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A Descriptive Study of Biofilm Formation and Antimicrobial Susceptibility Pattern of *Pseudomonas aeruginosa* Isolated from Clinical Samples

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ABSTRACT

Background: *Pseudomonas aeruginosa* is a clinically troublesome pathogen that frequently causes opportunistic infections and nosocomial outbreaks. Infection caused by multidrug-resistant, biofilm-forming Pseudomonas is a major cause of morbidity and mortality.
Materials and Methods: Antimicrobial susceptibility testing for all *P. aeruginosa* isolates from clinical samples was performed by the Kirby-Bauer disk diffusion method according to the Clinical and Laboratory Standards Institute guidelines. Congo red agar and the Christensen tube methods were used to test the biofilm production.
Results: This study included 260 isolates of *P. aeruginosa* from clinical samples; the majority of the isolates were from pus (58%), followed by urine (17%). It was shown that 66% of isolates produced biofilms. *P. aeruginosa* had the highest percentage of ceftazidime resistance (83%), followed by cefepime resistance (71%). When biofilm formation and antibiotic resistance were correlated among *P. aeruginosa* isolates, the percentage of biofilm-forming *P. aeruginosa* that were multidrug-resistant (83.9%) was considerably ($p < 0.001$) higher than that of non-biofilm producers.
Conclusion: This study indicates a strong association between the development of biofilms and resistance to various antibiotics. Misuse of antibiotics increases expenses for treatment, morbidity, and mortality. It is essential to enforce strict guidelines on antibiotic use, adopt measures to control infections, and establish programs to monitor organisms resistant to multiple drugs.

Keywords: *Pseudomonas aeruginosa*, biofilm production, and antimicrobial susceptibility pattern.

INTRODUCTION

Pseudomonas aeruginosa is a rod-shaped Gram-negative bacterium and is known to be a widespread and adaptable pathogen found in humans, animals, and the environment. Managing the extensive spread of *P. aeruginosa* strains in healthcare facilities is difficult. [1] *P. aeruginosa* is among the most

frequently encountered opportunistic pathogens that have clinical importance because of its ability to produce various virulence factors, its resistance to multiple drugs, and its ability to form biofilms. [2]

P. aeruginosa is a major factor in various infections that occur in both the community and healthcare settings. Typical infections acquired in the community include ulcerative keratitis, diabetic foot wounds, and otitis externa. In hospital settings, it leads to infections such as ventilator-associated pneumonia, urinary tract infections, bloodstream infections, surgical site infections, and skin infections in burn patients. [3] It can lead to severe, life-threatening infections in individuals who have cancer, diabetes, cystic fibrosis, weakened immune systems, or who have undergone major surgeries. [4]

The formation of biofilm is a significant virulence factor accountable for 65% of hospital-acquired infections and 60% of all bacterial infections in humans.[5] *P. aeruginosa* is a recognized biofilm producer that plays a vital role in its competition and survival. The capability of *P. aeruginosa* to form biofilms depends on the synthesis of three exopolysaccharides: Pel, Psl, and alginate. Among these, alginate is a key virulent factor that contributes to biofilm development. Biofilms are often referred to as microbial cities. [6]

Pseudomonas aeruginosa exhibits both intrinsic and acquired resistance to a variety of antibiotics, primarily as a result of the improper use of frequently prescribed antimicrobials. [7] *P. aeruginosa* is known for its ability to produce biofilms and extended-spectrum beta-lactamases (ESBLs), posing significant challenges for clinical microbiologists, healthcare providers, professionals in infection control, and researchers focused on the development of new antimicrobial agents.[8] Therefore, ongoing monitoring and regular antimicrobial susceptibility testing, in addition to identifying biofilm formation, will support healthcare providers in managing and averting multidrug-resistant infections caused by Pseudomonas. Therefore, the purpose of this study is to ascertain the frequency of biofilm formation and the antimicrobial susceptibility pattern in *P. aeruginosa* isolated from clinical samples sent to the microbiology laboratory for culture and sensitivity.

Aim: To study biofilm formation and antimicrobial susceptibility pattern of *P. aeruginosa* isolated from clinical samples.

Objectives: To isolate, identify, and determine the antimicrobial susceptibility pattern of *P. aeruginosa* from clinical samples. To determine the frequency and risk factors associated with biofilm production in *P. aeruginosa.*

MATERIALS & METHODS

This study was conducted in the Microbiology department of a tertiary care teaching hospital over a period of one year from September 2022 to August 2023 from the samples obtained for routine culture and sensitivity testing from both sexes with all age groups having clinical infections.

Study design: Descriptive study.
Place of study: Tertiary care teaching hospital.
Duration of study: 12 months.
Study group: All the patients attending various clinical departments of the hospital.

Pseudomonas aeruginosa obtained from various clinical samples sent to the laboratory for aerobic bacterial culture over the duration of the study was included in this study. The samples analyzed included pus, sputum, urine, blood, an endotracheal tube, and different body fluids such as pleural fluid, ascitic fluid, and cerebrospinal fluid (CSF). A total of 260 isolates of Pseudomonas aeruginosa were studied. For the microscopic examination, direct smears were prepared from the different samples and stained using the Gram stain technique according to the Modified Hucker's method. These smears were then screened for the presence of pus cells, epithelial cells, and any

microorganisms. All samples were inoculated onto well-dried nutrient agar, blood agar, and MacConkey agar using a sterile nichrome wire loop. The incubation of the plates was performed overnight in ambient air at a temperature of 37°C, after which colony morphology was observed. A colony smear was prepared from a single well-isolated colony and stained with Gram stain. The colony smear that displayed characteristics of Gram-negative, non-spore-forming rods was then further evaluated through motility and biochemical testing. All isolates were subjected to antimicrobial susceptibility testing on Muller-Hinton agar using the Kirby-Bauer disc diffusion method with commercially available discs.

Biofilm production:

Biofilm production was determined using the Congo red agar method and the tube method.

Congo red agar method:

A concentrated aqueous solution of Congo red was prepared and autoclaved at 121° C for a duration of 15 minutes, apart from the other components of the medium. Once the BHI agar with sucrose had cooled to 55° C, the autoclaved Congo red solution was mixed in and then poured into 90 mm Petri dishes. The isolated colonies were transferred to Congo red agar medium, and after overnight incubation, the colonies were observed. Colonies that appeared black were regarded as positive indicators of slime production. In contrast, non-slime producers typically maintained a pink color. [9,10]

Tube method:

This technique was introduced by Christensen and colleagues in 1982. The colonies were introduced into tryptic soy broth, which contained one percent glucose. Following an overnight period of incubation, the tubes were emptied and thoroughly rinsed three times with phosphate-buffered saline to eliminate planktonic flora. The tubes were then treated with crystal violet (0.1%). A visible blue layer forming inside the tube indicated a positive test. The line at the liquid-air interface, though stained blue, was not regarded as a positive result. [11,12]

Multidrug-resistant (MDR) phenotype is defined as P. aeruginosa, which is resistant to more than one antimicrobial agent in three or more antimicrobial categories. Pan-DR (PDR) phenotype is defined as a bacterium that is resistant to all antimicrobial agents in all antimicrobial categories. [13]

Statistical Analysis

Microsoft Excel was used for graphical and tabular depiction. Among the qualitative factors, the subject's age and sex were nominal data. Quantitative data were expressed using frequency and percentage. P-value was calculated, and $p<0.05$ was considered statistically significant.

RESULT

Among all culture-positive samples (5114), *Pseudomonas aeruginosa* was obtained from 260 samples, representing a rate of 5.08%. The isolates of *P. aeruginosa* were predominantly found in males (61%) in contrast to females (39%). The highest number of *Pseudomonas aeruginosa* was identified in patients between the ages of 41-60 years, and the lowest in the age group under 20 years old.

Table no. 1: Distribution of *P. aeruginosa* isolates from various clinical samples.

Clinical samples	No. of isolates	Percentage %
Pus	150	58%
Urine	45	17%
Sputum	30	12%
Blood	16	6%
ET tube tip	12	5%
Body fluids	7	2%
Total	260	100%

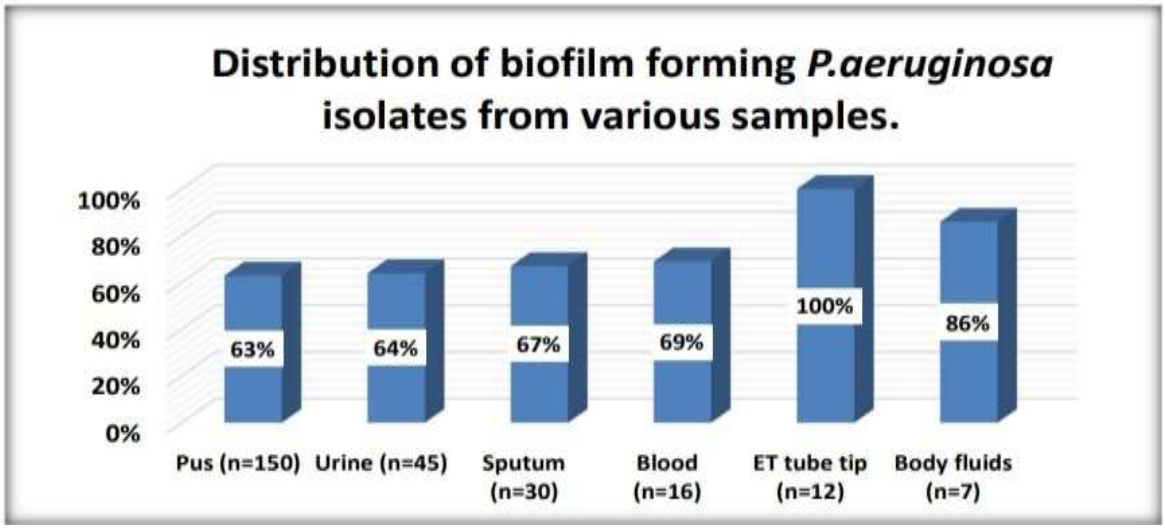
Table no.1 shows that *Pseudomonas aeruginosa* was isolated most commonly from pus (58%) followed by urine (17%).
P. aeruginosa isolates were more commonly obtained from specimens submitted by the surgery department (29%) followed by the intensive care unit (23%). Majority of the isolates (87%) recovered from patients attending inpatient departments than from outpatient departments (13%).

Table no. 2: Antibiotic resistance pattern of *P. aeruginosa* isolates.

Antibiotics	Abbreviation	Concentration	Resistant no. (%)
Piperacillin-tazobactam	PTZ	100/10 µg	50(19%)
Ceftazidime	CAZ	30µg	215(83%)
Cefepime	CPM	30µg	184(71%)
Aztreonam	AT	30µg	86(33%)
Imipenem	IPM	10µg	22(8%)
Meropenem	MRP	10µg	31(12%)
Amikacin	AK	30µg	164(63%)
Ciprofloxacin	CIP	5µg	173(67%)
Norfloxacin*	NX	10µg	17(38%)

*** For urinary isolates: no. of isolates- 45.**

Table no. 2 shows that *P. aeruginosa* revealed the highest resistance to ceftazidime (83%), followed by cefepime (71%), ciprofloxacin (67%), and amikacin (63%). Lower resistance was seen to imipenem (8%) and meropenem (12%).
Among *P. aeruginosa* strains, 173 (66%) were biofilm producers, and non-biofilm producing strains were 87 (34%).



Graph 1: The distribution of biofilm-forming *P. aeruginosa* isolates from various clinical samples.

Graph 1 illustrates that biofilm formation occurred more frequently in isolates from the ET tube (100%), body fluids (86%), and blood (69%). Patients in the intensive care unit exhibited the highest count of biofilm creators (80%). The majority of patients with biofilm producers had indwelling medical devices (67%), followed by comorbidities (42%), underwent surgical procedures (26%), and those with extended hospital stays (10%).

Table no. 3: Biofilm formation and antibiotic resistance among *Pseudomonas aeruginosa* isolates.

Antibiotics tested	Biofilm formation		Chi-square value(x^2)	Fishers Exact test	P value
	Positive (173)	Negative (87)			
Piperacillin-tazobactam (n=50)	49(98%)	1(2%)	_	0.0000001	<0.001
Ceftazidime (n=215)	160(74%)	55(26%)	34.646		<0.001
Cefepime (n=184)	136(74%)	48(26%)	15.376		<0.001
Aztreonam (n=86)	81(94%)	5(6%)	44.118		<0.001
Imipenem (n=22)	22(100%)	0		0.0035	<0.001
Meropenem (n=31)	29(94%)	2(6%)		0.0027	<0.001
Amikacin (n=164)	130(79%)	34(21%)	32.328		<0.001
Ciprofloxacin (n=173)	136(79%)	37(21%)	33.853		<0.001
Norfloxacin* (n=17)	15(88%)	2(12%)		0.047	<0.001

*** For urine sample: no. of isolates- 45.**

Table 3 shows that there was a highly significant difference in resistance to all the antibiotics tested between biofilm producer, and non-biofilm producers. ($p<0.001$)

Table no. 4: Correlation of biofilm formation and multidrug resistance in *Pseudomonas aeruginosa* isolates.

	Biofilm producer (n=173)	Non-biofilm producers (n=87)	Chi-square value (X^2)	P value
MDR isolates(n=143)	120(83.9%)	23(16.1%)	43.101	**<0.001**
Non-MDR isolates(n=117)	53(45%)	64(55%)		

Table no. 4 shows a correlation between biofilm formation and multidrug resistance among isolates of *P. aeruginosa,* revealing that a significantly higher number of biofilm-forming *P. aeruginosa* strains showed multidrug resistance (83.9%) as compared to non-biofilm producers with a highly significant difference ($p<0.001$).

DISCUSSION

Pseudomonas aeruginosa is the most frequently encountered non-fermenting bacterium isolated from clinical samples and poses significant challenges in treating infections acquired from the community as well as those occurring in hospital settings. In India, the occurrence of *P. aeruginosa* infections ranges between 10.5% and 30%. This study identified the frequency of *P. aeruginosa* isolates to be 5.08%, which aligns closely with the findings from Bindu *et al.* [14] and Tadvi *et al.* [15] who reported rates of 5.5% and 4.5%, respectively. Other research conducted by Tahira *et al.* [16] and Anupurba *et al.* [17] recorded a higher prevalence rate of 32-40%. The differing rates of *P. aeruginosa* noted in various studies might be explained by factors such as the type of hospital, the specializations involved, the types of clinical samples, and the demographic characteristics of the study population.

The gender-wise distribution of *Pseudomonas aeruginosa* isolates indicates that these infections are more frequently observed in males (61%), in contrast to females (39%). This observation is consistent with the findings of Rakesh *et al.* [18] who also reported 61%, and Mallure *et al.* [19] who noted a prevalence of 56% among males. On the other hand, Anil *et al.* [20] noted a slight predominance of females at 55.17% compared to males at 44.83%. Factors such as outdoor activities, personal habits, occupation types, and exposure to environments like soil and water that harbor these organisms may contribute to the higher incidence in males.

The highest proportion of *P. aeruginosa* isolates was found in patients aged between 41 and 60 years, accounting for 41%,

followed by those over 60 years, who represented 31%. This finding aligns with the research conducted by Andhale *et al*. [21] where the majority of cases were in the age group of 41 to 60 years (46.66%), followed by individuals aged 60 to 80 years (26.66%). Contributing factors such as compromised immune systems, extended hospital stays, and various associated comorbidities may explain the vulnerability of this demographic.

The sample-wise distribution of *Pseudomonas aeruginosa* isolates revealed that most isolates were from pus samples (58%), followed by urine (17%), sputum (12%), blood (6%), tips of endotracheal tubes (5%), and body fluids (2%), as illustrated in [Table 1]. Consequently, wound infections caused by *Pseudomonas aeruginosa* are more common, followed by urinary and lower respiratory tract infections that significantly contribute to morbidity both within the local community and among hospital attendees. These findings correspond with studies by Handa *et al*. [22] (2024) and Gyawali *et al*. [23] (2020). Conversely, Tadvi *et al*. [15] and Rodrigues *et al*. [24] noted the highest rate of *P. aeruginosa* isolation from blood samples. Research conducted by Saha *et al*. [25] and Narayan *et al*. [26] indicated that sputum was the primary source, followed by urine. The discrepancies observed among these studies may stem from variations in the duration of the study, sample sizes, geographical contexts, and patient demographics.

The isolation rate of *Pseudomonas aeruginosa* was markedly higher in indoor patients, accounting for 87%, compared to merely 13% among outdoor patients. This observation mirrors findings by Tewari *et al*. [27] (2020) and Saha *et al*. [25] (2018), who suggested that longer hospital stays correlate directly with an increased incidence of *Pseudomonas aeruginosa* infections. Patients in the hospital for extended periods, particularly those undergoing surgical procedures, intubation, long-term medical treatment, and those with weakened immune systems, may account for the elevated rates of Pseudomonas infections among indoor patients.

P. aeruginosa is recognized as one of the leading pathogens responsible for hospital-acquired infections, particularly in critical care settings such as intensive care units (ICUs). In the current study, 23% of *Pseudomonas aeruginosa* infections were identified in the intensive care unit. Factors such as the extensive use of antibiotics, the presence of indwelling urinary catheters, mechanical ventilation, and prolonged hospital stays may contribute to the risk of infection in these high-dependency areas.

An increase in resistance to various anti-pseudomonal medications, particularly among strains found in hospitals, has been documented globally. In this investigation, *P. aeruginosa* displayed the highest level of resistance to ceftazidime (83%), followed by cefepime (71%), ciprofloxacin (67%), amikacin (63%), norfloxacin (38%), and aztreonam (33%). The resistance observed was lower for imipenem (8%) and meropenem (12%), with piperacillin-tazobactam showing a resistance of 19% [Table 2]. Bhandari *et al*. [28] reported a strikingly high resistance rate to ceftazidime (93.9%) and cefepime (98.5%). Conversely, Ranjan S *et al*. [29] noted a much lower resistance to ceftazidime (27%). The elevated levels of resistance against these antimicrobial agents may be attributed to the indiscriminate administration of third and fourth-generation cephalosporins as broad-spectrum empirical treatments and the production of extended-spectrum beta-lactamases (ESBL), which facilitate resistance through the hydrolysis of the beta-lactam ring of beta-lactam antibiotics. Additionally, as this hospital serves as a referral center, a significant number of patients have already been administered antimicrobials at private clinics before their referral. Our study discovered a relatively low resistance rate to imipenem (9%) and meropenem (12%). These findings align with those of Chattopadhyay *et al*. [30] (12% resistance to imipenem and 14% against meropenem) and Dash *et al*. [31] (6.4%

resistant to imipenem and 8% resistant to meropenem), indicating that carbapenems continue to be effective against *P. aeruginosa* in hospital environments. Nevertheless, several studies have reported an alarmingly high prevalence of carbapenem resistance; for instance, Khan *et al*. [32] found a 60% resistance to both imipenem and meropenem, while Kumari *et al*. [33] reported resistance rates of 53% and 63% to imipenem and meropenem, respectively. The lower resistance rate to carbapenems observed in this study could be attributable to their high cost, leading to limited usage by clinicians in our institution.

Biofilms are believed to account for 65% of infections acquired in hospitals and 60% of all human infections. In the current study, out of 260 *P. aeruginosa* isolates, 173 (66%) were identified as biofilm producers, whereas 87 (34%) were not. These findings are consistent with those of Charan *et al*. [34], who reported a 65% rate of biofilm production among *P. aeruginosa* isolates. All *P. aeruginosa* isolates retrieved from endotracheal (ET) tubes were biofilm producers (100%), with a significant 86% from body fluids. The formation of biofilms in ET tubes is influenced by various factors, such as the polyvinylchloride plastic surface of the tubes and the accumulation of tracheobronchial secretions due to insufficient host defense mechanisms within the inner lumen. In a related study conducted by Neopane *et al*. [35], 100% of *P. aeruginosa* isolates from the ET tube demonstrated biofilm production, along with 80% from urine, 77.8% from sputum, 69.2% from pus, and 66.7% from blood.

Biofilms that develop on implanted medical devices are responsible for a significant proportion, approximately 50–70%, of infections acquired in hospitals and pose substantial treatment challenges. In our investigation, we found that a majority of patients with biofilm-producing organisms had indwelling medical devices (67%), were affected by underlying health conditions (42%), underwent surgical interventions (26%), and experienced extended hospital stays (10%). The predominant group of patients with indwelling medical devices exhibited other associated risk factors, including surgical procedures and longer hospitalization periods.

In our present research, 55% of the isolates identified were multidrug-resistant *Pseudomonas aeruginosa*. This observation aligns with findings from similar studies conducted by Gurung *et al*. [36] and Hadadi *et al*. [37] who reported rates of 57% and 62.5% for MDR *P. aeruginosa*, respectively.

Notably, we found that a significantly greater proportion of biofilm-forming *P. aeruginosa* exhibited MDR (83.9%) in comparison to non-biofilm producers, resulting in a statistically significant difference ($p<0.001$) [Table 4]. These statistically meaningful results ($p< 0.001$) underscore a strong association between biofilm formation and resistance to multiple drugs. Comparable results from Rouchelle *et al*. [38] demonstrated a significant statistical correlation between biofilm production and multidrug resistance, with 72.3% of biofilm producers classified as multidrug resistant.

Our research also indicated that 6% (16 out of 260) of *P. aeruginosa* isolates were classified as pan-drug resistant. These findings are in agreement with those of Gill *et al*. [39] who reported a 3% prevalence of pan-drug-resistant isolates.

CONCLUSION

Pseudomonas aeruginosa is a frequently encountered pathogen in hospitals that often develops resistance to multiple drugs and can lead to severe infections in critically ill individuals. Because the use of indwelling devices is often linked to the formation of biofilms, it is crucial to reduce their use whenever possible. If these devices are necessary, stringent care is essential to prevent infections acquired in hospitals, as these infections are typically more prone to being resistant to multiple drugs and are associated with biofilms.

Resistance to multiple drugs is a widespread and increasing issue in healthcare, posing a risk to public well-being, resulting in

significant illness and death, along with a higher economic cost due to inappropriate antibiotic use, especially when used excessively. To help stop the transmission of microorganisms, regular hand washing should be encouraged. Patients should receive improved surgical and medical treatment during their hospital stay.

Among the antibiotics tested, imipenem and meropenem showed the least resistance against *P. aeruginosa*. Consequently, the use of these drugs should be limited to serious infections acquired in hospitals to prevent the rapid emergence of resistant strains. Therefore, we suggest routine examination of biofilm development and keeping track of the antimicrobial resistance patterns of *P. aeruginosa*. This will assist in formulating an effective antimicrobial approach in clinical situations while addressing infections caused by this organism.

Declaration by Authors

Ethical Approval: Approved

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