

EVALUATION OF BIOFILM PRODUCTION BY *PSEUDOMONAS AERUGINOSA* ISOLATES RECOVERED FROM CYSTIC FIBROSIS AND NON-CYSTIC FIBROSIS PATIENTS

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ABSTRACT

Cystic fibrosis (CF) patients typically suffer of persistent and recurrent lung infections caused by *Pseudomonas aeruginosa* that many times possess ability for the biofilm production. Here, biofilm production among *P. aeruginosa* isolates recovered from sputum of CF and non-CF patients was evaluated. Most isolates were biofilm-producing independently of the patient's condition.

Key words: *Pseudomonas aeruginosa*, biofilm, cystic fibrosis

Cystic fibrosis (CF) is a genetic and chronic disease that affects the lower respiratory tract. This disease is characterized by excessive mucus production that covers the epithelium (2).

Patients with CF present recurrent and persistent lung infections caused by *Pseudomonas aeruginosa*. In most of cases, the antimicrobial therapy is unable to eradicate the pseudomads infection. Despite also unclear, there is evidence that the biofilm formation is responsible, in part, for therapeutic failure (6, 7).

Additionally, the biofilm mode of growth makes the bacteria more resistant to antimicrobial agents due to: 1) low penetration of the antimicrobial inside matrix; 2) alteration in the rate of bacterial growth; and 3) horizontal transference of elements genetically mobiles that possess genes of antimicrobial resistance (5, 8). Beside this, quorum sensing (QS) seems to be related to biofilm formation under certain

growth conditions as such as medium of culture, temperature and atmosphere of incubation (4, 7).

In the study presented here we evaluated the ability of biofilm formation among *P. aeruginosa* isolates recovered from cystic (CF+) and non-cystic fibrosis (CF-) patients.

A total of 74 *P. aeruginosa* isolates recovered from sputum of patients hospitalized at Hospital de Clínicas de Porto Alegre (HCPA) were analyzed. All the isolates were identified by the use of WalkAway[®] 96SI system (Siemens), oxidase test and production of pigments. Of these, 35 and 39 isolates were recovered from CF+ and CF- patients, respectively. The age of the CF+ ranging from four to fifty-two years old. Among *P. aeruginosa* isolates from CF+, 30 isolates (85.7%) presented colony with mucoid aspect. Only five isolates recovered from younger patients did not present this mucoid phenotype. Patients without cystic fibrosis diagnosis did not present any

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chronical diseases but two patients that were admitted to intensive care unit were submitted to mechanical ventilation.

Quantitative determination of biofilm was made using a microtitre plate assay in accordance with Stepanovic *et al* (10), with some modifications. Briefly, three to five colonies were suspended in 5 ml of Trypticase Soy Broth (TSB) and incubated during 18h at $35\pm 1^\circ\text{C}$, without shaking. After incubation, the stationary-phase culture was vortexed and thereafter diluted 1:100 in TSB with 0.25% glucose, and 200 μl of this solution was incubated in 96-well plates during 18h at $35\pm 1^\circ\text{C}$. Media with suspended bacteria was then removed; the plates were carefully washed four times with water and air-dried before staining with 200 μl of 0.9% crystal violet solution (Sigma, Stockholm, Sweden) for 15 min. After removing the dye solution and washing with water, the attached dye was solubilized with 95% ethanol and the optical density of the adherent biofilm was determined twice with a filter of 450/630nm in a microtitre plate reader (OrthoReader II, Ortho diagnostic systems, New Jersey, USA). In the experiments, we used TSB with 0.25% glucose as a negative control (background absorbance). All isolates were tested at least three times in triplicate.

For interpretation of the biofilm results, the isolates were classified as follow: non-producing, weak, moderate and strong-producing, based on the following optic density (OD) average values: $\text{OD}(\text{isolate}) \leq \text{OD}(\text{control})$ = non-biofilm-producing; $\text{OD}(\text{control}) \leq \text{OD}(\text{isolate}) \leq 2\text{OD}(\text{control})$ = weak-producing; $2\text{OD}(\text{control}) \leq \text{OD}(\text{isolate}) \leq 4\text{OD}(\text{control})$ = moderate-producing; $4\text{OD}(\text{control}) \leq \text{OD}(\text{isolate})$ = strong-

producing (10).

Pseudomonas aeruginosa PAO1 (biofilm-producing) and *P. aeruginosa* ATCC 27853 (non-biofilm-producing) were used as controls.

Biofilm production is often associated with microbial virulence and resistance (4). Although the mechanism is still poorly elucidated, some studies reported a great ability for biofilm production among *P. aeruginosa* isolates from CF+ mainly by their capacity of adaptation into cystic fibrosis lung (1, 3, 9). Similarly to other studies, we noted that most of the isolates from CF+ were able to produce biofilm “in vitro”. On the other hand, a high proportion of biofilm-producing isolates among that recovered from CF- can indicate that this characteristic is an important virulence factor that can be attributed to *P. aeruginosa* isolates from sputa, independently of the physiologic condition of the patient since we did not observe a statistically significant association between the presence of cystic fibrosis and biofilm production ($p=0.6205$).

The $\text{OD}_{450/630}$ values obtained to classify the isolates as non-producing, weak and moderate-biofilm-producing were $\text{OD}_{450/630} \leq 0,071$; $0,071 < \text{OD}_{450/630} \leq 0,142$ and $0,142 < \text{OD}_{450/630} \leq 0,284$; respectively (Figure 1), considering a standard deviation value of 0.0034. A total of 74 isolates were analyzed and among all of them, the biofilm production was observed in 68% (50/74) of the isolates being the most classified as weak-producing (Table 1). Only two isolates, both from CF+, showed $\text{OD}_{450/630}$ values of 0.156 (strain 34) and 0.1986 (strain 35) and were classified as moderate-biofilm-producing (Table 1 and Figure 1).

Table 1. Status of biofilm production among *Pseudomonas aeruginosa* isolates recovered from CF+ and CF- patients.

Cystic fibrosis (n° of isolates)	Biofilm status			
	non-producing	weak	moderate	strong
Yes (35)	10	23	2	-
No (39)	14	25	-	-

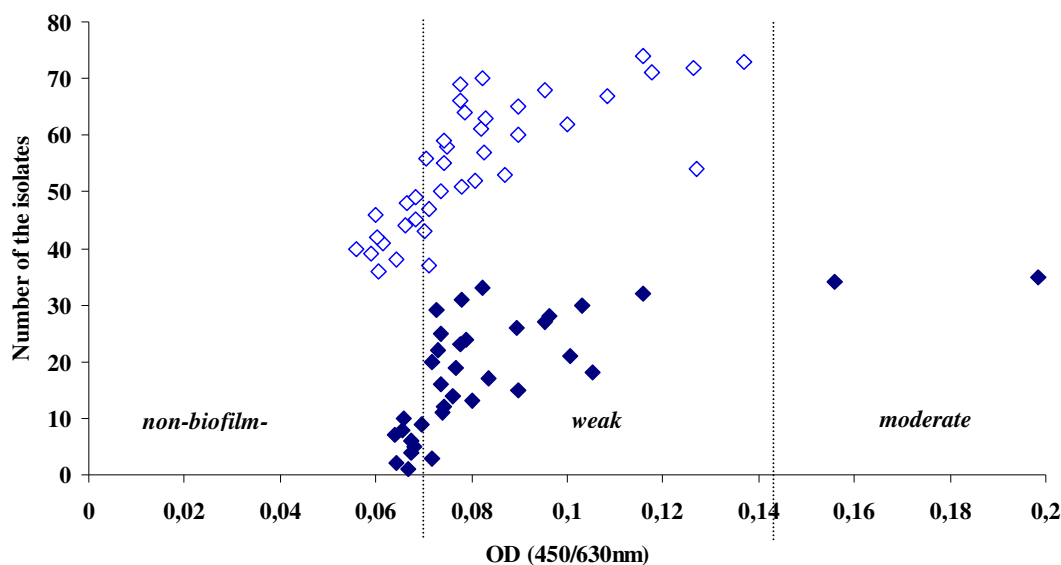


Figure 1. Biofilm production by *Pseudomonas aeruginosa* isolates. Black square represents CF+ and empty square represents CF- patients. Isolates yielding $OD_{450/630} \leq 0,071$; $0,071 < OD_{450/630} \leq 0,142$ and $0,142 < OD_{450/630} \leq 0,284$ were considered to be non-producing, weak and moderate-biofilm-producing, respectively.

Among isolates from CF+, 71.4% (25/35) were biofilm-producing while among isolates from CF-, 64.1% (25/39) were biofilm-producing.

An important aspect of this study was that we used the microtiter assay as standard technique for evaluating of biofilm production. It is known that exist many techniques (electronic and confocal microscopy, Calgary device, flow continuous cell and others) that can be used for this aim and, for this reason, the interpretation of the results obtained from different methodologies can suffer some alterations (weak to moderate or moderate to strong, for example) (5, 10).

In conclusion, our study demonstrated that most of *P. aeruginosa* isolates from cystic fibrosis have capacity to produce biofilm “in vitro”. Despite of this, most of them were classified as weak biofilm-producing by the methodology applied (microtiter assay). Although the cystic fibrosis isolates possess ability and flexibility to produce biofilm our results showed a high capacity of biofilm production “in vitro” among

non-cystic fibrosis isolates as well. Further investigations including a higher number of isolates can be useful to elucidate the correlation between biofilm production and clinical condition of the patient.

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