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# BIOFILM PRODUCTION BY *STAPHYLOCOCCUS AUREUS* STRAINS ISOLATED FROM CYSTIC FIBROSIN PATIENTS

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*Cystic fibrosis is a serious genetic disorder affecting respiratory, gastrointestinal and genital tracts. The most common causes of health status deterioration of patients with cystic fibrosis are still considered to be bacterial infections. Staphylococcus aureus is one of the most common pathogens isolated from the air passages of these patients. The purpose of our study was to determine the ability and intensity of the biofilm production by 59 of Staphylococcus aureus strains isolated from the air passages of patients with cystic fibrosis in the Cystic fibrosis center at the University Hospital Hradec Kralové. We also evaluated biofilm production and its intensity in 59 Staphylococcus aureus strains isolated from the air passages of control group patients. The control group was formed by patients with nosocomial pneumonia hospitalized in the University Hospital. For assessing*

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*the biofilm production, we used modified microtiter-plate test by Christensen. We divided the intensity of biofilm production into four categories according to the measured optical density — negative, weak, medium and strong. Out of total 59 Staphylococcus aureus strains isolated from the air passages of cystic fibrosis patients 45 (77.2 %) strains produced biofilm in various degrees: 21 (35.6 %) weakly, 6 (10.2 %) medium and 18 (30.5 %) strongly. Similar results were determined for strains isolated from control group: 22 (37.3 %) weak, 13 (22.0 %) medium and 9 (15.3 %) strong biofilm producing strains. We found no statistically significant difference in the biofilm formation between both groups of Staphylococcus aureus strains.*

## Introduction

Cystic fibrosis (CF) is the most common lethal hereditary disorder in Caucasian population. It is a multiorgan disease that affects especially respiratory, gastrointestinal and reproductive system. The first clinical symptoms of CF may occur in early stage of childhood and become worse with increasing age. The hallmark of the lung pathology in CF is an alteration of the air passage environment leading to intermittent and subsequently chronic colonization of the air passages by different types of bacterial and fungal microorganisms. The most common bacterial isolates are *Pseudomonas (P.) aeruginosa*, *Burkholderia (B.) cepacia*, *Staphylococcus (S.) aureus* and *Haemophilus (H.) influenzae*. Now, about 80 % of the CF patient population reaches adulthood. The predicted median age of survival for people with CF is their late 30s [1,2].

*P. aeruginosa* is an opportunistic pathogen that accounts for 10 % of nosocomial infections. Although initial colonization of the air passages leads to intermittent infections, *P. aeruginosa* is rapidly adapted to the microenvironment of the air passages. This results in chronic infection that cannot be eradicated by current antibiotics [3]. In the air passages, *P. aeruginosa* may generate aggregates encapsulated in alginate which are similar to bacterial biofilms. The progression of the lung disease during the chronic infection is a result of massive immune response and may lead to permanent lung damage [4]. Chronic *P. aeruginosa* infection is the major cause of morbidity in CF patients [3]. Incidence of *P. aeruginosa* is associated with increasing age of CF patients; about 75 to 80 % of the adults with CF have chronic *P. aeruginosa* infection [3,5,6].

*Burkholderia cepacia* complex (Bcc) also belongs to serious pathogens in CF patients, although the prevalence and incidence decreased from 3.6 % to 3.1 % and 1.3 % to 0.8 %, respectively, during the years 1995 to 2005 [7]. Bcc has an identical phenotype but different genotype and comprises at least 17 closely related species. The most important are *Burkholderia multivorans* a *Burkholderia cenocepacia* which account for 90 % Bcc infections in CF patients [8]. Variable

and unpredictable clinical outcomes of Bcc infections range from asymptomatic carriage to fatal bacterial invasion manifested by a progressive necrotizing pneumonia. The latter one is known as "cepacia syndrome" and occurs in 20 % of CF patients [9]. The Bcc is primarily resistant to many antibiotics. There is a high risk of the epidemic distribution of Bcc between CF patients [10,11].

*H. influenzae* is isolated especially from the air passages of children with CF. In 1995 to 2005 the prevalence in children 2 to 5 years old increased from 22 % to 34 % [7]. Most of *H. influenzae* strains isolated from the air passages of CF patients are non-encapsulated; therefore these strains cannot be classified into 6 serotypes (designated a-f) [12]. The role of *H. influenzae* in pathogenesis of CF has not been elucidated yet. Recent studies indicate that most of CF patients are colonized by the different *H. influenzae* strains. Persistence of *H. influenzae* is linked with its ability of the biofilm formation [13].

The most common pathogens mentioned above and including *S. aureus* are more frequently accompanied by a variety of new pathogens like *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans*, nontuberculous mycobacteria and other rare gramnegative pathogens. Among fungi isolated from the air passages of CF patients belong *Aspergillus* species and *Candida albicans* [14,15].

*S. aureus* is also usually one of the first pathogens, which can be cultivated from the CF patient air passages [16,17]. Chronic infections caused by *S. aureus* strains had been the major cause of mortality of CF patients since the discovery of antibiotics in 1940s [18]. Strategy of adaptability and persistence of *S. aureus* in lungs of CF patients is based on biofilm formation, possible intracellular survival and occurrence of its different phenotype, the SCVs — small colony variants [19].

Biofilm formation by *S. aureus* and other bacteria is a cyclic process, which usually begins by attraction of the bacterial cells to the surface due to chemical bonds and physical forces. The mechanism of the chemical bonding can be explained by concentration gradients which are facilitated by different kinds of chemoattractants (amino acids, sugars, proteins and other macromolecules) [20]. Physical forces include surface electrostatic charges, Van der Waals attraction and hydrophobic interactions [21].

Attraction of bacterial cells leads to the attachment of the bacteria to the various host surfaces [22]. Microbes are bound to the surface due to adhesive molecules. *S. aureus* possesses a large number of surface associated adhesins to adhere to the host matrix proteins. These adhesins belong to family of MSCRAMMs (microbial surface components recognizing adhesive matrix molecules), which is represented especially by clumping factor, collagen-binding protein and teichoic acid [22,23]. These molecules interact with host matrix, which consists of glycoproteins (fibronectin, vitronectin and fibrinogen) and other macromolecules like polysaccharides and lipids [24]. Expression of adhesive molecules and subsequent regulation of biofilm formation is influenced by a complex of *S. aureus* regulatory factors. Major importance is ascribed to the

accessory gene regulator *agr* and the staphylococcal accessory regulator *sarA*, which belong to molecules of quorum-sensing system [25,26].

After successful attachment more bacterial cells adhere to already attached bacteria, leading to intercellular aggregation [27]. The process follows through accumulation phase and multiplication of bacteria. The biofilm formation is accomplished by a maturation phase. Bacterial cells alter metabolic pathways and morphology in order to adapt the biofilm microenvironment. Typical three-dimensional mushroom-like structure is established. The major role in the aggregation of bacterial cells belongs to an adhesive exopolysaccharide matrix (polysaccharide intercellular adhesin, PIA), whose primary component is a polymer of *N*-acetylglucosamine. PIA in addition to proteins and teichoic acid forms a predominant part of the extracellular matrix of biofilm [28,29]. The cycle of biofilm formation is terminated by the dispersion of bacterial cells from the biofilm to enable dissemination of bacteria in host organism. The dispersion is controlled by the above mentioned quorum-sensing system and also by the various mechanical forces, e.g., fluid movement [30].

Major problem of bacterial biofilm is its troublesome eradication from the host organism, including implanted medical devices and various host tissues like respiratory tract of CF patients. Bacteria living in biofilm are protected against antibiotics and host defence mechanisms (e.g., antimicrobial peptides, neutrophil phagocytosis) [31]. The minimum inhibitory concentrations and minimum bactericidal concentrations of antibiotics to biofilm-growing bacteria may be 100 to1000 fold higher compared with planktonic bacteria [32-34]. However, antibiotic susceptibility as well as sensitivity to host defence mechanisms is usually relatively quickly restored when bacteria are dispersed from a biofilm formation [35,36].

In this study, we investigated the biofilm formation by *S. aureus* strains isolated from CF patients air passages. We also assembled the control group of *S. aureus* strains isolated from inflammatory changes in air passages (not colonization) from the intensive care unit patients with nosocomial pneumonia often receiving mechanical ventilation and from the air passages of healthy individuals.

## Materials and Methods

### Bacterial Strains

We tested 59 *S. aureus* strains isolated from the air passages of CF patients (CF strains) in the Cystic fibrosis centre at the University Hospital Hradec Kralové. For comparison, 59 *S. aureus* control strains isolated from the respiratory tract of non-cystic fibrosis patients (NCF strains) were used. These patients were hospitalized also at the University Hospital Hradec Králové, concretely in intensive care units and pulmonary clinic. Isolates were collected during the year 2010 and at the

beginning of the year 2011.

*S. aureus* strains were identified on the basis of a characteristic growth on blood agar, microscopic observation and free coagulase detection (ITEST plus s.r.o., CZ). The biotype of each strain was determined by an automated microbiology system VITEK 2 (bioMeriéux SA, FR). When necessary, strains were long-term stored at –80 °C in Cryobank (bacterial storage system; ITEST plus s.r.o.).

## Biofilm Assay

The biofilm formation was evaluated by a modified microtiter-plate test by Christensen using the sterile flat-bottomed 96-well polystyrene microtiter plate (Anicrin s.r.l., IT). The tested strains were inoculated on the blood agar with 5 % sheep blood (Bio-Rad Laboratories Inc., FR) and incubated aerobically on the blood agar at 36 ± 1 °C for 22-24 hours. Fresh cultures of *S. aureus* were used for a suspension equal to a McFarland standard of 1 (approximately equivalent to $3\times10^8$ bacterial cells per 1 ml) prepared in physiological saline. The turbidity of the bacterial suspension was measured by DENSI-LA-METER (ERBA-Lachema s.r.o., CZ).

To each well of a microtiter plate we added 180 µl of sterile BHI broth and subsequently 20 µl bacterial suspension. Microtiter plate was covered with a lid and incubated at 36 ± 1 °C for 22-24 hours. Each bacterial strain was tested in duplicate.

A negative control (sterility) consisting of uninoculated broth was included in each assay.

After incubation, the liquid content of each well was carefully removed and the well was washed with a sterile physiological saline four times. The subsequent drying of the biofilm layer in well was carried out at 36 ± 1 °C for 22-24 hours. The biofilm layer was stained with 160 µl crystal violet (Trios, spol. s.r.o., CZ) at 25 °C for 20 min. After removing the dye, the wells were washed gently with distilled water and dried at 36 ± 1 °C for 3-4 hours. This procedure was followed by adding 160 µl 95 % ethanol to each well, and the microtiter plate was then incubated for 1 hour at 25 °C. The optical density of each well was measured spectrofotometrically at 620 nm using a microtiter plate reader Multiscan FC (Thermo scientific Inc., Finland).

The strains isolated from the air passages of CF and NCF patients were divided into four subsets on the basis of their average absorbance by Gelosia *et al*. [37]. The strains with absorbance ≤ 0.12 were evaluated as biofilm negative, with absorbance between 0.12 and 0.24 as biofilm weakly positive, with absorbance between 0.24 and 0.36 as biofilm medium positive, and with absorbance ≥ 0.36 as biofilm strongly positive. In order to investigate the statistically significant

difference in biofilm formation between the strains isolated from the air passages of CF and NCF patients, the Mann–Whitney Rank Sum Test was used.

## Results and Discussion

Out of total 59 *S. aureus* strains isolated from the respiratory tract of CF patients, 47 (83 %) strains were isolated from sputum, 8 (14 %) from deep throat swabs, and 2 (3 %) from nose swabs. In the control group, 59 *S. aureus* strains isolated from the NCF patients: 12 (20 %) of strains were isolated from sputum, 14 (24 %) from laryngeal swab, 7 (12 %) from pharyngeal swab, 11 (19 %) from tracheal aspirate, 7 (12 %) from bronchoalveolar lavage and 8 (13 %) from other parts of respiratory tract. Median age of CF and NCF patients was 16.5 and 67.0 years, respectively.

The biofilm formation was determined in 76.3 % of *S. aureus* strains isolated from the air passages of CF and in 74.6 % of *S. aureus* strains isolated from the air passages of NCF patients. The distribution of strains into various subsets is reported in Table I. Some of the CF strains isolated in our study had the SCV (small colony variant) phenotype and belonged among strong producers of biofilm. There was no statistically significant difference in the biofilm formation between both groups of *S. aureus* strains.

Table I Evaluation of biofilm production of CF strains and NCF strains

| Biofilm formation | Absorbance (*A*) | CF strains | NCF strains |
|---|---|---|---|
| Negative | $A \leq 0.12$ | 14 (23.7 %) | 15 (25.4 %) |
| Weakly positive | $0.12 < A \leq 0.24$ | 21 (35.6 %) | 22 (37.3 %) |
| Medium positive | $0.24 < A \leq 0.36$ | 6 (10.2 %) | 13 (22.0 %) |
| Strongly positive | $A > 0.36$ | 18 (30.5 %) | 9 (15.3 %) |

CF strains – strains isolated from the respiratory tract of patients with cystic fibrosis
NCF strains – strains isolated from the respiratory tract of non-cystic fibrosis patients

As mentioned above, an ability of the biofilm formation was determined in most of *S. aureus* strains isolated from both groups of patients. Our results are lower than the observations of Pietruczuk–Padzik *et al*. [38], who reported 100 % of *S. aureus* strains isolated from CF patients as biofilm positive. However, authors of the mentioned study used different evaluation of the biofilm formation.

As observed by Semczuk *et al*. [39], *S. aureus* strains isolated from the air passages of CF patients are more prone to the biofilm formation than *S. aureus* strains from healthy individuals. In our study, this conclusion was not confirmed. This difference is most likely caused by the selection of a group of NCF patients. 54.2 % of NCF strains were isolated from the air passages of healthy individuals,

however, 45.8 % of NCF strains were isolated from the air passages of patients with nosocomial pneumonia.

Different results may also be caused by our modification of Christensen method. In our study, inoculated microtiter plates were incubated aerobically at 36 ± 1 °C for 22-24 h. The same conditions were reported by Stepanovic *et al*. [40]. On the other hand, Oliveira *et al*. [41] showed biofilm positivity of some *S. aureus* strains after prolonged incubation (48 h). Other discrepancies might be caused by the washing step. Some authors washed wells two [42], three [43,44] or four times [27]. We recommend washing wells of microtiter plates four times to avoid false-positive results.

However, we noticed the difference (not statistically significant) among subsets of biofilm strongly positive strains. The strong biofilm formation was determined in 30.5 % of CF strains and merely in 15.3 % of NCF strains. A stronger biofilm formation of *S. aureus* isolated from the air passages of CF patients is associated with an adaptation of this versatile bacterium to the specific micro-environment of the respiratory tract of CF patients [16,45].

A high level of resistance among bacteria in a biofilm might be interpreted by several mechanisms. Antibiotics penetrate into biofilm layers slowly or incompletely due to extracellular polysaccharide substance resulting in reduced exposure of biofilm bacteria to antibiotics. The reduced penetration of antibiotics, such as glycopeptides, enables bacteria to successfully inactivate the antibiotics [35,46,47].

In specific micro-environment of bacterial biofilm, a low pH value which influences antibiotic efficacy is often established. In deeper biofilm layers, anaerobic conditions may be present. Microbes in an anaerobic section can be protected from the aminoglycosides by means of oxygen inavailability which is needed to modulate action of these antibiotics [34-36]. Stress responses resulting in slow growth or stationary-phase of bacteria may be induced in biofilm by the lack of nutrients or accumulation of acidic waste products. If bacteria in a biofilm have low cell wall synthesis, antibiotics will be unable to destroy them [36]. Stress response of bacteria in a biofilm might also lead to an elevated expression of efflux pumps. Moreover, the presence of a subpopulation of persisting bacteria in a biofilm may account for broad antibiotic and host defense resistance [33,34,48].

## Conclusion

The key point for improving the quality of life of patients with cystic fibrosis is a successfully managed prevention and therapy based on a proper evaluation of lung infection etiopathogenesis. The biofilm formation by *S. aureus* in CF patients is a serious problem making the therapy of acute exacerbations of chronic infections difficult. For successfully managed treatment of CF patient infections,

the use of effective antimicrobial agents with an adequate concentration and sufficient penetration into the biofilm is necessary.

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