Exploring the Tolerance of Multidrug Resistant Pseudomonas Aeruginosa to Cefotaxime: Risk Factors and Implications

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**Abstract :**Multidrug-resistant bacteria, prevalent in healthcare settings, pose significant challenges due to their resistance to multiple antimicrobial agents. The purpose of the study was to investigate biochemical properties of *Pseudomonas aeruginosa* and evaluate its hemolytic activity, lipid peroxidation levels, and the anti-biofilm effects of Cefotaxime. Biochemical analysis confirmed key characteristics of *P. aeruginosa* including motility, non-lactose fermentation, urease production, and positive oxidase and catalase activities, reflecting the bacterium's metabolic versatility. Hemolysis assays demonstrated a concentration-dependent cytotoxic effect, with 54% hemolysis observed at 250 µg/mL and complete hemolysis 100% at 1000 µg/mL, indicating significant membrane disruption. Lipid peroxidation analysis, which served as an indicator of oxidative stress, revealed an increase in peroxidation rates as Cefotaxime concentration increased, ranging from 47% at 250 µg/mL to 100% at 1000 µg/mL. Cefotaxime also exhibited potent antibiofilm activity, effectively inhibiting biofilm formation within 24 hours and reducing biofilm-associated bacterial loads over 48 hours. Although Cefotaxime showed promising antibiofilm effects, overcoming biofilm-associated multidrug resistance in *P. aeruginosa* is critical to support of further therapeutic applications particularly in preventing infections on medical devices where biofilm formation poses a persistent threat.

**Keywords:** Multidrug-resistant; Pseudomonas aeruginosa; Hemolysis assay; Biofilm

# Introduction

Multidrug-resistant (MDR) bacteria, commonly associated with healthcare environments, present a major health challenge [(N. R. Kumar et al., 2024)](https://paperpile.com/c/tU1JXi/rc3Dn). MDR bacteria are characterized by their resistance to an antimicrobial drug from various classes of antimicrobial compounds [(El Zowalaty et al., 2015; Niveditha et al., 2021)](https://paperpile.com/c/tU1JXi/DMULk+rhYqZ). The excessive and improper utilization of antibiotics in healthcare has driven the rise of multidrug-resistant bacteria, leading to infections acquired in hospitals [(Riley, 2019)](https://paperpile.com/c/tU1JXi/491N1). Multidrug-resistant (MDR) infections are rapidly evolving into a worldwide healthcare concern [(Morris & Cerceo, 2020)](https://paperpile.com/c/tU1JXi/Ivuzi). Identified a resistance to multiple antimicrobial agents in vitro susceptibility tests, with MDR Gram-positive and negative bacteria commonly defined as resistant to various bactericidal agents [(Breijyeh et al., 2020)](https://paperpile.com/c/tU1JXi/GWVd2)[(G. & Ganapathy, 2022; I. L. Kumar & Ramesh, 2021)](https://paperpile.com/c/tU1JXi/Q7G9i+dleHT)). Infections caused by multidrug resistant gram negative bacteria cause serious public health issues worldwide, with certain regions regions are frequently reporting *Pseudomonas aeruginosa* strains that exhibiting multidrug resistance [(Al-Orphaly et al., 2021; Ravella Venkatasubramanyam et al., 2024)](https://paperpile.com/c/tU1JXi/5HUX8+HyE27). *Acinetobacter baumannii* and *Pseudomonas aeruginosa* which are resistant to multiple drugs, persist in hospitals and are easily spread through healthcare workers' hands, making them a major concern [(Weinberg et al., 2020)](https://paperpile.com/c/tU1JXi/LAAvq)[(*Evaluation Composite Restoration Posterior Teeth Proanthocyanidin Pretreatment Liner Using Fédération Dentaire Internationale Criteria: Split-Mouth Randomized Controlled Trial*, n.d.; Pranati et al., 2021; Sakthi et al, 2021)](https://paperpile.com/c/tU1JXi/O3YOT+N0aWq+ZHp67). A common nosocomial *Pseudomonas aeruginosa* is often resistant to various drugs, with prevalence rates consistently varying [(Recio et al., 2020)](https://paperpile.com/c/tU1JXi/Do79G). *P.aeruginosa* comprises a group of bacteria with significant antibiotic resistance, often leading to hospital-acquired infections, particularly in immunocompromised [(F. Hussein, 2024)](https://paperpile.com/c/tU1JXi/Q2JF4). Resistance in *P. aeruginosa* is difficult to treat and is defined as resistance to all of the following antibiotics ceftazidime, cefotaxime and ciprofloxacin [(Karruli et al., 2023)](https://paperpile.com/c/tU1JXi/Pgs0y). *Pseudomonas aeruginosa* causes life-threatening infections and has developed resistance to numerous antibiotics, primarily due to the overuse and misuse of these medications [(Jawad, 2016)](https://paperpile.com/c/tU1JXi/0DlCk). The porin protein in *Pseudomonas aeruginosa* serves as the main entry route for carbapenems into the cell and contains a binding site-specific antibiotic [(Kohler et al., 2019)](https://paperpile.com/c/tU1JXi/NHshR). Cefotaxime, a third-generation cephalosporin, is widely used in human medicine to treat severe bacterial infections like meningitis [(Cabellos et al., 2022)](https://paperpile.com/c/tU1JXi/bMRH). Cefotaxime short half-life of around hours following its conversion to deacetyl-cefotaxime, allowing for dosing intervals of periods of six hours[(Dallefeld et al., 2019)](https://paperpile.com/c/tU1JXi/bF9Ib)[(Keerthana & Ramesh, 2021; Murugesan, 2021; Tiwari & Jain, 2021)](https://paperpile.com/c/tU1JXi/zVpdg+X3LTg+mNFNB)[(Keerthana & Ramesh, 2021; Murugesan, 2021; Subramanian et al., 2021; Tiwari & Jain, 2021)](https://paperpile.com/c/tU1JXi/zVpdg+X3LTg+mNFNB+RgqSQ). When intravenous ceftriaxone is administered with calcium containing solutions in neonates, calcium ceftriaxone precipitates can form in the lungs and kidneys [(Donnelly et al., 2017)](https://paperpile.com/c/tU1JXi/AV5RZ)[(Ajay et al., 2023; Chokkattu et al., 2023; Padarthi et al., 2023)](https://paperpile.com/c/tU1JXi/KJR4f+WStbi+0ZvCh). Cefotaxime is commonly chosen to treat infections such as UTIs, and soft tissue infections, especially when penicillin is ineffective or inappropriate[(Lupia et al., 2022; Noufal et al., 2021)](https://paperpile.com/c/tU1JXi/djTsF+iQS8N). Cefotaxime is widely used for its broad-spectrum activity and is recommended as an empiric or first-line treatment for severe infections according to the official recommendations for antimicrobial use in infectious disease, issued by the government, provide essential treatment protocols [(Gondane & Pawar, 2023)](https://paperpile.com/c/tU1JXi/joTVn).

# Materials and methods

The isolation of *P. aeruginosa* to use MacConkey agar to differentiate *P. aeruginosa* from oral pathogen samples. The pathogens were re-streaked on the same agar medium to identify the pathogen. Incubation of inoculated culture media at 45°C selects for thermophilic *P. aeruginosa* strains.

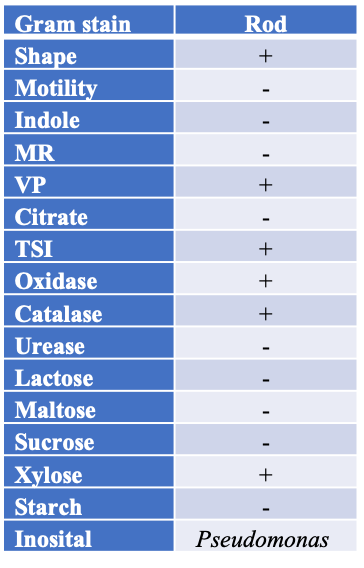
*Pseudomonas aeruginosa* isolates were identified using traditional biochemical methods, including the IMViC tests, as outlined in Bergey’s Manual [(Buchanan and Gibbons., 1975)](https://www.scirp.org/reference/referencespapers?referenceid=2224276). These tests were used to assess several key characteristics, including indole production, methyl red test, catalase activity, citrate utilization, Voges-Proskauer test, hydrogen sulfide (H₂S) production, lactose fermentation, urease activity, and inositol fermentation. Additionally, tests for the utilization of starch, sucrose, xylose, and maltose, as well as triple sugar iron (TSI) agar, were performed.The in vitro hemolytic activity was assessed using a modified spectrophotometric method [(Dathe et al., 1998)](https://paperpile.com/c/tU1JXi/6reZw). Human red blood cells were incubated with serial dilutions of the bacterial isolates, and the cells were washed multiple times using phosphate-buffered saline (PBS) through centrifugation at 3,000 rpm for 3 minutes. After washing, the optical density of the supernatant was compared to that of the PBS control. The red blood cells were counted using a hemocytometer, and hemolytic activity was measured by incubating the cells at room temperature for 1 hour with bacterial concentrations of 250 µg/ml and 1000 µg/ml. After incubation, the samples were centrifuged at 10,000 g for 5 minutes. The absorbance of the supernatant was then measured at 570 nm to determine the extent of hemolysis.For bacterial strain growth, *P. aeruginosa* was cultured in suitable media and incubated at 35°C for 24 hours. The cell mass was collected and transferred to polypropylene tubes, with a control maintained under similar conditions without treatment for comparison. After incubation, the bacterial suspension was centrifuged at 15,000 rpm for 5 minutes at 4°C to separate the supernatant. The resulting cell pellets were washed with sterile distilled water, and the wet weight of the pellet was recorded.

To assess lipid peroxidation, thiobarbituric acid-reactive substances (TBARS) were quantified using a procedure that involved grinding 1.0 g of the cell pellet and mixing it with 20 ml of 0.1% TCA solution. The mixture was then centrifuged at 10,000 rpm for 15 minutes. A 1 ml aliquot of the supernatant was combined with 4 ml of a 20% TCA solution containing 0.6% thiobarbituric acid and incubated at 95°C for 30 minutes. After rapid cooling on ice, the sample was centrifuged again at 10,000 rpm for 10 minutes. The absorbance of the supernatant was measured at 532 nm using a UV-Vis spectrophotometer [(Na et al., 2002)](https://paperpile.com/c/tU1JXi/PnoUl).  *P. aeruginosa*, at a density of 104cells/ml, was cultured in six-well plates to form a biofilm over 24 hours. The biofilm was then exposed to cefotaxime for 12 to 48 hours, followed by staining with propidium iodide and acridine orange to differentiate live and dead cells after 48 hours[(Shinde et al., 2021)](https://paperpile.com/c/tU1JXi/OIDzX).

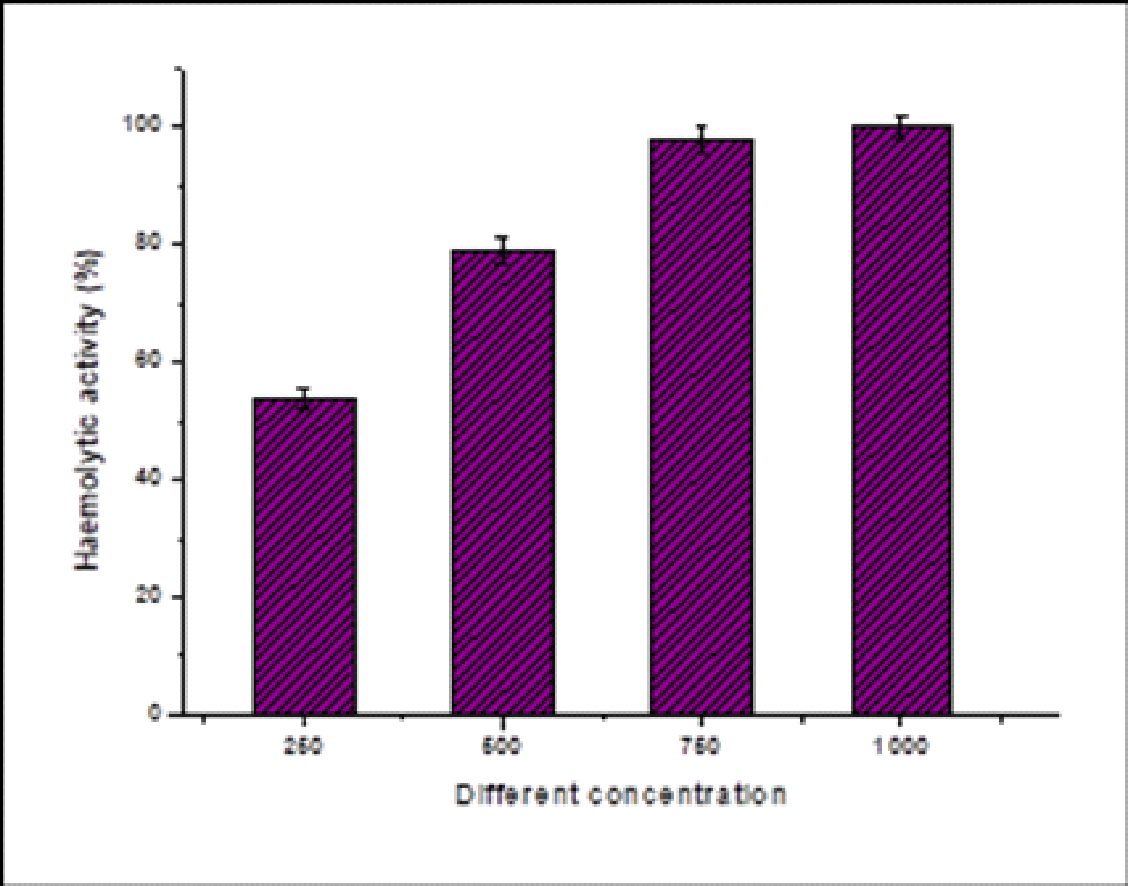
# Results

*Pseudomonas* species are a group of bacteria known for their high rates of antibiotic resistance and are commonly associated with hospital-acquired infections(Nikalje et al., 2024) (Chehelgerdi et al., 2023). The organism is characterized as a Gram-negative, rod-shaped bacterium with motility, indicative of flagellar presence. Biochemical tests reveal that the pathogen does not produce indole, and it yields negative results for both the MR and VP tests. The absence of mixed-acid fermentation and acetoin production from glucose. Positive citrate utilization organism's ability to utilize citrate as the sole carbon source. Additionally, the pathogen exhibits positive reactions for both oxidase and catalase, confirming its involvement in aerobic respiration and hydrogen peroxide decomposition. Urease activity is also positive, indicating the bacterium capacity to hydrolyze urea to ammonia and carbon dioxide. The pathogen does not ferment lactose, maltose, sucrose, or xylose. Hydrolyze starch, as evidenced by a positive starch hydrolysis test. The Triple Sugar Iron (TSI) test results are negative, reflecting a lack of fermentation of the tested sugars and the production of hydrogen sulfide. The inositol utilization test also yielded a negative result (Table 1).

**Table1.** Biochemical identification of pathogen

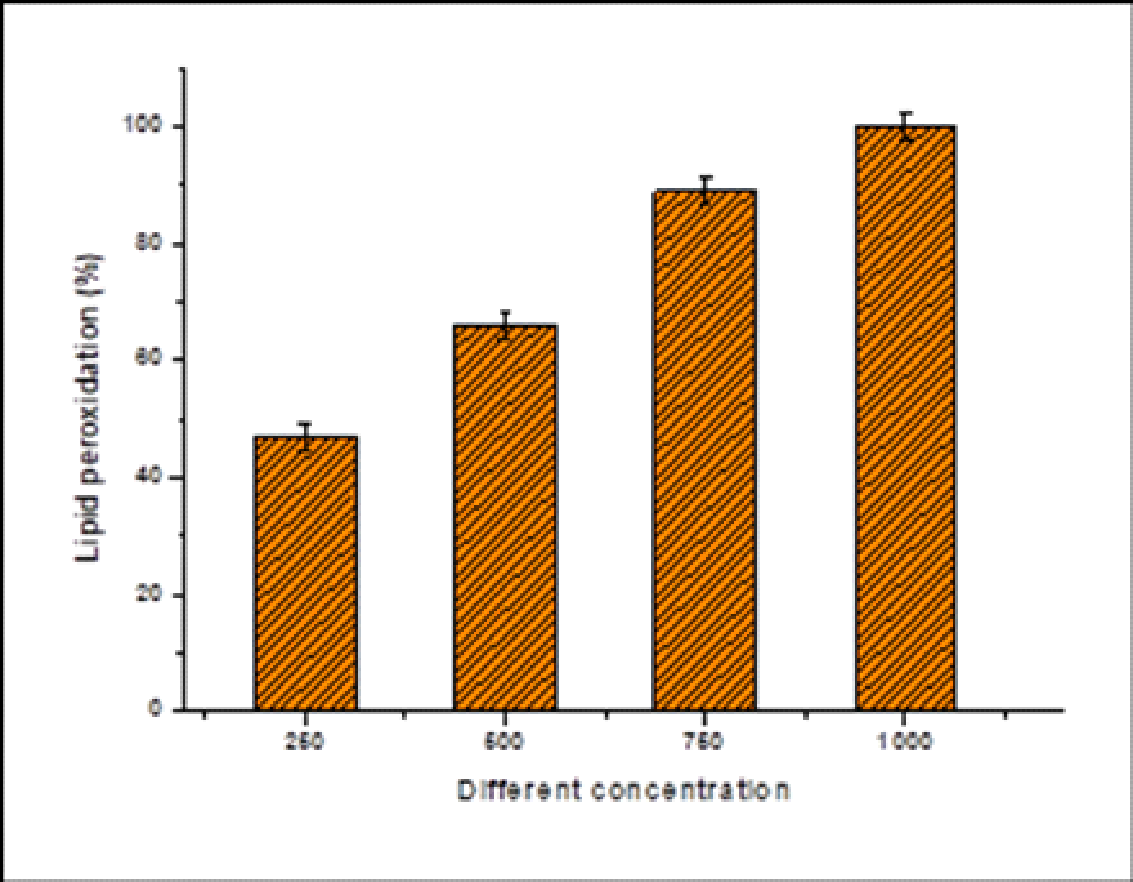


The haemolytic activity was performed to determine the toxicity of the cell free extract of the pathogen *P. aeruginosa*. Cell lysate against *Pseudomonas aeruginosa* across a range of concentrations at 250 µg/ml, the lysate caused 54% haemolysis, indicating moderate disruption of the bacterial cell membranes. At the maximum concentration of 1000 µg/ml, the lysate induced full haemolysis, achieving 100% activity (Fig. 1). These results demonstrate the potent haemolytic capabilities of the cell lysate against *Pseudomonas aeruginosa*, with the extent of membrane disruption directly proportional to the toxin of pathogen concentration increases the cell red blood cells are lysate.



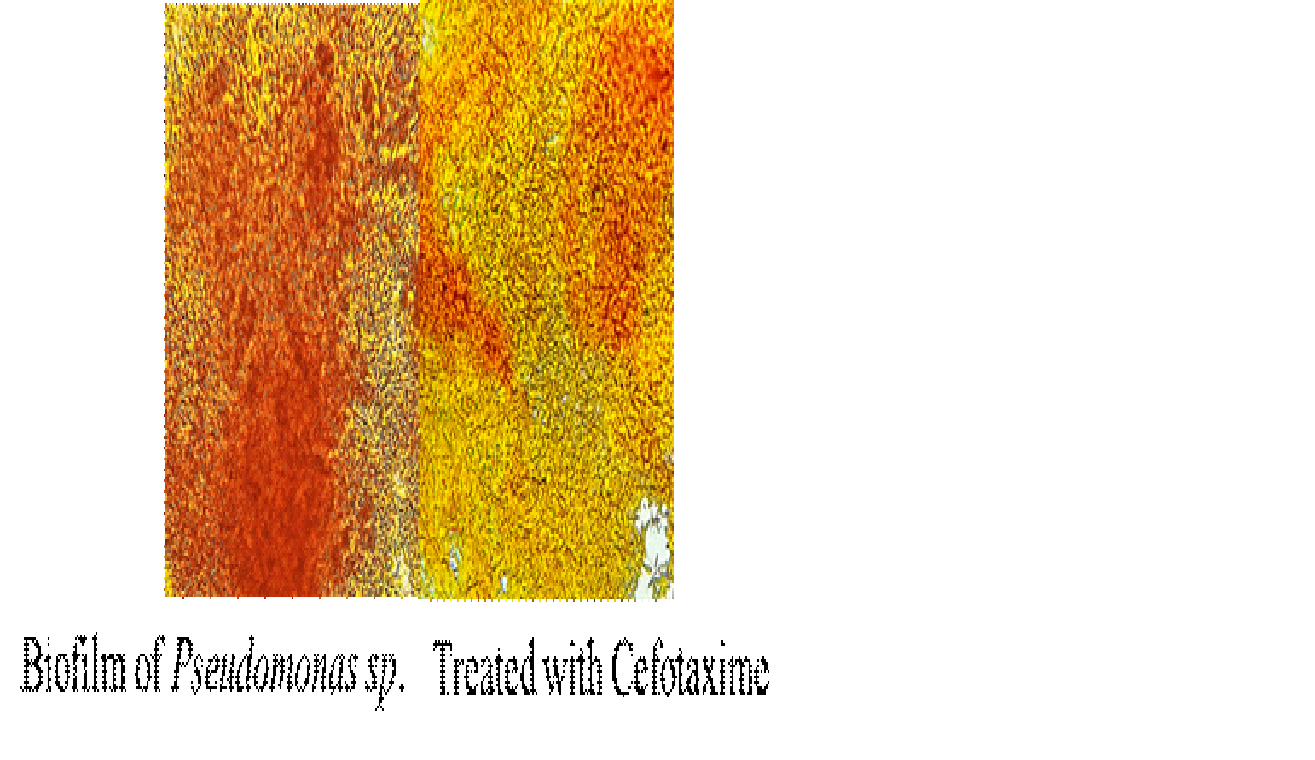
**Figure 2.** Haemolytic activity with different concentration of cefotaxime.

The study examined the impact of an antibiotic on lipid peroxidation in cell lysates at different concentrations: 250, 500, 750, and 1000 µg/mL. Results indicated that lipid peroxidation, a marker of oxidative stress, increased as the antibiotic concentration rose. Specifically, lipid peroxidation was 47% at 250 µg/mL, 66% at 500 µg/mL, 89% at 750 µg/mL, and reached 100% at 1000 µg/mL. These findings suggest a concentration-dependent relationship, where higher doses of the antibiotic lead to greater oxidative stress and potential cellular damage in the lysates.



**Figure 2.** Lipid peroxidation activity with different concentration of cefotaxime.

The present study demonstrates that Cefotaxime has a bactericidal action against *P. aeruginosa*, particularly in terms of biofilm inhibition. It can inhibit bacterial biofilms within 24 hours and decrease the number of *P. aeruginosa* in biofilms after Cefotaxime (100μg/ml) treatment (Fig. 2). Furthermore, Cefotaxime has been shown to effectively inhibit biofilms that form on the surface of medical devices and cause infection.



**Figure 3**. Biofilm activity *pseudomonas sp.* with treated cefotaxime.

# Discussion

The various carbohydrate fermentations and inositol utilization *Pseudomonas* pathogen as a species[(Dharman et al., 2023; S. Sindhu et al., 2023; Sreenivasagan et al., 2023)](https://paperpile.com/c/tU1JXi/LWJzl+OAyfI+eME3N). Previous study *Pseudomonas aeruginosa* isolates underwent a series of biochemical tests, including oxidase-positive, catalase as indicated by the rapid emergence of purple colour which turned dark in 10 seconds, confirming the production of oxidase [(Al-Bayati et al., 2021)](https://paperpile.com/c/tU1JXi/6qapr). Isolates were confirmed to be catalase-positive, exhibiting the efficacy to produce the enzyme catalase[(Al-Fridawy et al., 2020)](https://paperpile.com/c/tU1JXi/nkB98). Among all isolates, only L-2644 showed β-hemolytic activity across both blood types and at both tested temperatures [(Mogrovejo et al., 2020)](https://paperpile.com/c/tU1JXi/DOgy5). It binds to the host's red blood cells and forms pores in the membrane, disrupting cellular balance and causing cell death. [(Santagostino et al., 2021)](https://paperpile.com/c/tU1JXi/225sK). The hemolytic activity observed in the tested compounds was primarily determined by the cation structure, whereas the anion type had minimal impact on their hemotoxicity [(Ermolaev et al., 2021)](https://paperpile.com/c/tU1JXi/eWDig). Lipid peroxidation is a key stress response in bacteria, and Withaferin A counters *P. aeruginosa* by reducing ROS, lipid damage, inflammation, and boosting antioxidants in zebrafish [(Murugan et al., 2022)](https://paperpile.com/c/tU1JXi/dL10p).The formation of a strong biofilm is important for *Pseudomonas aeruginosa* to persist, outcompete other microbes, and maintain dominance in the diverse microbial community found in the lungs of individuals with cystic fibrosis (Oluyombo et al., 2019). *Pseudomonas aeruginosa* can colonize various surfaces, including medical devices, contact lenses, and larger equipment in the food industry [(Coughlan et al., 2016)](https://paperpile.com/c/tU1JXi/HdDsk). The molecular mechanisms that contribute to bacterial tolerance to antimicrobials within biofilms, is essential for creating effective methods to manage, prevent, and eradicate infections associated with biofilms[(Ramakrishnan et al., 2023; Shenoy & Maiti, 2023; J. S. Sindhu et al., 2023)](https://paperpile.com/c/tU1JXi/ED99r+LdLON+tAnCF).Gram-negative bacteria possess two membrane layers, which contribute to their heightened resistance to pollutants compared to Gram-positive bacteria Imron et al. According to [(Kamali et al., 2020)](https://paperpile.com/c/tU1JXi/RuGmc), *Pseudomonas aeruginosa* strains that produced biofilm were predominantly classified as non-multidrug resistant (non-MDR)[(Kasabwala et al., 2021; Rajeshkumar & Lakshmi, 2021; Varghese et al., 2023)](https://paperpile.com/c/tU1JXi/y4O0U+89pd1+QeTtX). Biofilm-forming *Pseudomonas aeruginosa* are significant in pathogenesis because the biofilm limits antibiotic penetration, making it difficult to eliminate the bacteria from surfaces [(Abdulhaq et al., 2020)](https://paperpile.com/c/tU1JXi/FHEaw). The biofilm antimicrobial resistance was tested by cultivating the bacterial strain for 72 hours in a polystyrene flow-cell chamber[(Wu et al., 2020)](https://paperpile.com/c/tU1JXi/AESgw). Biofilm formation was assessed under shaking and static conditions that mimic the urinary bladder environment, but no notable differences were found between the groups of isolates[(Desai et al., 2021)](https://paperpile.com/c/tU1JXi/JMXYQ).

# Conclusion

This study highlights the resilience and pathogenicity of *P. aeruginosa*, known for its antibiotic resistance and capable of forming biofilms. Biochemical analysis confirmed its metabolic versatility and virulence factors, such as hemolytic activity and the induction of oxidative stress, which contribute to its survival and damage to host cells. The biofilm-forming capacity of *P. aeruginosa* on medical devices poses a significant challenge in treatment, as biofilms protect the bacteria from antibiotic action. Although Cefotaxime showed effectiveness in inhibiting biofilm formation, the study underscores the critical need for innovative approaches to manage and prevent persistent *P. aeruginosa* infections, particularly in healthcare settings.

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