBL-producing isolates are prevalent [15].

Routine disk susceptibility testing cannot always detect MBL; it only detects resistance to carbapenems. Resistance to carbapenems can be mediated by multiple mechanisms. The detection of MBL is important as it calls for preventive measures to stop the spread of MDR strains [10]. Early detection of MBL-producing organisms is critical as it allows for the prompt use of appropriate antibiotics to effectively control infection [16].

The present study showed 62.5% of the isolates as MBL positive, which is lower than other studies such as Pandurangan S et al., with 32.5% Pandya NP et al., who reported 96.3% of MBL positive isolates, and Shenoy KA et al., with 93.24%, [10,17,18]. The lesser occurrence of MBL isolates in this study may be attributed to the presence of other mechanisms such as AmpC co-production, loss of porin channels, expression of efflux pumps, and the presence of altered PBPs. Various factors such as over-the-counter use of antibiotics, irrational use of antibiotics, easy accessibility to higher antibiotics, lack of adequate health measures, and improper sanitation and living conditions are considered crucial for the development and spread of carbapenemase-producing organisms [11].

In the present study, both MHT and MBLs were positive in 52.5% of the isolates, whereas Pandurangan S et al., reported only 6% of isolates showing positivity for both methods. In the present study, 20% of the isolates tested negative for both methods, despite being resistant to carbapenems by disk diffusion. This could be attributed to the overproduction of ESBL or AmpC hyperproducers with porin loss. Pandurangan S et al., reported 43% of isolates testing negative for both tests [10].

Early detection of MBL-producing Gram-negative bacteria and determining their antibiotic sensitivity are of crucial importance for initiating appropriate treatment [19]. Since the MHT is a cost-effective and easy-to-perform test, it could serve as an important alternative to molecular methods in resource-limited areas [20]. Therefore, the MHT and DDT can be utilised in the microbiology laboratory to detect carbapenemase production. Clinically, these tests can guide antimicrobial therapy and help control the spread of carbapenemase-producing infections, especially in severe and life-threatening cases [11].

Limitation(s)

Variation in patient backgrounds and patient care is the major limitation of the present study, as it may influence the outcome of the results. However, a large sample size can help to mitigate this limitation and provide more comprehensive findings.

CONCLUSION(S)

Carbapenemase production in Gram-negative organisms has diminished the clinical effectiveness of carbapenem drugs, presenting a significant challenge in the treatment of severe infections. In summary, the majority of carbapenem-resistant isolates in the present study were from the Enterobacteriaceae family. These isolates were subjected to the MHT and DDT to identify strains producing carbapenemase and metallo-beta-lactamase, which exhibited resistance to a broad range of antibiotics. The present study enables the early detection of these MDR strains, ensuring appropriate management.

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PARTICULARS OF CONTRIBUTORS:
1. Assistant Professor, Department of Microbiology, Rangaraya Medical College, Kakinada, Andhra Pradesh, India.
2. Professor and Head, Department of Microbiology, Government Medical College, Machilipatnam, Andhra Pradesh, India.
3. Assistant Professor, Department of Microbiology, NRI Institute of Medical Sciences, Visakhapatnam, Andhra Pradesh, India.
4. Assistant Professor, Department of Microbiology, Rangaraya Medical College, Kakinada, Andhra Pradesh, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Rajeswari Pilli,
House No. 70-1A-15, Flat No. T1, Aishwarya Rainbow Apartment, Vidiyalavari Street, Ramanayyapeta, Kakinada-533001, Andhra Pradesh, India.
E-mail: rajeswari1807@gmail.com

PLAGIARISM CHECKING METHODS: [Jain H et al.]
- Plagiarism X-checker: Jul 01, 2023
- Manual Googling: Nov 21, 2023
- iThenticate Software: Nov 24, 2023 (22%)

ETYMOLOGY: Author Origin

EMENDATIONS: 6

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