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Original article

EVALUATION OF BIOFILM PRODUCTION IN *STAPHYLOCOCCUS AUREUS* FROM CYSTIC FIBROSIS PATIENTS

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Abstract

Cystic fibrosis (CF) is a multisystemic, life-limiting autosomal recessive disease. It is characterized by impaired chloride transport, which results in mucus retention that predisposes patients to chronic respiratory infections. Among the most common pathogens isolated from CF patients is *Staphylococcus aureus*, which possesses the ability to form biofilms. This ability is a key factor contributing to antimicrobial resistance and persistence. This study aimed to evaluate biofilm production in 41 clinical isolates of *S. aureus* from CF patients and 11 non-CF controls, using the quantitative colorimetric microtiter plate assay. Results showed that 51% (21/41) of CF isolates were methicillin-sensitive *S. aureus* (MSSA), while 49% (20/41) were methicillin-resistant *S. aureus* (MRSA). Among non-CF isolates, 27.3% (3/11) were MRSA and 72.7% (8/11) were MSSA. Biofilm production was observed in 32% (13/41) of CF *S. aureus* isolates. Among these, 46% (6/13) were MSSA and 54% (7/13) were MRSA. The biofilm phenotypes of CF isolates were distributed as follows: 6.8% strong, 24.4% moderate, and 68.3% weak or non-producers. In non-CF isolates, 18.2% (2/11) were biofilm producers, with one strong and one moderate producer; the remaining 82% (9/11) were weak or non-producers. These findings highlight the clinical relevance of biofilm production in CF-associated *S. aureus* infections and underscore the need for tailored antimicrobial strategies.

Keywords: biofilm, *Staphylococcus aureus*, MSSA, MRSA, CF

Introduction

Cystic fibrosis (CF) is a common life-limiting autosomal recessive genetic disorder, with the highest prevalence in Europe, North America, and Australia[1,2]. The frequency of this disorder is highly variable and often depends on ethnic and geographic origin of the affected patients. The disease is caused by mutation of a gene that encodes a chloride-conducting transmembrane channel called the cystic fibrosis transmembrane conductance regulator (CFTR), which regulates anion transport and mucociliary clearance in the airways. Mutations in this gene lead to dysfunctional chloride channels[3]. Dysfunctional CFTR disrupts mucociliary clearance in the respiratory tract. Functional failure of CFTR results in mucus

retention and chronic infection and subsequently in local airway inflammation that is harmful to the lungs. This ultimately leads to respiratory failure, which is the principal cause of mortality in CF patients. Cystic fibrosis affects several body systems. Thickened and dehydrated secretions also persist in the pancreas, gastrointestinal tract, hepatobiliary system, and reproductive tract, which is the cause of pancreatic insufficiency, malabsorption, and male infertility[4]. Advances in early diagnosis through newborn screening and correction of pancreatic insufficiency and undernutrition, inhaled antibiotics, airway clearance techniques, and the implementation of CFTR modulators have resulted in remarkable improvements in the quality of life and clinical outcomes in patients with cystic fibrosis, with median life expectancy now older than 40 years. Despite these advances, pulmonary disease remains the major cause of morbidity and mortality in CF. A hallmark of CF pathology is the establishment of polymicrobial infections within the thickened airway mucus, often involving opportunistic pathogens such as *Staphylococcus aureus, Pseudomonas aeruginosa, Haemophilus influenzae, Burkholderia cepacia* complex, and *Stenotrophomonas maltophilia*. *Staphylococcus aureus* frequently establishes early colonization in childhood, while *Pseudomonas aeruginosa* often dominates later in adolescence and adulthood[5].

Staphylococcus aureus is a human pathogen which causes skin and soft tissue infections, necrotizing pneumonia, bacteremia, catheter-related infections and ventilator-associated pneumonia, and infections in cystic fibrosis patients (CF)[6]. This microbe secretes many virulence factors. It also manifests multidrug-resistant profile. A significant feature that protects *S. aureus* against host defenses and eradication is the capability of *S. aureus to produce* biofilm and persist within the host. Biofilm is defined as a complex, diverse, structured microbial community embedded in a matrix formed by extracellular polymeric substances, whose major components are extracellular DNA, extracellular polysaccharides and structural proteins[7]. Biofilm provides protection against host immune responses and allows reduced antibiotic penetration, leading to emergence of persister cells and subsequent development of chronic and persistent infections[8]. Therefore, this must be considered in the management of biofilm-associated diseases like CF. *S. aureus* is the most commonly identified opportunistic pathogen in children and the overall CF population with 60 to 80% of CF patients under 20 years old being colonized according to French and American cystic fibrosis registries. After initial colonization, a subset of patients, representing 36% of patients according to data from the European Cystic Fibrosis Society patient registry, will become chronically colonized[9].

In this study, we aimed to analyze the ability of 41 clinical isolates of *S. aureus* obtained from CF patients and 11 non-CF controls of *S. aureus* to produce biofilm.

Material and methods

Bacterial isolates

A total of 41 clinical isolates of *S. aureus isolated* from respiratory tract of CF patients and 11 non-CF controls of *S. aureus* were included in this study. The study was performed during a 6 month-period in 2023 (February 1 - June 30). The CF patients were treated at the University Clinic for Children's Diseases in Skopje, and non-CF patients had different respiratory tract diseases, treated at outpatient settings. Identification of staphylococci was performed using standard microbiological methods, including culture on blood agar, catalase and DNA-ase biochemical test, followed by confirmation with the Vitek-2 automated system, using biochemical and antimicrobial susceptibility testing cards (bioMérieux, France).

Informed consent for this study was waived due to the retrospective use of anonymized bacterial isolates.

Biofilm quantification assay

Quantitative assessment of biofilm production was performed by the colorimetric microtiter plate assay as described previously by Stepanović *et al*.[10] (Figure 1). After verifying the purity of the strain, a few colonies with identical morphology were suspended in physiological saline. The turbidity of the bacterial suspension was adjusted to 0.5 McFarland standard. The obtained suspension should be vortexed for at least 1 min. Suspensions were diluted 1:100 in 200 μL of tryptic soy broth (TSB) containing 1% glucose, and were then transferred into the sterile flat-bottomed 96-well polystyrene microplates for 24 h at 35-37°C. The plates were carefully washed three times with sterile phosphate buffered saline (PBS, pH 7.3) after 24 h of incubation at 37°C. Fixation of the adherent biofilms was performed by 99% methanol for 15 min; the solutions were removed, and the plate was air-dried. The staining of biofilms was performed with 200 μl of 0.9% crystal violet solution (Sigma, Stockholm, Sweden) for 15 min at room temperature. Then, the dye was rinsed by water and the plates were allowed to air dry. Biofilm in each well was destained by treatment with 200 μL of 95% ethanol for 30 min at room temperature. Biofilm quantification was determined by measuring optical density (OD) at 570 nm using a microtiter plate ELISA reader (BioTek, Bad Friedrichshall, Germany). All experiments were performed in triplicate and repeated three times. Mean OD values were calculated for all tested strains and negative controls. The cut–off value (ODc) was defined as three standard deviations (SD) above the mean OD of the negative controls. For each microtiter plate the cut–off value was determined.

The strains were classified in four categories for easier interpretation of the results, based on previously calculated mean OD values: non-biofilm producer (OD<ODc); weak biofilm producer (ODc < OD < 2 × ODc); moderate biofilm producer (2 × ODc<OD<4 × ODc); strong biofilm producer (4 × ODc < OD) (Figure 1). SPSS software, version 28, was used for data analysis. Association of two or more set of variables was analyzed using the Chi – square test. A p value < 0.05 was considered as statistically significant.

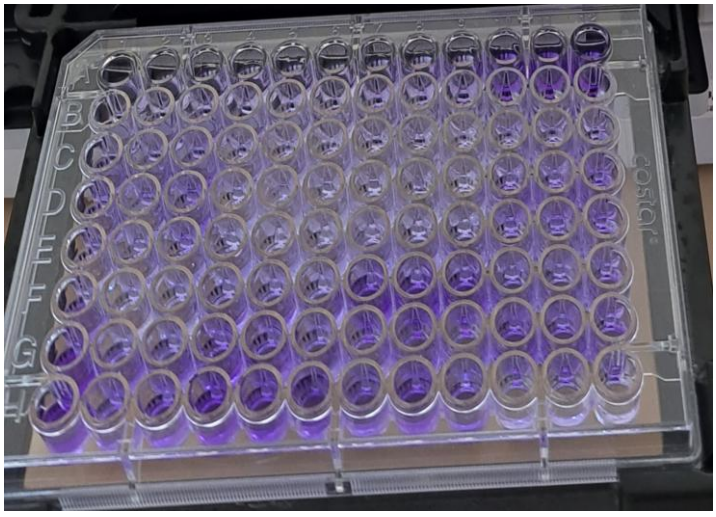

Fig. 1. Biofilm formation by *Staphylococcus aureus* isolates using the tissue culture plate (TCP) method

Results

Quantitative assessment of biofilm production was performed by the colorimetric microtiter method, in 96-well polystyrene microtiter plates, which used crystal violet for staining of biofilm. The intensity of staining corresponds to biofilm biomass, with darker wells indicating stronger biofilm production. Isolates were categorized as strong, moderate, weak, or non-biofilm producers based on optical density thresholds.

A total of 52 clinical isolates of *Staphylococcus aureus* from CF and non-CF patients were analyzed in this study. Forty-one isolate originated from CF patients, and 11 isolates were from non-CF controls. Non-CF control demographic data were not available for age and gender distribution. The demographic characteristics of the study population are presented in Table 1. Of the total 41 CF isolates, 22 (54%) were isolates from males and 19 (46%) were from female patients. Among CF patients, 54% were male and 85% were under 20 years of age. Methicillin-resistant *S. aureus* (MRSA) was identified in 49% of CF isolates compared to 27% of non-CF controls, though this difference was not statistically significant (p = 0.311). The p-value of 0.311 suggests a trend toward higher MRSA prevalence in CF (49% *vs*. 27%), but this could reasonably occur by chance.

Table 1. Demographic and clinical characteristics of study population

Characteristic	CF Patients (n=41)	Non-CF Controls (n=11)	p-value
Gender, n (%)			
Male	22 (54%)	–	–
Female	19 (46%)	–	–
Age Group, n (%)			
0–10 years	20 (49%)	–	–
11–20 years	15 (36%)	–	–
21–30 years	4 (10%)	–	–
>30 years	2 (5%)	–	–
Methicillin Susceptibility, n (%)			**0.311**
MSSA	21 (51%)	8 (73%)	
MRSA	20 (49%)	3 (27%)	
Total	41 (100%)	11 (100%)	

Biofilm production was observed in 32% (13/41) of CF *S. aureus* isolates compared to 18% (2/11) of non-CF *S. aureus* isolates, representing a 14 percentage-point difference (Table 2). However, statistical analysis using Fisher's exact test revealed no significant difference between groups (p=0.477). The odds ratio (OR) of 2.09 (95% CI: 0.40–12.18) suggests that CF isolates may be approximately twice as likely to produce biofilm., compared to non-CF isolates. However, the wide confidence interval (CI) crossing 1.0 (0.40–12.18), indicates substantial uncertainty. The true effect could range from CF isolates being 60% less likely to produce biofilm (OR=0.40) to being 12 times more likely (OR=12.18), which means that, while 32% of CF isolates produced biofilm compared to 18% of non-CF isolates (a 14-percentage point difference), this difference could be due to random chance given our sample size.

Table 2. Biofilm production in CF *vs*. non-CF *S. aureus* isolates

Group	Biofilm Producers	Non-Producers	Total	p-value
CF Patients	13(32%)	28(68%)	41	0.477
Non-CF Controls	2(18%)	9(82%)	11	
Total	15	37	52	

*Fisher's exact test; OR=2.09 (95% CI: 0.40-12.18)

Our data show that in our specific CF patient isolates of *S. aureus,* biofilm producers were detected in 35% of MRSA strains and 29% in MSSA isolates (Table 3). MRSA shows numerically higher biofilm production (35% *vs*. 29%), but this 6 percentage-point difference is not statistically significant. When comparing MSSA and MRSA isolates, biofilm production rates were similar (29% *vs*. 35%, p=0.747). The p-value of 0.747 indicates no evidence of a significant difference in biofilm production between MSSA and MRSA in CF patients, and there is a high probability this difference occurred by chance. The p value 0.747 shows that

only 13 total biofilm producing isolates had a low power to detect real differences. OR of 1.35 demonstrate that MRSA isolates had 35% higher odds of biofilm production than MSSA. The wide confidence interval (CI) crosses 1.0; hence, we cannot be very confident that this difference is due only to random variation, or there is a high uncertainty regarding the true effect.

Table 3. Biofilm production by methicillin susceptibility (CF isolates only)

Phenotype	Biofilm+	Biofilm-	Total	p-value
MSSA	6(29%)	15(71%)	21	0.747
MRSA	7(35%)	13(65%)	20	
Total	13	28	41	

*Fisher's exact test; OR=1.345 (95% CI: 0.20-2.77)

The distribution of biofilm phenotypes into 3 categories, regarding their strength for biofilm production, is shown in Table 4. Both groups showed similar patterns, mostly weak/non-producers (68% CF *vs*. 82% non-CF), and few strong producers (7% *vs*. 9%). Biofilm phenotypes were confirmed in 18.2% of non-CF isolates of *S. aureus* (2/11). Biofilm production was confirmed in one isolate of methicillin sensitive *S. aureus,* which was placed in the category of moderate producers, and in one isolate of methicillin resistant *S. aureus,* which was a strong biofilm producer.

The comparison of biofilm strength categories using the Mann-Whitney U test indicated no evidence that biofilm strength categories were distributed differently between CF and non-CF groups (U=217, p=0.881). Both CF and non-CF isolates were predominantly classified as weak or non-biofilm producers (68% and 82%, respectively), with few strong biofilm producers in either group (7% *vs*. 9%). Furthermore, not only is the prevalence of biofilm production similar, but the intensity of biofilm production (when present) is also similar between groups.

Table 4. Distribution of biofilm strength categories

Biofilm Strength	CF Patients (n=41)	Non-CF Controls (n=11)	Total (n=52)
Strong	3(7%)	1(9%)	4(8%)
Moderate	10(24%)	1(9%)	11(21%)
Weak/None	28(68%)	9(82%)	37(71%)
Total	41(100%)	11(100%)	52(100%)

*Mann-Whitney U test: U=217, p=0.881

Table 5. Complete statistical summary of all comparisons

Comparison	Test Used	p-value	Statistical Significance	Effect Size (95% CI)
CF *vs*. non-CF (biofilm)	Fisher's exact	0.477	No	OR = 2.09 (0.40-12.18)
MSSA *vs*. MRSA (CF only)	Fisher's exact	0.747	No	OR = 0.74 (0.20-2.77)
Biofilm strength distribution	Mann-Whitney U	0.881	No	U = 217
MRSA prevalence	Fisher's exact	0.311	No	OR = 2.54 (0.58-12.28)

Discussion

Staphylococcus aureus remains one of the most prevalent pathogens in cystic fibrosis, particularly during childhood and adolescence, with colonization rates exceeding 60-80% in young CF patients according to registry data[7]. Our study provides a phenotypic analysis of biofilm production in *S. aureus* isolates from CF patients compared to non-CF controls, with particular attention to methicillin susceptibility patterns. We observed a higher biofilm production (32% *vs*. 18%) by CF isolates and a higher prevalence of MRSA among CF isolates (49%) compared to non-CF isolates (27%). These trends align with established literature about CF lung environment selecting for certain bacterial phenotypes[11]. While our study observed trends suggesting higher biofilm production and MRSA prevalence in CF isolates compared to non-CF isolates, these differences did not reach statistical significance (all $p>0.30$). Statistical analysis using Fisher's exact test revealed no significant differences both in biofilm production between CF isolates and non-CF isolates ($p=0.477$) or between MSSA and MRSA strains from CF patients ($p=0.747$). The odds ratios for these comparisons (2.09 and 0.74, respectively) had wide confidence intervals crossing 1.0, indicating substantial uncertainty and lack of statistical precision. Similarly, the observed trend toward higher MRSA prevalence in CF isolates (49% *vs*. 27%) did not reach significance ($p=0.311$), probably because of the limited statistical power due to sample size constraints, particularly in the non-CF control group (n=11). These non-significant findings should be interpreted in light of the study's limited statistical power. Post-hoc power analysis indicates our study had only approximately 25% power to detect the observed effect sizes at $\alpha = 0.05$, and suggests approximately 200 total isolates would be needed to achieve 80% power for our main comparisons. Therefore, while our findings are consistent with the biological plausibility of CF environmental selection pressures, they require confirmation in larger cohorts[7].

Many epidemiological patterns show increasing MRSA colonization in CF populations, with reported prevalence ranging from 20% to 30% in many centers[12]. The clinical significance of MRSA in CF remains an area of active investigation, with some studies suggesting associations with more rapid lung function decline and increased treatment requirements[13]. Contrary to reports suggesting enhanced biofilm production in MRSA strains, particularly certain pandemic clones, our study found comparable rates of biofilm production between MSSA (29%) and MRSA (35%) isolates from CF patients, with no statistical significance ($p = 0.747$) [14]. This finding contrasts with some earlier studies that suggested enhanced biofilm production in MRSA strains, particularly those belonging to pandemic clones like USA300[12]. This finding also aligns with more recent CF-specific literature suggesting that biofilm regulatory mechanisms in the CF lung may operate independently of methicillin resistance determinants[11]. This may be due to differences in the predominant genetic lineages of MRSA in our region compared to those in studies reporting higher biofilm association. The unique selective pressures of the CF airway, including chronic inflammation, antibiotic exposure, and mucus-rich environment, may drive biofilm formation through pathways distinct from those associated with healthcare or community-acquired MRSA strains[15].

Although biofilm production did not differ significantly between CF and non-CF isolates (32% *vs*. 18%, $p=0.477$), a limitation likely due to sample size, the observed trends align with the well-documented adaptive mechanisms of *S. aureus* in the CF airway, including a shift toward biofilm-producing phenotypes. This concept is supported by literature indicating that *S. aureus* adapts to the CF airway through various mechanisms, which can include enhanced biofilm production[16]. In particular, biofilm production in MRSA is a major concern, as it synergizes with multidrug resistance, complicating eradication and being linked to worse clinical outcomes. It is noteworthy that some studies report very high rates of biofilm production in CF MRSA (85.6%)[17], a proportion markedly higher than the 35% observed in our CF MRSA isolates. This discrepancy highlights the substantial heterogeneity in biofilm-

producing capacity among *S. aureus* strains across different CF populations and may reflect differences in methodology, patient demographics, or the genetic background of prevalent clones.

The distribution of biofilm strength among CF isolates revealed that most biofilm-positive strains exhibited moderate (24.4%) rather than strong (7%) biofilm production, with the majority of isolates (68%) classified as weak or non-producers. This spectrum of biofilm-producing capacity is consistent with previous reports on *S. aureus* in CF contexts. The relatively low proportion of strong biofilm producers in our cohort may reflect the younger age distribution of our CF patients (85% under 20 years), as biofilm production and chronic adaptation in *S. aureus* may evolve over time in the CF airway[9].

Several limitations of our study warrant consideration. First, as we already mentioned, the modest sample size, particularly in the non-CF control group, limits the statistical power to detect potentially meaningful differences. Second, the *in vitro* biofilm assay using crystal violet staining, while standardized and widely used, may not fully capture the complexity of biofilm production in the *in vivo* CF lung environment, where multiple microbial species, host factors, and anatomical structures interact. Third, the cross-sectional design prevents assessment of temporal changes in biofilm phenotypes within individual patients over time.

Despite these limitations, our findings contribute to the growing body of literature on *S. aureus* adaptations in CF. Observed trends, though not statistically significant in direct comparisons, suggest biological patterns that merit further investigation in larger, longitudinal cohorts. Future studies incorporating genetic characterization of biofilm-related genes (such as those encoding polysaccharide intercellular adhesin, surface proteins, and regulatory systems) could provide insights into the molecular basis of biofilm formation in CF isolates of *S. aureus.*

Conclusion

This study represents a phenotypic evaluation of biofilm production in *Staphylococcus aureus* isolates from CF patients in our clinical setting. Our findings demonstrated that approximately one-third of CF *S. aureus* isolates exhibited biofilm-producing capacity *in vitro*, with comparable rates observed between MSSA and MRSA strains. While statistical analysis revealed no significant differences in biofilm production between CF and non-CF isolates or between MSSA and MRSA, the observed trends align with the clinical understanding of *S. aureus* as an adaptable pathogen in the CF airway.

The predominance of moderate rather than strong biofilm producers in our cohort, combined with the high proportion of weak or non-producers, suggests heterogeneous biofilm-producing capacity among CF *S. aureus* isolates. This heterogeneity may reflect different stages of adaptation to the CF lung environment or strain-specific characteristics.

These findings underscore that biofilm production represents an important virulence determinant in CF-associated *S. aureus* infections, irrespective of methicillin susceptibility. The clinical implications extend beyond antibiotic resistance profiles to include persistence mechanisms that challenge conventional antimicrobial therapies. Our results highlight the need for continued investigation into biofilm-targeting strategies and personalized approaches to managing *S. aureus* infections in CF patients, particularly as CFTR modulator therapies alter the pulmonary microenvironment and potentially influence microbial adaptations.

Future longitudinal studies with larger sample sizes, incorporating both phenotypic and genotypic analyses, are warranted to elucidate the evolution of biofilm-producing phenotypes over time and their correlation with clinical outcomes in the CF population.

Conflict of interest statement. None declared.

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