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ANTIBIOTIC RESISTANCE PATTERNS OF ESBL AND MBL PRODUCING GRAM-NEGATIVE BACTERIA IN A TERTIARY CARE HOSPITAL

Medical Microbiology

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ABSTRACT

Background: The increasing prevalence of antibiotic resistance among Gram-negative bacteria poses a significant challenge to clinical management, particularly in healthcare settings. This study aimed to assess the prevalence and antibiotic susceptibility patterns of Extended-Spectrum Beta-Lactamase (ESBL) and Metallo-Beta-Lactamase (MBL) producing Gram-negative bacterial isolates from various clinical samples at a tertiary care hospital in South Delhi. **Methodology:** A total of 280 Gram-negative isolates were collected from inpatient and outpatient departments, with urine and pus samples comprising the majority. ESBL and MBL production was screened using VITEK and confirmed phenotypically via disk diffusion and Imipenem-EDTA Combined Disk Synergy tests respectively. Genotypic confirmation was done for ESBL producers using PCR targeting blaTEM, blaSHV, and blaCTX-M genes. **Result:** Out of the 280 isolates which were tested, **167**(59.6%) were found to be potential ESBL producers. Among them, *E.coli* was screened positive for ESBL production in **95** (56.8%) isolates followed by *Klebsiella* in **38** (22.7%). Among MBL producers, *Pseudomonas aeruginosa* (41.8%) and *Acinetobacter baumannii* (25.45%) were dominant. Antibiotic susceptibility testing revealed high resistance to third-generation cephalosporins and carbapenems, while nitrofurantoin and colistin showed better efficacy. **Conclusion:** The study emphasizes the urgent need for a multi faceted approach to combat antibiotic resistance in healthcare settings. Key strategies include enhancing antibiotic stewardship programs, implementing routine screening for ESBL and MBL producing organisms and enforcing strict infection control measure. Judicious use of colistin and ongoing surveillance of resistance patterns are also critical. By adopting these measures, healthcare institutions can improve patient outcomes and preserve the effectivenesss of existing antibiotics.

KEYWORDS

Extended-Spectrum Beta-Lactamase (ESBL), Metallo-Beta-Lactamase (MBL), Antibiotic Resistance, Antimicrobial Susceptibility Testing (AST), DDDT

INTRODUCTION

Gram-negative bacteria are recognized as significant pathogens responsible for various infections across all age groups, both in hospital and community settings. Among these, *Escherichia coli* and *Klebsiella pneumoniae* are particularly concerning, having been classified as top-priority pathogens by the World Health Organization (WHO) due to their increasing resistance to commonly prescribed antibiotics. It is estimated that 70% of bacterial infections in hospitals are caused by drug-resistant pathogens, complicating treatment and increasing morbidity and mortality rates (**1**).

Beta-lactam antibiotics have been the mainstay in treating infections caused by gram-negative bacteria. These antibiotics inhibit bacterial cell wall synthesis, a critical function for bacterial survival. However, resistance mechanisms, particularly the production of beta-lactamase enzymes such as Extended-Spectrum Beta-Lactamases (ESBLs) and Metallo-Beta-Lactamases (MBLs), have emerged, severely limiting the efficacy of these drugs (**2**). ESBLs hydrolyze a wide range of beta-lactam antibiotics, while MBLs, classified under Ambler Class B, degrade almost all beta-lactam agents, including carbapenems, which are often considered last-resort antibiotics (**3**).

The evolutionary history of gram-negative bacteria shows their remarkable adaptability, driven by horizontal gene transfer and mutation (**4**). These processes, along with environmental factors like pollution and the selective pressure from widespread antibiotic use, have led to the emergence of resistant strains that pose significant challenges to healthcare systems globally (**5**). As early as the 18th century, gram-negative bacteria were distinguished by the Gram-staining technique, developed by Hans Christian Gram in 1884, which paved the way for their detailed study (**6**). Throughout the 20th century, groundbreaking work by scientists like Louis Pasteur and Robert Koch helped in the identification and understanding of numerous gram-negative species, including the pathogenic *Escherichia coli* and *Salmonella typhi* (**7**).

One of the major public health crises caused by these bacteria is their resistance to antibiotics. In the 1980s, an outbreak of *E. coli* O157linked to contaminated food underscored the dangers of these pathogens, highlighting the need for improved detection and food safety measures (**8**). Similarly, *Acinetobacter baumannii*, an opportunistic pathogen, became notorious for its multidrug resistance, particularly during the Iraq and Afghanistan conflicts, earning the nickname "Iraqibacter" (**9**).

Studies have shown that the emergence of antibiotic resistance in gram-negative bacteria is multifactorial, involving several resistance mechanisms, such as the production of ESBLs and MBLs (**10**). The discovery of plasmid-mediated MBLs in *Pseudomonas aeruginosa* in 1991 marked a turning point in understanding how rapidly these resistant genes could spread in healthcare settings (**11**). Since then, numerous studies have documented the rising prevalence of these enzymes, especially in pathogens like *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and members of the Enterobacteriaceae family (**12**).

Despite the extensive research on the prevalence and mechanisms of ESBL- and MBL-producing gram-negative bacteria, significant gaps remain in understanding the regional and institutional variability of these resistance patterns, particularly in resource-constrained settings like South Asia. Many previous studies have focused on either phenotypic or genotypic detection methods, but comparative analyses of these techniques in real-world clinical settings are limited. Moreover, while the global spread of resistance mechanisms such as NDM-1 has been well-documented, localized data on emerging resistancesuch asESBL and MBL trends, especially in tertiary care hospitals catering to diverse patient populations, are scarce. Addressing these gaps is crucial to developing context-specific diagnostic protocols and containment strategies for combating multidrug-resistant gram-negative pathogens.

Given the global health implications, the present study investigated the prevalence of ESBL- and MBL-producing gram-negative bacterial isolates in a tertiary care hospital in South Delhi. The study also evaluated different phenotypic and genotypic methods for detecting these resistance mechanisms, with the goal of informing more effective treatment strategies and preventing the spread of multidrug-resistant organisms within healthcare facilities. By contributing to the growing body of literature on antibiotic resistance, this study will provide valuable insights for the development of targeted interventions, particularly in resource-limited settings.

MATERIALS AND METHODS

Study Area

This cross-sectional study was conducted in the Department of Microbiology, Hamdard Institute of Medical Sciences and Research (HIMSR), and the associated HAHC Hospital, Jamia Hamdard, over duration of six months. The study focused on gram-negative bacterial isolates obtained from various clinical samples, including blood, urine,

pus, and body fluids. A total of 280 samples were estimated using the sample size formula:

$n=(z)^2 \times p(1-p)/d^2$

Where,
n = sample size
z = level of confidence according to the standard normal distribution (99%)
p = estimated proportion of the population that presents the characteristic
d = tolerated margin of error

Ethical approval for the study was obtained from the Ethics and Research Committee of Jamia Hamdard University.

Inclusion And Exclusion Criteria
The study population included both paediatric and adult patients attending various clinical departments of the hospital. Duplicate samples were excluded from the study.

Antimicrobial Susceptibility Testing
The gram-negative isolates were subjected to antibiotic susceptibility testing using the VITEK 2 Compact system (Biomerieux), an automated identification and susceptibility testing system.

Phenotypic Methods for Detection of ESBLs (13, 14)
Screening Test
The screening for ESBL production was done using the VITEK system and standard disk-diffusion method, following CLSI guidelines.

Procedure:
- A 0.5 McFarland standard turbidity bacterial suspension was prepared by selecting 3-5 freshly grown pure colonies.
- This suspension was inoculated on Mueller-Hinton Agar (MHA) plates using a sterile cotton swab.
- Disks of three third-generation cephalosporins—Cefotaxime (30 µg), Ceftazidime (30 µg), and Ceftriaxone (30 µg)—were placed at a distance of 20 mm on the plate.
- Plates were incubated at 35°C for 16–18 hours.

Interpretation:
Zone diameters of Cefotaxime (≤27 mm), Ceftazidime (≤22 mm), and Ceftriaxone (≤25 mm) were considered suspicious for ESBL production.

Phenotypic Confirmatory Test
Positive isolates from the screening test were further confirmed using the Double Disk Diffusion Test.

Procedure:
o A 0.5 McFarland standard suspension was inoculated on an MHA plate.
o Disks containing ceftazidime (30 µg) and ceftazidime-clavulanic acid (30 µg/10 µg) were placed 20-25 mm apart, along with cefotaxime (30 µg) and cefotaxime-clavulanic acid (30 µg/10 µg).
o The plates were incubated at 35°C for 16–18 hours.

Interpretation:
oA ≥5 mm increase in zone diameter for any antimicrobial agent in combination with clavulanic acid compared to its standalone counterpart was considered positive for ESBL production.

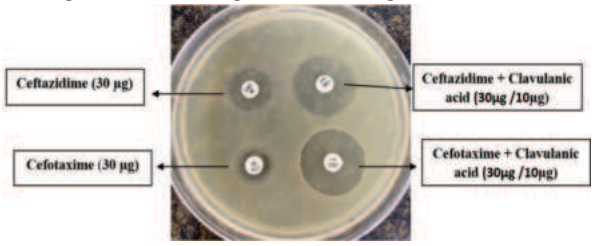


Fig 1: Confirmatory Test for ESBL Detection

Genotypic Method For Detection Of ESBLs
The phenotypically confirmed ESBL-producing isolates were further tested genotypically via PCR to detect the presence of blaTEM, blaSHV, and blaCTX-M genes.

DNA Extraction (15)
Bacterial DNA was extracted using the phenol-chloroform method:
- Colonies from overnight cultures were inoculated into BHI broth and incubated.
- The cells were harvested, and the pellet was suspended in EDTA buffer and subjected to phenol-chloroform extraction.
- The DNA was precipitated using ethanol, washed, and dissolved in TE buffer. DNA purity was confirmed using a Nanodrop spectrophotometer.

PCR Amplification
Singleplex PCR was used to detect the blaTEM, blaSHV, and blaCTX-M genes. Primers specific for each gene were used for amplification.

Thermal Profiling for TEM and CTX-M gene
- Initial Denaturation was done at 94ºC for 5 min.
- Denaturation done at 94ºC for 30 sec.
- Annealing done at 54ºC for 1 min.
- Extension done at 72ºC for 2 min.
- Final extension at 72º C for 5 min.

Thermal Profiling for SHV gene
- Initial Denaturation was done at 94ºC for 5 min.
- Denaturation done at 94ºC for 30 sec.
- Annealing done at 54ºC for 30 sec.
- Extension done at 72ºC for 1 min.
- Final extension at 72º C for 10 min.

Gel Electrophoresis
The amplified PCR products were visualized using agarose gel electrophoresis. After running the samples, bands were visualized under UV light in the Gel-Doc system.

Phenotypic Method for Detection of MBLs (16)
Screening Test
The isolates were screened for MBL production using the VITEK system and disk diffusion test. Imipenem, meropenem, and ertapenem resistance was considered indicative of MBL production.

Phenotypic Confirmatory Test
The Imipenem-EDTA combined disk synergy test was used for confirmation.

Procedure:
Imipenem and meropenem disks were placed on the MHA plate, with EDTA added to one disk of each.

The plates were incubated, and a ≥7 mm increase in zone size with the EDTA disk indicated MBL production.

Quality Control
- **ESBL:** *Klebsiella pneumoniae* (ATCC 700603) as the positive control and *E. coli* (ATCC 25922) as the negative control.
- **MBL:** *Pseudomonas aeruginosa* (ATCC 105663) as the positive control and *Pseudomonas aeruginosa* (ATCC 27853) as the negative control.

This comprehensive methodology provided a robust framework for detecting and analyzing ESBL- and MBL-producing gram-negative bacteria in clinical isolates.

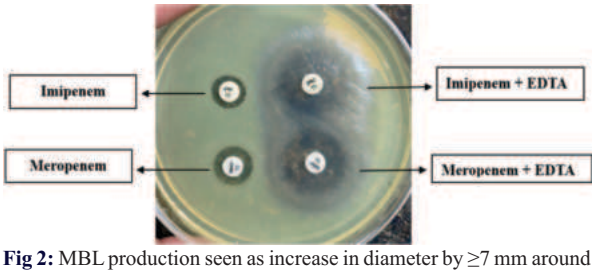


Fig 2: MBL production seen as increase in diameter by ≥7 mm around IMP+EDTA and

RESULTS
This study aimed to assess the prevalence and antibiotic susceptibility patterns of Extended-Spectrum Beta-Lactamase (ESBL) and Metallo-Beta-Lactamase (MBL) producing gram-negative bacterial isolates obtained from various clinical specimens at a tertiary care hospital. A

total of 280 isolates were collected from inpatient (IPD) and outpatient (OPD) departments and subjected to screening and confirmatory tests for ESBL and MBL production. The findings provide significant insights into the distribution of these resistant pathogens across different sample types, their prevalence in various age groups and genders, and the associated antibiotic resistance patterns.

Prevalence Of Gram-Negative Bacteria Across Clinical Specimens

A total of 280 gram-negative bacterial isolates were collected from inpatient (IPD) and outpatient (OPD) samples during the study period. The specimens included urine, pus, blood, stool, body fluids, and miscellaneous samples. The highest number of isolates were obtained from urine samples, representing 47.8% of the total, followed by pus samples (30.7%), and blood (7.8%). Stool, body fluids, and miscellaneous samples contributed a smaller proportion of isolates (2.85%, 6.07%, and 4.64%, respectively) (**Figure 3**).

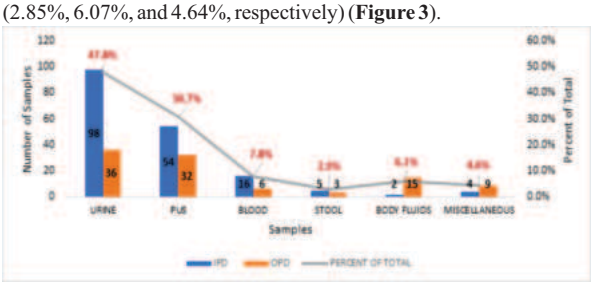


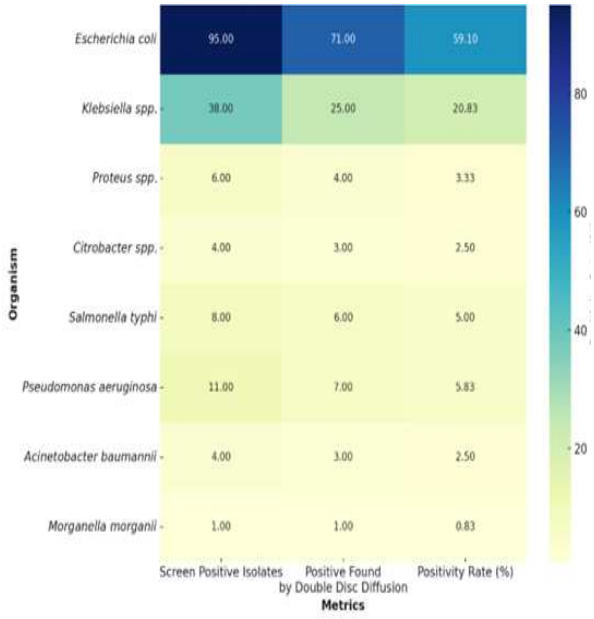
Figure 3: Distribution of Gram-Negative isolates among different clinical specimens obtained from IPD and OPD patients

Detection of ESBL-Producing Isolates

Out of the 280 isolates screened for ESBL production using the VITEK and disk diffusion methods, 167 isolates (59.6%) were identified as potential ESBL producers. Among these, *Escherichia coli* was the predominant ESBL producer, with 95 out of 167 isolates (56.8%) testing positive. *Klebsiella spp.*, another significant pathogen, accounted for 22.7% of the ESBL-positive isolates, while other organisms such as *Proteus spp.*, *Citrobacter spp.*, *Salmonella typhi*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* showed lower percentages of ESBL production (**Table 1**).

A phenotypic confirmatory test (Double Disk Diffusion Test) was performed on the 167 screen-positive isolates, confirming 120 (71.9%) as ESBL producers. The highest number of confirmed ESBL producers was seen in urine samples (46.6%), followed by pus (24.1%) and blood (10%). The high prevalence of ESBL producers in urine samples aligns with the finding that *E. coli* is the leading cause of UTIs, often complicated by resistance mechanisms such as ESBL production **(Figure 4)**.

Table 1: Detection of ESBL in Screen positive isolates by Phenotypic Confirmatory Test (Double Disk Diffusion Test)



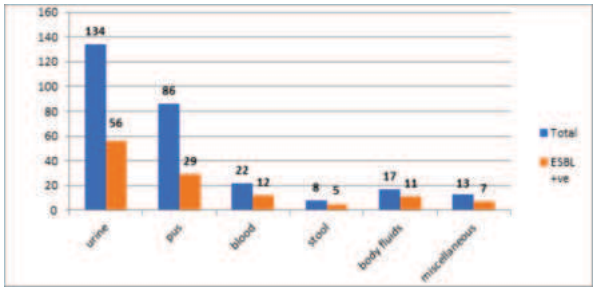


Figure 4: Distribution of ESBLs among different clinical specimens

Age and Gender Distribution of ESBL Producers

The age distribution of patients with ESBL-producing isolates revealed that the highest number of cases occurred in the 31–40-year age group, accounting for 23.3% of ESBL producers. This was followed by the 21–30-year age group (15.8%) and the 41–50-year age group (15%). The least number of cases were seen in patients aged 81–90 years (3.33%). This distribution suggests that middle-aged adults are more susceptible to infections caused by ESBL-producing organisms, possibly due to increased healthcare exposure, underlying comorbidities, or surgical interventions in this age group.

Gender-wise, females accounted for 55.8% of the ESBL-positive cases, while males represented 44.1%. The higher proportion of females could be attributed to the increased incidence of UTIs in women, which are frequently caused by ESBL-producing *E. coli*. In contrast, males may have been more affected by ESBL producers in respiratory and soft tissue infections.

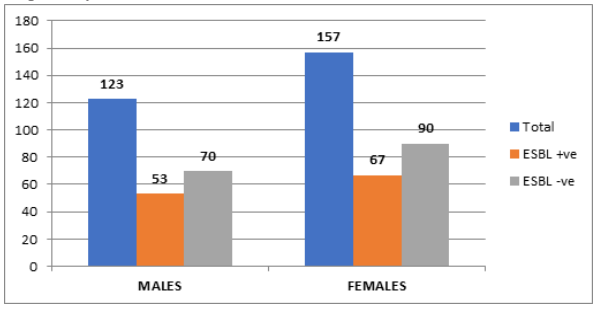


Figure 5: Gender Comparison among ESBL isolates

Detection of MBL-Producing Isolates

The study also screened the isolates for Metallo-Beta-Lactamase (MBL) production. Out of the 280 isolates, 55 (19.6%) were identified as potential MBL producers. *Pseudomonas aeruginosa* was the predominant MBL producer, accounting for 41.8% of the MBL-positive isolates. This was followed by *Acinetobacter baumannii* (25.45%), a pathogen commonly associated with multidrug resistance in hospital-acquired infections. *Escherichia coli* and *Klebsiella spp.* were less frequently identified as MBL producers, with 18.1% and 14.5%, respectively.

The phenotypic confirmatory test (Imipenem-EDTA Combined Disk Synergy Test) confirmed 47 out of 55 isolates as MBL producers. The distribution of MBL producers across clinical specimens showed that 55.31% were isolated from pus samples, while 25.5% were from urine samples.

Antibiotic Susceptibility Patterns

The antibiotic susceptibility pattern revealed significant resistance among both ESBL and MBL producers to multiple classes of antibiotics. Among the ESBL producers, resistance to cephalosporins such as ceftazidime (96.6%) and ceftriaxone (92.5%) was particularly high, which is expected as these antibiotics are substrates for ESBL enzymes. However, nitrofurantoin (85.7%) and imipenem (75.8%) showed good efficacy against ESBL-producing isolates, making them valuable options for treatment in infections caused by these resistant organisms.

For MBL producers, resistance was observed to be highest with imipenem (89.3%) and meropenem (83%), which are carbapenems often used as last-resort treatments for multidrug-resistant gram-negative infections. The resistance to these critical antibiotics

highlights the challenge of treating infections caused by MBL-producing organisms. Fortunately, colistin demonstrated excellent activity, with 95.7% sensitivity, offering a potential therapeutic option for infections caused by carbapenem-resistant organisms.

Table 2: Antibiotic Susceptibility Pattern Among ESBL And Non ESBL Producers

Antibiotics	ESBL producers N=120		Non ESBL producers N=160	
	Sensitive	Resistant	Sensitive	Resistant
Ampicillin	0%	100%	14.11%	85.89%
Amoxicillin/Clavulanic Acid	37.5%	61.66%	45.65%	54.3%
Pipercillin/Tazobactam	57.5%	42.5%	51.8%	48.12%
Ceftazidime	3.33%	96.6%	73.1%	26.8%
Ceftriaxone	7.5%	92.5%	83.12%	16.87%
Cefoxitin	33.6%	66.3%	83.5%	16.5%
Cefoperazone/Sulbactam	58.2%	41.8%	71.2%	28.8%
Amikacin	68.3%	31.6%	89.3%	10.7%
Gentamicin	65.8%	34.2%	70.6%	29.4%
Ciprofloxacin	19.16%	80.83%	54.3%	45.6%
Norfloxacin	26.7%	73.3%	44.7%	55.2%
Imipenem	75.8%	24.2%	88.75%	11.25%
Nitrofurantoin	85.7%	14.3%	89.74%	10.26%
Trimethoprim/Sulfamethoxazole	55.8%	44.2%	49.3%	50.6%

Table 3: Antibiotic Susceptibility Pattern Among MBL Producers

Antibiotics	MBL producers (N=47)	
	Sensitive (n_1) % = n_1/N X 100	Resistant (n_2) % = n_2/N X 100
Amoxicillin/Clavulanic Acid	19 (40.4%)	28 (59.6%)
Pipercillin/Tazobactam	29 (61.8%)	18 (38.2%)
Ceftazidime	23 (49%)	24 (51%)
Ceftriaxone	20 (42.56)	27 (57.44%)
Amikacin	14 (29.8%)	33 (70.2%)
Gentamicin	17 (36.17%)	30 (63.83%)
Ciprofloxacin	30 (63.83%)	17 (36.17%)
Meropenem	8 (17%)	39 (83%)
Imipenem	5 (10.7%)	42 (89.3%)
Nitrofurantoin (n=12)	9 (75%)	3 (25%)
Trimethoprim/Sulfamethoxazole	20 (42.55%)	27 (57.45%)
Colistin	45 (95.7%)	2 (4.3%)

Molecular Detection Of ESBL Genes

Molecular analysis of 25 randomly selected ESBL-positive isolates revealed that 56% harbored the blaCTX-M gene, while 44% had the blaTEM gene. No isolates were found to carry the blaSHV gene. In contrast, among the non-ESBL producers, 12% carried the blaTEM gene, and 4% had the blaCTX-M gene. The absence of the blaSHV gene suggests that this gene may be less prevalent in the region or among the studied population.

Table 4: Molecular Detection Of Genes Among ESBL Producing Isolates

Positive by PCR for ESBL genes	Number amplified in ESBL producers N_1=25	Number amplified in non ESBL producers N_2=25
bla TEM	11 (44%)	3(12%)
bla SHV	0	0
bla CTX-M	14 (56%)	1(4%)

DISCUSSION

The rise in antibiotic resistance among gram-negative bacteria (GNB) exemplifies their ability to acquire and express genetic material conferring resistance to multiple antibiotics. The rapid evolution of resistance, compounded by inappropriate antibiotic use, surpasses the pace of novel antibiotic discovery. Widespread use of beta-lactam antibiotics has particularly driven the emergence of highly resistant strains, such as Extended-Spectrum Beta-Lactamase (ESBL) and Metallo-Beta-Lactamase (MBL) producers, in both community and hospital settings.

This study, conducted in the microbiology laboratory of HAHC Hospital, Jamia Hamdard, South Delhi, investigated the prevalence and antibiotic susceptibility patterns of ESBL and MBL producers among 280 gram-negative isolates collected from October 2023 to March 2024. Among these, 42.85% were ESBL producers, and 16.7% were MBL producers, as confirmed by phenotypic tests. The prevalence of ESBL producers aligns with prior studies reporting rates of 6.6–68% in India **(17)**. *E. coli* and *Klebsiella spp*. emerged as predominant ESBL producers, particularly in urinary isolates.

MBL prevalence, observed in 16.7% of isolates, was consistent with rates reported in previous studies from India. A study done by Ahir et al. in Gujarat found that 11.42% of *P.aeruginosa* harbor MBLs**(18)**. *P. aeruginosa*, *Acinetobacter spp., K. pneumoniae*, and *E. coli* were the most common MBL producers, with pus and urine samples showing the highest positivity rates. ESBL prevalence was higher in females (55.8%), while MBL prevalence was more common in males (65.9%). Age-wise, ESBL rates were highest in the 31–40 age group.

Antibiotic susceptibility tests revealed high resistance to commonly used antibiotics, with carbapenems, amikacin, and nitrofurantoin showing the best efficacy against ESBL producers. Similar study done by Soltani et al. denoted amikacin and imipenem are the choicest drugs for positive ESBL strains **(19)**. Conversely, MBL producers displayed extensive resistance, with colistin being the most effective option.

PCR analysis identified CTX-M as the most prevalent ESBL gene (56%), followed by TEM (44%), while SHV was undetected. The results are in accordance with the study done by Ahmed et al. in 2013 in which CTX-M is the predominant gene type (71.4% in *E.coli* and 68.4% in *Klebsiella*) followed by TEM (55.1% in *E.coli* and 58% *Klebsiella*) **(20)**. Notably, some phenotypically ESBL-negative isolates carried ESBL genes, highlighting the complexity of resistance mechanisms and the need for improved detection methods.

CONCLUSION

This study highlights the significant prevalence of ESBL and MBL-producing gram-negative bacterial isolates in a tertiary care hospital, with *Escherichia coli* and *Klebsiella pneumoniae* being the predominant ESBL producers, and *Pseudomonas aeruginosa* and *Acinetobacter baumannii* leading among MBL producers. The widespread resistance to commonly used antibiotics, particularly third-generation cephalosporins and carbapenems, poses a major challenge for treatment. Colistin remains one of the few effective antibiotics against MBL producers, while nitrofurantoin and imipenem were valuable options against ESBL infections.

To mitigate the growing threat of antibiotic resistance, it is essential to implement comprehensive antibiotic stewardship programs within healthcare institutions. These programs should promote the rational use of antibiotics, specifically limiting the unnecessary administration of third-generation cephalosporins and carbapenems, which contribute to the rise of resistant strains. Empirical antibiotic therapy must be guided by local antibiograms and adapted based on the specific susceptibility patterns observed in each hospital.

Routine screening for ESBL and MBL producers, especially in high-risk areas such as intensive care units, should become standard practice to enable early detection and timely intervention. Early identification of these resistant pathogens will help inform appropriate treatment and prevent their further spread. In addition, stringent infection control measures, including adherence to hand hygiene protocols, the use of personal protective equipment (PPE), and rigorous environmental cleaning, are vital to minimize the transmission of resistant organisms, particularly in surgical and ICU settings where MBL producers are more prevalent.

The study also underscores the need for judicious use of colistin, which showed high efficacy against MBL producers. However, the use of colistin should be carefully monitored to avoid the development of resistance. Hospitals and healthcare providers should explore newer antibiotics and therapeutic strategies that are effective against multi-drug-resistant organisms. Moreover, strengthening public health surveillance systems to monitor the prevalence and resistance patterns of ESBL and MBL producers is crucial. Surveillance data can inform national and local policies, ensuring that infection control strategies and antibiotic usage are continuously updated and optimized based on emerging trends.

In conclusion, addressing the challenge of antibiotic resistance requires a multi-faceted approach, combining improved antibiotic

stewardship, rigorous infection control, routine screening, and ongoing surveillance. These strategies will help to combat the spread of ESBL and MBL producers, ultimately improving patient outcomes and preserving the efficacy of available antibiotics.

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