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High throughput determination of the biofilm prevention concentration for *Pseudomonas aeruginosa* biofilms using a synthetic cystic fibrosis sputum medium

Amber De Bleeckere [a], Sara Van den Bossche [a], Pieter-Jan De Sutter [b], Tine Beirens [a], Aurélie Crabbé [a], Tom Coenye [a,*]

[a] *Laboratory of Pharmaceutical Microbiology, Ghent University, Ghent, Belgium*
[b] *Laboratory of Medical Biochemistry and Clinical Analysis, Ghent University, Ghent, Belgium*

A B S T R A C T

The presence of *Pseudomonas aeruginosa* biofilms in cystic fibrosis (CF) patients suffering from chronic lung infections contributes to the failure of antimicrobial therapy. Conventionally, the minimal inhibitory concentration (MIC) is determined to assess the antimicrobial susceptibility of a pathogen, however this parameter fails to predict success in treating biofilm-associated infections. In the present study we developed a high throughput method to determine the antimicrobial concentration required to prevent *P. aeruginosa* biofilm formation, using a synthetic cystic fibrosis sputum medium (SCFM2).

Biofilms were grown in SCFM2 for 24 h in the presence of antibiotics (tobramycin, ciprofloxacin or colistin), whereafter biofilms were disrupted and a resazurin staining was used to quantify the number of surviving metabolically active cells. In parallel, the content of all wells was plated to determine the number of colony forming units (CFU). Biofilm preventing concentrations (BPCs) were compared to MICs and minimal bactericidal concentrations (MBCs) determined according to EUCAST guidelines. Correlations between the resazurin-derived fluorescence and CFU counts were assessed with Kendall's Tau Rank tests.

A significant correlation between fluorescence and CFU counts was observed for 9 out of 10 strains investigated, suggesting the fluorometric assay is a reliable alternative to plating for most *P. aeruginosa* isolates to determine biofilm susceptibility in relevant conditions. For all isolates a clear difference between MICs and BPCs of all three antibiotics was observed, with the BPCs being consistently higher than the MICs. Additionally, the extent of this difference appeared to be antibiotic-dependent.

Our findings suggest that this high throughput assay could be a valuable addition to evaluate the antimicrobial susceptibility in *P. aeruginosa* biofilms in the context of CF.

1. Introduction

Cystic fibrosis (CF) is a hereditary disease resulting in accumulation of thick and viscous mucus in many organs, including the airways [1]. This mucus contains high concentrations of mucin, DNA and nutrients (e.g. carbohydrates, lipids and amino acids) and is therefore an ideal environment for the growth of opportunistic pathogens like *Pseudomonas aeruginosa*, which is frequently recovered from the respiratory tract of CF patients [2,3]. In CF sputum, *P. aeruginosa* forms biofilm aggregates [4] and this biofilm formation is an important reason for failure of antimicrobial therapy [5–7].

The antimicrobial susceptibility of bacterial pathogens is typically assessed by disk diffusion or by determining the minimal inhibitory concentration (MIC) using broth dilution or gradient strip methods [8–10]. However, a poor correlation is observed between the results of MIC testing and the ability of an antibiotic to control chronic lung infections *in vivo* [11]. This can at least partially be attributed to the CF lung microenvironment, which contributes to biofilm heterogeneity and metabolic adaptations, and thereby has considerable impact on antibiotic susceptibility [12,13]. Indeed, there are marked differences in gene expression between *P. aeruginosa* biofilms grown *in vitro* and those found in human infections. These differences in transcriptome impact bacterial growth and metabolic activity, resulting in enhanced antimicrobial tolerance. Examples of genes showing differential expression include *mexX* and *mexY* (linked to efflux of antibiotics; induced *in vivo*) and *oprD* and *oprF* (linked to antibiotic uptake; induced *in vitro*). In addition, the observed downregulation of genes linked to several metabolic pathways (including the TCA cycle, fatty acid catabolism, and amino acid biosynthesis) can indirectly impact antibiotic efficacy [14]. Additionally, it has been shown that genetically distinct populations of *P. aeruginosa* share a common gene expression profile when cultured in patho-physiological conditions, such as CF sputum and an explanted CF

* Corresponding author.
E-mail address: tom.coenye@ugent.be (T. Coenye).

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lung [15,16]. This highlights the importance of including specific aspects of the microenvironment in *in vitro* models, as this might increase their ability to predict the antimicrobial susceptibility of pathogens *in vivo* [13,14] e.g. by using culture media that more closely resemble the *in vivo* nutritional environment than standard media.

An example of such a culture medium is the synthetic cystic fibrosis sputum medium (SCFM2) that simulates the CF lung environment. It contains amino acids, ions and sugars in the same concentrations as found in CF sputum. In addition, it is supplemented with other components (eDNA, DOPC, *N-acetyl*glucosamine and mucin) that contribute to the viscosity and allow the formation of medium embedded biofilm aggregates [17,18]. When *P. aeruginosa* is cultured in this growth medium, a gene expression profile very similar to the one observed in expectorated sputum of CF patients is observed [17]. In addition, it has been shown that the same *P. aeruginosa* genes are essential for growth in SCFM2 and in expectorated sputum of CF patients [18] and the overall physiology of *P. aeruginosa* in the context of CF is captured well in this medium [19]. This medium can be applied in micro-titer plate assays and in this way the antimicrobial susceptibility can be determined in a high throughput way, which is critical for implementation in routine clinical practice.

The aim of the present study was to develop a novel high throughput antimicrobial susceptibility assay for *P. aeruginosa* in the context of CF, using a microtiter plate-based assay with SCFM2 as a growth medium, coupled to a resazurin-based viability staining. MICs and minimal bactericidal concentrations (MBCs) were determined using conventional approaches and compared to the biofilm preventing concentration (BPC) [20] obtained with the novel approach.

2. Methods

2.1. Bacterial isolates, culture conditions, chemicals

Ten *P. aeruginosa* isolates were included in this study: three CF reference isolates AA2 (LMG 27630), DK2 (LMG 27626) and LESB58 (LMG 27622) [21], a hyper-biofilm forming isolate (CF127) [22] and six isolates recovered from chronically colonized CF patients receiving care at the Ghent University Hospital (this study was approved by the Ethics Committee of Ghent University Hospital, registration number B670201836204). Pure cultures were obtained by growth on Tryptic Soy Agar (TSA, Neogen, Heywood, UK) for 24 h at 37 °C. Overnight cultures were grown in Mueller Hinton Broth (MHB, Lab M, Moss Hall, UK) for 16 h at 37 °C. The following antibiotics were used: tobramycin (TCI Europe, Zwijndrecht, Belgium), ciprofloxacin (Merck Life Science, Darmstadt, Germany) and colistin sulphate (TCI Europe). For tobramycin and colistin, stock solutions of 5 mg/mL were prepared and for ciprofloxacin a stock solution of 3.2 mg/mL with 70 μL 1 M HCl was prepared. All stock solutions were prepared in MilliQ water, filter sterilized (PES, 0.22 μm, VWR, Haasrode, Belgium) and stored at 4–7 °C for maximum one week prior to use. SCFM2 was prepared as described previously [18], with the modification that mucin was autoclaved first instead of sterilized by UV exposure.

2.2. Determination of minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs)

The MICs of tobramycin, ciprofloxacin and colistin were determined using broth microdilution as described in the EUCAST guidelines. In summary, an inoculum of 5×10^5 CFU/mL was incubated in a final volume of 200 μL under static conditions [8]. MBCs were determined by plating the entire content of the wells (200 μL) containing the MIC and the consecutive wells with higher antibiotic concentrations on TSA. After 24 h of incubation at 37 °C, growth was assessed, and the MBC was recorded as the lowest concentration that did not lead to growth after plating. All experiments were performed in duplicate and repeated three times, each time starting from a fresh pure culture. Median values of these triplicates were used as MIC and MBC values for data analysis and interpretation. A maximum variation of a twofold dilution was observed.

2.3. Determination of the biofilm preventing concentration (BPC)

After overnight incubation at 37 °C in MHB, cultures were diluted to a final inoculum of 5×10^7 CFU/mL, in a 96 well-plate (U-bottom, Greiner Bio-One, Frickenhausen, Germany), containing serial twofold dilutions in SCFM2 of the different antibiotics. The antibiotic concentrations used for BPC determination are listed in Table S1. Non-treated controls (containing only bacteria) and blanks (containing only SCFM2) were included in each experiment. After 24 h of incubation under static conditions at 37 °C, the biofilms were first disrupted by sonication (40 kHz for 5 min) (Hei-MIX Titramax 100/101/1000, Heidolph Instruments, Schwabach, Germany) and shaking (900 rpm for 5 min) (Branson 3510, Branson Ultrasonics, Danbury, USA). Subsequently, the metabolic activity was quantified using a resazurin staining (CellTiter-Blue, CTB) (Promega, Leiden, The Netherlands). The resazurin solution was prepared by diluting 2.1 mL stock solution of CTB with 10.5 mL physiological saline (0.9% NaCl). 120 μL of this CTB solution was added to each well. The plate was covered from light and incubated under static conditions for 1 h at 37 °C. Subsequently, the fluorescence was measured (excitation wavelength: 560 nm and emission wavelength: 590 nm) using an EnVision plate reader (PerkinElmer, Waltham, U.S.). The BPC_{fluo} (i.e. the BPC measured with this fluorometric assay) was defined as the lowest concentration of an antibiotic that reduces the resazurin-derived fluorescence with at least 90% compared to a non-treated control after 24 h of exposure to the antibiotics. After each fluorescence measurement, the entire content of all wells was diluted and plated on TSA. After 24 h of incubation at 37 °C, colonies were counted and the number of CFU was calculated. The BPC_{CFU} (i.e. the BPC determined by plating) was defined as the lowest concentration of an antibiotic that reduces the number of CFU in a biofilm with at least 90% compared to non-treated control within 24 h of incubation. All experiments were performed in duplicate and repeated three times, each time starting from a fresh pure culture. Median values of these triplicates were used as BPC_{fluo} and BPC_{CFU} values for data analysis and interpretation.

2.4. Statistical analysis

To determine whether the CTB-derived fluorescence can be used as an alternative to plating for determining the number of CFU, Kendall's Tau correlations (r_τ) between fluorescence and CFU counts were calculated. Values for non-treated controls were also included. Datapoints below the limit of detection, i.e. fluorescence values equal to one and CFU counts less or equal to 10^2 CFU/mL, were excluded. Additionally, the average fold error (AFE) and absolute AFE (AAFE) of the new assay compared to the standard one, were calculated with equations (1) and (2),

$$AFE = 10^{\frac{1}{n}\sum log\left(\frac{BPC_{fluo}}{BPC_{CFU}}\right)} \quad \text{Eq.1}$$

$$AAFE = 10^{\frac{1}{n}\sum \left|log\left(\frac{BPC_{fluo}}{BPC_{CFU}}\right)\right|} \quad \text{Eq.2}$$

where n is the number of antibiotic-isolate pairs and BPC_{fluo} and BPC_{CFU} values are the median BPC values of antibiotic-isolate pairs. A difference of one twofold dilution between replicate measurements of BPC was deemed acceptable. As a consequence, an AFE between 0.5 and 2 indicates that the fluorometric assay results in BPC_{fluo} values that are not consistently smaller or larger than the BPC_{CFU} values (i.e. no systematic bias). An AAFE smaller than 2 indicates BPC_{fluo} values do not consistently deviate from the BPC_{CFU} values, regardless of direction (overall

bias). In addition, the proportion of unbiased measurements (i.e. observations where BPC_{fluo} equals BPC_{CFU}) was calculated, along with the proportion of measurements outside the tolerance range (ie. observations where BPC_{fluo} and BPC_{CFU} differ with more than a twofold dilution). The correlations between the standard susceptibility endpoint parameters MIC and MBC and the biofilm susceptibility endpoint parameter BPC_{fluo} were also determined using the Kendall's Tau correlation test [23]. To assess whether the ratios BPC_{fluo}/MIC and BPC_{fluo}/MBC differed between antibiotics, Kruskal-Wallis tests were performed with post-hoc contrasts (Wilcoxon) and Holm's corrections for multiple testing. All statistical analyses were performed using SPSS statistics software (version 28, IBM, New York, US).

3. Results

3.1. Resazurin-derived fluorescence values correlate well with CFU counts

MIC, MBC and BPC values of tobramycin, ciprofloxacin and colistin were determined for 10 *P. aeruginosa* isolates and are shown in Table 1. The BPC was determined based on metabolic activity (BPC_{fluo}) and CFU counts (BPC_{CFU}). The MICs ranged from 0.5 to 16 μg/mL for tobramycin, from 0.0625 to 8 μg/mL for ciprofloxacin, and from 0.25 to 8 μg/mL for colistin. The MBC was typically higher than the MIC, between 1 and 64 μg/mL for tobramycin, between 0.5 and 128 μg/mL for ciprofloxacin and between 2 and 64 μg/mL for colistin. Both the BPC_{fluo} and the BPC_{CFU} were consistently higher than the MIC. For tobramycin BPC_{fluo} and BPC_{CFU} were identical for almost all isolates, except for two isolates the BPC_{fluo} was twofold higher than the BPC_{CFU} and BPCs ranged from 2 to 128 μg/mL. In contrast, for ciprofloxacin both values differed frequently. Specifically, for only two isolates the BPC_{fluo} equaled the BPC_{CFU} and for six isolates the BPC_{fluo} was twofold higher than the BPC_{CFU}, however a twofold dilution difference was deemed acceptable. Only for isolate P2I2 there was a fourfold difference between BPC_{fluo} and BPC_{CFU}. The BPC_{fluo} ranged from 0.5 to 128 μg/mL, while the BPC_{CFU} ranged from 0.5 to 64 μg/mL. In the case of colistin, the BPC_{fluo} ranged from 8 to 512 μg/mL and the BPC_{CFU} from 4 to 256 μg/mL; for four isolates the BPC_{fluo} equaled the BPC_{CFU} while for five isolates a twofold increase in BPC_{fluo} compared to the BPC_{CFU} was observed. Surprisingly, when resazurin was added to CF127 grown in SCFM2 for 24 h at 37 °C, a fluorescent signal similar to the signal of the blanks (containing only SCFM2) was obtained, suggesting this isolate does not convert resazurin to resorufin. This of course precluded determining the BPC_{fluo} for this isolate. When the resazurin staining was applied on CF127 grown in MHB for 24 h at 37 °C, a strong fluorescent signal was recorded (data not shown), suggesting the inability to convert resazurin to resorufin is context-dependent. Determining the BPC with the fluorometric assay (results are available after approx. 1 h) is considerably faster than via plating (results are available after approx. 24 h). Results obtained with both approaches were compared to determine whether the BPC_{fluo} can be used as a valid alternative measurement. The BPC_{fluo} was equal to the BPC_{CFU} in half of the cases and differed within a twofold dilution in the other cases, which is considered acceptable. Only for P2I2 treated with ciprofloxacin, the BPC_{fluo} differed from the BPC_{CFU} with a fourfold dilution (Table 1); overall a strong correlation was found between BPC_{fluo} and BPC_{CFU} ($r_\tau = 0.87$, $p < 0.001$) (Fig. S1). For each isolate a statistically significant ($p < 0.05$) correlation between fluorescence values and CFU values (both log-transformed) was also observed (Fig. 1), although the strength of this correlation is isolate dependent; for six isolates strong correlations ($r_\tau \geq 0.3$) were observed and for the three clinical isolates P2I1, P2I2 and P3I1 moderate correlations ($0.3 > r_\tau \geq 0.2$) were observed [24]. When including all datapoints in the Kendall tau's correlation test, even stronger correlations were observed ($r_\tau > 0.5$, $p < 0.001$) (Fig. S2). 25.03% of the measurements obtained with the plate-count technique were equal to or below the limit of detection; for the fluorometric assay this was 17.09%. These datapoints were observed for multiple replicates per strain and were always measured at the highest antibiotic concentrations tested. To assess the potential bias introduced by using the BPC_{fluo} as a proxy for the BPC_{CFU}, the AFE and AAFE were calculated, as measures for systematic and overall bias, respectively (Table S2). The AFE and AAFE for all antibiotics combined were both 1.47, indicating a slight positive bias of the BPC_{fluo} assays. This bias was largest for ciprofloxacin (AFE and AAFE = 1.85) and smallest for tobramycin (AFE and AAFE = 1.17). In total, 48% of the measurements were unbiased. However, this bias is acceptable as it does not exceed the precision of the methods used (i.e. a single twofold dilution, $0.5 < AFE < 2$) and only 4% of the measurements is found outside the tolerance range. These results indicate the resazurin-based BPC determination is a valid alternative to plating.

3.2. The fluorometric derived biofilm preventing concentration (BPC_{fluo}) is consistently higher than the minimal inhibitory concentration (MIC)

Next, we compared the BPC_{fluo} with the MIC. A clear difference between MIC and BPC_{fluo} of all three antibiotics was observed for all

Table 1
Overview of median MIC, MBC, BPC_{fluo} and BPC_{CFU} values (in μg/mL) of tobramycin, ciprofloxacin, and colistin for the 10 *P. aeruginosa* isolates investigated. *ND, not determined.

Tobramycin										
	LMG 27630	LMG 27626	LMG 27622	CF127	P1I1	P1I2	P2I1	P2I2	P3I1	P3I2
MIC	1	0.5	4	0.5	0.5	16	8	0.5	2	8
MBC	1	2	8	2	16	64	32	2	16	32
BPC_{fluo}	8	16	32	ND*	32	128	128	2	32	32
BPC_{CFU}	8	16	32	16	16	64	128	2	32	32
Ciprofloxacin										
	LMG 27630	LMG 27626	LMG 27622	CF127	P1I1	P1I2	P2I1	P2I2	P3I1	P3I2
MIC	0.25	0.0625	4	0.125	1	2	8	8	2	4
MBC	0.5	1	16	0.5	8	16	128	128	16	16
BPC_{fluo}	1	0.5	16	ND*	16	16	64	128	16	32
BPC_{CFU}	0.5	0.5	8	1	8	8	64	32	8	16
Colistin										
	LMG 27630	LMG 27626	LMG 27622	CF127	P1I1	P1I2	P2I1	P2I2	P3I1	P3I2
MIC	2	2	8	1	1	0.5	0.5	0.5	0.25	0.25
MBC	2	8	64	2	4	8	2	8	2	4
BPC_{fluo}	64	16	512	ND*	16	8	16	8	8	8
BPC_{CFU}	32	16	256	16	8	8	8	8	4	8

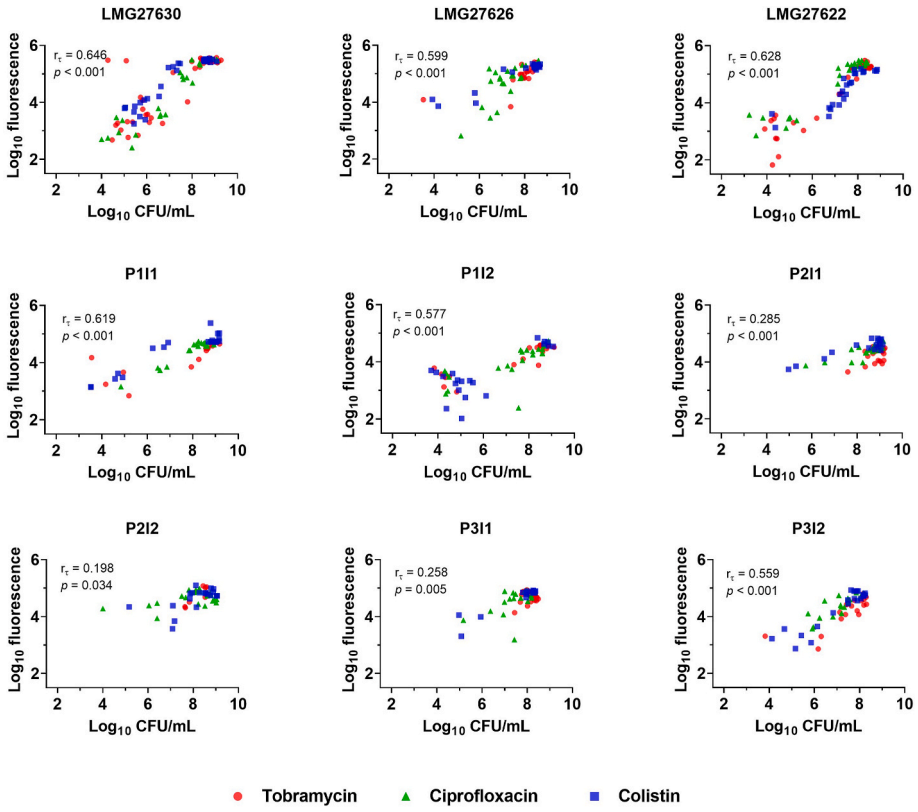

Fig. 1. Relation between $\log_{10}$ CFU/mL values and $\log_{10}$ fluorescence values for all the replicates tested (n = 3) of the nine investigated *P. aeruginosa* isolates (CF127 excluded) at the different antibiotic concentrations (for the concentration range for each strain see Table S1), along with corresponding Kendall's Tau correlation coefficients (r_τ) and *p*-values. Datapoints below or equal to the limit of detection, i.e. $\log_{10}$ CFU/mL ≤ 2 (25.03%) and $\log_{10}$ fluorescence ≤ 0 (17.09%) were excluded.

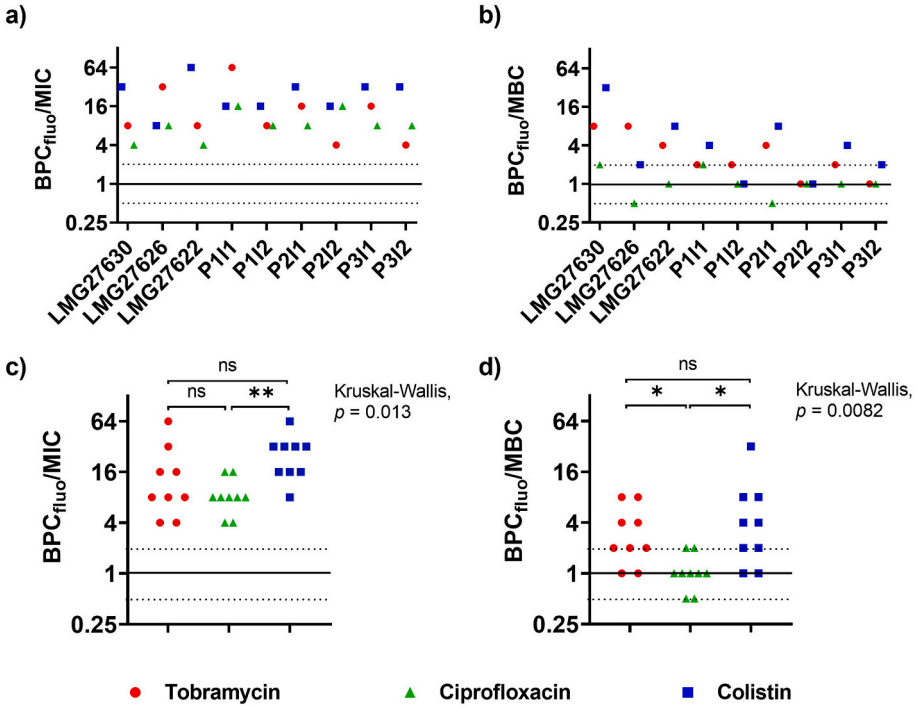

Fig. 2. BPC_{fluo}/MIC ratios (a, c) and BPC_{fluo}/MBC ratios (b, d) for tobramycin, ciprofloxacin and colistin. Panels (a) and (b): grouped per isolate (nine *P. aeruginosa* isolates investigated, CF127 excluded). Panels (c) and (d): grouped per antibiotic. The solid vertical black line is the line of unity (BPC_{fluo} = MIC) and dotted black lines represent twofold margin around this value. ns: not significant ($p_{adjusted} \geq 0.05$), *: $p_{adjusted} < 0.05$, **: $p_{adjusted} < 0.01$.

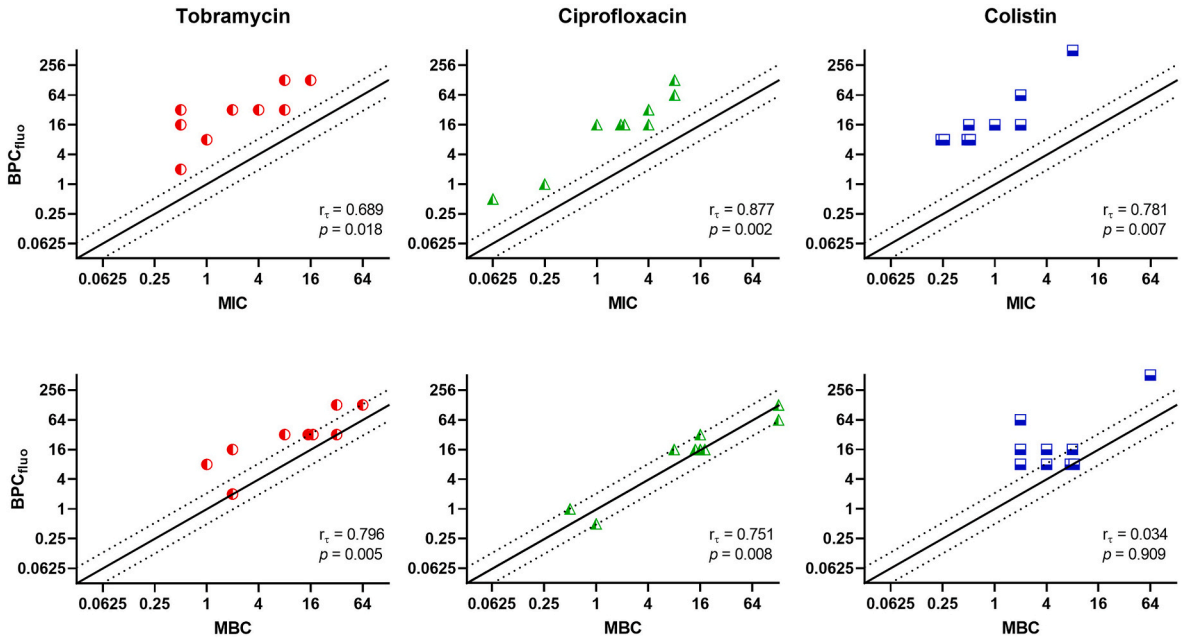

Fig. 3. Relation between MIC values and BPC_{fluo} values (above) and MBC values and BPC_{fluo} values (below) per antibiotic for the nine *P. aeruginosa* isolates (CF127 excluded), along with corresponding Kendall's Tau correlation coefficients (r_τ) and *p*-values. The solid black line represents the line of unity and dotted black lines represent twofold margin.

isolates, with all BPC_{fluo} values being at least fourfold higher than the observed MIC values (Figs. 2 and 3). BPC_{fluo}/MIC ratios were significantly affected by the type of antibiotic (Kruskal-Wallis $H(2) = 8.72$, $p = 0.013$). Post-hoc contrasts (Wilcoxon comparisons) of the mean ranks between the antibiotics showed that BPC_{fluo}/MIC ratios were significantly higher for colistin than for ciprofloxacin (Holm's method $p_{adjusted} = 0.009$). Other differences between antibiotics were not significant ($p_{adjusted} > 0.05$) (Fig. 2). This implies that the extent to which the BPC_{fluo} differs from the MIC is to a certain extent antibiotic-dependent. To assess the possible correlation between the MIC and BPC_{fluo}, Kendall's Tau correlation coefficients were determined per antibiotic (Fig. 3). Strong correlations were found between the MIC and the BPC_{fluo} for all antibiotics ($r_\tau \geq 0.69$, $p < 0.05$).

3.3. The fluorometric derived biofilm preventing concentration (BPC_{fluo}) is not consistently higher than the minimal bactericidal concentration (MBC)

The BPC_{fluo} was not always higher than the MBC, with some ratios being equal or even smaller than one, indicating an MBC that is higher than the BPC_{fluo} (Figs. 2 and 3). In addition, when the BPC_{fluo} was higher than the MBC, the extent of this difference was much smaller than the difference with the MIC. Nonetheless, comparable trends were observed in terms of antibiotic dependency, with colistin resulting in the largest ratio for most of the isolates and ciprofloxacin giving the smallest ratio. Moreover, none of the BPC_{fluo} values of ciprofloxacin differed by only a factor of two from the observed MBC values. In contrast, for tobramycin four of the observations (44%) differed by a factor of more than two and for colistin five of the observations (56%) differed by a factor of more than two (Fig. 3). BPC_{fluo}/MBC ratios were significantly affected by the type of antibiotic (Kruskal-Wallis $H(2) = 9.60$, $p = 0.008$). Post-hoc contrasts (Wilcoxon comparisons) of the mean ranks between antibiotics showed that the BPC_{fluo}/MBC ratio was significantly smaller for ciprofloxacin compared to colistin or tobramycin (Holm's method $p_{adjusted} = 0.024$ for both), but no significant difference between BPC_{fluo}/MBC ratios of tobramycin and colistin was found ($p_{adjusted} > 0.05$) (Fig. 2). To assess the possible correlation between the MBC and BPC_{fluo}, Kendall's Tau correlation coefficients were determined per antibiotic and are shown in Fig. 3. Strong correlations were found between the MBC and the BPC_{fluo} ($r_\tau \geq 0.81$, $p < 0.01$) for tobramycin and ciprofloxacin. For colistin however, no correlation was observed ($r_\tau = 0.04$, $p = 0.91$).

4. Discussion

The goal of the present study was to develop a high throughput method to evaluate the antibiotic susceptibility of *P. aeruginosa* under conditions that mimic both the *in vivo* biofilm phenotype and the nutritional microenvironment of the CF lung and can easily be implemented in routine clinical practice. Using this method, we observed that BPC_{fluo} values were consistently higher than MIC values, while this was not the case for the MBC. This was expected, as a higher concentration of an antibiotic is typically required to kill planktonic cells, compared to the concentration needed to inhibit their growth. Significant correlations between the BPC_{fluo} values and both the MIC and the MBC were observed. Nevertheless, the collected data was deemed too sparse and antibiotic-dependent to generalize into a regression model able to predict BPC_{fluo} based on MIC or MBC values. Both the difference between BPC_{fluo} and MIC, as well as the difference between BPC_{fluo} and MBC appeared to be antibiotic-dependent to some extent, with the differences being overall largest for colistin and smallest for ciprofloxacin. This can be attributed to the positive charge of tobramycin and colistin at neutral pH, leading to intercalation with the eDNA in SCFM2. In contrast, ciprofloxacin is negatively charged at neutral pH, possibly permitting a better penetration within the biofilm. Over the past decade, multiple studies on susceptibility testing in sputum culture media have been reported, resulting in variable findings regarding its influence on antibiotic efficacy [25–29]. However, results from these studies are difficult to compare for several reasons. Firstly, the choice of the type of sputum culture medium varies between the studies. Multiple sputum culture media have been developed to better mimic the *in vivo* microenvironment of the CF lung, including the artificial sputum medium (ASM) [30], the synthetic cystic fibrosis sputum medium SCFM and the modified version SCFM2, with the latter being the most expensive and time consuming to prepare [15,27,28]. Nonetheless, when *P. aeruginosa* is cultured in SCFM2, nearly identical essential genomes were found when

compared to genomes of *P. aeruginosa* when extracted from natural CF patient sputum [18], which is why we selected it for the present study. The cultivation time of the biofilms prior to antibiotic treatment is another variable; this ranged from 24 h to 72 h in different studies. Some of these studies not only measured inhibitory concentrations but also tracked the eradication of mature biofilms by antibiotics [27,29]. To reduce the time to obtain an antibiotic susceptibility read-out, we cultured the isolates in SCFM2 in the presence of antibiotics to determine their inhibitory effect on biofilm formation. This not only shortens the duration of the assay, but it also allows to determine the inhibitory effect of an antibiotic on the formation of a biofilm, in line with the approach for the MIC assay with planktonic cells.

A resazurin staining was used to determine the BPC_{fluo}, as a rapid alternative to plating. While a resazurin-based viability staining has been used in other studies [23,25,26], results obtained with this approach have not been compared to results obtained with culture-based approaches. Our results show that the correlation between BPC_{fluo} and BPC_{CFU} values was strong and significant. In addition, only 4% of the measurements was situated outside the tolerance range of twofold dilutions, indicating a high accuracy, and meaning that the BPC_{fluo} is a valid alternative measure to the BPC_{CFU} determined via plating. In addition, a higher percentage of the measurements obtained with the plate-count technique was below the limit of detection, suggesting the fluorometric assay has a higher sensitivity than the plate-count technique, although the differences are small. When resazurin was added to a culture of the hyper biofilm forming isolate CF127 in SCFM2, only a very low fluorescent signal, was obtained. In contrast, when CF127 was cultured in MHB, high fluorescence was observed. This indicates that CF127 is not able to convert resazurin into resorufin when grown in SCFM2, which could be related to the hyper-biofilm forming character of CF127, an isolate that is known to produce excessive amounts of the polysaccharides PeI and PsI, leading to dense biofilm matrices [31]. Data obtained with CF127 indicate that it will not be possible to apply the new approach to all *P. aeruginosa* isolates and drop-outs may occur.

In conclusion, by mimicking key aspects of both the pathogen phenotype and host microenvironment, it is anticipated that the response of *P. aeruginosa* to antibiotics using the newly developed method is more predictive of its susceptibility in the patient, compared to conventional methods that are currently used in clinical microbiology laboratories. We believe that our assay could be a valuable tool in routine clinical practice, as it is easy to implement and provides results within the same timeframe as MIC determinations. However, additional studies with a larger number of strains (including additional hyper-biofilm formers) and other (classes of) antibiotics will be required to confirm the applicability of our approach. Previous clinical studies suggested there is no evidence to base treatment regimens on biofilm susceptibility testing over conventional susceptibility testing in the context of CF, as these do not result in a better clinical outcome [32–34]. However, in these studies the Calgary Biofilm Device was used, in which biofilms are grown in the general growth medium Cation Adjusted MHB on the surface of plastic pegs in a 96-well plate [35], which is very different from how they grow *in vivo*. As the microenvironment plays an important role in determining antimicrobial susceptibility (as well as other aspects of biofilm biology) [14,36], we believe our approach using SCFM2 as a culture medium might more accurately predict the *in vivo* susceptibility, which could ultimately result in a better clinical outcome after antimicrobial treatment. However, biofilm-specific clinical breakpoints (including breakpoints for inhalation therapy, which is the recommended option in the treatment of chronic lung infection [37]) are not yet available and will need to be established. Subsequent follow-up studies will then be required to determine whether antibiotics selected based on this biofilm-based susceptibility testing will lead to better clinical outcomes.

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CRediT authorship contribution statement

Amber De Bleeckere: Conceptualization, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Sara Van den Bossche:** Investigation, Resources. **Pieter-Jan De Sutter:** Formal analysis, Investigation. **Tine Beirens:** Investigation. **Aurélie Crabbé:** Conceptualization, Writing – review & editing, Funding acquisition. **Tom Coenye:** Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Tom Coenye reports financial support was provided by Belgian Cystic Fibrosis Association. Tom Coenye is senior editor of the journal 'Biofilm'.

The author reports no conflicts of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioflm.2023.100106.

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