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Phenotypic Detection of ESBL, AmpC, and Hidden MBL Production in Clinical Isolates of Pseudomonas Aeruginosa

Dr. Irfan Hussain Gauri[1], Ivangi Agarwal[2], Dr. Bhagirath Ram Bishnoi (Professor)[3], Dr. Abhishek Binnani (Professor)[4], Dr. B.P Sharma (Ex. Professor)[5]

[1]Senior Resident, Department of Microbiology & Immunology Sardar Patel Medical College Medical College, Bikaner, Rajasthan, INDIA
[2]MSc. Medical Microbiology, Department of Microbiology & Immunology, Sardar Patel Medical College Medical College, Bikaner, Rajasthan, INDIA
[3]Department of Microbiology & Immunology, Sardar Patel Medical College Medical College, Bikaner, Rajasthan, INDIA
[4]Department of Microbiology & Immunology, Sardar Patel Medical College Medical College, Bikaner, Rajasthan, INDIA
[5]Department of Microbiology & Immunology, Sardar Patel Medical College Medical College, Bikaner, Rajasthan, INDIA

Corresponding Author:

Dr. Irfan Hussain Gauri
Senior Resident, Department of Microbiology & Immunology Sardar Patel Medical College Medical College, Bikaner, Rajasthan, INDIA
Email:
drhussain94615@gmail.com

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ABSTRACT

Background: Pseudomonas aeruginosa is an important opportunistic nosocomial pathogen with marked intrinsic and acquired antimicrobial resistance. Production of Extended-Spectrum β-Lactamases (ESBLs), AmpC β-lactamases, and Metallo-β-Lactamases (MBLs) contributes significantly to multidrug resistance and limits therapeutic options.
Methods: A prospective hospital-based study was conducted over seven months at a tertiary care teaching hospital in northwestern India. Among 100 non-duplicate Pseudomonas isolates, 44 were confirmed as P. aeruginosa. Antimicrobial susceptibility testing was performed using the Kirby–Bauer disk diffusion method according to CLSI guidelines. ESBL, AmpC, and MBL production were detected phenotypically using the Combination Disk Diffusion Test, Cefoxitin-Cloxacillin Double Disk Synergy Test (CC-DDST), and Imipenem-EDTA Combined Disk Test (IECDT), respectively.
Results: Most isolates were obtained from inpatients (90.9%), predominantly from urine (36.36%) and pus/wound specimens (15.91%). ESBL production was detected in 100% of isolates, AmpC in 90.9%, and MBL in 56.8%. Co-production of ESBL, AmpC, and MBL was observed in 56.8% of isolates. High resistance rates were noted for Cefoxitin (95.45%), Ceftazidime (88.64%), and Piperacillin-Tazobactam (65.91%). Although overall Imipenem resistance was 34.09%, 56% (14/25) of confirmed MBL-producing isolates appeared susceptible to Imipenem on routine disk diffusion testing, indicating the presence of "hidden" MBLs. Colistin and Polymyxin-B showed the highest susceptibility (88.64%).
Conclusion: A high prevalence of ESBL, AmpC, and MBL co-production was observed among clinical isolates of P. aeruginosa. The detection of phenotypically hidden MBLs highlights the limitations of routine susceptibility testing alone and emphasizes the need for specific phenotypic confirmatory tests to support appropriate antimicrobial therapy and stewardship practices.

Keywords: *Pseudomonas aeruginosa; ESBL; AmpC; MBL; antimicrobial resistance; phenotypic detection.*

INTRODUCTION

Pseudomonas aeruginosa is an important opportunistic Gram-negative pathogen responsible for a wide spectrum of healthcare-associated infections, particularly in immunocompromised and critically ill patients[1]. It is frequently implicated in ventilator-associated pneumonia, catheter-associated urinary tract infections, burn wound infections, septicemia, and

other severe nosocomial infections. The organism can survive in diverse hospital environments because of its minimal nutritional requirements and ability to persist in moist surfaces such as sinks, ventilators, and medical equipment[2].

The treatment of *P. aeruginosa* infections has become increasingly challenging due to its intrinsic and acquired resistance to multiple antimicrobial agents. The World Health Organization has classified carbapenem-resistant *P. aeruginosa* as a "Priority 1: Critical" pathogen because of the growing global burden of antimicrobial resistance. Resistance in *P. aeruginosa* is mediated through multiple mechanisms, including decreased outer membrane permeability, efflux pump overexpression, target-site mutations, and production of β-lactamase enzymes[3,4].

Among these mechanisms, β-lactamase production remains one of the most clinically significant causes of resistance to β-lactam antibiotics[5]. Extended-Spectrum β-Lactamases (ESBLs) hydrolyze penicillins, cephalosporins, and monobactams, whereas AmpC β-lactamases confer resistance to cephamycins and are not inhibited by conventional β-lactamase inhibitors[6]. Metallo-β-Lactamases (MBLs), including IMP, VIM, and NDM types, hydrolyze carbapenems and most β-lactam antibiotics except monobactams. The emergence of these enzymes has severely limited therapeutic options for multidrug-resistant (MDR) *P. aeruginosa* infections[7].

The simultaneous co-production of ESBL, AmpC, and MBL enzymes further complicates antimicrobial therapy and laboratory detection. Co-existing resistance mechanisms may mask individual enzyme phenotypes during routine susceptibility testing, leading to false-negative results and inappropriate antimicrobial therapy[8]. In particular, isolates harboring low-level or "hidden" MBLs may appear susceptible to carbapenems in standard disk diffusion testing despite possessing clinically significant carbapenemase activity[9].

In resource-limited settings, molecular characterization of resistance genes is often not feasible because of financial and technical constraints. Therefore, reliable and cost-effective phenotypic methods remain essential for the detection of β-lactamase-mediated resistance[10]. Although India is recognized as a high-burden region for multidrug-resistant Gram-negative pathogens, data regarding the co-production of ESBL, AmpC, and MBL enzymes among *P. aeruginosa* isolates remain limited in many tertiary care centers[11].

The present study was undertaken to determine the prevalence of ESBL, AmpC, and MBL production among clinical isolates of *P. aeruginosa*, evaluate their co-production patterns, and analyze the associated antimicrobial susceptibility profiles in a tertiary care teaching hospital in northwestern India. The findings may contribute to improved diagnostic practices, antimicrobial stewardship strategies, and infection control measures in healthcare settings[12].

MATERIALS AND METHODS

Study Design and Setting

A prospective, hospital-based observational surveillance study was conducted at the Bacteriology Laboratory of the Department of Microbiology at a major tertiary care referral center located in North-West Rajasthan, India. The study was executed over a continuous seven-month period. The research protocol adhered strictly to the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines to ensure transparent and comprehensive reporting.

Sample Collection and Patient Demographics

Clinical specimens were systematically collected from consecutive patients presenting to the Outpatient Departments (OPD) and those admitted to various Inpatient Departments (IPD), including specialized Intensive Care Units (ICUs) and surgical wards. The diverse array of clinical specimens analyzed included mid-stream clean-catch urine, purulent exudates and wound swabs, whole blood, cerebrospinal fluid (CSF), expectorated sputum, throat swabs, tracheal aspirates, and high vaginal swabs.

To maintain the statistical integrity of the prevalence data, stringent inclusion and exclusion criteria were applied. All consecutive, non-duplicate isolates of *Pseudomonas* spp. recovered from clinically significant infections were included in the primary cohort. Conversely, repeat isolates obtained from the same patient during a single hospital admission, as well as polymicrobial cultures exhibiting three or more distinct organism types lacking a clear predominant pathogen, were strictly excluded from the analysis. Comprehensive demographic and clinical metadata were prospectively extracted from patient medical records utilizing a standardized questionnaire. Documented variables included patient age, biological sex, admission status (IPD vs. OPD), underlying comorbidities (e.g., immunosuppression, malignancies), history of recent surgical interventions, presence of severe burns, utilization of invasive medical devices (central venous catheters, urethral catheters, mechanical ventilators), prior exposure to broad-spectrum antimicrobial therapy, and the duration of hospital stay.

Microbiological Isolation and Identification

All collected clinical specimens were transported to the laboratory without delay and processed following standard microbiological protocols. Specimens were aseptically inoculated onto Nutrient Agar, MacConkey Agar, and 5% Sheep Blood Agar plates[13,14]. Urine samples were additionally inoculated onto Hi-Chrome UTI differential media (Hi-Media,

Mumbai, India) to facilitate rapid presumptive identification. All culture plates were incubated aerobically at 37°C for 18 to 24 hours[15].

Suspected *Pseudomonas* colonies were initially identified based on characteristic colony morphology: the appearance of non-lactose fermenting (pale) colonies on MacConkey agar, and large, irregular, flat colonies with a matte surface, serrated edges, and frequent β-hemolysis on Blood Agar[16]. The production of characteristic water-soluble pigments—pyocyanin (blue-green) and pyoverdine (yellow-green fluorescence)—coupled with a distinct sweet, grape-like or aminoacetophenone odor, provided a strong presumptive identification of *P. aeruginosa*[17].

Confirmatory identification at the species level was established through a comprehensive battery of standard biochemical assays. Isolates were confirmed as *P. aeruginosa* based on the following definitive phenotypic profile: Gram-negative, slender, motile bacilli demonstrating strictly oxidative metabolism on Hugh and Leifson's Oxidative-Fermentative (OF) medium; robustly positive reactions for both catalase and cytochrome oxidase; positive for citrate utilization; negative for indole production, methyl red (MR), and Voges-Proskauer (VP) tests; negative for urease production; and exhibiting an alkaline slant/alkaline butt (K/K) reaction on Triple Sugar Iron (TSI) agar with an absolute absence of hydrogen sulfide (H_2S) or gas production[18,19]. Furthermore, the isolates demonstrated the capacity for growth at 42°C and positive nitrate reduction capabilities. Out of an initial pool of 100 *Pseudomonas* spp. isolates, 44 isolates met all rigorous biochemical criteria for *P. aeruginosa* and were systematically selected for subsequent antimicrobial susceptibility testing and specific β-lactamase characterization[20].

Antimicrobial Susceptibility Testing (AST)

In vitro antimicrobial susceptibility profiling of the confirmed *P. aeruginosa* isolates was performed using the standardized Kirby-Bauer disk diffusion method on Mueller-Hinton Agar (MHA), in strict compliance with the interpretive criteria and methodologies outlined by the Clinical and Laboratory Standards Institute (CLSI) guidelines (M100 document series).

Standardized bacterial inoculum was prepared by suspending 3 to 5 isolated colonies from an overnight culture into 0.9% sterile physiological saline. The turbidity of the suspension was meticulously adjusted to match a 0.5 McFarland standard (yielding a bacterial concentration of approximately 1.5 X 10^8 CFU/mL). A sterile cotton swab was utilized to inoculate the entire surface of the MHA plates to achieve confluent lawn growth[21,22].

A comprehensive panel of 12 anti-pseudomonal antibiotic disks (obtained from Hi-Media Laboratories, Mumbai, India) encompassing diverse pharmacological classes was applied to the inoculated agar using sterile forceps[23]. The evaluated antimicrobial agents and their respective disk potencies included:

- **Penicillins:** Penicillin G (6 µg)
- **β-lactam/β-lactamase inhibitor combinations:** Piperacillin-Tazobactam (100/10 µg)
- **Cephalosporins:** Ceftazidime (30 µg), Cefoxitin (30 µg)
- **Monobactams:** Aztreonam (30 µg)
- **Carbapenems:** Imipenem (10 µg)
- **Aminoglycosides:** Amikacin (30 µg), Tobramycin (30 µg)
- **Fluoroquinolones:** Norfloxacin (10 µg)
- **Polymyxins:** Colistin (10 µg), Polymyxin-B Sulphate (300 Units)
- **Nitrofurans:** Nitrofurantoin (300 µg; tested exclusively on urinary isolates).

Following the application of the disks, the plates were incubated aerobically at 37°C for 16 to 18 hours. The diameters of the resulting zones of growth inhibition were measured using a calibrated precision caliper[24]. The measured zones were categorically interpreted as Susceptible (S), Intermediate (I), or Resistant (R) according to the established CLSI clinical breakpoints. For the purpose of aggregated statistical analysis and to reflect clinical reality, isolates categorized as 'Intermediate' were grouped with 'Resistant' isolates under the overarching classification of "Non-susceptible." To ensure the precision and reliability of the disk diffusion assays, *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 were utilized concurrently as standardized quality control strains for every batch of testing[25,26].

Phenotypic Detection of β-Lactamases

All 44 confirmed *P. aeruginosa* isolates underwent a rigorous, sequential protocol of phenotypic screening and specific inhibitor-based confirmatory testing to delineate the production of ESBL, AmpC, and MBL enzymes.

1. Detection of Extended-Spectrum β-Lactamases (ESBL)

Screening: Isolates were initially screened for potential ESBL production based on reduced in vitro susceptibility to third-generation cephalosporins. Following CLSI recommendations, isolates exhibiting an inhibition zone diameter of ≤ 22mm for Ceftazidime (30 µg) or ≤ 27 mm for Cefotaxime (30 µg) were flagged as presumptive ESBL producers.

Phenotypic Confirmation: Presumptive isolates were subjected to the Combination Disk Diffusion Test (CDDT). A standard Ceftazidime disk (30 µg) and a combination disk containing Ceftazidime supplemented with Clavulanic acid

(30/10 µg) were placed at a center-to-center distance of 25 mm on a MHA plate previously inoculated with the standardized test organism suspension (0.5 McFarland). Following an overnight incubation at 37°C, the zones of inhibition were compared. An absolute increase in the inhibition zone diameter of ≥ 5 mm around the Ceftazidime-Clavulanic acid combination disk, relative to the zone around the unsupplemented Ceftazidime disk, was interpreted as definitive phenotypic confirmation of ESBL production[27].

2. Detection of AmpC β-Lactamases

Screening: The production of AmpC cephalosporinases was initially suspected in isolates demonstrating reduced susceptibility to cephamycins. Specifically, an inhibition zone size of ≤ 18 mm around a Cefoxitin (30 µg) disk served as the primary screening trigger.

Phenotypic Confirmation: Because AmpC enzymes resist inhibition by clavulanic acid, standard ESBL confirmatory tests are inadequate. Therefore, the Cefoxitin-Cloxacillin Double Disk Synergy Test (CC-DDST) was employed, leveraging the specific inhibitory capability of cloxacillin against AmpC enzymes. A standard Cefoxitin disk (30 µg) and a customized disk containing Cefoxitin supplemented with Cloxacillin (30 µg / 200 µg) were placed 25 mm apart on an inoculated MHA plate. Following 16–18 hours of incubation at 37°C, an expansion of the inhibition zone diameter by ≥ 5 mm around the Cefoxitin-Cloxacillin disk, compared to the zone around the Cefoxitin disk alone, was recorded as a confirmed positive result for AmpC β-lactamase production[28].

3. Detection of Metallo-β-Lactamases (MBL)

Screening: Isolates exhibiting diminished susceptibility to carbapenems, indicated by an inhibition zone diameter of ≤ 19 mm around an Imipenem (10 µg) disk, were identified as potential MBL producers. However, to comprehensively capture covert or "hidden" MBL production, confirmatory testing was systematically applied to the entire cohort.

Phenotypic Confirmation: The Imipenem-EDTA Combined Disk Test (IECDT) was utilized to detect the specific zinc-dependent hydrolytic activity of MBLs. Two Imipenem (10 µg) disks were placed at a distance of 25 mm (center-to-center) on the inoculated MHA plate. To one of the Imipenem disks, 10 µL of a specifically prepared 0.5 M anhydrous EDTA solution (adjusted to pH 8.0 using NaOH, resulting in a final disk concentration of 750 µg EDTA) was meticulously added. After 16 to 18 hours of incubation at 37°C, an absolute increase in the inhibition zone diameter of ≥ 7 mm around the Imipenem-EDTA combination disk, in comparison to the unsupplemented Imipenem disk, was interpreted as definitive confirmation of MBL production[28].

Statistical Analysis

The collected clinical, demographic, and microbiological data were systematically digitized and analyzed. Given the explicit observational and descriptive architecture of the study, continuous variables were summarized using central tendencies, while categorical and nominal data—encompassing patient demographics, prevalence rates of specific β-lactamases, co-production frequencies, and antimicrobial resistance profiles—were expressed as absolute frequencies and corresponding percentages.

RESULTS

Demographic and Clinical Correlates

Over the designated seven-month study period, a total of 100 non-duplicate, consecutive *Pseudomonas* species isolates were successfully recovered from various clinical specimens. Through exhaustive biochemical profiling, 44 isolates (44% of the total *Pseudomonas* yield) were definitively identified as *Pseudomonas aeruginosa* and formed the analytical core of this study.

Demographic analysis of the patient cohort harboring the *P. aeruginosa* isolates revealed a pronounced male preponderance. Males accounted for 66% (n=29) of the cases, whereas females constituted 34% (n=15), resulting in a male-to-female ratio of approximately 1.9:1. The age distribution indicated that the burden of infection was disproportionately borne by the elderly population. The highest isolation rate occurred in patients aged >60 years, representing 34% (n=15) of the cohort. This was closely followed by the 41–60 years age group at 32% (n=14). Pediatric and young adult patients aged ≤ 20 years accounted for 23% (n=10) of the isolates, while the 21–40 years demographic exhibited the lowest isolation frequency at 11% (n=5).

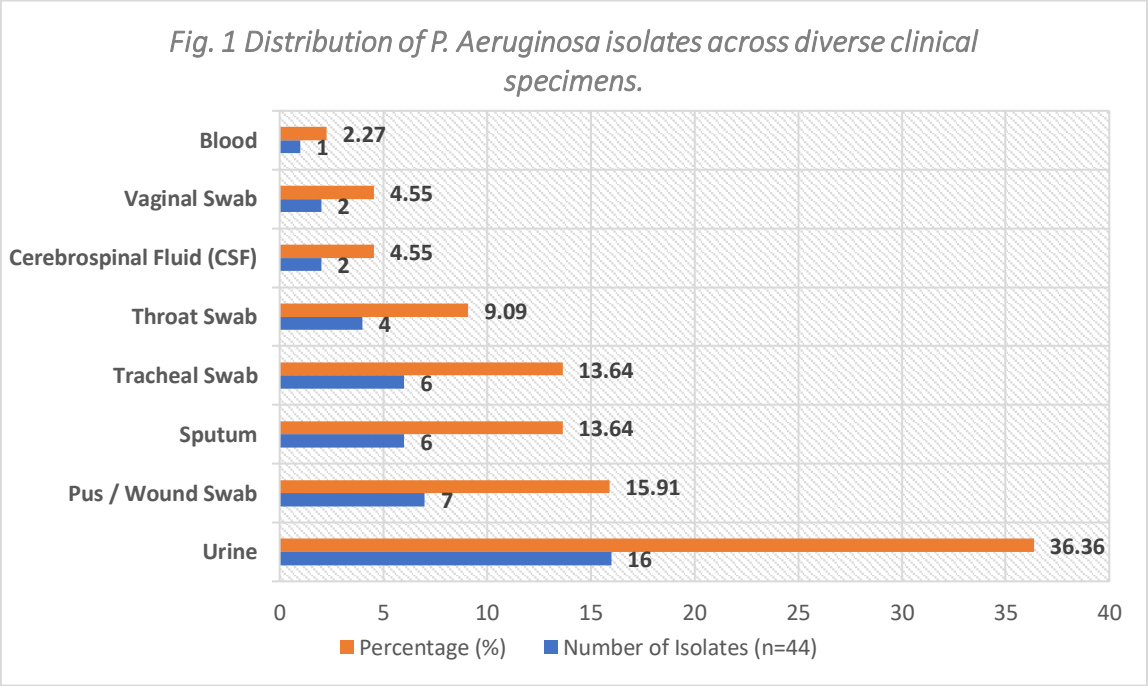
The overwhelming majority of the *P. aeruginosa* isolates were recovered from patients admitted to the Inpatient Department (IPD), accounting for 90.9% (n=40) of the total yield. In stark contrast, only 9.1% (n=4) of the isolates were sourced from patients attending the Outpatient Department (OPD). This distribution heavily underscores the established role of *P. aeruginosa* as a primary agent of nosocomial, rather than community-acquired, infections.

Distribution Across Clinical Specimens

The distribution of the 44 *P. aeruginosa* isolates across various clinical specimen types highlighted the pathogen's predilection for the urinary tract and suppurative wound environments (**Table 1**). Urine specimens constituted the largest

single source, yielding 36.36% (n=16) of the isolates. Purulent exudates and wound swabs accounted for 15.91% (n=7). Infections of the respiratory tract were also prominent; expectorated sputum and tracheal aspirates each contributed 13.64% (n=6) of the isolates, representing a combined respiratory burden of 27.28%. Lower isolation frequencies were observed in throat swabs (9.09%), cerebrospinal fluid (4.55%), vaginal swabs (4.55%), and blood cultures (2.27%).

Table 1: Distribution of P. aeruginosa isolates across diverse clinical specimens.		
Clinical Specimen Category	**Number of Isolates (n=44)**	**Percentage (%)**
Urine	16	36.36
Pus / Wound Swab	7	15.91
Sputum	6	13.64
Tracheal Swab	6	13.64
Throat Swab	4	9.09
Cerebrospinal Fluid (CSF)	2	4.55
Vaginal Swab	2	4.55
Blood	1	2.27



Analysis of Clinical Risk Factors

A detailed evaluation of the predisposing clinical risk factors demonstrated that invasive medical instrumentation and compromised physiological states were the primary drivers of *P. aeruginosa* acquisition. The presence of an indwelling urinary catheter was the most significantly associated risk factor, identified in 36.36% (n=16) of the patients. Admission to the Intensive Care Unit (ICU) correlated with 20.45% (n=9) of the infections. Furthermore, patients subjected to endotracheal intubation and mechanical ventilation accounted for 13.64% (n=6) of the isolates. Advanced age, recognized both as an isolated risk factor and in conjunction with other clinical vulnerabilities, was a major determinant, implicated in 36.36% (n=16) of the total cohort. Immunosuppression (6.82%), recent surgical interventions (4.55%), and severe thermal burns (4.55%) constituted the remaining prominent risk categories.

Prevalence and Co-Production of β-Lactamases

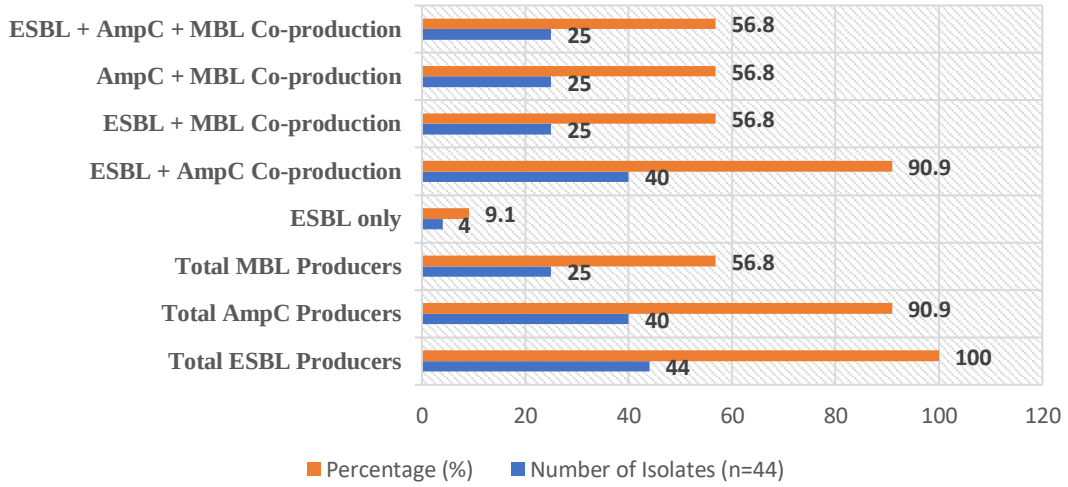
Phenotypic confirmatory testing uncovered an alarming state of β-lactamase hyper-endemicity within the study hospital. As detailed in **Table 2**, an absolute penetrance of ESBL production was observed; 100% (n=44) of the *P. aeruginosa* clinical isolates were confirmed as ESBL producers utilizing the CDDT method. Furthermore, the specific CC-DDST protocol confirmed the production of AmpC β-lactamases in an exceptional 90.9% (n=40) of the isolates. The production of Metallo-β-Lactamases (MBLs), confirmed through the IECDT utilizing EDTA chelation, was detected in a staggering 56.8% (n=25) of the cohort.

The dynamics of enzyme co-production presented a formidable resistance architecture. Not a single isolate in the entire study cohort produced AmpC or MBL in strict isolation without concurrent ESBL activity. The co-production of ESBL and AmpC was ubiquitous among the AmpC-positive strains, representing 90.9% (n=40) of all isolates. Most critically, more than half of the *P. aeruginosa* cohort—56.8% (n=25)—demonstrated the simultaneous co-production of all three major

enzyme classes (ESBL + AmpC + MBL) . Only a marginal fraction of the cohort, 9.1% (n=4), produced ESBL alone in the absence of accompanying AmpC or MBL enzymes.

Table 2: Phenotypic prevalence and complex co-production patterns of ESBL, AmpC, and MBL enzymes among P. aeruginosa isolates.		
β-Lactamase Phenotype	**Number of Isolates (n=44)**	**Percentage (%)**
Individual Prevalence		
Total ESBL Producers	44	100.0
Total AmpC Producers	40	90.9
Total MBL Producers	25	56.8
Co-production Patterns		
ESBL only	4	9.1
ESBL + AmpC Co-production	40	90.9
ESBL + MBL Co-production	25	56.8
AmpC + MBL Co-production	25	56.8
ESBL + AmpC + MBL Co-production	25	56.8

Fig. 2 Phenotypic prevalence and complex co-production patterns of ESBL, AmpC, and MBL enzymes among P. aeruginosa isolates.



Antimicrobial Susceptibility and Resistance Profiling

The comprehensive in vitro antibiogram of the 44 *P. aeruginosa* isolates revealed severe restrictions in the therapeutic armamentarium, directly mirroring the profound enzymatic resistance mechanisms present (**Table 3**).

The cephalosporin class exhibited near-total failure. Resistance to Cefoxitin (a cephamycin utilized to screen for AmpC activity) reached 95.45%, and resistance to Ceftazidime (a third-generation anti-pseudomonal cephalosporin) stood at an alarming 88.64%. Resistance to Penicillin was similarly high at 72.73%.

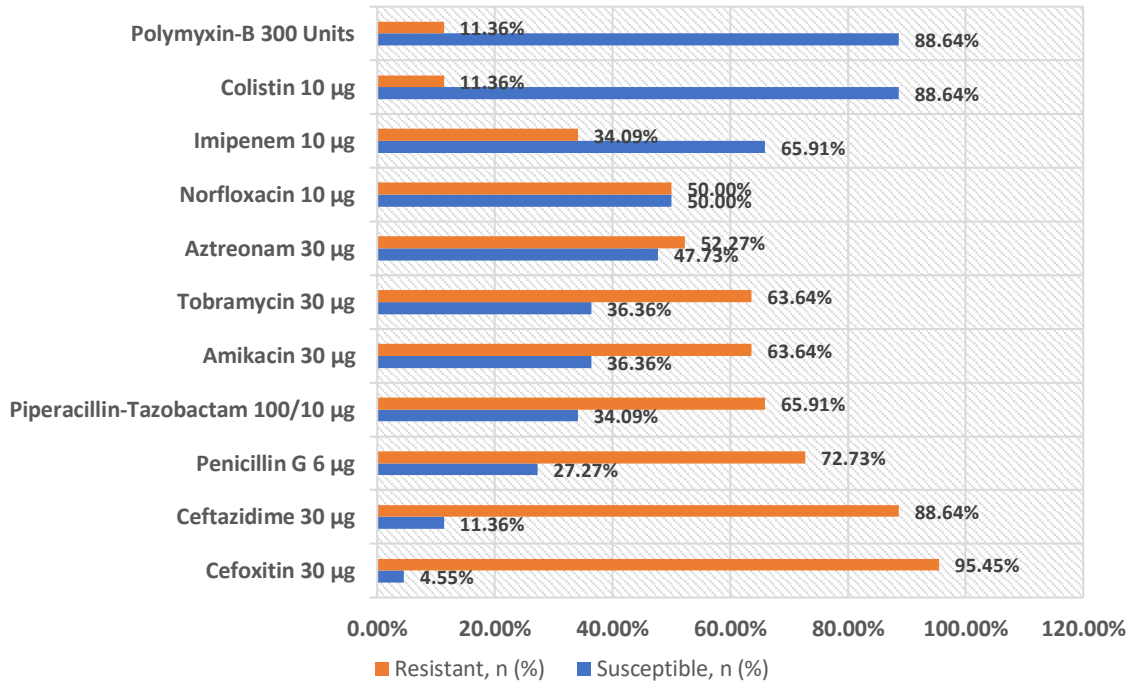
Crucially, Piperacillin-Tazobactam, a combination frequently deployed as a first-line empirical agent for suspected pseudomonal infections, demonstrated a severely compromised efficacy, recording a resistance rate of 65.91%. The aminoglycoside antibiotics afforded limited utility; both Amikacin and Tobramycin registered identical resistance rates of 63.64%. Aztreonam, a monobactam generally stable against MBL hydrolysis but vulnerable to ESBLs and AmpC, exhibited a 52.27% resistance rate. The fluoroquinolone representative, Norfloxacin, showed a 50% resistance rate.

Within the carbapenem class, overall in vitro resistance to Imipenem was recorded at 34.09% (n=15). However, the polypeptide antibiotics—Colistin and Polymyxin-B Sulphate—emerged as the most robust therapeutic options, retaining an 88.64% susceptibility rate across the cohort (with 11.36% expressing resistance) . Nitrofurantoin, evaluated exclusively on the 16 urinary isolates, yielded an absolute resistance rate of 100% within that specific subset.

Table 3: Aggregate antimicrobial susceptibility and resistance profiles of the 44 P. aeruginosa clinical isolates. (Nitrofurantoin excluded from the general matrix as it was restricted to urinary isolates, demonstrating 16/16 resistance).

Antimicrobial Agent/Disk Potency	Susceptible, n (%)	Resistant, n (%)
Cefoxitin 30 µg	4.55%	95.45%
Ceftazidime 30 µg	11.36%	88.64%
Penicillin G 6 µg	27.27%	72.73%
Piperacillin-Tazobactam 100/10 µg	34.09%	65.91%
Amikacin 30 µg	36.36%	63.64%
Tobramycin 30 µg	36.36%	63.64%
Aztreonam 30 µg	47.73%	52.27%
Norfloxacin 10 µg	50.00%	50.00%
Imipenem 10 µg	65.91%	34.09%
Colistin 10 µg	88.64%	11.36%
Polymyxin-B 300 Units	88.64%	11.36%

Fig. 3 Antimicrobial susceptibility and resistance profiles of the 44 P. Aeruginosa clinical isolates.



Analysis of the "Hidden MBL" Phenomenon

A highly critical observation emerged upon cross-tabulating phenotypic enzyme production with the standard unsupplemented antibiogram results, specifically concerning carbapenem efficacy. Among the 25 isolates definitively confirmed to produce MBLs via the EDTA synergy test, routine disk diffusion testing utilizing Imipenem (10 µg) categorized only 11 isolates (44%) as overtly resistant. Astonishingly, the remaining 14 isolates (56%) of the confirmed MBL-producing cohort appeared fully susceptible to Imipenem in standard in vitro testing.

Furthermore, an inverse observation was noted: while the total number of Imipenem-resistant isolates in the entire study was 15, only 11 of these were MBL producers. This indicates that 4 isolates expressed Imipenem resistance through alternative, non-MBL mechanisms, likely involving the hyper-expression of AmpC coupled with the mutational loss of the OprD outer membrane porin. The high prevalence of MBL-harboring isolates that failed to express phenotypic carbapenem resistance in routine assays underscores a profound diagnostic vulnerability characterized by "hidden" or covert MBL expression.

DISCUSSION

The present study highlights a high prevalence of β-lactamase-mediated resistance among clinical isolates of *Pseudomonas aeruginosa* in a tertiary care hospital setting. The near-universal production of ESBLs, along with a high frequency of AmpC and MBL co-production, reflects the growing burden of multidrug-resistant (MDR) *P. aeruginosa* and emphasizes the challenges associated with diagnosis and antimicrobial therapy[29,30].

A marked male predominance (66%) and higher isolation rates among elderly patients (>60 years) were observed in the present study, findings comparable to earlier studies by Anupurba et al. and Senthamarai et al. The majority of isolates were recovered from inpatients (90.9%), particularly from patients with urinary catheterization, ICU admission, and mechanical ventilation, supporting the established role of *P. aeruginosa* as a major nosocomial pathogen associated with invasive procedures and prolonged hospitalization[31]. Biofilm formation on indwelling medical devices may further contribute to persistence and antimicrobial resistance[32].

The prevalence of ESBL production in the present study was 100%, while AmpC and MBL production were detected in 90.9% and 56.8% of isolates, respectively[33]. The high rate of ESBL and AmpC co-production significantly limits the effectiveness of cephalosporins and β-lactam/β-lactamase inhibitor combinations. Similar trends have been reported in previous Indian studies, although the prevalence observed in the current study is comparatively higher. The predominance of isolates from hospitalized patients with prior antimicrobial exposure may have contributed to this elevated resistance pattern[34].

The coexistence of multiple β-lactamases presents important diagnostic challenges. Hyperproduction of AmpC enzymes may mask ESBL detection in routine phenotypic assays, leading to false-negative results. In this study, the Cefoxitin-Cloxacillin Double Disk Synergy Test (CC-DDST) proved useful for identifying AmpC production and unmasking underlying resistance mechanisms[35]. These findings support the routine use of specific inhibitor-based phenotypic methods in clinical microbiology laboratories, particularly in resource-limited settings where molecular techniques are not routinely available.

A notable finding of the study was the high prevalence of MBL-producing isolates that appeared susceptible to Imipenem on routine disk diffusion testing[36]. Among the 25 confirmed MBL producers, 14 isolates (56%) demonstrated apparent in vitro susceptibility to Imipenem, indicating the presence of "hidden" MBLs. This finding has significant clinical implications, as reliance on routine susceptibility testing alone may lead to inappropriate carbapenem therapy and subsequent treatment failure. Therefore, confirmatory phenotypic testing using EDTA-based methods should be considered essential for accurate detection of MBL production[37].

Antimicrobial susceptibility testing revealed high resistance rates to Cefoxitin, Ceftazidime, Piperacillin-Tazobactam, aminoglycosides, and fluoroquinolones, indicating limited therapeutic options for MDR *P. aeruginosa* infections. Colistin and Polymyxin-B retained the highest activity, with 88.64% susceptibility. However, the emergence of resistance even to polymyxins is concerning because these agents are often considered last-resort therapies for severe MDR infections[38,39].
The findings of this study emphasize the urgent need for strengthened antimicrobial stewardship, rational antibiotic use, and strict infection control practices. Routine phenotypic screening for ESBL, AmpC, and MBL production should be integrated into diagnostic protocols to improve therapeutic decision-making and reduce the spread of multidrug-resistant strains. Further molecular studies with larger sample sizes are recommended to characterize the underlying resistance genes and monitor evolving resistance trends.

Limitations of the Study
While providing vital, highly actionable phenotypic mapping of a regional resistance crisis, this study acknowledges certain methodological limitations. The reliance on phenotypic detection methods, although highly cost-effective and clinically relevant for resource-constrained settings, cannot delineate the exact molecular epidemiology (e.g., differentiating between *bla*CTX-M, *bla*NDM, *bla*VIM, or specific *ampC* alleles). Furthermore, the sample size of 44 confirmed *P. aeruginosa* isolates, sourced from a single geographic tertiary center over seven months, may limit the broad statistical generalizability of the exact prevalence percentages to other regions across the subcontinent. Nevertheless, the identified mechanisms, the alarming rates of co-production, and the critical discovery of hidden MBLs firmly align with, and starkly illuminate, broader global resistance trajectories, offering indispensable insights for immediate clinical application.

CONCLUSION
The clinical landscape of *Pseudomonas aeruginosa* infections at this tertiary care center is defined by extreme antimicrobial resistance, driven by the hyper-endemic co-production of ESBL, AmpC, and MBL enzymes. The simultaneous expression of all three major β-lactamase classes in over half the patient cohort severely incapacitates standard empirical β-lactam therapy, crucially including the widely utilized Piperacillin-Tazobactam combination. Most alarmingly, the identification of a significant cohort of "hidden MBL" producers—strains that harbor destructive metalloenzymes yet appear deceptively susceptible to carbapenems in standard disk diffusion assays—exposes a critical diagnostic vulnerability that can precipitate catastrophic clinical failure if unrecognized.

To avert the widespread emergence of untreatable, pan-drug resistant phenotypes, clinical microbiology laboratories must transcend basic primary susceptibility testing. It is imperative to institutionalize routine, specific phenotypic inhibitor-based screening protocols (such as Cloxacillin and EDTA synergy assays) to reliably unmask hidden resistance. Concurrently, aggressive institutional antimicrobial stewardship, stringent infection control measures targeting device-

associated biofilms, and the highly prudent, restricted preservation of last-resort polymyxins are non-negotiable mandates for contemporary clinical practice and public health survival.

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