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# Correlation between Biofilm Formation and Antibiotic Resistance in Pseudomonas aeruginosa Isolated from Various Clinical Specimens

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ORIGINAL STUDY

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# Correlation between Biofilm Formation and Antibiotic Resistance in *Pseudomonas aeruginosa* Isolated from Various Clinical Specimens

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**Abstract**

**Background: *Pseudomonas aeruginosa*, is a Gram-negative nosocomial pathogen implicated in various human infections, primarily associated with healthcare services. Biofilms are known for their resistance to antimicrobial agents.**

**Objectives: The study aims to investigate the correlation between biofilm formation and antibiotic resistance in isolates.**

**Materials and Methods: The samples were collected from (110) patients admitted to two main hospitals in Hilla city: Al-Hilla General Teaching Hospital, and Mergan Teaching Hospital. The specimens were collected from various clinical sites such as wounds, ears, and urine.**

**Results: Out of (70) positive cultures cultured on Cerimide agar medium, only 15 (13.6%) specimens showed positive identification as *P. aeruginosa*. Out of the total 110 specimens, 70 (63.6%) showed positive bacterial cultures. The results showed that all *P. aeruginosa* isolates were resistant to 12 antimicrobial agents. Except for colistin and ciprofloxacin, the results showed the highest rate of resistance against Ticarcillin, Ticarcillin /clavulanic acid, Piperacillin, Amikacin Ceftazidime, Cefepime, Imipenem, and Gentamycin. *P. aeruginosa* isolates 2 (13.33%) isolates had strong biofilm formation, moderate biofilm was observed in 6 (40%) isolates, and 5 (33.3%) isolates had weak or no biofilm formation 2 (13.3%).**

**Conclusion: The formation and persistence of biofilms can result in elevated transfer of antibiotic resistance. A relationship was observed between, Antibiotic resistant and the level of biofilm formation.**

***Keywords:*** Pseudomonas aeruginosa, Biofilm formation, Antibiotic resistance

## 1. Introduction

*Pseudomonas aeruginosa*, a Gram-negative microorganism, ranks third among the most common causes of nosocomial infections, following *Staphylococcus aureus* and *Escherichia coli* [1]. It particularly poses a significant risk in patients with conditions such as cystic fibrosis (CF), burns, wounds, skin infections, urinary tract infections (UTIs), immunodeficiency, and those undergoing artificial ventilation [2]. Over recent years, widespread antibiotic usage has led to *P. aeruginosa* developing resistance to various broad-spectrum antibiotics [3].

Various factors contribute to this resistance, such as the microorganism's permeability to antibiotics, the presence of efflux pumps, alterations in microbial receptors for antibiotics, and the production of beta-lactamase enzymes [4]. Another significant factor is the formation of biofilms by *P. aeruginosa*. Biofilms, which are communities of microorganisms attached to surfaces, serve as a protective shield against antibacterial agents like disinfectants, heat, and drying [5]. They persist on surfaces, especially in hospital settings, contributing to contamination and the spread of infectious diseases.

The structure of *P. aeruginosa* biofilms further enhances antibiotic resistance due to several reasons. These include limited penetration of antibiotics into the biofilm matrix [6], altered chemical environments within the biofilm [7], and cell differentiation within

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the biofilm community [8]. These mechanisms, stemming from the multicellular nature of biofilms, lead to increased antibiotic resistance and pose challenges for treatment strategies [9, 10].

## 2. Material and methods

### 2.1. Clinical specimens & culture characteristics

A cross-sectional study was conducted, involving 110 clinical specimens collected from patients who attended two main hospitals in Hilla city: Al-Hilla General Teaching Hospital and Mergan Teaching Hospital, over a period of four months from August to November 2023. The specimens were obtained from various infection sites including wounds, ears, urine, and blood. Upon collection, the samples were immediately transferred to the laboratory. Each sample was streaked on MacConkey agar and nutrient agar, then incubated aerobically at 37°C for 24 hours. Colonies with various morphologies were isolated, and bacteria were stained with Gram stain and examined under a light microscope. Colonies containing bacilli were subcultured on Cetrimide agar and incubated for 24 hours at 37°C.The growth and colors of bacterial colonies were observed to confirm the presence of *Pseudomonas aeruginosa* using the Vitek 2 compact system (BioMerieux, France).

### 2.2. Antibiotic susceptibility testing

Antibiotic sensitivity testing was performed on all isolates using the VITEK 2 system by bioMerieux. Traditionally, the Kirby-Bauer disc diffusion assay has been used for this purpose, but it is laborious and prone to inconsistencies, subjectivity, and human error. The VITEK 2 system has revolutionized antibiotic susceptibility testing (AST) with its rapid and automated fluorescence-based technology, allowing the determination of the lowest inhibitory concentration (MIC) through the analysis of bacterial growth kinetics with antibiotics in test cards [11]. The detection of antimicrobial activity using the Vitek 2 system followed the method outlined by bioMérieux, France. Bacteria were inoculated on MacConkey agar plates and incubated at 37°C for 24 hours. A bacterial suspension was prepared from the resulting growth by transferring 1-3 colonies to test tubes containing 3ml of normal saline, and the suspension turbidity was adjusted to a McFarland standard of 0.5 [12]. The susceptibility of *P. aeruginosa* isolates to antibiotics including Amikacin, Cefepime, Ceftazidime, Ciprofloxacin, Colistin, Gentamicin, Imipenem, Meropenem, Piperacillin, Ticarcillin, Ticarcillin/Clavulanic Acid, and Tobramycin, was tested using the VITEK 2 Compact system Gram-Negative Susceptibility with software version 5.01 and AST-GN76 (P. aeruginosa cards), following the manufacturer's instructions.

### 2.3. Biofilm formation assay

Microtiter Plate Biofilm Formation Assay was used according to [13, 14]. The procedure steps are: Straining were classified as follows: OD < ODc = non Adherent, ODc < OD < 2 × ODc = weakly adherent, 2 × ODc < OD < 4 × ODc = moderately adherent, 4 × ODc < OD = strongly adherent.

### 2.4. Ethical approval

The essential ethical approval from the ethical committee at Hilla Surgical Teaching Hospital was obtained. Moreover, all subjects involved in this work were informed, and the agreement required for conducting the experiments and publishing this work was obtained from each one prior to the collection of samples, approved by a local ethics committee (at the College of Medicine University of Babylon number 8425 on 14/11/2023.

## 3. Results

### 3.1. Distribution of Pseudomonas aeruginosa in various specimens

It was revealed that out of the total 110 specimens, 70 (63.6%) showed positive bacterial cultures. No growth was observed in the remaining 40 (36.4%) specimens, suggesting the presence of microorganisms that may be difficult to culture, such as viruses, fungi, or other agents, or possibly due to differences in the size and nature of the specimens (Fig. 1). Among the 70 positive cultures, when cultured on Cetrimide agar medium (a selective medium), only 15 (13.6%) specimens were identified as *P. aeruginosa*. This study illustrated the distribution of *P. aeruginosa* isolates, as shown in (Fig. 1).

The percentage rate of *P. aeruginosa* isolates from the site of infection indicated its prevalence and potential clinical implications, as shown in (Table 1).

### 3.2. Antibiotic susceptibility of P. aeruginosa isolates

Fig. 2 displays the antibiotic susceptibility of *P. aeruginosa* isolates using Vitek 2 system.

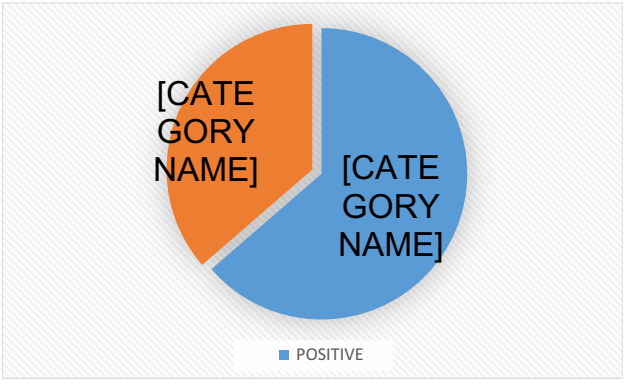


*Fig. 1. Frequency distribution for positive bacterial culture among other etiological agents associated with isolated specimens.*

*Table 1. Distribution of P. aeruginosa isolates based on the type of specimens.*

| Site of infection | No. of specimens | No. of *P. aeruginosa* isolates | (%) |
|---|---|---|---|
| Urine | 37 | 6 | 40 |
| Wound | 23 | 4 | 26.7 |
| Ear | 35 | 5 | 33.3 |
| Blood | 15 | 0 | 0 |
| Total | 110 | 15 | 100 |

*Table 2. Determination of biofilm formation ability of all p. aeruginosa clinical isolates by using tissue culture plate method.*

| Values | NBF | WBF | MBF | SBF |
|---|---|---|---|---|
| N. | 2 | 5 | 6 | 2 |
| % | 13.33% | 33.33% | 40.00% | 13.33% |
| P value | 0.334 | | | |

NBF = Non-biofilm-former, WBF = Weak-biofilm-former, MBF = Moderate-biofilm-former, SBF = Strong-biofilm-former.
*represents a significant difference at $p < 0.05$.

### 3.3. Detection of biofilm formation using Tissue culture plate method

A total of (15) isolates of *P. aeruginosa* were tested for their ability to produce biofilm. From these isolates, 2 (13.33%) isolates had a strong biofilm formation capacity, moderate biofilm was shown in 6 (40.00%) isolates, and 5 (33.33%) isolates showed weak or no biofilm formation, 2 (13.33 %) as shown in Table 2.

### 3.4. Correlation between antimicrobial susceptibility and biofilm formation

In light of the results, the connection between antibiotic susceptibility and biofilm formation is shown in Table 3.

*Table 3. Correlation between antimicrobial susceptibility and biofilm formation among P. aeruginosa clinical isolates.*

| Biofilm formation | Ticarcillin | | Ticarcillin \ Clavulanic Acid | | Piperacillin | | Ceftazidime | | Cefepime | | Imipenem | | Meropenem | | Amikacin | | Gentamicin | | Tobramycin | | Ciprofloxacin | | Colistin | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| N. | R | S | R | S | R | S | R | S | R | S | R | S | R | S | R | S | R | S | R | S | R | S | R | S |
| NBF (2) | 2 | 0 | 2 | 0 | 2 | 0 | 2 | 0 | 2 | 0 | 2 | 0 | 2 | 0 | 2 | 0 | 2 | 0 | 2 | 0 | 2 | 0 | 1 | 1 |
| WBF (5) | 5 | 0 | 5 | 0 | 5 | 0 | 5 | 0 | 5 | 0 | 5 | 0 | 5 | 0 | 5 | 0 | 5 | 0 | 5 | 0 | 4 | 1 | 2 | 3 |
| MBF (6) | 6 | 0 | 6 | 0 | 6 | 0 | 5 | 1 | 5 | 1 | 5 | 1 | 5 | 1 | 6 | 0 | 5 | 1 | 4 | 2 | 6 | 0 | 1 | 5 |
| SBF (2) | 2 | 0 | 2 | 0 | 2 | 0 | 2 | 0 | 2 | 0 | 2 | 0 | 1 | 1 | 2 | 0 | 2 | 0 | 1 | 1 | 1 | 1 | 1 | 1 |
| P value | 1 | | 1 | | 1 | | 0.658 | | 0.658 | | 0.658 | | 0.326 | | 1 | | 0.658 | | 0.315 | | 0.290 | | 0.717 | |

*Represent the significant difference at $p < 0.05$. NBF = Non-biofilm-former, WBF = Weak-biofilm-former, MBF = Moderate-biofilm-former, SBF = Strong-biofilm-former.

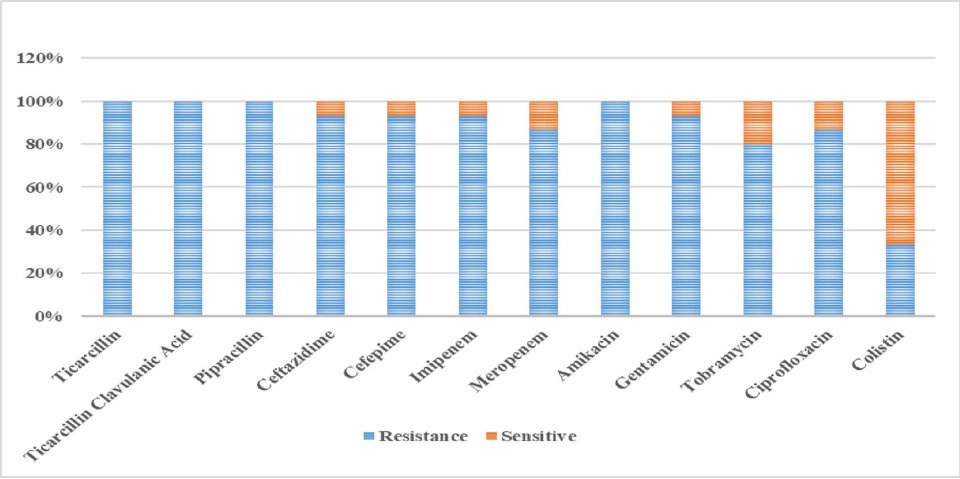


*Fig. 2. Antibiotic susceptibility of P. aeruginosa isolates.*

## 4. Discussion

The results of this study indicated that *P. aeruginosa* was highly isolated from urine with 6 isolates (40%) followed by ear infections with 5 isolates (33.3 %) and wounds with 4 isolates (26.7%). While there are no isolates were found in blood specimens. Al-Charrakh *et al*. [15] studied *P. aeruginosa* isolated from various clinical samples from public and private hospitals in Baghdad, Iraq, and found that 75 isolates belonged to *P. aeruginosa.* The isolates were distributed as follows: burn (22), ear (14), sputum (13), wound (7), urine (5), blood (5), nasal swab (4), eye (3), and biopsy (2). This indicates a higher rate of ear and wound isolates compared to this study and a lower rate of isolates from urine compared to this study. While [16] reported a high isolation rate of *P. aeruginosa* from wounds (60%) and ear infections (40%) compared to other infection sites (urine and burn). Results from study [17] revealed that the most common *P. aeruginosa* isolates were obtained from purulent specimens collected from skin wounds and burns, followed by isolates from urine and ear discharge specimens.

We found that 27% of *P. aeruginosa* was isolated from urine samples, 19.7% from ear infections, and only 13.5% from wounds. *P. aeruginosa* is the most common bacterial isolate in mild to severe from of external otitis and chronic supportive otitis media and wounds. While the lower percentage of isolates detected in wound samples may be due to *P. aeruginosa* not being the primary cause of urinary tract infection, and the causes may be other bacterial isolates. *P. aeruginosa* is a common cause of burns and wound inflammation because it thrives in humid and wet hospital environments [18]. Infections caused by *P. aeruginosa* is often facilitated by the breakdown of the body's physical barriers to infection, such as the skin or mucous membranes, or by a lack of immunity. This bacterium has minimal nutritional requirements and can tolerate a wide range of physical conditions, including temperatures up to 41°C [19]. Additionally, *P. aeruginosa* has been identified as a leading cause of middle ear infections, particularly chronic otitis media, which is characterized by infection and discharge.

Antibiotic susceptibility tests were conducted for all *P. aeruginosa* isolates. Alhayali *et al*., showed that a higher resistance of 42.5% was observed against Ticarcillin/Clavulanic acid and Netilmicin. The lowest 15% of the resistance was observed against Piperacillin/ Tazobactam. The resistance percentages for Amikacin, Aztreonam, Cefepime and Ciprofloxacin were 37.5%. Meropenem and Ceftazidime showed resistance rates of 35% and 32.5% with Imipenem. The isolates were resistant to Gentamicin and Levofloxacin at 30% and 10(25%) were resistant to 6 classes of antibiotics: aminoglycosides class (amikacin, tobramycin and gentamicin), cephalosporin's class (Cefepime, ceftazidime, and cefetriaxone), carbepenems class (imipenem and meropenem), fluoroquinolons class (norfloxacin, Ciprofloxacin), $\beta$- Lactamase inhibitors class (piperacillin, carbencillin). *P. aeruginosa* is recognized as one of the main causes of nosocomial infections (NIs). To combat the spread of this resistant microorganism and implement effective infection prevention measures, it is essential to monitor its susceptibility to antimicrobial agents among isolates [20].

Reza Haidari *et al*., demonstrated that over 50.0% of *P. aeruginosa* isolates were resistant to piperacillin

(59.8%), ceftazidime (59.8%), aztreonam (54.9%), tobramycin (52.0%), and gentamicin (51.0%). These findings are consistent with previous reports from Nepal [21], Iran [22], and Ethiopia [23]. However, other studies [24–26] reported lower resistance rates for ceftazidime and ciprofloxacin compared to our findings. These differences may stem from various factors such as the type and ethnicity of the population studied, the source of isolates, the methods used for measuring antibiotic susceptibility, the presence or absence of antibiotic consumption surveillance programs, surveillance of antibiotic use in agriculture, livestock, and other industries, as well as differences in regional epidemiology [27].

The tissue culture plate is considered the gold standard method for biofilm detection. TCP method is a more quantitative and reliable method for the detection of biofilm-forming microorganisms compared to other methods. In this study, some of the isolates were tested for their ability to form biofilm by the TCP method.

Reem *et al*., showed that all the isolates were tested for the ability to form biofilm by the TCP method. The results showed that 15 (68.2%) isolates exhibited strong biofilm formation, moderate biofilm was observed in 3 (13,6%) isolates, and 4 (18.2 %)isolates displayed weak or no biofilm formation. Saxena *et al.* [14] showed that the number of isolates which exhibited strong biofilm formations was (6.25%) while weak biofilm producers accounted for (60%). On the contrary, Nasirmoghadas *et al*. [28] revealed that the biofilm formation was observed in 92.4% of the isolates.

Another study conducted by Da Costa Lima *et al*., revealed that 75% of the isolates were biofilm producers. Biofilms are sticky populations of microorganisms enclosed by a matrix composed of self-secreted extracellular polysaccharides or slime. These biofilms act as effective barriers against antibacterial agents [29]. Biofilms are three-dimensional bacterial communities attached to living or nonliving surfaces. Biofilm development begins with the formation of microcolonies, which subsequently mature depending on factors such as bacterial cell density and nutrient availability.

The formation of biofilms is seen as a survival strategy to combat environmental stressors like pH, UV damage, H2O2, metal toxicity, and the human immune response to bacterial infection, including phagocytosis [30]. There is a growing understanding that many chronic infections are related to biofilms. In fact, the National Institutes of Health estimate that approximately 80% of medical bacterial infections treated by physicians in the developed world are caused by organisms growing in biofilms [31]. The presence of non-biofilm-producing isolates may be attributed to heterogeneity in bacterial origins such as genetic characterization, sources of isolates, and environmental conditions, as well as the absence of quorum sensing, which represents the initial steps in biofilm formation, or the absence of genes responsible for biofilm formation. Biofilm formation is a key survival strategy used by *P. aeruginosa thrives* in harsh environments, including exposure to antibiotic agents and host immune responses [32]. These sessile populations of microorganisms, enclosed by the self-secreted extracellular polysaccharide matrix, or slime, are typically more resistant to antibiotics compared to planktonic cells.

Analysis of biofilm formation potential identified almost all *P. aeruginosa* isolates as dominant biofilm formers. The statistical analysis examining the link between antibiotic resistance and biofilm formation showed that biofilm production in multidrug-resistant isolates was higher than in drug-susceptible isolates. In this study, a total of 15 isolates of *P. aeruginosa* were tested for their ability to produce biofilm. Out of these isolates, 2 (13.3%) exhibited strong biofilm formation, moderate biofilm was observed in 6 (40%) isolates, and 5 (33.3%) isolates displayed weak biofilm formation, with only 2 (13.3%) showing no biofilm formation (Table 2).

The familiar mechanisms of antibiotic resistance, such as efflux pumps, modifying enzymes, and target mutations, do not seem to be responsible for the protection of bacteria in a biofilm.

In accordance with the present results, a separate study [33] indicated that 96.2% of isolates, both multidrug-resistant (MDR) and non-MDR, had the ability to form biofilms. The same study also found that 58.6% of MDR clinical isolates were strong biofilm producers, indicating a significant correlation between MDR status and biofilm formation. However, previous studies from different regions of the world have shown a lower prevalence of biofilm formation and no association between biofilm production and antibiotic resistance [34]. This discrepancy may be attributed to other mechanisms, such as the presence of purines, plasmid acquisition, chromosomal mutation, and efflux pumps, involved in antibiotic resistance [35].

## 5. Conclusion

Based on the correlation of study findings, it was observed that biofilm formation was higher among isolates with a resistant phenotype. Resistance to antimicrobial agents and the ability to grow as a biofilm are the main problems in the treatment of infections triggered by P. *aeruginosa*.

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