Original Article

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Microbiology Section

# Unveiling the Weapons of Superbug *Pseudomonas*: A Cross-sectional Observational Study on Virulence Factors and Antimicrobial Resistance of a Lurking Pathogen

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## ABSTRACT

**Introduction:** *Pseudomonas* is an opportunistic pathogen that thrives in a wide range of environments and poses a severe threat. Known for acquiring additional resistance mechanisms, it has become a formidable challenge in healthcare. Understanding its weapons and defenses is key to unveiling its strategies and identifying vulnerabilities to disarm this dangerous pathogen.

**Aim:** To characterise clinical isolates of *Pseudomonas* species in terms of speciation, expression of key virulence factors, and antimicrobial resistance profiles.

**Materials and Methods:** This cross-sectional observational study was conducted at Indira Gandhi Government Medical College and Mayo Hospital, Nagpur Maharashtra, India, from January 2020 to December 2020. A total of 500 *Pseudomonas* isolates were tested for speciation, virulence factors, and antibiotic susceptibility using standard laboratory tests. The biofilm detection methods were analysed using Chi-square test and Antibiotic Susceptibility Testing (AST) was done using Z test. A p-value of <0.05 was considered statistically significant.

**Results:** Out of 500 *Pseudomonas* isolates, a maximum of 282 (56.4%) were from pus, while fewer than 12 (2.4%) were from other specimens. A total of 416 (83.2%) samples were pigment producers. Among these, 392 (84.12%) *P. aeruginosa* were identified as pigment producers. *P. putida* and *P. fluorescens* were also pigment producers, while *P. stutzeri* did not produce any pigment. The Tissue Culture Plate (TCP) method was more quantitative, with 244 (52.36%) being a reliable method for detecting biofilm. Out of 500 *Pseudomonas* isolates, 332 (66.4%) were resistant to Ceftazidime; 200 (40%) were identified as Extended-Spectrum Beta-Lactamase (ESBL) producers; 104 (20.8%) were resistant to imipenem; and 58 (11.6%) were classified as MBL. Out of 218 (43.6%) AmpC producers, 94 (18.8%) were inducible, while the remaining 124 (24.8%) were non inducible. Co-expression of beta-lactamases showing AmpC and ESBL was found in 58 (11.6%) isolates of *Pseudomonas*.

**Conclusion:** *Pseudomonas*, armed with a wide array of virulence factors and resistance mechanisms, can evade host defenses and resist antimicrobial treatments, presenting significant challenges in healthcare settings. Efforts to curb this superbug include implementing infection prevention practices and using novel antibiotics and inhibitors targeting biofilm formation, which are vital to staying ahead of this adaptable adversary.

**Keywords:** Antibiotic resistance, Biofilm, Quorum sensing

## INTRODUCTION

*Pseudomonas* is a gram-negative bacillus that belongs to the family Pseudomonadaceae [1]. The genus *Pseudomonas* contains more than 140 species; of these, 25 species are associated with humans, including *P. aeruginosa*, *P. fluorescens*, *P. putida*, *P. cepacia*, *P. stutzeri*, *P. maltophilia*, and *P. putrefaciens* [2]. *Pseudomonas* presents a serious therapeutic challenge for the treatment of both community-acquired and nosocomial infections. *Pseudomonas* species are both invasive and toxigenic. According to Pollack (2000), the three stages of infection are: 1) bacterial attachment and colonisation; 2) local infection; and 3) bloodstream dissemination and systemic disease.

The importance of colonisation and adherence is most evident when studied in the context of respiratory tract infections in patients with cystic fibrosis and in those complicated by mechanical ventilation. The production of extracellular proteases adds to the organism's virulence by assisting in bacterial adherence and invasion [3]. Infections caused by *P. aeruginosa* are often difficult to treat due to its virulence, intrinsic and acquired antibiotic resistance, and a relatively limited choice of effective antimicrobial agents [4]. Pyocyanin is detectable in large quantities in the sputum of patients with cystic fibrosis [5] and in ear secretions of chronic otitis media caused by *P. aeruginosa* [6]. Biofilms have been reported to contribute to the pathogenicity of *P. aeruginosa*, leading to persistent and recurrent infections [7].

The selection of the appropriate antibiotic to initiate therapy is essential for optimising clinical outcomes [8]. Unfortunately, choosing the most appropriate antibiotic is complicated by *P. aeruginosa*'s ability to develop resistance to multiple classes of antibacterial agents, even during the course of treating an infection. This problem is exacerbated by the development of resistance during therapy, a complication that has been shown to double the length of hospitalisation and the overall cost of patient care [9]. Antibiotic resistance has been recognised as a global health issue. *Pseudomonas aeruginosa* is one of the most challenging organisms involved in a variety of infections. The World Health Organisation (WHO) published a list of highly antibiotic-resistant bacteria that are in need of priority for research and development of new antibiotics. This list was divided into three levels, with the most critical being carbapenem-resistant *Pseudomonas aeruginosa* [10].

*Pseudomonas* infections are common in immunocompromised patients with diabetes. This is particularly important in countries like India, which has become the diabetic capital of the world in the last few decades. Therefore, it is essential to study this microorganism, especially in clinical settings like tertiary care hospitals. With this background, this study presents an integrated approach in characterising clinical *Pseudomonas* isolates by concurrently evaluating species distribution, key virulence factors, and a comprehensive profile of beta-lactamase-mediated resistance, including the co-production of ESBL and AmpC enzymes. The use of a quantitative biofilm detection method and the generation of region-specific data from a tertiary care centre in Central India provide valuable insights for infection control and antimicrobial stewardship strategies.

The aim of the study was to characterise clinical isolates of *Pseudomonas* species by assessing their virulence factors and antibiotic resistance patterns, with a special focus on beta-lactamase-mediated resistance and the co-expression of ESBL and AmpC enzymes.

**Primary objectives:**

1. To isolate and identify *Pseudomonas* species from various clinical specimens and classify them at the species level.
2. To assess the presence of virulence factors in *Pseudomonas* isolates, including pigment production (pyoverdine, pyocyanin, pyrorubin) and biofilm-forming ability.
3. To evaluate the antimicrobial susceptibility profile of *Pseudomonas* isolates, with particular emphasis on resistance to ceftazidime and imipenem, and to detect beta-lactamase-mediated resistance mechanisms such as ESBL, AmpC, and Metallo-Beta-Lactamase (MBL) production.

**Secondary objectives:** To determine the prevalence of co-expression of multiple beta-lactamases, particularly ESBL and AmpC enzymes, among the *Pseudomonas* isolates.

## MATERIALS AND METHODS

This cross-sectional observational study was conducted in the Department of Microbiology at Indira Gandhi Government Medical College and Mayo Hospital, Nagpur Maharashtra, India, over a period of one year, from January 2020 to December 2020. The study was approved by the Institutional Ethics Committee (IEC) of Indira Gandhi Government Medical College and Mayo Hospital, Nagpur (IEC Approval Number: IGGMC/Pharm/IEC/199/2018). Informed consent was deemed not applicable, as the study involved the analysis of de-identified, routine clinical microbiology laboratory isolates.

**Sample size calculation:** The required sample size was calculated using the formula [11].

$$N = \frac{Z^2 \times p \times (1-p)}{d^2}$$

where, N is the required sample size,

Z is the standard normal variates at 95% confidence level (1.96), prevalence (43.6 % i.e., 44% prevalence based on previous study conducted in 2018 at Nagpur, Maharashtra - [12].

and d is the allowable margin of error (0.05).

Accordingly,

$$N = \frac{(1.96)^2 \times 0.44 \times 0.53}{(0.05)^2} = 358$$

A post-hoc sample size estimation was performed to validate the adequacy of the included sample (N=500).

**Inclusion criteria:** Pure isolates of *Pseudomonas* spp. obtained from various clinical specimens, such as pus, sputum, urine, wound swab, Bronchoalveolar Lavage (BAL), Endotracheal Tube (ET) aspirates, and ear swabs received from Outpatient Departments (OPD), hospital wards, and Intensive Care Units (ICUs) were included in the study.

**Exclusion criteria:** Duplicate isolates from the same patient or sample site, mixed cultures where *Pseudomonas* was not the predominant pathogen, isolates with incomplete laboratory records or insufficient sample volume for complete analysis were excluded from the study.

### Study Procedure

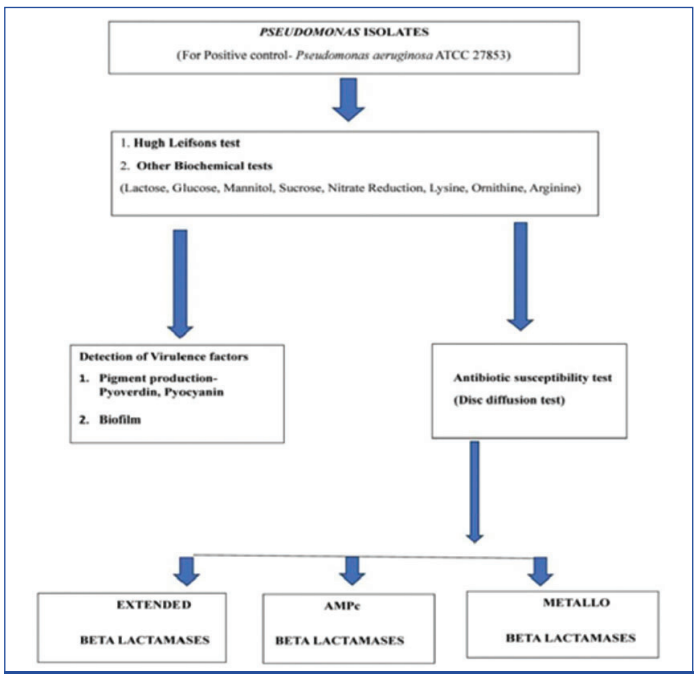
Isolates were identified as *Pseudomonas* based on standard microbiological techniques, including gram staining, colony morphology, and a series of conventional biochemical tests (e.g., oxidase, catalase, citrate utilisation, nitrate reduction, motility). Speciation was conducted using phenotypic and biochemical characteristics.

The study parameters included the evaluation of species distribution and key virulence factors such as pigment production, gelatinase production, and biofilm formation. Biofilm production was assessed quantitatively using the TCP method. Antimicrobial susceptibility testing was performed using the Kirby-Bauer disk diffusion method on Mueller-Hinton agar and interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines [13].

Further characterisation included the detection of acquired resistance mechanisms such as:

- ESBL production
- AmpC beta-lactamase (both inducible and non-inducible types)
- MBL production
- Co-expression of ESBL and AmpC enzymes.

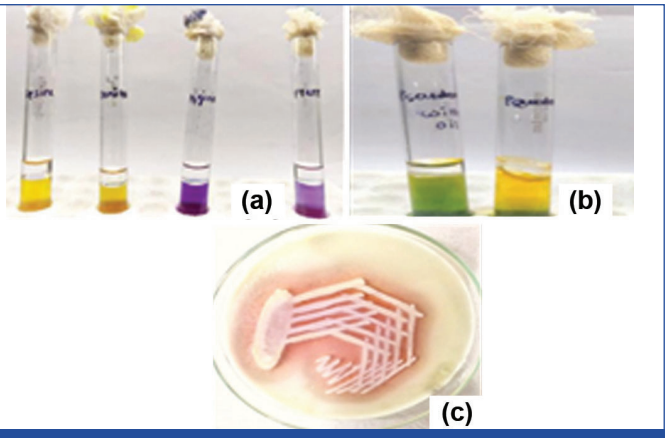
The protocol used to carry out the study is summarised in [Table/Fig-1]. The isolate tested positive for arginine dihydrolase [14], oxidative fermentation (Hugh-Leifson's test) [15], and gelatin hydrolysis [15], as indicated in [Table/Fig-2a-c]. The speciation results of the *Pseudomonas* isolates are shown in [Table/Fig-3] according to their biochemical traits.



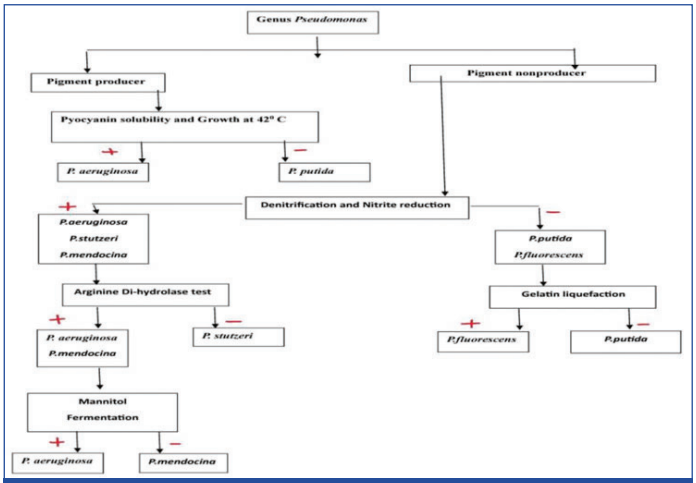

**[Table/Fig-1]:** Schematic representation of the study procedure.

### Study of Virulence Factors of *Pseudomonas* Spp.

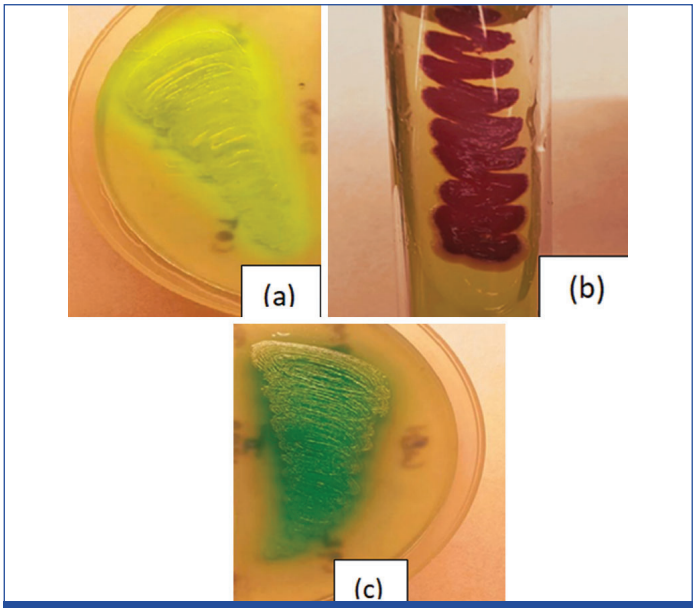
1. **Pyoverdin Pigment Production [15]:** Pyoverdin is a water-soluble pigment that is a characteristic feature of the fluorescent group. Peptone water and nutrient agar were inoculated with a colony of *Pseudomonas* isolates, and pigment production was observed. The *Pseudomonas* species produced pigments, as shown in [Table/Fig-4].
2. **Pyocyanin Solubility in Chloroform [15]:** To an overnight broth culture of the strain in peptone water, five drops of chloroform were added. After shaking the tube vigorously, it was allowed




**[Table/Fig-2]:** Showing biochemical tests a) Arginine Di-hydrolase test found positive [13]; b) Oxidative fermentation (Hugh Leifsons Test) [14]; c) Positive gelatin hydrolysis test [15].




**[Table/Fig-3]:** Speciation of genus *pseudomonas*.

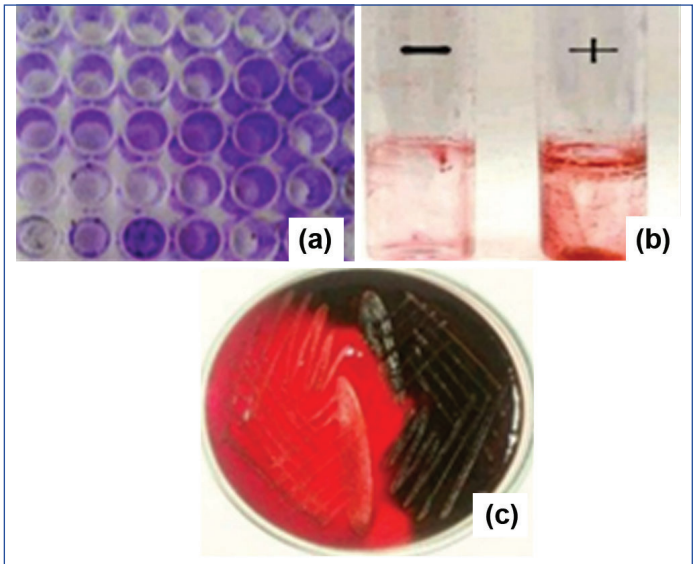



**[Table/Fig-4]:** Nutrient agar showing pigment production a) Pyoverdin; b) Pyocyanin; c) Pyorubin.

to stand to separate the broth and chloroform phases. After separation, the presence of pyocyanin pigment was indicated by a blue color in the chloroform phase at the bottom of the tube. The results of the isolated *Pseudomonas* species' pyocyanin pigment production are shown in [Table/Fig-5].

3. **Biofilm detection:** All of the *Pseudomonas* isolates were tested for biofilm production by TCP [16], Tube Method (TM) [15], and Congo Red Agar (CRA) [17]. The method is demonstrated as in [Table/Fig-6a-c] for biofilm production by TCP, Tube Method, and Congo Red Method, respectively.

**[Table/Fig-5]:** Pyocyanin pigment production [15].




**[Table/Fig-6]:** Biofilm production a) Tissue Culture Plate (TCP) [16]; b) Tube method [15]; c) Congo Red Method [17].

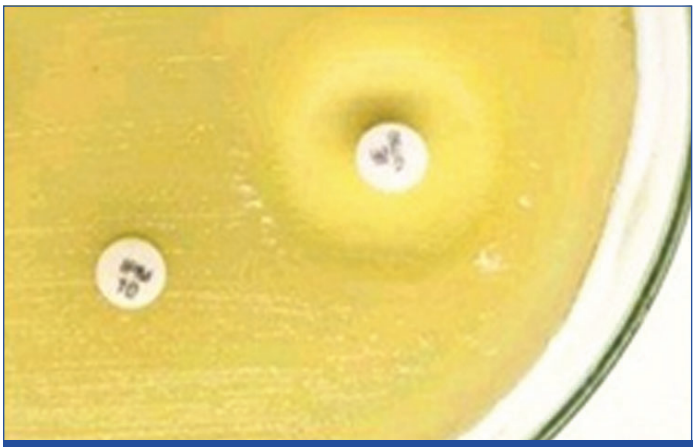
## Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed on *Pseudomonas* spp. using the Kirby-Bauer disk diffusion method and interpreted according to CLSI guidelines 2020 [13]. Testing was validated with the reference strain *Pseudomonas aeruginosa* ATCC 27853.

1. **Testing for ESBL production:** This was conducted using the combined disc diffusion test as shown in [Table/Fig-7] [18]. In [Table/Fig-7], isolates showing a zone diameter of Ceftazidime-Clavulanic acid (CAC) that was greater by 5 mm than the zone diameter of the Ceftazidime disk (CAZ) were indicated as ESBL positive [17].

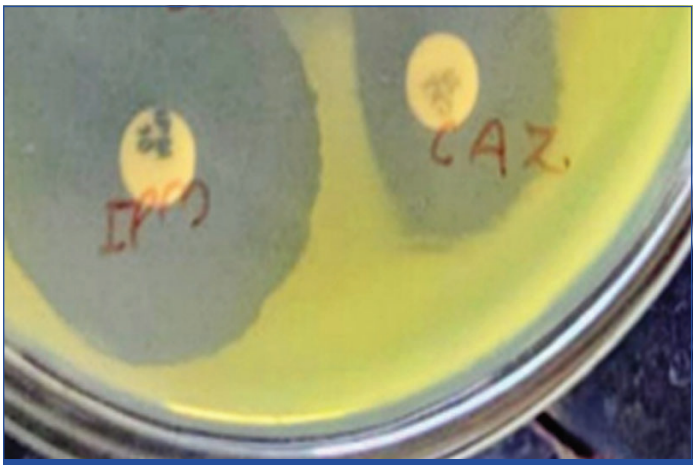
**[Table/Fig-7]** Isolate showing zone diameter of Ceftazidime-Clavulanic acid (CAC) was more by 5mm in zone diameter of Ceftazidime disk (CAZ) indicated, ESBL positive [18].

2. **Metallo-beta-Lactamase (MBLs) production:** MBL production was assessed by the disc potentiation test as shown in [Table/Fig-8] [19].



**[Table/Fig-8]:** Isolate showing zone diameter of Imipenem- EDTA was more by 5mm in zone diameter of Imipenem disk, indicated MBL positive [19].

3. **Testing of AmpC Beta-Lactamases:** This was detected by the ceftazidime-imipenem antagonism test as shown in [Table/Fig-9] [20].




**[Table/Fig-9]:** Isolate showing blunting of Ceftazidime (30ug) zone of inhibition adjacent to Imipenem disk (10ug), indicated inducible Amp C positive [20].

## STATISTICAL ANALYSIS

These findings were recorded using Microsoft Excel 2010. Statistical analysis was performed using SPSS (version 20.0) for Windows (SPSS Science, Chicago, IL, USA). The biofilm detection methods were analysed using the Chi-square test, and antibiotic susceptibility patterns were evaluated using the Z test, p-values ≤0.05 considered statistically significant.

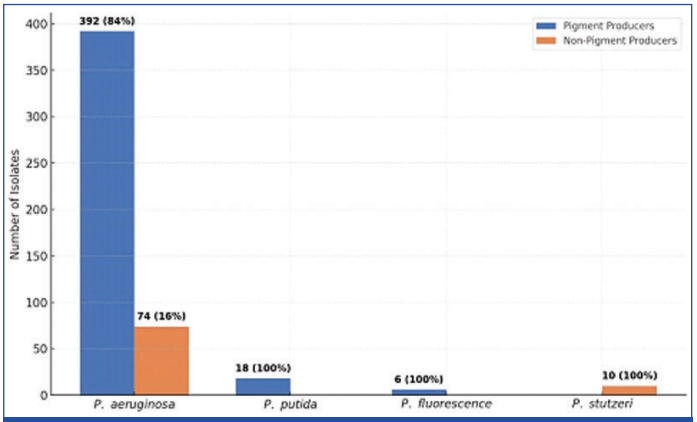
## RESULTS

The distribution of clinical specimens from which *Pseudomonas* species were isolated is summarised in [Table/Fig-10]. The most commonly isolated species was *Pseudomonas aeruginosa* (466 isolates, 93.2%). The unique phenotypic traits among *Pseudomonas* species are highlighted in [Table/Fig-11], which details the pigment production characteristics of the various species. [Table/Fig-12] shows the evaluation of *Pseudomonas aeruginosa* biofilm formation using various detection techniques (n=466). Accordingly, [Table/Fig-13] displays the production of biofilms in non *aeruginosa Pseudomonas* species separately, illustrating the methodological differences in biofilm detection between species.

The antibiotic susceptibility profile of the isolates, displaying the percentage of resistance and sensitivity to tested antimicrobial agents, is found in [Table/Fig-14]. The antibiotic susceptibility patterns of *P. aeruginosa* isolates are depicted in [Table/Fig-15] (n=466). The antibiotic susceptibility patterns of non aeruginosa

| Specimens (N=500) | *P. fluorescens* | *P. aeruginosa* | *P. putida* | *P. stutzeri* |
|---|---|---|---|---|
| Pus,ear swab, wound swab | 4 (66.66%) | 266 (62.33%) | 8 (44.44%) | 4 (40%) |
| Blood | 2 (33.33%) | 104 (22.31%) | 2 (11.11%) | 4 (40%) |
| ET Aspirate, sputum, pleural fluid | 0 | 52 (11.15%) | 2 (11.11%) | 0 |
| Urine | 0 | 32 (6.87%) | 6 (33.33%) | 2 (20%) |
| Others (drain fluids, corneal scrapping) | 0 | 12 (2.58%) | 0 | 0 |
| Total | 6 (1.2%) | 466 (93.2%) | 18 (3.6%) | 10 (2%) |

**[Table/Fig-10]:** Clinical specimens wise distribution of various species of *Pseudomonas* (N=500).




**[Table/Fig-11]:** Pigment production in various species of *Pseudomonas* (N=500).

| Biofilm detection method | No. of biofilm producers (%) | No. of biofilm non producers (%) | Total | Chi-square value | p-value | Degree of Freedom |
|---|---|---|---|---|---|---|
| Congo Red agar Plate Method (CRP) | 126 (27.04%) | 340 (72.96%) | n=466 | 63.2 | 0.0001 | 2 |
| Tube Method (TM) | 200 (42.92%) | 266 (57.08%) | | | | |
| Tissue Culture Plate method (TCP) | 244 (52.36%) | 222 (47.64%) | | | | |

**[Table/Fig-12]:** Biofilm production in *Pseudomonas aeruginosa* by various methods (n=466).

| Method of detection | Biofilm producers | | |
|---|---|---|---|
| | *P. putida* (n=18) | *P. fluorescens* (n=6) | *P. stutzeri* (n=10) |
| Tissue Culture Plate (TCP) method | 10 (55.56%) | 4 (66.67%) | 6 (60%) |
| Tube method (TM) | 8 (44.44%) | 4 (66.67%) | 6 (60%) |
| Congo Red Method (CRP) | 8 (44.44%) | 2 (33.33%) | 4 (40%) |

**[Table/Fig-13]:** Biofilm production in other *Pseudomonas* isolates by various methods.

isolates are depicted in [Table/Fig-16]. The detection of ESBLs in all *Pseudomonas* isolates (N=500) is shown in [Table/Fig-17]. MBL and AmpC beta-lactamase detection results are described in [Table/Fig-18,19], respectively. The co-expression of various beta-lactamases in *Pseudomonas* isolates is represented in [Table/Fig-20].

## DISCUSSION

*Pseudomonas* poses a significant clinical burden, particularly in immunocompromised and hospitalised patients. *P. aeruginosa* is

| Antibiotic | Resistant | Percentage (%) | Sensitive | Percentage (%) |
|---|---|---|---|---|
| Ceftazidime | 312 | 67% | 154 | 33% |
| Ciprofloxacin | 326 | 70% | 140 | 30% |
| Cefepime | 326 | 70% | 140 | 30% |
| Meropenem | 320 | 69% | 146 | 31% |
| Gentamicin | 316 | 68% | 150 | 32% |
| Pi-Tazobactam | 312 | 67% | 154 | 33% |
| Piperacillin | 302 | 65% | 164 | 35% |
| Tobramycin | 302 | 65% | 164 | 35% |
| Cotrimoxazole | 288 | 62% | 178 | 38% |
| Amikacin | 260 | 56% | 206 | 44% |
| Imipenem | 102 | 22% | 364 | 78% |

**[Table/Fig-14]:** Antibiotic susceptibility profile showing resistance and sensitivity rates of isolates.

| Isolates | Resistant to Imipenem | MBL |
|---|---|---|
| *P. aeruginosa* (n=466) | 102 (21.89%) | 58 (12.45%) |
| *P. putida* (n=18) | 2 (11.11%) | 0 |
| *P. fluorescens* (n=6) | 0 | 0 |
| *P. stutzeri* (n=10) | 0 | 0 |
| Total | 104 (20.8%) | 58 (11.6%) |

**[Table/Fig-18]:** MBL detection in *Pseudomonas* isolates (N=500).

the second most common cause of nosocomial pneumonia (17%), the third most common cause of urinary tract infections (7%), the fourth most common cause of surgical-site infections (8%), and the fifth most common isolate overall (9%) from all sites [14]. It exhibits intrinsic resistance due to its outer membrane and can acquire additional resistance via horizontal gene transfer. Efflux pumps and biofilm formation further enhance antibiotic resistance.

| Antibiotics | Cipro | CPM | MRP | GEN | PIT | PI | TOBRA | COT | AK | IMP |
|---|---|---|---|---|---|---|---|---|---|---|
| Ceftazidime (CAZ) | 2.83 (0.0022) | 2.83 (0.0022) | 3.26 (0.0005) | 3.53 (0.0002) | 3.81 (0.0001) | 4.49 (0.001) | 4.49 (0.0001) | 5.43 (0.0001) | 7.24 (0.0001) | 17.16 (0.0001) |
| Ciprofloxacin (CIPRO) | -- | 0 (0.5) | 0.42 (0.335) | 0.707 (0.239) | 0.986 (0.161) | 1.67 (0.046) | 1.67 (0.046) | 2.62 (0.0043) | 4.47 (0.0001) | 14.72 (0.0001) |
| Cefepime (CPM) | | -- | 0.42 (0.335) | 0.707 (0.239) | 0.986 (0.161) | 1.67 (0.046) | 1.67 (0.046) | 2.62 (0.0043) | 4.47 (0.001) | 14.72 (0.0001) |
| Meropenem (MRP) | | | -- | 0.28 (0.389) | 0.56 (0.287) | 1.25 (0.105) | 1.25 (0.105) | 2.20 (0.013) | 4.05 (0.0001) | 14.34 (0.0001) |
| Gentamicin (GEN) | | | | -- | 0.27 (0.389) | 0.97 (0.165) | 0.97 (0.165) | 1.92 (0.027) | 3.77 (0.0001) | 14.09 (0.0001) |
| Pi-tazobactam (PIT) | | | | | --- | 0.69 (0.244) | 0.69 (0.244) | 1.64 (0.0503) | 3.49 (0.0002) | 13.84 (0.0001) |
| Piperacillin (PI) | | | | | | --- | 0 (0.5) | 0.95 (0.1706) | 2.81 (0.002) | 13.22 (0.0001) |
| Tobramycin (TOBRA) | | | | | | | ---- | 0.95 (0.1706) | 2.81 (0.002) | 13.22 (0.0001) |
| Cotrimoxazole (COT) | | | | | | | | ---- | 1.86 (0.031) | 12.35 (0.0001) |
| Amikacin (AK) | | | | | | | | | ---- | 10.61 (0.0001) |

**[Table/Fig-15]:** Antibiotic susceptibility pattern of the *P. aeruginosa* isolates. (n=466).
(p-value =By using Z-test p-value ≤0.05 considered as statistically significant while p-value ≥0.05 considered as not significant)
1. CAZ: Ceftazidime; CIPRO: Ciprofloxacin; CPM: Cefepime; MRP: Meropenem; GEN: Gentamycin; PIT: Piperacillin-Tazobactam; PI: Piperacillin; TOBRA: Tobramycin; COT: Cotrimoxazole; AK: Amikacin; IMP: Imipenem
2. The values shown below each antibiotic name are Z values, with p-values mentioned in brackets.
3. These values represent the statistical comparison results between resistance rates among different antibiotic

| | *P. putida* (n=18) | | *P. fluorescens* (n=6) | | *P. stutzeri* (n=10) | |
|---|---|---|---|---|---|---|
| Antimicrobial Agents | Sensitive | Resistant | Sensitive | Resistant | Sensitive | Resistant |
| Piperacillin | 4 (22.22%) | 14 (77.78%) | 2 (33.33%) | 4 (66.67%) | 2 (20%) | 8 (80%) |
| Ciprofloxacin | 6 (33.33%) | 12 (66.67%) | 2 (33.33%) | 4 (66.67%) | 4 (40%) | 6 (60%) |
| Gentamicin | 8 (44.45%) | 10 (55.55%) | 2 (33.33%) | 4 (66.67%) | 4 (40%) | 6 (60%) |
| Amikacin | 10 (55.56%) | 8 (44.44%) | 4 (66.67%) | 2 (33.33%) | 6 (60%) | 4 (40%) |
| Tobramycin | 8 (44.45%) | 10 (55.55%) | 2 (33.33%) | 4 (66.67%) | 4 (40%) | 6 (60%) |
| Cotrimoxazole | 8 (44.45%) | 10 (55.55%) | 2 (33.33%) | 4 (66.67%) | 4 (40%) | 6 (60%) |
| Cefepime | 6 (33.33%) | 12 (66.67%) | 4 (66.67%) | 2 (33.33%) | 6 (60%) | 4 (40%) |
| Piperacillin-Tazobactam | 10 (55.56%) | 8 (44.44%) | 5 (83.33%) | 1 (16.67%) | 7 (70%) | 3 (30%) |
| Ceftazidime | 8 (44.45%) | 10 (55.55%) | 2 (33.33%) | 4 (66.67%) | 4 (40%) | 6 (60%) |
| Meropenem | 12 (66.67%) | 6 (33.33%) | 4 (66.67%) | 2 (33.33%) | 10 (100%) | 0 |
| Imipenem | 16 (88.89%) | 2 (11.11%) | 6 (100%) | 0 | 10 (100%) | 0 |

**[Table/Fig-16]:** Antibiotic susceptibility pattern of the other isolates.

| Isolates | Resistant to Ceftazidime | ESBL |
|---|---|---|
| *P. aeruginosa* (n=466) | 312 (66.95%) | 186 (39.91%) |
| *P. putida* (n=18) | 10 (55.55%) | 8 (44.45%) |
| *P. fluorescens* (n=6) | 4 (66.67%) | 4 (66.67%) |
| *P. stutzeri* (n=10) | 6 (60%) | 2 (20%) |
| Total | 332 (66.4%) | 200 (40%) |

**[Table/Fig-17]:** ESBL detection in *Pseudomonas* isolates (N=500).

Virulence factors such as pigments, exotoxins, elastase, and type III secretion systems contribute to tissue damage and immune evasion. Pigments like pyocyanin generate reactive oxygen species and modulate host responses. Quorum sensing coordinates these pathogenic traits based on population density [21].

*P. aeruginosa* is most prevalent in patients with burns, cystic fibrosis, organ transplants, and intravenous drug abusers. These infections occur in areas where moisture tends to accumulate, such as in indwelling catheters, burns, and external ears. In the present study,

| Species | AmpC detection | | |
|---|---|---|---|
| | Inducible | Non inducible | Total |
| *P. aeruginosa* | 88 (93.61%) | 114 (91.94%) | 202 (92.66%) |
| *P. putida* | 4 (4.26%) | 6 (4.83%) | 10 (4.59%) |
| *P. fluorescence* | 2 (2.13%) | 2 (1.61%) | 4 (1.83%) |
| *P. stutzeri* | 0 | 2 (1.61%) | 2 (0.92%) |
| Total (% out of 500) | 94 (18.8%) | 124 (24.8%) | 218 (43.6%) |

**[Table/Fig-19]:** AmpC detection in *Pseudomonas* (N=500).

| Co-expression | Number |
|---|---|
| *P. aeruginosa* (n=466) | 54 (93.1%) |
| *P. putida* (n=18) | 4 (6.9%) |
| *P. fluorescens* (n=6) | 0 |
| *P. stutzeri* (n=10) | 0 |
| Amp C and ESBL in total (n=500) | 58 (11.6%) |

**[Table/Fig-20]:** Co-expression of various Beta lactamases in *Pseudomonas* isolates.

among *Pseudomonas* species, *P. aeruginosa* (466 isolates, 93.2%) was the most frequently observed strain, while *P. fluorescens* (6 isolates, 1.2%) was less commonly observed in a total of 500 samples The proportion of *P. aeruginosa* in Patel HK et al., was 99.1%, comparable to the present study but disproportionate when compared to the study by Juyal D et al., (77%) [22,23]. *P. fluorescens* has been reported to cause bloodstream infections associated with the use of syringes preloaded with heparin intravenously. The percentage of *P. fluorescens* in the present study was comparable to Yan JJ et al., (0.95%) and lower than Sidhu S (3.2%) [24,25]. Out of 466 samples of *P. aeruginosa*, 52 (11.15%) isolates were from respiratory specimens, which was lower than that reported by Javiya A et al., (41.1%) [26], but comparable to Patel HK et al., [22], who reported 10.1% isolation from respiratory specimens.

*P. putida* was the second most common species causing infections, with a proportion of 18 (3.6%) in 500 isolated samples, which was lower than that of Yan JJ et al., (7.8%) and Sidhu S (6.8%), while it was higher than that of Ergin C and Mutlu G, (1.04%) [24,25,27].

Various species of *Pseudomonas* produce a number of pigments that diffuse freely into their surroundings and mediate tissue injury [24,25,27]. The highest rate of pigment production was shown by *P. aeruginosa*, which produces pyocyanin, resulting in characteristic bluish pus. Pyocyanin is one of the most important virulence factors of *Pseudomonas* species, playing a critical role in lung infections. It is also recognised as a quorum-sensing signaling molecule in *Pseudomonas* strains, significantly contributing to pathogenesis and increasing the severity of infections. Out of a total of 500 isolated samples, 416 (83.2%) produced pigments. A total of 392 (84.12%) of 466 isolates from *P. aeruginosa* were identified as pigment producers. All isolates from *P. putida* and *P. fluorescens* produced pigments, while none of the samples from *P. stutzeri* showed pigment production. These results are comparable to those of a study by Finlayson EA and Brown PD, which found pigment production in 82.5% of isolates [28].

The first event to initiate an infection is adherence and colonisation through biofilm formation. This ability contributes to developing resistance to multiple antibiotics and disinfectants in the pathogenesis of *Pseudomonas*. *Pseudomonas* species cause biofilm-related infections like endocarditis, urinary tract infections, and septic arthritis. The biofilm produced on indwelling medical devices often leads to recurrent, untreatable infections and the failure of these devices. Therefore, detecting biofilm is crucial for addressing chronic and recurrent infections.

Afreenish H et al., tested 110 clinical isolates for biofilm production, finding that 71 (63.3%) produced biofilms using the TCP method, 54 (49.1%) using the Tube method (TM), and 4 (3.6%) using the CRP method [21]. In the study by Nagaveni et al., the TCP and TM methods detected biofilms in 9 (36%) samples, while the CRP method identified 3 (12%) [29]. The TCP method is considered more quantitative and reliable for detecting biofilm-producing *Pseudomonas* species and is used as a screening method for biofilm detection. It is regarded as the gold standard method, while the Tube method and CRA method are both qualitative for biofilm detection. The sensitivity pattern of *P. aeruginosa* isolates varies with findings by Juyal D et al., [23].

In a study by Cho CH and Lee SB [30], *P. putida* showed resistance to Tobramycin (0%), Ceftazidime (12.5%), and Ciprofloxacin (12.5%); Imipenem (18.7%), Piperacillin (25%), Piperacillin/Tazobactam (25%), and Ticarcillin (100%) same findings were noted in present study. A study conducted in India by Trivedi MK et al., [31] found that all *P. fluorescens* isolates were 100% susceptible to Ceftazidime, Piperacillin/Tazobactam, Gentamicin, Tobramycin, and Colistin.

A previous study by Bisharat N et al., indicated that *Pseudomonas* stutzeri had susceptibility rates of 99% to Gentamicin and Ofloxacin, 98% to Amikacin and Imipenem, 97% to Ciprofloxacin and Meropenem, 95% to Ceftazidime, and 93% to Piperacillin, among others [32]. In the present study, out of 500 *Pseudomonas* isolates, 332 (66.4%) were resistant to Ceftazidime, and 200 (40%) were ESBL producers, as determined by the Combined Disc Diffusion Test [Table/Fig-17]. Agarwal R et al., detected 30 (20.27%) of the total 141 Pseudomonas samples producing ESBL, whereas a higher percentage was noted by Shahcheraghi F et al., [33,34]. Out of 500 *Pseudomonas* isolates, 104 (20.8%) were resistant to Imipenem, and 58 (11.6%) were recognised as MBL producers. These findings are comparable to those of Hemalatha V et al., who reported a 14% resistance rate, but differ from Irfan S et al., which reported a 59.5% resistance rate [35,36]. Beta-lactamase-producing organisms can lead to significant therapeutic failures if they remain undetected. Clinicians treating infections based on available antibiotic tests face risks, particularly with infections by AmpC-producing organisms, especially *P. aeruginosa*, which is increasingly problematic due to treatment failures.

In the current study, out of 500 strains of *Pseudomonas*, 218 (43.6%) were found to be AmpC producers based on the AmpC disc test. Among these, 94 (18.8%) were inducible, while the remaining 124 (24.8%) were non inducible. This rate is much lower than that found in the study by Gencer S et al., which reported a 53% prevalence, while lower proportions were observed in studies by Basak S et al., which reported 19% [Table/Fig-18] [37,38].

Extensive resistance to antimicrobials presents a significant challenge and poses threats to the management of infections. This resistance arises from factors such as injudicious use of antibiotics, the absence of a fixed antibiotic policy, easy over-the-counter availability of antimicrobials, and the extensive use of broad-spectrum antibiotics. Such considerations are particularly important in the context of *Pseudomonas* infections, which are common in patients with diabetes mellitus (DM), burns, nosocomial infections, and those who are immunocompromised. The high incidence of beta-lactamase production due to multiple mechanisms in *Pseudomonas* is alarming and necessitates urgent action from both therapeutic and infection control perspectives.

In present study, various co-expressions of beta-lactamases in *Pseudomonas* isolates were observed. Present study found the coexistence of AmpC and ESBL in 58 (11.6%) isolates of *Pseudomonas*, which was lower than the findings observed by Chatterjee SS et al., (2010) [39]. The co-expressions of ESBL and AmpC were the highest among all co-expressions, totaling 29 (11.6%).

The production of multiple beta-lactamases by *Pseudomonas* has significant therapeutic consequences, posing a considerable clinical challenge if it remains undetected. Given that these organisms also harbour other drug resistance genes, the only viable treatment

options currently are Polymyxin B and Colistin. Therefore, early identification of infections caused by these organisms is essential, as appropriate treatments may help to slow down the emergence of resistant strains and reduce mortality rates in hospitalised patients. This highlights the necessity for the detection of isolates that produce these enzymes to avoid therapeutic failures and nosocomial outbreaks.

In response to the growing antimicrobial resistance exhibited by *Pseudomonas* aeruginosa, several innovative therapeutic strategies are under investigation. These include the development of novel antimicrobials and adjuvant compounds aimed at restoring the efficacy of existing antibiotics through synergistic mechanisms. Strategies such as disrupting biofilm architecture and inhibiting quorum sensing pathways are being explored to prevent chronic colonisation and attenuate virulence. Moreover, alternative approaches like bacteriophage therapy, antimicrobial peptides, and host-directed therapies are gaining attention as potential adjuncts or replacements for traditional antibiotic regimens. Successful personalised phage therapies have been reported for patients suffering from chronic, life-threatening infections caused by MDR *P. aeruginosa* [40]. All these strategies hold promise in addressing the limitations of current treatment options and improving clinical outcomes.

This study emphasises the clinical burden posed by *Pseudomonas* species, especially *P. aeruginosa*, due to their ability to produce multiple β-lactamases and form biofilms. These features significantly reduce treatment efficacy and complicate infection management.

Incorporating routine detection of resistance mechanisms and virulence traits into diagnostic protocols can support timely, targeted therapy while reinforcing infection control measures in hospital environments. Further studies are warranted to explore the molecular drivers of resistance and virulence in *Pseudomonas* spp. The development of novel therapeutic strategies, including anti-biofilm compounds and next-generation β-lactamase inhibitors, may enhance treatment outcomes. Integrating rapid diagnostic platforms with antimicrobial stewardship efforts will be crucial in mitigating the impact of these adaptable and resistant pathogens.

### Limitation(s)

1. The laboratory-based design precluded assessment of clinical outcomes.
2. Data from a single centre may limit external validity.
3. Resistance mechanisms were identified phenotypically, and molecular confirmation was not performed.
4. The cross-sectional nature restricted evaluation of temporal resistance trends.

## CONCLUSION(S)

This study underscores the clinical significance of *Pseudomonas* species, particularly *P. aeruginosa*, as a resilient opportunistic pathogen with diverse virulence factors and increasing antimicrobial resistance. The high prevalence of biofilm formation and co-expression of β-lactamases highlights the therapeutic complexity posed by these isolates, especially in critical care settings. The persistence of *Pseudomonas* in hospital environments necessitates stringent infection control, vigilant surveillance, and integration of antibiofilm strategies. Emerging interventions such as quorum-sensing inhibitors, bacteriophages, and biofilm-targeted vaccines represent promising alternatives. Given the rising resistance to key antimicrobial classes, there is an urgent need for continued research into the molecular mechanisms underlying resistance and virulence. A comprehensive understanding of these factors is essential for guiding novel therapeutic development, refining diagnostic capabilities, and informing effective antimicrobial stewardship in tertiary care hospitals.

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