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Prevalence of Metallo Beta-Lactamase in Non-Fermentative Gram-Negative Bacilli from Clinical Isolates by Phenotypic Methods in a Tertiary Care Hospital

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Abstract

Background: Non-Fermentative Gram-Negative Bacilli (NFGNB), particularly Pseudomonas aeruginosa and Acinetobacter species, are major contributors to hospital-acquired infections and are increasingly associated with multidrug resistance due to Metallo-β-lactamase (MBL) production. These enzymes confer resistance to carbapenems and pose significant therapeutic challenges. **Objective:** To determine the prevalence of MBL-producing NFGNB from clinical specimens using phenotypic methods in a tertiary care hospital in Chennai. **Methods:** A cross-sectional study was conducted from August to October 2023, including 148 non- duplicate clinical isolates of NFGNB. Phenotypic detection of MBL was performed using the Combination Disk Test (CDT) and Double Disk Synergy Test (DDST). Antimicrobial susceptibility testing was conducted using the Kirby-Bauer disk diffusion method as per CLSI guidelines. **Results:** Among the isolates, 51% were Pseudomonas aeruginosa and 49% were Acinetobacter spp. CDT detected MBL production in 63.5% of isolates, while DDST detected 59.5%. MBL production was higher in Acinetobacter spp. (59.5%) compared to P. aeruginosa (40.4%). The kappa value (κ = 0.6–0.7) indicated good agreement between CDT and DDST. High rates of resistance to imipenem and meropenem were noted. COPD emerged as a borderline significant factor (p=0.058) influencing MBL positivity. **Conclusion:** The high prevalence of MBL-producing NFGNB highlights the urgent need for routine phenotypic screening to guide antimicrobial therapy and implement effective infection control strategies. CDT and DDST remain reliable, cost-effective methods in resource-limited settings. Further molecular studies are recommended for confirmation and surveillance.

Keywords: Acinetobacter Species, Combination Disk Test (CDT), Double Disk Synergy Test (DDST), Metallo-β-lactamase (MBL), Non-Fermentative Gram-Negative Bacilli (NFGNB), *Pseudomonas aeruginosa*

1. Introduction

Gram-negative organisms are frequent causes of hospital-acquired infections. Common among these are *Klebsiella* species, *Escherichia coli*, *Pseudomonas* species, and *Acinetobacter* species. *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Acinetobacter baumannii* are the most common gram-negative, non-fermentative bacteria encountered in the laboratory from various clinical specimens[1].

The mainstay of treatment for these organisms is beta-lactam antibiotics with Imipenem and Meropenemas reserved drugs for MDRO[2]. In recent years acquired Metallobeta-Lactamase (MBL) producing organisms has increased[3]. Metallo-β-Lactamases (MBL) are a group of enzymes that induce the hydrolysis of a broad set of β-lactam drugs including carbapenems and are also not susceptible to beta-lactamase inhibitors like clavulanate[3]. The genes encoding MBL production are carried on highly mobile plasmids. This allows easy

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dissemination. Invasive infection with MBL producers leads to higher morbidity and mortality[4]. There are five different types of MBL namely IMP, VIM, SPM, GIM, and SIM[5]. Early detection and reporting of MBL is essential in a hospital setting for effective infection control. Though genotypic methods are available, due to the ease of availability and cost-effectiveness of phenotypic methods - the IMP-EDTA combined dick test and Double disk synergy test were implemented[6]. Phenotypic testing method which can be easily adopted in routine clinical laboratory testing.

2. Aim and Objectives

To study the prevalence of the Metallo beta lactamase-producing gram-negative No fermenters (NF-GNB) isolated from the clinical samples in a tertiary care hospital in Chennai.

3. Review of Literature

Non-fermentative Gram-negative bacilli are opportunistic pathogens, commonly involved in nosocomial infections, especially in Intensive Care Units (ICUs). Studies have shown a significant increase in the prevalence of MBL-producing strains among NFGNB in recent years. In a study conducted by Sahu *et al.,*[7] MBL production was observed in Pseudomonas aeruginosa (30%), *Acinetobacter baumannii* (25%), and *Stenotrophomonas maltophilia* (10%) isolates from clinical samples. Similarly, a study by Kumar *et al.,* found a higher prevalence of MBL-producing *Pseudomonas aeruginosa* (38%) and *Acinetobacter baumannii* (27%) in a hospital setting[8]. These findings highlight the growing concern of MBL-mediated resistance in NFGNB, which compromise the effectiveness of commonly used antibiotics, including carbapenems.

Several factors contribute to the high prevalence of MBL-producing NFGNB, including the overuse and misuse of antibiotics, inadequate infection control practices, and the ability of these organisms to acquire and transfer resistance genes. Furthermore, the emergence of carbapenem-resistant *Pseudomonas aeruginosa* and Acinetobacter baumannii in hospital-acquired infections underscores the importance of monitoring MBL production in these pathogens[9]. MBL-producing NFGNB (38.5%) increased in Device Associated Infection (DAI) among the most commonly encountered organisms *Pseudomonas aeruginosa* and Acinetobacter species in high-risk areas, especially in ICUs[10].

The detection of MBL production in clinical isolates is crucial for appropriate antimicrobial therapy. Phenotypic methods are widely employed in clinical microbiology laboratories due to their relative ease, cost-effectiveness, and ability to provide rapid results. Among the various phenotypic methods, the combination of disk diffusion and the Double Disk Synergy (DDS) test is commonly used for detecting MBL production[11]. The principle is that if the organism produces MBL, there will be a synergy effect between the carbapenem and EDTA, resulting in an enhanced inhibition zone around the carbapenem disc. This synergistic effect occurs because the EDTA binds the zinc ion required for the enzymatic activity of MBLs, thereby enhancing the activity of the carbapenem[11].

Numerous studies have demonstrated the reliability of the DDS method for detecting MBL-producing NFGNB. For instance, a study by Jayalakshmi *et al.,*[12] reported that the DDS method had a sensitivity of 91.5% and a specificity of 98.2% for detecting MBLs in *Pseudomonas aeruginosa* isolates. Another study by Sahoo *et al.,*[13] highlighted the high sensitivity (95%) and specificity (97%) of the DDS method for MBL detection in *Acinetobacter baumannii*.

Phenotypic methods, including the combination of disk diffusion and DDS methods, offer several advantages. These methods are simple, inexpensive, and relatively easy to perform in routine microbiology laboratories. Additionally, they do not require specialized equipment, making them accessible in resource-limited settings. Moreover, these methods provide rapid results, enabling clinicians to initiate appropriate antibiotic therapy without delay[11].

4. Material and Methods

Study design: Cross-sectional descriptive study
Study place: ESIC Medical College and Hospitals, Department of Microbiology Duration of the study: 01.08.2023 to 31.10.2023 Ethical committee approval No: IEC/2023/2/39 Inform
consent obtained Inclusion Criteria:
All the non-duplicate gram-negative no fermenters isolated from clinical samples like tissue, pus, wound

swab, blood, endotracheal aspirate, bronchial Veola lavage, urine, and sterile body fluids. Sample size N=4pq/(d)2 Expected proportion of happening cases (p)=70% (from three months Hospital statistics of ESIC Medical College and hospital, KK Nagar, Chennai)
p=Incidence% q= 100-p
d=10% N=96
Required Sample size is 96 Samples.

5. Methodology

Samples received for bacterial culture and sensitivity were processed as per the standard operating procedure of the laboratory. The isolates were identified either by conventional or automated system – VITEK2 and the antibiotic susceptibility testing was done by Kirby Bauer disk diffusion method for the following drugs -*Pseudomonas aeruginosa* -Ceftazidime (30µg), Cefepime (30µg), *Piperacillin- tazobactam* (100/10µg), levofloxacin(5µg), Ciprofloxacin(5µg), Tobramycin(10µg), Gentamycin(10µg), Amikacin (30µg), Aztreonam (30µg), Imipenem (10µg), Meropenem (10µg). Acinetobacter species - Ceftazidime (30µg), Cefepime (30µg), Piperacillin-tazobactam (100/10µg), levofloxacin (5µg), Ciprofloxacin (5µg), Gentamycin (10µg), Amikacin (30µg), Cefotaxime (30µg), Imipenem (10µg), Meropenem (10µg), Cotrimoxazole (1.25/23.75). The susceptibility test results were interpreted as susceptible, intermediate, and resistant categories based on the inhibition zone diameter according to the Clinical and Laboratory Standards Institute (CLSI 2023)[14]. Among these isolates, whichever was found to be imipenem resistant (<23mm of zone size by disk diffusion) were subjected to phenotypic screening methods for Metallo β-lactamase production by combination Disk and double disc synergy test and clinical details of those patients were collected.

5.1 MBL Screening Methods

5.1.1 Combination Disk Test

A suspension of Imipenem-resistant gram- negative non fermented isolates was prepared after adjusting the turbidity to 0.5 Mac Farland. Within 15 minutes of the inoculum suspension, organisms

were plated on the MHA plate by lawn culture technique. Two discs - imipenem (IMP) (10µg) and Imipenem (10µg) with 10µl of 0.5M (750µg) anhydrous Ethylene Diamine Tetra Acetic Acid (EDTA) were placed 25mm apart and incubated overnight at 37°C. An increment in zone diameter of >7mm surrounding the IMP-EDTA disk compared to the imipenem disk alone was considered positive for MBL.

5.1.2 Double Disk Synergy Test

For the double disc synergy test, the test organisms were inoculated on MHA as above. Two discs- animipenem (10µg) disc and another blank disc to which 10µl of 0.5 MEDTA is added–were placed 20mm apart from center to center. Enhancement of the zone of inhibition in the area between imipenem and EDTA disc in comparison to zone size on the far side of the drug was considered a positive result.

6. Results (Including Observations)

A total of 148 samples were included in this study; the sex distribution showed a predominance of males (61.1%) compared to females (38.9%) as in (Figure 1). Patients were admitted across multiple wards, ICU:20.8%, Chest Ward:16.8%, Medical-Surgical Ward (MS):19.5%, Female Surgical (FS):14.1%, Male Medical (MM):8.1%, Female Medical (FM):9.4%, ENT Ward:0.7%. (Figure 2) Several common comorbid conditions were identified in this study, T2DM:35.6%, HTN:18.8%, COPD:13.4%, LRTI:24.8%, Diabetic Foot:25.5%, UTI:11.4%, Venous Ulcer:8.1%, Acute Pulmonary Edema:6.0% (Figure 3).

Utilization of medical devices was included in this study, ET tube: 12.1%, Urinary catheter: 18.1%, Centralline:1.3%, Nodevice:79.6%—indicating many patients were not on invasive medical devices (Figure 4).

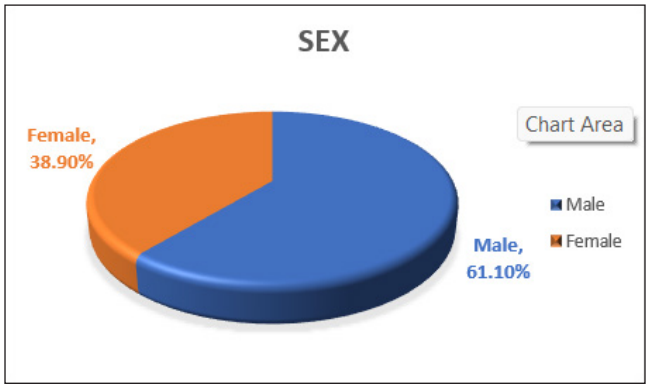

Figure 1. Sex distribution of the study population.

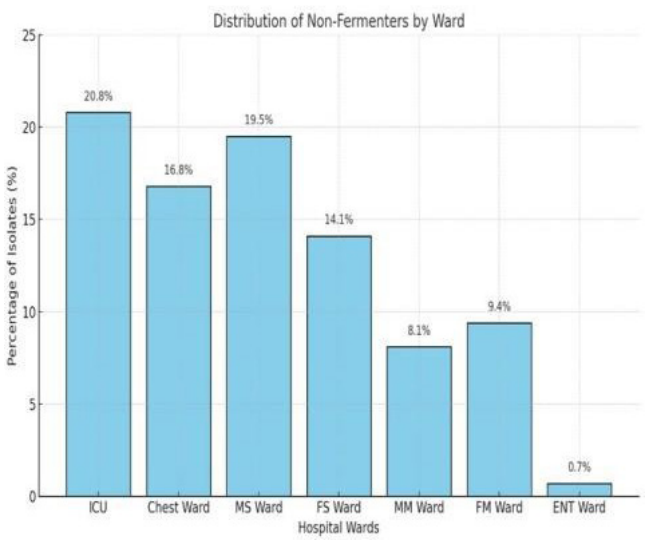

Figure 2. Distribution of non-fermentors by ward.

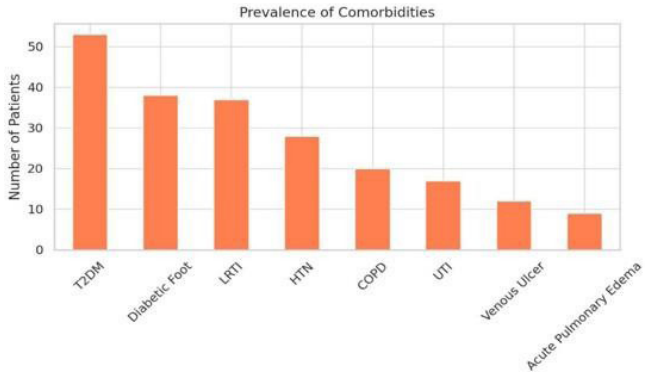

Figure 3. Comorbidities of study population.

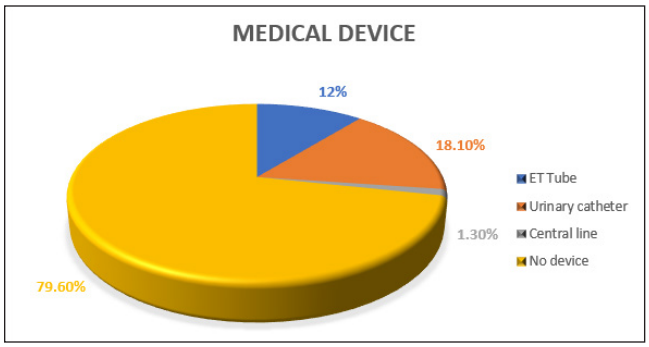

Figure 4. Distribution of medical devices in study population.

The length of hospital stay varied from 0 to 14 days, with 24.2% of patients staying for 4 days and 20.8% for 5 days, suggesting a generally short hospitalization period for most patients (Figure 5).

Frequencies of DURATION OF HOSPITAL STAY

DURATION OF HOSPITAL STAY	Counts	% of Total	Cumulative %
0	13	8.7%	8.7%
11	3	2.0%	10.7%
13	1	0.7%	11.4%
14	3	2.0%	13.4%
2	1	0.7%	14.1%
3	10	6.7%	20.8%
4	36	24.2%	45.0%
5	31	20.8%	65.8%
6	15	10.1%	75.8%
7	12	8.1%	83.9%
8	10	6.7%	90.6%
9	13	8.7%	99.3%
o	1	0.7%	100.0%

Figure 5. Duration of hospital stay in study population.

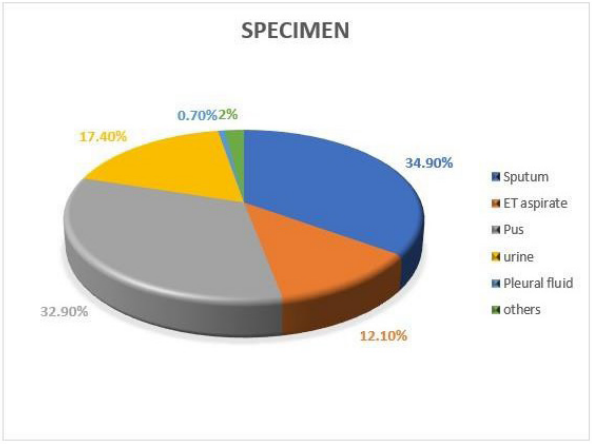

Figure 6. Sample profile of study population.

In this study multiple samples were included, Sputum (34.9%) and ET aspirate (12.1%) were the most frequently collected respiratory samples. Pus swabs (32.9%), urine (17.4%), and pleural fluid (0.7%) represented other sources of microbiological culture (Figure 6).

Out of the 148 samples, 51% of *Pseudomonas aeruginosa* (75) and 49% of Acinetobacter species (73) were isolated (Figure 7).

The antimicrobial resistance pattern was also noted in this study, most of the isolates were multidrug-resistant. In *Pseudomonas aeruginosa* (n=75) among that Aztreonam 72%, *Piperacillin tazobactam* 30.66%, Imipenem 30.66%, Meropenem 22.66%, Gentamyc in 25.33%, Ciprofloxacin 26.6%, Amikacin 24%, Ceftazidime 32%, Tobramycin 25.33%, Levofloxacin 22.66%, Cefepime 9.33%. (Table 1) (Figure 8)

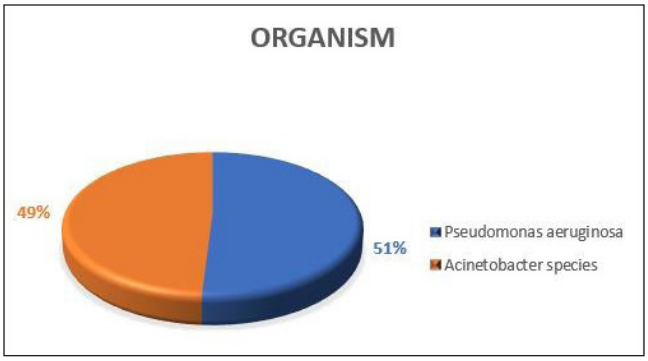

Figure 7. Distribution of organism.

Table 1. Anti-microbial resistance pattern

Pseudomonas aeruginosa	
Aztreonam	72%
Piperacillin Tazobactam	30.66%
Imipenem	30.66%
Meropenem	22.66%
Gentamycin	25.33%
Ciprofloxacin	26.6%
Amikacin	24%
Ceftazidime	32%
Tobramycin	25.33%
Levofloxacin	22.66%
Cefepime	9.33%
Acinetobacter species	
Cefotaxime	64.28%
Piperacillin Tazobactam	83.56%
Imipenem	76.71%
Meropenem	75%
Gentamycin	78%
Ciprofloxacin	76.71%
Amikacin	75%
Ceftazidime	23.28%
Trimethoprim- Sulfamethoxazole	64.38%

Acinetobacter species (n=73), Cefotaxime 64.28%, *Piperacillin tazobactam* 83.56%, Imipenem 76.71%, Meropenem 75%, Gentamycin 78%, Ciprofloxacin 76.71%, Amikacin 75%, Ceftazidime 23.28%, Trimethoprim-Sulfamethoxazole 64.38% (Table1) (Figure 8).

MBL was detected more in Acinetobacter species (59.5%) than in *Pseudomonas aeruginosa* (40.4%). 63.5% (n=94) were detected as MBL by combination

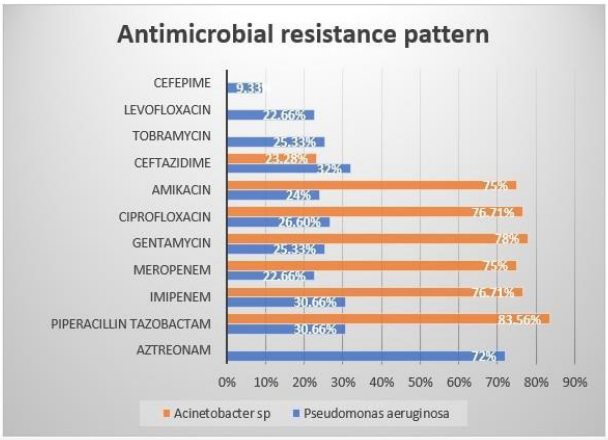

Figure 8. Anti-microbial resistance pattern.

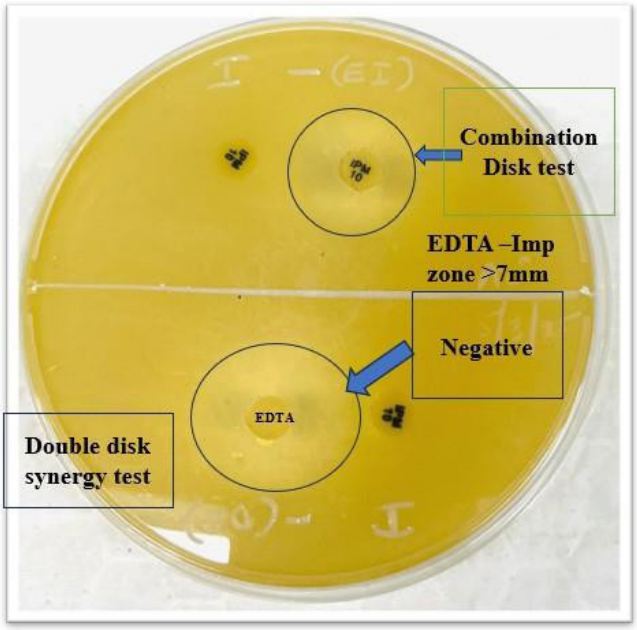

Figure 9. MBL screening test (1. Combination disk test, 2. Double disk synergy test).

disc test whereas 59.5%(n=88) were detected as MBL by Double disc synergy test. Kappa statistics were used to assess agreement between phenotypic resistance testing methods (combination disk and double disk synergy test). The Kappa value was moderate to substantial (κ between 0.81 and 0.86). Almost perfect concordance (κ ≈ 0.86) between observers (0.857) and tests (0.858) was verified through agreement analysis. Excellent agreement is indicated by kappa values higher than 0.81, according to Landis and Koch criteria. This shows that, when utilized in standardized laboratory conditions, the Imp–EDTA CDT is

a reliable phenotypic method for detecting metallo-β-lactamase production.

Logistic Regression Analysis A binary logistic regression model was used to evaluate factors influencing a positive combination disk test result (indicative of MBL production).

Age: Estimate = 0.0361, p = 0.378–Not significant, Sex (female vs. male): Estimate = -0.8287, p = 0.482–Not significant, COPD: Estimate = 3.0597, p = 0.058–Border line significance.

Other predictors including ICU admission, presence in specific wards, and other comorbidities showed extremely high standard errors and non-significant p-values (often p =1.000). COPD was the only factor approaching statistically significant. Sex, age, and hospital ward were not significantly associated with positive test outcomes.

7. Discussion

The present study underscores the growing concern regarding the emergence of multidrug- resistant *Pseudomonas aeruginosa* and *Acinetobacter* species in clinical settings. More than half of the isolates demonstrated resistance to key antibiotics, including carbapenems such as imipenem and meropenem. This high rate of resistance aligns with global trends where overuse of antibiotics and lack of stringent infection control measures have facilitated the dissemination of resistant strains. Similarly, Falagas *et al*[7]., and Kumar *et al*[8]. The anti-microbial susceptibility data revealed alarming resistance levels to drugs like aztreonam, cefotaxime, and trimethoprim- sulfamethoxazole. Similarly, Sharma R *et al*[15].

The dominance of *Pseudomonas aeruginosa* (51%) and *Acinetobacter* spp. (49%) among isolates confirms their role as the principal pathogens in nosocomial infections, particularly in patients with respiratory tract infections and wound infections. Kumarasamy *et al.*, and Nordmann *et al.,* showed similar[17,18]. Respiratory specimens such as sputum and endotracheal aspirates constituted the majority of isolates, reflecting the role of invasive procedures and ventilator- associated complications in these infections. Similarly, Nordmann *et al.,* 2019 study also showed[17]. In this present study, the combination disk test (63.5%) detected more MBL producers than the double disk synergy test which is similar to Chowdhury *et al.,*[16] and MBL was detected more in *Acinetobacter* species (59.5%) than *Pseudomonas aeruginosa* (40.4%). The observed Kappa value (κ≈ 0.6–0.7) indicates good agreement between the phenotypic detection methods—namely, the combination disk test and the double disk synergy test—highlighting their effectiveness, accessibility, and ease of implementation in routine microbiology laboratories, particularly in resource-limited settings where molecular diagnostics may not be available. Young *et al*[19]., and Keiser *et al*[20]., did the same.

In logistic regression analysis, comorbid conditions such as COPD showed a borderline significant association with MBL production (p = 0.058). This suggests that chronic pulmonary conditions, possibly due to recurrent hospital visits and antibiotic exposure, may predispose patients to colonization or infection with resistant organisms[21]. COPD requires hospitalization; however, it is necessary that these patients are not exposed to higher antibiotics or administration of antibiotics without culture studies need to be stopped to prevent AmR in hospital settings. Colistin is the last resort, however rot appropriate in patients with renal dysfunction[22]. There is a need for timely testing and reporting of newer drugs as these infections when untreated can result in sepsis contributing to mortality.

7.1 Use of Newer Drugs for MBL producing NFGNB

The newer drugs like Cefiderocol, a siderophore Cephalosporin, are effective against MBL producers due to their unique iron-transport entry and β-lactamase stability. Aztreonam–avibactam is a promising combination that overcomes MBL and co-produced β-lactamases, showing synergy in resistant infections. Other agents like colistin, tigecycline, fosfomycin, and eravacycline are often used in combination regimens, though limitations such as toxicity and poor efficacy in bloodstream infections persist. Meropenem–vaborbactam and imipenem–relebactam have limited utility against MBLs.

Overall, newer drugs like cefiderocol and emerging combinations (e.g., aztreonam–avibactam) offer hope in managing these difficult infections, especially when guided by rapid diagnostics and stewardship efforts.

7.2 Clinical Implications

- Routine screening for MBL production is critical for timely infection control interventions[19,20]. CDT and DDS can serve as front line diagnostic tools to identify resistance and guide antibiotic therapy. Surveillance of high-risk units like the ICU and monitoring of patients with chronic comorbidities are essential to prevent outbreaks[8,23].
- The high prevalence of MBL (63.5%) emphasizes the importance of early diagnosis and tailored treatment in preventing complications

7.3 Limitations and Recommendations

The study was limited by its cross-sectional design and single-center nature. The absence of molecular confirmation of MBL genesis another limitation. Future research should include genotypic methods for validation and longitudinal studies are needed to assess outcomes and transmission dynamics[18,20].

8. Summary and Conclusion

The prevalence of MBL-producing NFGNB is increasing, threatening the health care clinical outcomes of patients. It is important to perform routine screening of MBL–producing organisms in diagnostic laboratories to reduce the dissemination of such strains in hospital patients and manage antibiotics. Though the combination disc test and Double disc synergy test are simple to detect the MBL producers, further confirmation with genotypic methods is crucial.

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