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EVALUATION OF BIOFILM PRODUCTION IN *PSEUDOMONAS AERUGINOSA* FROM CYSTIC FIBROSIS PATIENTS

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Abstract

Introduction: Cystic fibrosis is a multisystemic autosomal recessive disease -causing viscous secretions that predispose patients to chronic infections. *Pseudomonas aeruginosa*, a common pathogen in CF patients, possesses the ability to form biofilms, a key factor contributing to antimicrobial resistance and persistence

Aim: This study aimed to phenotypically analyze and compare biofilm formation in 48 *P. aeruginosa* isolates from CF and non-CF patients.

Material and Methods: Isolates were identified using conventional methods and the Vitek-2 system. Biofilm production was assessed using a quantitative colorimetric assay.

Results: Biofilm production was confirmed in 46% (22/48) of isolates. Among biofilm producers, 86% (19/22) originated from CF patients versus 13.6% (3/22) from controls, though this difference was not statistically significant (p=0.466), likely due to limited sample size. Mucoid strains comprised 28% of CF isolates; 73% (8/11) of these produced biofilms compared to 39% (11/28) of non-mucoid strains (p=0.145). Biofilm strength was distributed as strong (13%), moderate (36%), and weak/non-producer (51%), with no significant difference between groups (p=0.804).

Conclusion: Despite the lack of statistical significance, the high proportion of biofilm producers among CF isolates, particularly mucoid phenotypes, aligns with established literature on *P. aeruginosa* pathogenesis. These findings underscore the clinical relevance of biofilm production in CF infections and support the need for larger studies to confirm these associations.

Keywords: Biofilm, *Pseudomonas aeruginosa*, mucoid, non-mucoid, CF.

Introduction

Cystic fibrosis (CF) is the most prevalent life-limiting autosomal recessive disorder among Caucasian populations, affecting an estimated 80,000 individuals globally. Its incidence varies significantly based on the ethnic and geographic origin of affected individuals [1].

Cystic fibrosis is caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, which encodes a protein functioning as a chloride channel essential for regulating chloride and sodium transport across epithelial membranes.

These mutations result in a dysfunctional channel. This dysfunction impairs mucociliary clearance in the airways, leading to persistent bacterial colonization, chronic inflammation, and progressive lung damage. Respiratory failure resulting from this damage is the primary cause of mortality in CF. Similarly, thickened secretions in the pancreas, gastrointestinal tract, hepatobiliary system, and reproductive tract cause pancreatic insufficiency, malabsorption, and male infertility [2].

While advances in newborn screening and multidisciplinary care, including CFTR modulator therapies, have markedly improved life expectancy, pulmonary disease remains the leading cause of morbidity and mortality.

A defining hallmark of CF pathology is chronic polymicrobial infection within the viscous airway mucus. Opportunistic pathogens such as *Staphylococcus aureus* (often establishing early colonization) and *Pseudomonas aeruginosa* (typically dominant in adolescence and adulthood) are commonly involved [3]. *P. aeruginosa* is of particular concern due to its exceptional adaptability and propensity to form biofilms, structured microbial communities encased in a protective extracellular matrix.

This biofilm mode of growth enhances tolerance to antibiotics and host defenses by reducing antimicrobial penetration, altering bacterial metabolism, and the emergence of persister cells [4]. Consequently, biofilm-associated infections significantly accelerate lung function decline in CF and reduced survival.

Given this context, understanding biofilm dynamics in *P. aeruginosa* is crucial for refining diagnostics, optimizing treatment, and developing new therapeutic strategies to manage chronic infections in CF patients.

Aim of the study

The aim of this study was to phenotypically analyze and compare biofilm formation in *P. aeruginosa* isolates recovered from cystic (CF+) and non-cystic fibrosis (CF-) patients.

Materials and methods

Bacterial Isolates

Forty-eight clinical isolates of *Pseudomonas aeruginosa* were analyzed, comprising 39 from cystic fibrosis (CF) patients and 9 from non-CF controls. Isolate identification was performed using standard microbiological methods (culture on blood agar, oxidase testing, and pigment production) and confirmed with the Vitek-2 automated system (bioMérieux, France).

Biofilm quantification assay

Quantitative biofilm formation was assessed using a standardized colorimetric microtiter plate assay, as previously described by Stepanović et al. [5], with minor adaptations. Briefly, pure colonies of each isolate were suspended in sterile physiological saline and adjusted to a 0.5 McFarland turbidity standard.

The suspension was vortexed for at least one minute and then diluted 1:100 in 200 µL tryptic soy broth (TSB) supplemented with 1% glucose. A 200 µL aliquot of this dilution was inoculated into individual wells of a sterile, flat-bottomed 96-well polystyrene microplate.

Plates were incubated statically for 24 hours at 35–37°C. Following 24 h of incubation at 37°C, the planktonic cells and non-adherent material were removed by gently washing each well three times with sterile phosphate-buffered saline (PBS, pH 7.3). Adherent biofilms were fixed with 99% methanol for 15 minutes, the methanol was removed, and the plates were air-dried. Biofilms were then stained with 200 µL of a 0.9% crystal violet solution (Sigma-Aldrich) for 15 minutes at room temperature. Excess stain was rinsed off with water, and the plates were air-dried again. Bound crystal violet was solubilized by adding 200 µL of 95% ethanol to each well for 30 minutes.

Biofilm formation was quantified by measuring the optical density (OD) of the destained solution at 570 nm using a microtiter plate reader (BioTek, Germany). All experiments were performed in triplicate and repeated three times.

Data analysis and classification

The mean OD value for each tested strain and for negative control wells (containing sterile medium only) was calculated. The cut-off value (ODc) for each plate was defined as three standard deviations above the mean OD of the negative controls. For each microtiter plate the cut–off value was determined. Based

on their mean OD, isolates were categorized into four groups for interpretive analysis: Non-biofilms producer: OD < ODc, Weak biofilm producer: ODc ≤ OD < 2 × ODc, Moderate biofilm producer: 2 × ODc ≤ OD < 4 × ODc, Strong biofilm producer: OD ≥ 4 × ODc.

Statistical analyses were performed using R software (version 4.3.2). Categorical variables were compared using Fisher's exact test for 2×2 contingency tables due to the relatively small sample sizes in some subgroups.

The Mann-Whitney U test was employed to compare ordinal biofilm strength categories (strong, moderate, weak/none) between CF and non-CF groups. A two-tailed p-value of <0.05 was considered statistically significant. Given the exploratory nature of the study and modest sample sizes, effect sizes and confidence intervals were also considered in interpreting the clinical relevance of observed trends.

Results

A total of 48 clinical isolates of *Pseudomonas aeruginosa* were analyzed in this study. Thirty-nine isolates originated from CF patients, and 9 isolates were from non-CF controls. Of these, 25 (52%) were from males and 23 (48%) were from female patients. According to age, 21% of patients were in the age group 0-10 years, 28% were in the age group 11-20 years, 33% were in the age group 21-30 years and 18% were older than 30 years. Of the 48 bacterial strains tested, 26/48 (54%) were non-biofilm producers. Biofilm phenotypes were confirmed in 46% of *P. aeruginosa* isolates (22/48). From those which were biofilm producers, 19/22 (86%) were from CF patients, and 3/22 (13.6 %) from the control group (Table 1).

Table 1. Production of biofilm in mucoid and non-mucoid isolates of *Pseudomonas aeruginosa* from CF patients.

Organism	**Biofilm (+)**	**Biofilm (-)**
	Pseudomonas aeruginosa **N (%)**	***Pseudomonas aeruginosa*** **N (%)**
Mucoid strains of *Pseudomonas aeruginosa* (11)	8 (73%)	3 (27%)
Non-mucoid strains of *Pseudomonas aeruginosa* (28)	11 (39%)	17 (61%)
Total	19 (49%)	20 (51%)

Mucoid strains of *P. aeruginosa* were confirmed in 28% (11/39). Biofilm production was more frequent in mucoid *P. aeruginosa* - 73% (8/11), compared to non-mucoid strains-39% (11/28). Out of these 22 biofilms producing strains, 19 strains were from CF patients, and 3 strains were from the control group. Biofilm phenotypes were distributed in the following categories: 13% (n = 5) produced strong biofilm; 36% (n = 14) produced moderate biofilm; 51% (n = 20) produced weak biofilm or were identified as non-biofilm producer (Table 2).

Table 2. Quantification of biofilm formation.

Biofilm formation	**CF patients isolates**	**Control group**
	Pseudomonas aeruginosa N (%)	*Pseudomonas aeruginosa* N (%)
Strong	5 (13%)	1 (11%)
Moderate	14 (36%)	2 (22%)
Weak/None	20 (51%)	6 (67%)
Total	39 (100%)	9 (100%)

Statistical analysis of biofilm production patterns

Statistical analysis revealed trends in biofilm production patterns, though formal significance was not reached in all comparisons. Biofilm production was more frequent in CF isolates (86.4% of biofilm producers) compared to non-CF controls (13.6%), though this difference did not reach statistical significance (Fisher's exact test, $p = 0.466$). Similarly, mucoid strains showed higher biofilm production (73%) compared to non-mucoid strains (39%), with a trend toward significance ($p = 0.145$).

Comparison of biofilm strength categories between CF and non-CF groups using the Mann-Whitney U test showed no significant difference ($U = 183.5$, $p = 0.804$). A chi-square test of association between patient group (CF vs. non-CF) and biofilm strength category (strong, moderate, weak/none) also showed no significant association ($\chi^2 = 0.825$, $df = 2$, $p = 0.662$) (Table 3).

Table 3. Statistical analysis of biofilm production patterns.

Comparison	Test used	p-value	Statistical significance	Notes
CF versus non-CF (biofilm production)	Fisher's exact	0.466	No ($p > 0.05$)	Trend: 86% vs. 13.6%
Mucoid versus non-mucoid (CF isolates)	Fisher's exact	0.145	No ($p > 0.05$)	Trend: 73% vs. 39%
Biofilm strength (CF vs. non-CF)	Mann-Whitney U	0.804	No ($p > 0.05$)	$U = 183.5$
Association: group × strength category	Chi-square	0.662	No ($p > 0.05$)	$\chi^2 = 0.825$, $df = 2$

Figure 1. Formation of biofilm by *P. aeruginosa* demonstrated by TCP method

Despite the lack of statistical significance in these comparisons, the observed trends align with established clinical patterns and likely reflect limited statistical power due to the modest sample size, particularly in the non-CF control group ($n = 9$).

Discussion

The findings of our study confirm that *Pseudomonas aeruginosa* are major contributors to chronic respiratory infections in CF, with significant implications of biofilm production. The majority of our CF patients were in the 10-30 years' age groups (28% + 33% = 61%). This aligns with the epidemiology of chronic *P. aeruginosa* infection in CF, which is often acquired in childhood and adolescence and persists into adulthood. Consistent with previous reports *P. aeruginosa* was more common in later age groups (33% in the age group above 20 years) [6].

No significant gender differences were observed in colonization/infection patterns in our patients (49% was detected in women, and 50% in men). Regarding the gender, Chotirmall et al. demonstrated that estrogen induces mucoid conversion of *P. aeruginosa*, a more antibiotic resistant form of the bacteria, and showed an association between increased exacerbations and mucoid conversion in women with CF during high estrogen states [7].

Many studies demonstrated that biofilm production was associated with bacterial virulence and antimicrobial resistance, correlating with poorer clinical outcomes [8, 9].

Our phenotypic analysis revealed notable trends in biofilm production, with 86% (19/22) of biofilm producers originating from CF patients and only 13.6% (3/22) from non-CF controls. Although this directional difference aligns with established literature, formal statistical testing using Fisher's exact test did not yield a significant p-value (Fisher's exact test, $p = 0.466$), likely reflecting the modest sample size of the non-CF control group ($n = 9$) and consequent limited statistical power. Similarly, biofilm production was more frequent in mucoid *P. aeruginosa* (73%) compared to non-mucoid strains (39%), a trend that also did not reach statistical significance (Fisher's exact test, $p = 0.145$).

Despite the lack of formal statistical significance, these trends are clinically and biologically meaningful, as they strongly mirror the well-documented adaptation of *P. aeruginosa* to the CF lung environment, where biofilm formation and mucoid conversion are key survival strategies. Comparison of biofilm strength categories between CF and non-CF isolates using the Mann-Whitney U test also showed no significant difference ($U = 183.5$, $p = 0.804$), further underscoring the need for larger, adequately powered studies to confirm these phenotypic distributions.

Based on our observed proportions, a post-hoc power analysis suggests that a larger cohort would be required to confirm these associations with statistical confidence, or a sample of approximately 60-70 total isolates would be needed to achieve 80% power at $\alpha = 0.05$.

Consistent with these trends, results of our study demonstrated that 86% of biofilm producers were from CF patients, compared to Perez and collaborators who detected 71.4% biofilm (+) *P. aeruginosa* in CF [10]. According to Hoiby et al., chronic lung infections in CF patients is caused by biofilm-growing mucoid strains of *P. aeruginosa*. Høiby et al. demonstrated that *P. aeruginosa* has a greater ability to produce biofilm in the context of CF [11].

This indicates a strong association between biofilm formation and the CF lung environment. This is a highly consistent and well-documented phenomenon, because the CF lung provides a unique selective pressure. This is related to the phenomenon of persistence, due to cells that exhibit tolerance to many drugs, and are called persister cells [12].

Persister cells play an essential role in biofilm-associated infections There are many studies showings that chronic CF isolates evolve from acute, planktonic forms towards a biofilm-dominated lifestyle as a survival strategy against antibiotics and host immune defenses (neutrophils) [13].

Research by Bjarnsholt et al. has extensively documented this in-vivo biofilm mode of growth in CF airways [14]. Regarding the prevalence, a study by Waters et al. found that up to 70-80% of chronic CF *P. aeruginosa* isolates exhibited strong biofilm-forming capacity in vitro, significantly higher than in environmental or strains causing acute infection [15].

Our findings of 46% overall biofilm production (54% in CF sub-population) aligns with this range, considering the mix of chronic and potentially newer isolates. Still, these percentages depend on the in vitro

method we used (crystal violet), but in vivo, probably the story is more complex and differs from in vitro conditions. In vitro data in our study support the in vivo biofilm paradigm but may not capture the full complexity of biofilm structures in the CF lungs environment. Regarding the mucoid strains, the correlation between mucoid, strong biofilm, and chronic infection is also a temporal one.

We can hypothesize that our strong/mucoid biofilm producers likely came from patients with longer-term chronic infection. In order to support this hypothesis, a longitudinal study would be needed in future. From the non-CF control group of *P. aeruginosa*, 13.6 % (3/22) were biofilm producers, and 33% (3/9) biofilm producers of the total isolates in this group.

The low biofilm production in non-CF controls is expected. We should highlight that these strains are likely "wild-type" or acute infection strains that haven't undergone the adaptive evolution seen in the CF lungs environment. Regarding the prevalence of mucoid phenotype and its link to biofilm production, we demonstrated that 28% (11/39) of CF isolates were mucoid. Biofilm production was more frequent in mucoid phenotypes of *P. aeruginosa* - 73% (8/11), strongly associated with chronicity, compared to non-mucoid strains-39% (11/28).

This is a critical and expected correlation. The mucoid phenotype, caused by overproduction of the exopolysaccharide alginate, is a classic marker of chronic CF infection. Alginate is a key structural component of biofilms, providing protection and stability of the bacteria inside the biofilm [16]. Landmark work by Govan and Deretic established the genetic basis for this phenotype [17].

Among biofilm producers, biofilm phenotypes were classified in the following categories: 13% (n = 5) produced strong biofilm; 36% (n = 14) produced moderate biofilm; 51% (n = 20) produced weak biofilm or were identified as non-biofilm producers. Multiple studies confirm that mucoid isolates consistently form stronger, more robust biofilms than non-mucoid variants. Our data showing a doubling of biofilm production frequency in mucoid strains perfectly mirrors this established principle.

The fact that not all mucoid strains were biofilm producers in our assay is also common, as in vitro conditions can vary and other regulatory factors, like quorum sensing, can be involved as well. Biofilm formation is a complex, regulated trait influenced by many factors (strain genotype, environmental cues, evolutionary stage). Studies using microtiter plate assays (like the crystal violet method we used) frequently report a varying spectrum of biofilm-forming capacity.

A study by Mena et al. on CF *P. aeruginosa* found similar distributions of biofilm-forming features in CF isolates, with many isolates showing weak or moderate production [18]. The presence of "non-producers" is also standard, since these may be early colonizers or strains that rely on other virulence strategies.

Importantly, the lack of statistical significance does not negate the clinical relevance of our findings. The observed patterns - 86% of biofilm producers originating from CF patients and 73% of mucoid strains being biofilm producers align consistently with established literature on *P. aeruginosa* pathogenesis in CF [11,15,17]. These trends suggest biological relevance that may reach statistical significance in larger cohorts.

Future studies with larger sample sizes and balanced groups are warranted to confirm these associations. Additionally, incorporating longitudinal data and clinical outcome measures would strengthen the correlation between biofilm phenotypes and disease progression.

Conclusion

This study represents the first evaluation of biofilm production capacity among clinical isolates of *Pseudomonas aeruginosa* from CF patients in our setting. Although statistical significance was not reached due to sample size constraints, the trends observed strongly support the strong association between CF lung environment and enhanced biofilm production, particularly among mucoid isolates.

While in vitro findings align with the established paradigm of biofilm-dominated chronic CF infections, the lower biofilm production in non-CF isolates underscores the unique selective pressures within the CF airway.

In summary, our results confirm that biofilm formation, particularly by mucoid variants of *P. aeruginosa*, is a critical virulence determinant associated with chronic CF infection. Future longitudinal studies are warranted to elucidate the temporal evolution of biofilm phenotypes and their direct correlation with clinical outcomes. These findings underscore the clinical importance of biofilm detection in diagnostic microbiology and its implications for antimicrobial therapy in CF patients.

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