

# Biofilm production using distinct media and antimicrobial susceptibility profile of *Pseudomonas aeruginosa*

## ABSTRACT

Biofilm production is an important mechanism for bacterial survival and its occurrence together with antimicrobial resistance represents a challenge for clinical management. Here, we evaluated the ability for biofilm production among *P. aeruginosa* isolates from patients with or without cystic fibrosis (CF) using two distinct media, besides determining the antimicrobial susceptibility profile of these isolates for eight antimicrobial agents. The ability for biofilm production when TSB medium was used was higher than when used CF sputum media ( $p = 0.0198$ ). However, *P. aeruginosa* isolates from CF have demonstrated similar performance for biofilm production, independently of the medium used. Besides, among the biofilm-producing isolates, those recovered from CF were more resistant to the carbapenems (meropenem and imipenem) agents than those isolates from non-CF isolates.

**Keywords:** biofilm; *Pseudomonas aeruginosa*; cystic fibrosis; antimicrobial resistance.

## INTRODUCTION

*Pseudomonas aeruginosa* is one of the leading causes of nosocomial infections and it has a crucial role in cystic fibrosis (CF), being responsible for high level mortality and morbidity.<sup>1,2</sup> The lung of CF patients is characterized by high mucus production, which blocks clearance mechanisms. *P. aeruginosa* grows in CF lung as microcolonies promoting resistance to immunologic cells defense as well as persistence character in face of nutritional deficiency and oxygen deprivation.<sup>3,4</sup>

It is well recognized that biofilm mode of growth can promote resistance to antimicrobial agents, and its occurrence in the infection process has been considered a limiting factor for therapeutic success.<sup>5,6</sup> Moreover, there are just a few studies that evaluate biofilm production on mucus and sputum *in vitro* and under these conditions one could expect better correlation *in vivo* production.

The aim of the present study was to compare biofilm production among *P. aeruginosa* isolates using tryptic soy broth (TSB), as conventional medium, and a pool of CF sputum, as alternative medium in microti-

ter plate assay. Besides, it was also compared the antimicrobial susceptibility profile of *P. aeruginosa* biofilm-producing isolates recovered from CF and non-CF patients against eight antimicrobial agents, using TSB medium.

## MATERIAL AND METHODS

### Samples and media

A total of 124 *P. aeruginosa* isolates, 52 from CF (PCF+) and 72 from non-CF (PCF-) patients, recovered from sputum and endotracheal aspirates were evaluated.

Sputum specimens from randomly selected CF patients were collected into a sterile bottle to prepare a pool of sputum. To certify the quality of the sputum, an amount of each specimen was stained by Gram and microscopically inspected (100x) using the presence of < 10 epithelial cells per field as criterion. After, this pool was homogenized mechanically, autoclaved at 121°C during 15 min and stored at -20°C.

### Biofilm formation

Quantitative determination of biofilm was made using a microtiter plate assay in accordance with O'Toole et al.,<sup>7</sup> with some modifi-

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cations. In brief, three to five colonies were suspended in 5 mL of TSB and incubated overnight 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 overnight 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 200 µL of 0.9% crystal violet solution (Sigma, Stockholm, Sweden) was added 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 dual with 450/630 nm microtiter plate reader (OrthoReader II, Ortho diagnostic systems, New Jersey, USA). In the experiments, we have just 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 results, the isolates were classified as follow: non-producing, weak, moderate, and strong-producing, based on the following optic density (OD) average values:  $\text{OD (isolate)} \leq \text{OD (control)}$  = non-biofilm-producing;  $\text{OD (control)} \leq \text{OD (isolate)} \leq 2\text{OD (control)}$  = weak-producing;  $2\text{OD (control)} \leq \text{OD (isolate)} \leq 4\text{OD (control)}$  = moderate-producing;  $4\text{OD (control)} \leq \text{OD (isolate)}$  = strong-producing.<sup>8</sup>

### Control strains

A reference biofilm-producing strain of *Pseudomonas aeruginosa* PAO1 and a non-producing strain of *Pseudomonas aeruginosa* ATCC 27853 were used as controls.

### Antimicrobial susceptibility profile

Susceptibility profile to amikacin, aztreonam, cefepime, ceftazidime, ciprofloxacin, gentamicin, imipenem, meropenem and piperacillin-tazobactam was assessed by the

VITEK automated system (bioMérieux, Marcy l'Étoile, France) using GNS-655 cards (bioMérieux, Marcy l'Étoile, France) in accordance with manufacturer's instructions and breakpoints established by CLSI documents.<sup>9</sup> The antimicrobial susceptibility profile of the biofilm producers was evaluated based on the TSB results.

### Statistical analysis

Fisher's exact test was used to compare biofilm production by TSB and CF sputum media, as well as biofilm production of cystic fibrosis and non-cystic fibrosis patients in sputum media. A p-value < 0.05 was considered statistically significant.

## RESULTS

### Biofilm production

In TSB, 96.1% (50/52) of the PCF+ isolates have produced biofilm. Among PCF- isolates, 91.7% (66/72) have produced biofilm. A strong capacity to form biofilm has been observed in 56% (28/50) and in 39.4% (26/66) of the PCF+ and PCF- isolates, respectively. Using CF sputum, 92.3% (48/52) of the PCF+ isolates showed to be biofilm-producers and most of these were classified as moderate biofilm-producers (58.3%). Among PCF- isolates, 66.7% (48/72) were biofilm-producers and 46% (22/48) of these isolates were classified as strong biofilm-producers (Table 1). The difference in the ability of producing biofilm between PCF- and PCF+ isolates, 66.7% vs 92.3%, respectively, showed to be extremely significant ( $p = 0.0009$ ).

Of the 52 PCF+ isolates that were evaluated using CF sputum, only two were unable to produce biofilm. However, our results showed that biofilm production using TSB was higher than when used CF sputum ( $p = 0.0198$ ) (Table 1).

**Table 1. Biofilm production among *P. aeruginosa* isolates using two distinct media**

Source of the isolates (n°)	Biofilm production in TS				Biofilm production in CF sputum			
	Non producer	Weak	Moderate	Strong	Non producer	Weak	Moderate	Strong
PCF+ <sup>a</sup> (52)	2	6	16	28	4	8	28	12
PCF- <sup>b</sup> (72)	6	14	26	26	24	12	14	22
Subtotal (124)	8	20	42	54	28	20	42	34
Total (124)	8*		116*		28*		96*	

<sup>a</sup>PCF+, *Pseudomonas aeruginosa* from cystic fibrosis patients.

<sup>b</sup>PCF-, *Pseudomonas aeruginosa* from non-cystic fibrosis patients.

\* Biofilm production considering TSB and CF sputum were statistically significant ( $p = 0.0198$ ).

**Table 2. Antimicrobial susceptibility profile of biofilm producers of cystic fibrosis patients vs. non-cystic fibrosis patients using TSB medium<sup>a</sup>**

Antimicrobial agents	Cystic fibrosis patients (n <sup>b</sup> = 50)			Non-cystic fibrosis patients (n <sup>b</sup> = 66)		
	% susceptible <sup>c</sup>	% intermediate <sup>c</sup>	% resistant <sup>c</sup>	% susceptible <sup>c</sup>	% intermediate <sup>c</sup>	% resistant <sup>c</sup>
Amikacin	76	8	16	72.7	-	27.3
Aztreonam	52	16	32	57.7	9	33.3
Cefepime	28	40	32	30.3	24.2	45.5
Ceftazidime	40	20	40	30.3	24.2	45.5
Ciprofloxacin	52	-	48	54.5	-	45.5
Gentamicin	48	28	24	63.6	-	36.4
Imipenem	72	8	20	33.3	18.2	48.5
Meropenem	92	-	8	36.4	-	63.6
Piperacillin-tazobactam	68	-	32	81.8	-	18.2

<sup>a</sup>Considering any degree of biofilm production (weak, moderate or strong).

<sup>b</sup>n is the total number of isolates with biofilm producer status.

<sup>c</sup>Susceptible, intermediate and resistant phenotypes, from CLSI breakpoints.

### Antimicrobial susceptibility profile and biofilm production

Table 2 illustrates the antimicrobial susceptibility profile of *P. aeruginosa* biofilm-producing from PCF+ and PCF-. Isolates with any degree of biofilm production (weak, moderate or strong) were considered as producer. Ciprofloxacin was the least active antimicrobial agent against the panel of PCF+ isolates (48% resistant) followed by ceftazidime (40%) and aztreonam, cefepime and piperacillin-tazobactam (32%). A high proportion of PCF+ isolates displayed intermediate susceptibility to cefepime (40%). On the other hand, isolates from PCF- were more susceptible to piperacillin-tazobactam (81.8%) and more resistant to meropenem (63.6%). Eighteen PCF- isolates (25%) were susceptible to all eight antimicrobial agents tested. Interestingly, only six PCF+ isolates (11.5%) were susceptible to all eight antimicrobial agents tested.

### DISCUSSION

The biofilm mode of growth is an important bacterial survival strategy. *Pseudomonas aeruginosa* can produce biofilm in lungs of chronically infected cystic fibrosis patients, protecting the bacteria against antimicrobial agents and immune responses.<sup>10,11</sup> Biofilm starts growing on a surface involving a complex mechanism of interaction between bacteria and environment. This interaction, usually started by a primary adhesion, is reversible during the first couple of hours; later the adhesion becomes irreversible due to pili and polysaccharides of the bacteria. Bacterial adherence to the airway components, such as epithelial cells and mucus, has been considered to be a first step for colonization and sub-

sequent establishment of an infection process.<sup>6</sup> This mechanism is still poorly understood and a lot of possibilities such as impermeability of the matrix and bacterial persistent cells probably contribute for this dynamic status. Also, biofilm production has been related to the occurrence of genetic events such as hypermutation, transfer of genes and activation of distinct metabolic pathways.<sup>5</sup> It is described that *P. aeruginosa* can interact with elements like mucins, lecithin, eukaryotic DNA, salt, iron and other elements present in the mucus and contribute for the biofilm production.<sup>6</sup>

In this study, we have evaluated the use of two media as vehicle to perform the test for biofilm production. Using a pool of CF sputum, isolates from 48 PCF+ and 48 PCF- showed to be biofilm producing, while isolates from 4 PCF+ and 24 PCF- showed to be non-biofilm producing. Still, biofilm production in TSB was higher than in CF sputum ( $p = 0.0198$ ). This can be explained by *P. aeruginosa* growing as microcolonies surrounded by matrix, mainly alginate, on sputum elements; in this condition is not observed the full adherence to the microplate well. On the other hand, the sputum can be a less rich medium than TSB to support biofilm production in both groups. But, interestingly, we have observed that PCF+ isolates maintained their ability of biofilm production in both media. It is known that nutritionally rich conditions may be present in the CF airways, mainly amino acids. This is the main fact that promotes auxotrophic isolates. Besides, in the secretions of these patients substances that freely circulate in the airway environment are generated, especially in chronic processes. This condition may promote a selection of *P. aeruginosa* isolates with higher capacity for biofilm production.<sup>3</sup>

In this study, isolates with higher capacity to produce biofilm tended to have higher rates of resistance to the panel of antimicrobial agents tested, ranging from 86.6% (aztreonam) to 100% (amikacin) (data not shown). Delissalde and Amábile-Cuevas<sup>12</sup> have reported in their study a rate slightly higher against six antimicrobials, ranging from 62% to 92% of biofilm production among resistant isolates after 24 h of incubation. The authors stated that multiresistant isolates had a higher capacity to produce biofilm both at 8 and 24 h of incubation. Interestingly, in this study the minority of *P. aeruginosa* isolates, not including isolates from CF, was capable of producing biofilm.<sup>12</sup> In our study, of the 72 isolates from PCF-, six (8.3%) were unable to produce biofilm in TSB. Among isolates from PCF+, only two isolates (3.8%) were unable to produce biofilm when we used TSB as medium. On the other hand, 24 PCF-isolates were unable to produce biofilm when CF sputum was used as medium. Four isolates from PCF+ were unable to produce biofilm in CF sputum medium. Surprisingly, these four isolates were strong biofilm producers in TSB medium.

Knowledge of the susceptibility of *P. aeruginosa* to antimicrobial agents used for treatment not only serves as a guide to treatment for individual CF patients, but it is also valuable for identifying the prevalence of resistant strains within and among different treatment centers, given the mobility of some patients. Our results have demonstrated that isolates from PCF+ and PCF- possess distinct profiles against a panel of antimicrobials. In particular, very high levels of resistance to two main drugs (meropenem and imipenem) were evident among isolates from PCF-, casting doubt on the adequacy of their empirical use in this patient group. This difference between the susceptibility profiles can be explained by the nature of the infection, since isolates from PCF- were recovered from patients admitted in the intensive care unit, nosocomial setting with a great selective pressure for extended spectrum antimicrobials such as carbapenems. In fact, our results showed that isolates from PCF- displayed more resistance to carbapenems than isolates from PCF+. Surprisingly, susceptibility to all eight antimicrobial agents was higher among isolates from PCF- than isolates from PCF+ (25% vs 11.5%).

Invariably, these isolates were able to produce biofilm in distinct media. Although biofilm production has recently been studied more deeply, more studies are still needed to improve our understanding about the link between virulence factors and antimicrobial resistance.

We are aware of the fact that CF sputum medium used here is not entirely representative of CF lung environment because other active compounds and growth conditions, including anaerobic atmosphere, may be also present.<sup>4,6</sup> On the other hand, *P. aeruginosa* isolates from PCF+ apparently displayed, under distinct conditions, the same feature for the biofilm production.

Biofilm production and antimicrobial resistance are among the main bacterial defense apparatus during an infectious process, and both represent currently a great challenge to the therapeutic practice.

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