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In vitro biofilm formation and antibiotic susceptibility of *Pseudomonas aeruginosa* isolated from airways of patients with cystic fibrosis

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ABSTRACT

Introduction. *Pseudomonas aeruginosa* is the predominant cause of airway infections in patients with cystic fibrosis (CF) as a result of its ability to form biofilm. Resistance to antimicrobial agents is the most important feature of biofilm infection. The aim of this study was to evaluate biofilm formation and to compare antibiotic susceptibility of *P. aeruginosa* living in two modes of growth: planktonic and biofilm, isolated from respiratory tract of CF patients.

Material and Methods. Biofilm formation and biofilm susceptibility to antibiotics were determined using modified microtitere plate method. For susceptibility testing of planktonic culture to antibiotics serial microdilution broth method were used.

Results. More than 95% of isolates were capable to form biofilm. Isolates grown as biofilms were more resistant to tested antibiotics compared to those grown planktonically. Ciprofloxacin showed the highest activity against *P. aeruginosa* biofilm. In contrast, no bacteriostatic activity was obtain for the highest concentration of piperacillin tested against most of *P. aeruginosa* strains growing in a biofilm (BIC > 4096 mg/L).

Conclusions. Our study indicates the need to develop a standardized susceptibility testing method for biofilm mode of growth of pathogens. It appears that it is appropriate to introduce a biofilm susceptibility testing to routinely performed tests, as the effect of antibiotics on biofilm eradication may be variable and unpredictable.

Keywords: biofilm resistance, chronic infections, susceptibility testing.

Introduction

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacterium that grows in soil, water, as well as on plant and animal tissues. Because of ability of this bacterium to product multiple virulence factors facilitating invasion and colonization, *P. aeruginosa* is a major opportunistic human pathogen responsible for bacteremia in burn patients, urinary-tract infections in catheterized patients, and pneumonia in mechanically ventilated patients [1–3]. *P. aeruginosa* is also the predominant cause of lung infections in cystic fibrosis patients [4]. The pathogenesis of *P. aeruginosa* lung infections is multifactorial and depends on numerous virulence factors, including secretion of extracellular enzymes (e.g. elastase, phospholipase C, alkaline protease, exotoxin A, pyoverdine, neuraminidase) and the presence of cell associated factors, such as flagella, pili and lipopolysaccharide (LPS) [5, 6]. Another important factor contributing to the pathogenesis of *P. aeruginosa* severe infections is its tendency to form organized communities, known as a biofilm, on biotic and abiotic surfaces [7]. Natural resistance of *P. aeruginosa* to several group of antibiotics, and the resistance to disinfectants together with the ability to biofilm for-

mation make this bacterium responsible for high rates of morbidity and mortality [8, 9].

The discovery of bacteria existing in a biofilm form has led many researches to revisit the pathogenesis of chronic infections. Biofilm is three-dimensional structured, specialized community of adherent microorganisms enclosed in a self-produced extracellular polymeric substance (EPS). Resistance of biofilm towards component of host immune system as well as antimicrobial agents appears to be risk factor for persistent infections and makes them difficult to eradicate [8, 10].

Bacteria living in biofilm undergo a phenotypic shift in behavior, and large groups of bacterial genes are differentially regulated. In this way, biofilm community obtains numerous advantages, such as passive resistance, metabolic cooperation, byproduct influence, quorum sensing systems, an enlarged gene pool with more efficient DNA sharing, and many other features which help bacteria to adapt to environmental condition and protect them from effects of external factors [11–13].

Cystic fibrosis (CF) is a genetic disorder due to recessive mutations in the CF transmembrane regulator gene which regulates chloride transport across epithelia cells. These mutations lead to dysfunction of the CF transmembrane regulator protein which constitutes a part of a chloride channel. This can result in a built-up of thick, sticky mucus, among others, in the respiratory tract, pancreas, and reproductive organs leading to multiple organ disorders. The span life and quality of life are most often dependent on changes in respiratory tract. As a result of hyperinflammation and a reduced ability to remove bacteria by mucociliary action, bacteria colonize the lungs [14]. Chronic airway infections in CF patients differ significantly from acute pulmonary infection in non-CF patients, bloodstream or urinary tract infections. *P. aeruginosa* grows in the CF lung very slowly as chronic biofilm infections and despite the use of a range of antipseudomonal antibiotics, eradication of the infection is quite rare [15–17].

This study was performed to compare antibiotic susceptibility of *P. aeruginosa* isolated from respiratory tract CF patients living in two modes of growth: planktonic and biofilm.

Material and Methods

Investigated bacterial strains
Pseudomonas aeruginosa strains were obtained from Microbiological Laboratory of Transfiguration of the Lord Clinical Hospital. The strains originally isolated from respiratory tract of CF patient were frozen and stored in Microbank cryogenic vials (ProLabDiagnostics, Canada) at -70 ± 10°C. Before each experiment subcultures were prepared on Tryptic soy agar (bio-Mérieux, Poland).

Staphylococcus epidermidis ATCC 35984, with a proven biofilm-forming ability and *Staphylococcus epidermidis* ATCC 35983 – a non-biofilm producer were used as positive and negative controls for biofilm production, respectively. *Pseudomonas aeruginosa* ATCC 27853 was applied as the quality control strain to verify the test procedures for determination of MIC.

Antibiotics applied in MBC and BIC determination
Piperacillin (PIP), ceftazidime (CAZ), ciprofloxacin (CIP) and amikacin (AN) were obtained from Sigma-Aldrich (Poland). Stock solution from dry powders were prepared at a concentration of 4096 mg/L for piperacillin and 1024 mg/L for ceftazidime, amikacin and ciprofloxacin. The stock solutions were stored at -70 ± 10°C before experiments.

Biofilm formation assay
Biofilm formation was determined by the microtiter plate assay, as previously reported [18]. Briefly, 200 μL of an overnight cultures grown on tryptic soy agar (bio-Mérieux, France) suspended in a tryptic soy broth (bio-Mérieux, France) and adjusted to a turbidity of 0.5 MacFarland in tryptic soy broth (TSB) were inoculated into 96-well flat-bottom microtiter plates (Medlab-Products Ltd., Poland) and incubated. Following incubation at 37°C for 20 hours the cultures were removed and the wells were washed three times with 200 μL of phosphate buffered saline (PBS, pH = 7.4; Sigma-Aldrich, Poland) and dried at room temperature. Biofilms were stained with 0.1% crystal violet (Merck, Poland) for 15 minutes, washed with water and air dried overnight. The crystal violet from stained biofilm was resuspended in 250 μL of 95% ethanol. The optical density (OD) of adherent biofilm was measured using an Infinite M200 (Tecan) plate reader at a wavelength of 590 nm. Wells containing uninoculated TSB media served as a negative control. Tests were repeated three times. The interpretation of biofilm formation was done according to the Stepanovic criteria presented in **Table 1** [19].

Susceptibility testing of planktonic cells
The stock solution of each antibiotic was two-fold serially diluted in a Mueller Hinton II broth (MHB II; bio-Mérieux, Poland) to concentrations ranging from 256

Table 1. Classification of biofilm formation

OD values	Biofilm formation
≤ ODc	None
2 x ODc ≥ OD > ODc	Weak
4 x ODc ≥ OD > 2 x ODc	Moderate
> 4 x ODc	Strong

ODc = mean OD of control probes + 3SD, OD − optical density, SD − standard deviation

Table 2. Detection of biofilm formation by the microtiter plate method

Biofilm formation	No of isolates [%]	Isolate	Absorbance at 590 nm
None	1 [4.5]	51	0.200
Weak	1 [4.5]	14	0.419
Moderate	2 [9.0]	23, 26	0.517 − 0.694
Strong	18 [82.0]	4, 5, 9, 13, 16, 20, 24, 28, 29, 30, 31, 32, 33, 37, 38, 39, 40, 52	1.069 − > 3.00

to 0.5 mg/L. Aliquot of 100µL of each dilution were distributed into the wells of a sterile 96-well microtiter plate. An overnight bacterial culture was suspended in MHB II, adjusted to a 0.5 McFarland standard, corresponding to a concentration of 10^8 CFU/mL and diluted 1:100 in MHB II. Bacterial suspensions were added to all wells except the wells, which were used as sterility controls. Growth wells (with bacteria and without antibiotics) were also included. The final concentration of bacteria was c.a. 5×10^5 CFU/mL and final concentrations of antibiotics ranged from 0.25 mg/L to 128 mg/mL. The plates were prepared in triplicate and then incubated at 37ºC for 20 h. The MIC was determined as the lowest concentration of antibiotic that inhibited the visible growth of the tested microorganism.

Biofilm susceptibility assay
Antimicrobial susceptibility of *P. aeruginosa* biofilm assay was performed according to Moscowitz [20] with modifications. Briefly, bacterial biofilm was formed by immersing the wells of flat-bottom microtiter plates as described above. Negative control wells were filled with sterile medium. The 24-hour biofilms were washed three times with PBS solutions and air-dried. Serial two-fold dilutions of antibiotics, ranging from 4 mg/L to 4096 mg/L, were prepared in MHB II. Next, 100 µL of each concentration was added to each corresponding well and plates were incubated 18 h at 37ºC. Antibiotics were aspirated gently after incubation and plates were washed three times with sterile PBS solution. To each well 100 µl of MHB II was added and the plates were sonicated using sonicating water bath for 5 minutes to disrupt the biofilm. The optical density at 650 nm (OD_{0h}) was measured on a microtiter plate reader (Infi-

nite M200,Tecan) before and after incubation at 37°C for 6 h. Adequate biofilm growth for the positive control wells was defined as a mean OD_{650} difference (OD_{650} at 6 h minus the OD_{650} at 0 h). The biofilm inhibitory concentrations (BICs) were defined as the lowest concentrations of drug that resulted in an OD_{650} difference at or below 10% of the mean of two positive control well readings. The 10% cut-off represents a 1-log10 difference in growth after 6 h of incubation.

Statistical analyses
The results of antimicrobial activity were analyzed using Kruskal-Wallis test. *P* value <0,05 was considered as significant. The STATISTICA software was used in the statistical analyzes.

Results

Biofilm formation
The established cut-off values of ODc for assessment of biofilm formation for all strains were 0.157. Final OD value of tested strains was calculated as an average OD value of the strain reduced by ODc value. The interpretation of biofilm formation was performed according to the following criteria: OD ≤ 0.157 − none producer; 0.157 > OD ≤ 0.313 − weak producer, 0.313 > OD ≤ 0.626 − moderate producer and OD > 0.626 − strong producer (**Table 2**). Of the 22 isolates, 21 formed biofilm, of which only one was weak producer, two strains were moderate and 18 strong producers with an average OD_{650} value of 0.418 ± 0.078, 0.516 ± 0.027 − 0.694 ± 0.042 and 1.069 ± 0.540 − > 3.000 respectively. For the six strains OD value were above upper limit of measurement range of plate reader (OD > 3.000).

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Effect of PIP on biofilm and planktonic *P. aeruginosa* mode of growth

Significant difference was observed between the inhibitory effect of piperacillin on biofilm and planktonic culture of *P. aeruginosa* CF isolates (**Figure 1**). The MIC value ranged from 4 mg/L to 64 mg/L and according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) susceptibility breakpoints (>16 mg/L) only three strains were resistant to PIP. In contrast, no bacteriostatic activity was obtain for PIP at the highest concentration tested against most of *P. aeruginosa* strains growing in biofilm (BIC > 4096 mg/L) and the BIC was from 64 to > 1024 fold higher than MIC.

Effect of CAZ on biofilm and planktonic *P. aeruginosa* mode of growth

Figure 2 presents the MIC and BIC of ceftazidime for *P. aeruginosa* isolates. The obtained BIC values, ranged from 64 mg/L to 512 mg/L, and were 8 to 64 fold higher than MICs. There were no correlation in differences between planktonic and biofilm inhibition concentra-

tions and susceptibility assessed according EUCAST guidelines.

Effect of AN on biofilm and planktonic *P. aeruginosa* mode of growth

Determination of MIC for planktonic culture of *P. aeruginosa* showed that the majority of tested strains were susceptible to amikacin (MIC ranged 4–8 mg/L), six were intermediate susceptible (MIC 16 mg/L) and only one was resistant (MIC 64 mg/L) (**Figure 3**). Antibiofilm activity of amikacin for all tested isolates decreased and BIC value increased from 2 to 8-fold in relation to MIC.

Effect of CIP on biofilm and planktonic *P. aeruginosa* mode of growth

Planktonic cultures of *P. aeruginosa* isolates were inhibited at ciprofloxacin concentration of 4 mg/L to 16 mg/L (**Figure 4**). Despite the fact that *P. aeruginosa* isolates cultured in floating form were resistant to CIP (according to EUCAST breakpoints), the concentration inhibiting the growth of *P. aeruginosa* biofilm (BICs) were equal or two to four-fold higher than MICs.
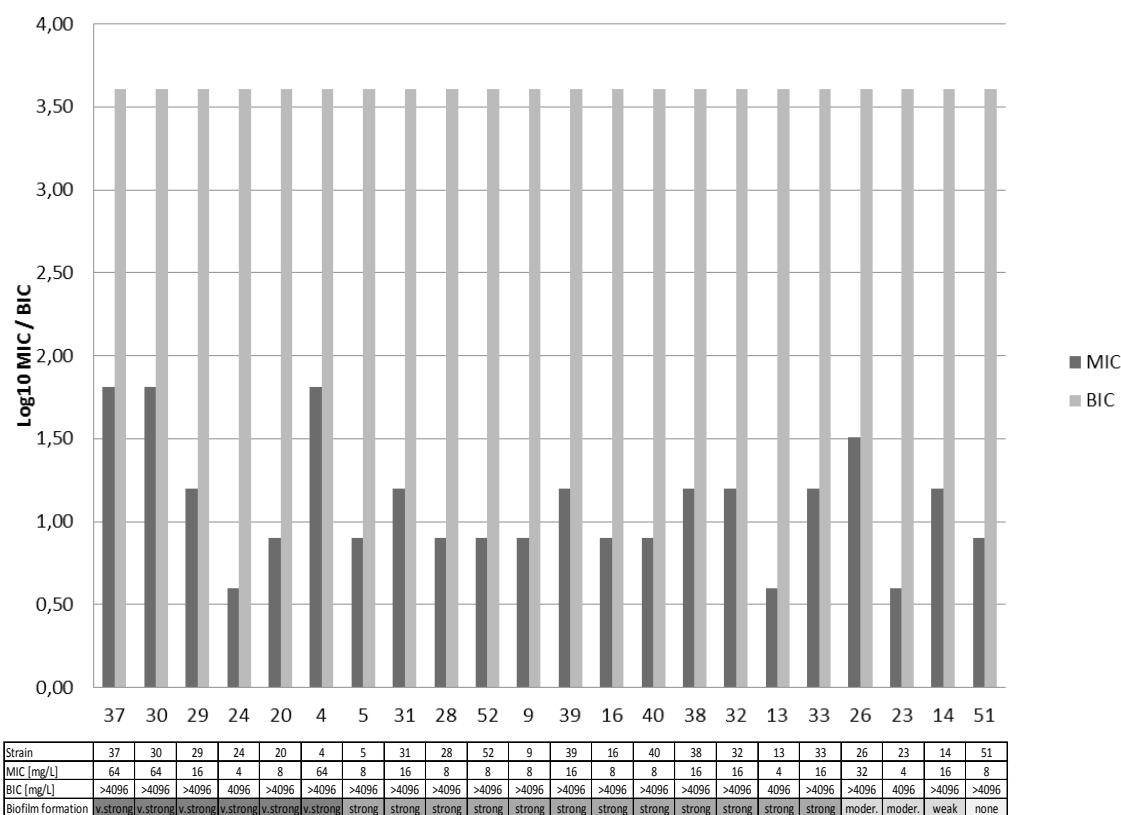

Strain	37	30	29	24	20	4	5	31	28	52	9	39	16	40	38	32	13	33	26	23	14	51
MIC [mg/L]	64	64	16	4	8	64	8	16	8	8	8	16	8	8	16	16	4	16	32	4	16	8
BIC [mg/L]	>4096	>4096	>4096	4096	>4096	>4096	>4096	>4096	>4096	>4096	>4096	>4096	>4096	>4096	>4096	>4096	4096	>4096	>4096	4096	>4096	>4096
Biofilm formation	v.strong	v.strong	v.strong	v.strong	v.strong	v.strong	strong	strong	strong	strong	strong	strong	strong	strong	strong	strong	strong	strong	moder.	moder.	weak	none

Figure 1. Comparison of planktonic (minimal inhibitory concentration, MIC,) and biofilm (biofilm inhibitory concentration, BIC) susceptibility of *Pseudomonas aeruginosa* isolates to piperacillin obtained from the airway of patients with cystic fibrosis. The MIC and BIC values are given in terms of $\log_{10}$ mg/L. Susceptibility to piperacillin was not correlated to the intensity of biofilm formation by the strains tested

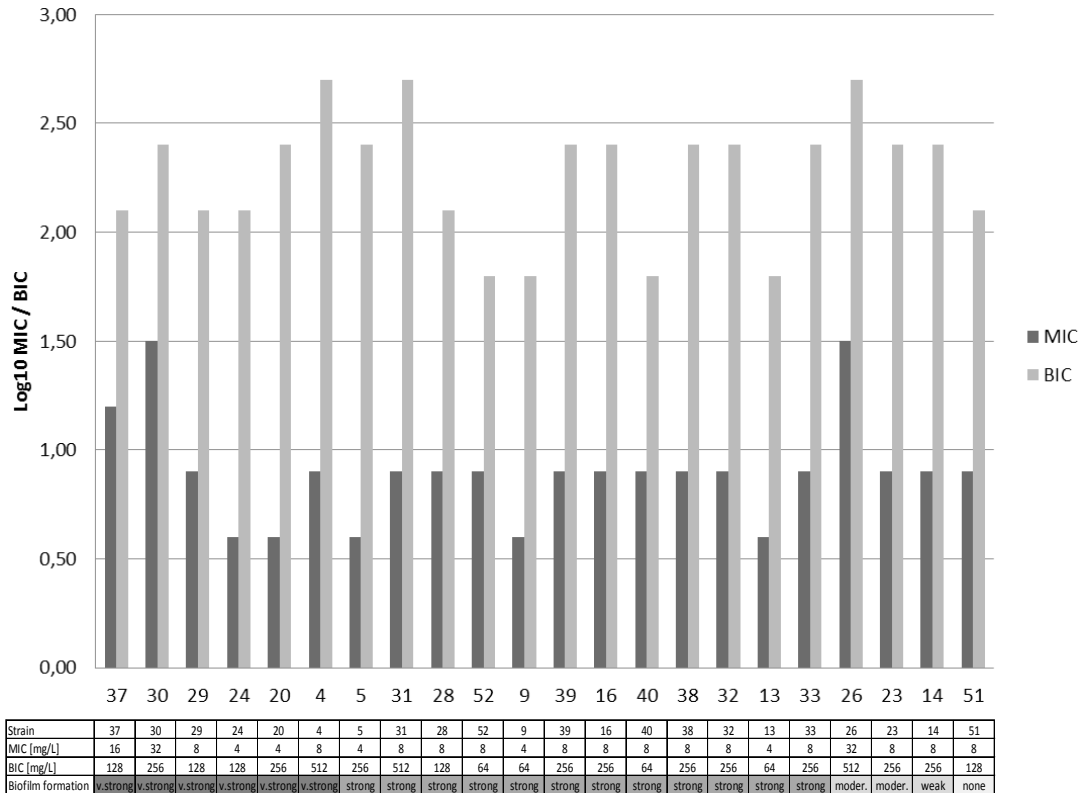| Strain	37	30	29	24	20	4	5	31	28	52	9	39	16	40	38	32	13	33	26	23	14	51
MIC [mg/L]	16	32	8	4	4	8	4	8	8	8	4	8	8	8	8	8	4	8	32	8	8	8
BIC [mg/L]	128	256	128	128	256	512	256	512	128	64	64	256	256	64	256	256	64	256	512	256	256	128
Biofilm formation	v.strong	v.strong	v.strong	v.strong	v.strong	v.strong	strong	strong	strong	strong	strong	strong	strong	strong	strong	strong	strong	strong	moder.	moder.	weak	none

Figure 2. Comparison of planktonic (minimal inhibitory concentration, MIC,) and biofilm (biofilm inhibitory concentration, BIC) susceptibility of *Pseudomonas aeruginosa* isolates to ceftazidime obtained from the airway of patients with cystic fibrosis. The MIC and BIC values are given in terms of $\log_{10}$ mg/L. Susceptibility to ceftazidime was not correlated to the intensity of biofilm formation by the strains tested
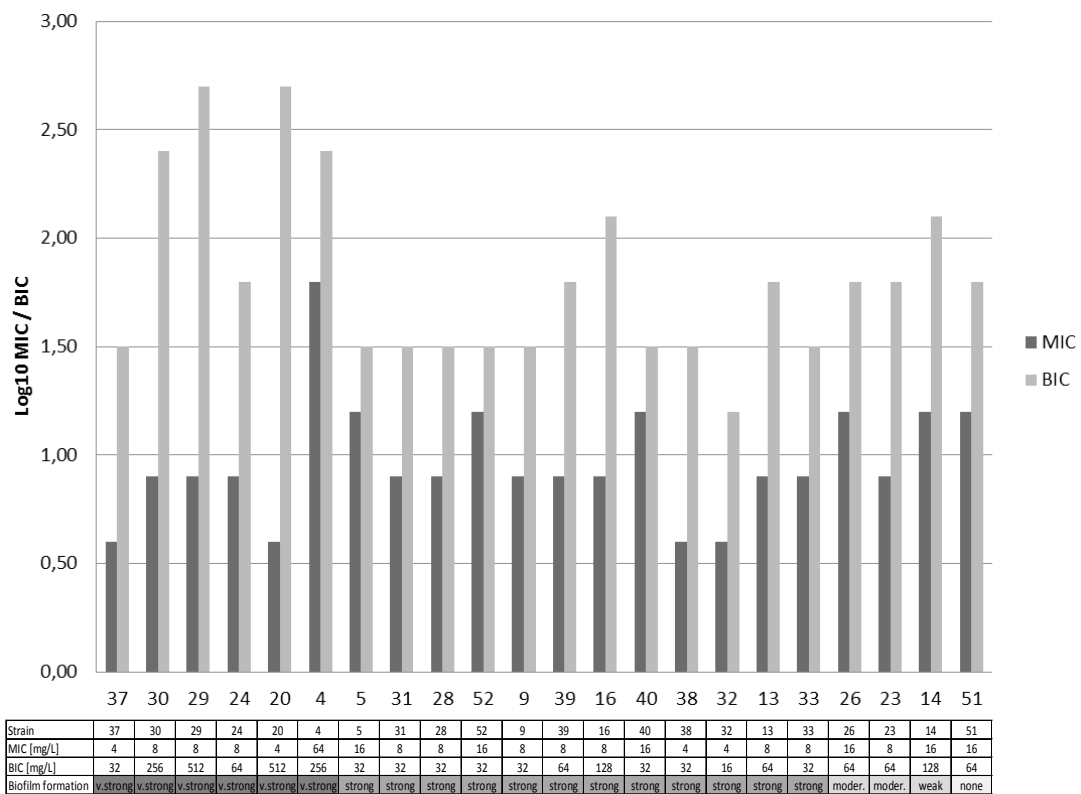

Strain	37	30	29	24	20	4	5	31	28	52	9	39	16	40	38	32	13	33	26	23	14	51
MIC [mg/L]	4	8	8	8	4	64	16	8	8	16	8	8	8	16	4	4	8	8	16	8	16	16
BIC [mg/L]	32	256	512	64	512	256	32	32	32	32	32	64	128	32	32	16	64	32	64	64	128	64
Biofilm formation	v.strong	v.strong	v.strong	v.strong	v.strong	v.strong	strong	strong	strong	strong	strong	strong	strong	strong	strong	strong	strong	strong	moder.	moder.	weak	none

Figure 3. Comparison of planktonic (minimal inhibitory concentration, MIC,) and biofilm (biofilm inhibitory concentration, BIC) susceptibility of *Pseudomonas aeruginosa* isolates to amikacin obtained from the airway of patients with cystic fibrosis. The MIC and BIC values are given in terms of $\log_{10}$ mg/L. Susceptibility to amikacin was not correlated to the intensity of biofilm formation by the strains tested
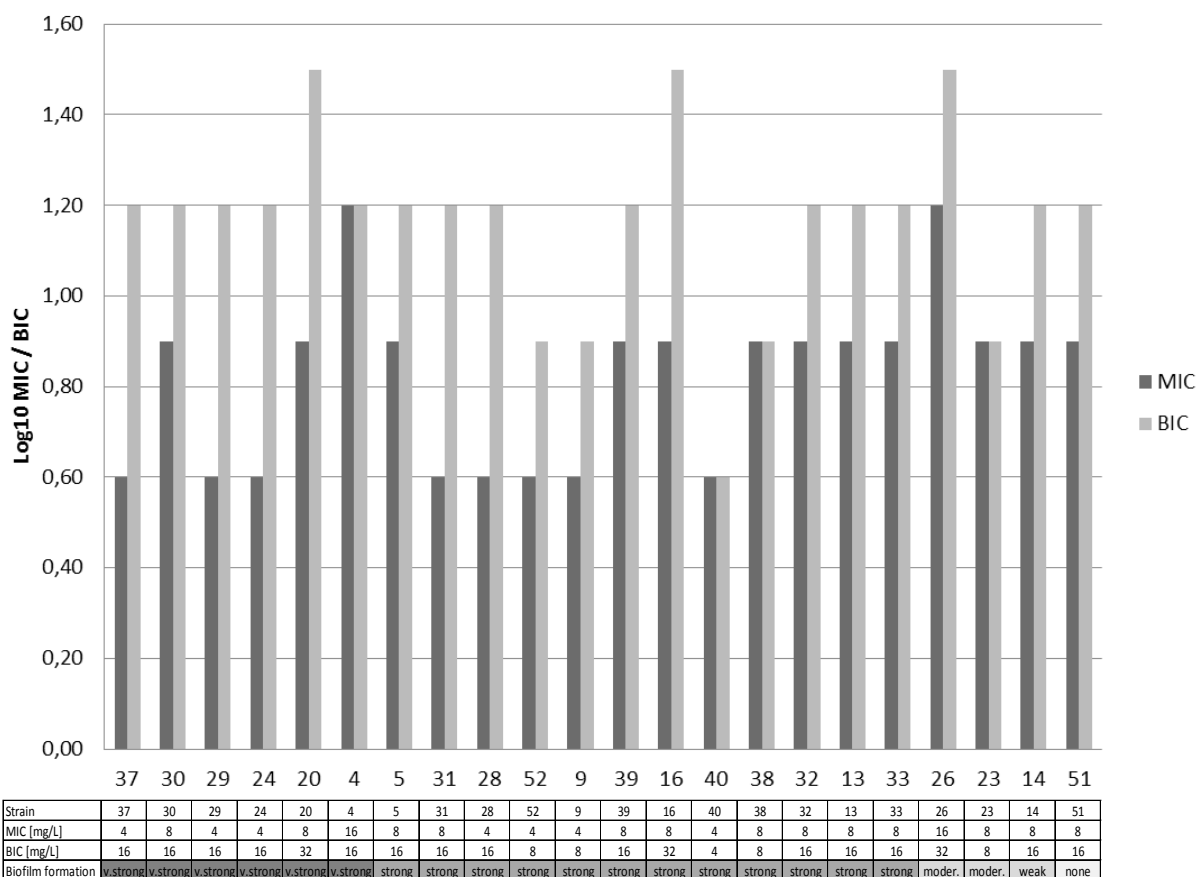
Figure 4. Comparison of planktonic (minimal inhibitory concentration, MIC,) and biofilm (biofilm inhibitory concentration, BIC) susceptibility of *Pseudomonas aeruginosa* isolates to ciprofloxacin obtained from the airway of patients with cystic fibrosis. The MIC and BIC values are given in terms of $\log_{10}$ mg/L. Susceptibility to ciprofloxacin was not correlated to the intensity of biofilm formation by the strains tested

Strain	37	30	29	24	20	4	5	31	28	52	9	39	16	40	38	32	13	33	26	23	14	51
MIC [mg/L]	4	8	4	4	8	16	8	8	4	4	4	8	8	4	8	8	8	8	16	8	8	8
BIC [mg/L]	16	16	16	16	32	16	16	16	16	8	8	16	32	4	8	16	16	16	32	8	16	16
Biofilm formation	v.strong	v.strong	v.strong	v.strong	v.strong	v.strong	strong	strong	strong	strong	strong	strong	strong	strong	strong	strong	strong	strong	moder.	moder.	weak	none

Correlation between biofilm formation and biofilm resistance

No significant correlation was observed between biofilm formation and BICs value for all tested antibiotics (**Figures 1–4**). No biofilm producer (strain No 51), weak (strain No 14) and moderate biofilm producers (strains No 23, 26) were equal to or more resistant to antibiotics than strong producers (e.g. strains No 13, 40, 9, 52, 28, 37).

Discussion

As bacteria were usually considered as free-living, unicellular organisms, we are aware now that they exist predominantly as adherent multicellular biofilms in diverse environmental niches. Most of the bacteria have the ability to form a biofilm on different surfaces and in various organs, such as implants, urinary catheters, teeth or lung tissue [21–23]. According to the data obtained from Centers for Disease Control and Prevention (CDC), biofilms are the background of at least 65% of all human bacterial infectious [24, 25]. Understanding bacterial physiology and the mechanisms of bacterial resistance to lethal concentrations of antibiotics it is crucial to elaborate on the effective eradication of resistant strains [26–29].

P. aeruginosa is a prime example of bacteria known to grown in a biofilm form [30–32]. Recent evidence indicating a biofilm mode of growth in the respiratory tract and the presence of biofilm quorum-sensing signals in the sputum of CF patients support the contestation that *P. aeruginosa* biofilms are present in an airway of cystic fibrosis patients [33, 34]. Many reports provided strong evidence for potential role of *P. aeruginosa* biofilm in pathogenesis of lung infections in CF patients. Bjarnsholt et al [35] detected both biofilm forming microcolonies and non adhered planktonic bacteria in samples of sputum from 77 chronic *P. aeruginosa* infected CF patients in that study and no other bacteria were isolated. In our study *in vitro* biofilm-forming capacity of *P. aeruginosa* isolated from airway CF patients were detected in more than 95% strains but with diverse intensity. The variability in biofilm formation amongst *P. aeruginosa* isolates was supported by others [7, 9, 13, 26, 36]. The reason for the variety in biofilm formation seems to be multifactorial [26, 35, 37].

Comparison of MICs and BICs demonstrated differences in activity of tested antibiotics. In general, sessile bacteria were inhibited by much higher concentrations of antibiotics than floating cells.

The huge increase in biofilm resistance to antibiotics occurred with piperacillin. Most isolates grown in planktonic culture were susceptible to piperacillin, however, we observed a loss of activity of PIP (BICs ≥ 4096 mg/L) when these isolates were grown as biofilms. For ceftazidime the BIC values for all strains were much more lower than for piperacillin, but still high (8 to 64 x MIC). The reason why these β-lactam antibiotics are not as active against biofilm as it is on planktonic cell is that β-lactam antibiotic required rapid cells growth to kill the bacteria.

Amikacin, the aminoglycoside antibiotic was slightly more effective against *P. aeruginosa* biofilm than ceftazidime. Fluoroquinolone – ciprofloxacin showed the lack of therapeutic useful activity (MIC higher than EUCAST clinical breakpoint) against planktonic cell, but against the biofilm-forming cells, compared with the other antibiotics, the inhibiting concentrations were lower.This is supported by some investigators [38, 39, 40] which indicated that β-lactam were less active antibiotics against sessile *P. aeruginosa,* in contrast to fluoroquinolones which were most active.

These results are in accordance with commonly accepted statement that biofilms are more resistant to antibiotics than planktonic cells [38, 41]. Currently we are aware that the resistance of bacteria living in the biofilm is not associated directly with mutations characteristic to specific strain. Several factors have been suggested to explain the biofilms resistance to antibiotics for example presence of exopolysaccharide substance that can slow the diffusion of antimicrobials. One hypothesis is that reduction in antibiotics penetration through the biofilm is owing to an alginate, synthesized by *P. aeruginosa* an exopolysaccharide, which acts as a barrier for biocides. The permeability studies of the alginate indicated that this factor was not the most important barrier for azithromycin, erythromycin and ceftazidime as their penetration rates were respectively 100%, 100% and 95%, and the bactericidal activity was low (bactericidal concentration ≥ 2560 mg/L) [42].

Biofilm resistance to antibiotics must thus be considered as a combination of the transfer limitation and other factors such as slow growth and decreased metabolic activity, neutralization of the antibiotics by biofilm matrix components (e.g. ability of negatively charged biofilm components to bind cationic compounds), and modifications in gene expression and cell physiology [7, 39, 43, 44].

Results of our study indicate that the effects of antibiotics on biofilm eradication may be variable and unpredictable. This makes it difficult for clinicians to choose the most active antibiotic. Antibiotics should be selected on individual bases, and the assessment of their effectiveness on both bacterial forms, planktonic and in a biofilm should be performed. Then an antibiotic therapy will have a chance of success. Our study suggest the need to develop and to introduce a standardized susceptibility testing method for biofilm mode of growth of pathogens into routinely performed tests as a part of clinical care, especially for patients suffered from CF.

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