

Antimicrobial susceptibility testing in biofilm-growing bacteria

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Abstract

Biofilms are organized bacterial communities embedded in an extracellular polymeric matrix attached to living or abiotic surfaces. The development of biofilms is currently recognized as one of the most relevant drivers of persistent infections. Among them, chronic respiratory infection by *Pseudomonas aeruginosa* in cystic fibrosis patients is probably the most intensively studied. The lack of correlation between conventional susceptibility test results and therapeutic success in chronic infections is probably a consequence of the use of planktonically growing instead of biofilm-growing bacteria. Therefore, several *in vitro* models to evaluate antimicrobial activity on biofilms have been implemented over the last decade. Microtitre plate-based assays, the Calgary device, substratum suspending reactors and the flow cell system are some of the most used *in vitro* biofilm models for susceptibility studies. Likewise, new pharmacodynamic parameters, including minimal biofilm inhibitory concentration, minimal biofilm-eradication concentration, biofilm bactericidal concentration, and biofilm-prevention concentration, have been defined in recent years to quantify antibiotic activity in biofilms. Using these parameters, several studies have shown very significant quantitative and qualitative differences for the effects of most antibiotics when acting on planktonic or biofilm bacteria. Nevertheless, standardization of the procedures, parameters and breakpoints, by official agencies, is needed before they are implemented in clinical microbiology laboratories for routine susceptibility testing. Research efforts should also be directed to obtaining a deeper understanding of biofilm resistance mechanisms, the evaluation of optimal pharmacokinetic/pharmacodynamic models for biofilm growth, and correlation with clinical outcome.

Keywords: Antibiotic, antimicrobial resistance, biofilm, pharmacokinetic/pharmacodynamic parameters, *Pseudomonas aeruginosa*, susceptibility testing

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Introduction

The development of biofilms is currently recognized as one of the most relevant drivers of persistent infections, and constitutes a major challenge for clinical microbiologists and clinicians [1]. The aims of this review are to describe the methods for antimicrobial susceptibility testing of biofilms, and to analyse the pharmacodynamic parameters obtained from these studies, in order to discuss their application in the clinical microbiology laboratory as a tool to guide therapeutic strategies.

Biofilms are defined as organized bacterial communities embedded in an extracellular polymeric matrix attached to living or abiotic surfaces. This social behaviour arises as an adaptation strategy for survival in hostile environments, including the human host [2].

The first stage of biofilm formation is adherence to a surface, helped by flagella and pili in Gram-negative bacteria [3,4] or surface proteins in Gram-positive bacteria [5]. After attachment, the biofilm proliferates and produces extracellular matrix, composed mainly of exopolysaccharides, and small amounts of protein, DNA, bacterial lytic products, and

compounds from the host [6,7]. Finally, a dispersal stage occurs, where some bacteria are released from the biofilm matrix to colonize new surfaces to start the cycle again [8].

Biofilm genesis requires cell–cell signalling for the differentiation of bacteria into complex communities. Quorum sensing regulates cell density, inducing changes in bacterial gene transcription, depending on the concentration of diffusible signal molecules such as second messengers, signalling molecules, and small RNAs [3].

Role of Biofilms in Infectious Diseases

According to the available information [8], up to 65–80% of all infections are associated with biofilm formation, highlighting their enormous clinical impact. Biofilms are typically implicated in chronic infections, in contrast to the planktonic bacteria involved in acute processes [1,9–11]. Chronic infections are characterized by the persistence of the aetiological agent, despite (in principle) adequate antibiotic therapy and host immune responses. Such types of infection have been found in almost all tissues of the human body, especially affecting patients with chronic wound infections and patients with chronic lung infections such as cystic fibrosis (CF) [12]. Biofilms are involved in >60% of chronic wound infections. The wounds may be colonized by one type of microorganism or, more frequently, by several species [13], the most common bacteria being *Staphylococcus aureus* and *Pseudomonas aeruginosa* [14,15].

Chronic respiratory infection by *P. aeruginosa* is the main cause of morbidity and mortality in CF patients, and nearly 80% of CF patients are at risk of developing it [16]. In CF, as well as in other chronic lung diseases such as bronchiectasis and chronic obstructive pulmonary disease [17,18], the biofilm mode of growth, together with the remarkable intrinsic antibiotic resistance of *P. aeruginosa* and the high prevalence of hypermutable strains [19,20], make eradication of chronic infection practically impossible.

P. aeruginosa also has an important role in ventilator-associated pneumonia development, which occurs in an approximately in one-third of intubated patients, and has been associated with biofilm formation in endotracheal tubes [21].

Biofilm-related infections have also been described on a wide range of biomedical devices, including prostheses, catheters, tracheal tubes, and cardiac valves [22–26]. The development of infection depends on the type and the length of use of the implant. In some cases, the attachment of bacteria to indwelling devices is favoured by host fibronectin and fibrinogen [27]. On the other hand, biofilms formed on this kind of surfaces may seed bacteria into the bloodstream, causing secondary infections.

Antimicrobial Resistance in Biofilms

One of the most important characteristic of biofilms is their increased tolerance to antimicrobial agents [28]. It has been proved that biofilms can tolerate up to 100–1000 times higher concentrations of antibiotics and disinfectants than planktonic cells [1,9,29–32]. Some of the several mechanisms that have been proposed over the years to contribute to biofilm phenotypic resistance are detailed below.

Decrease in antibiotic penetration

The exopolysaccharide matrix and biofilm structure reduce diffusion and act as a primary barrier, preventing the entrance of polar and charged antibiotics [33]. Alginate and extracellular DNA, which are components of the extracellular matrix, have shown antibiotic-chelating activity [34].

Different growth rates and nutrient gradients within the biofilm

Biofilms contain channels that allow the circulation of water, nutrients, and oxygen [35]. However, during biofilm formation, a gradient of available substances is established: the outer layers become aerobic and metabolically active, and the inner layers become anaerobic and nutrient-deficient, with a reduced growth rate [3,36–39]. Some antibiotics, such as fluoroquinolones, β -lactams, and aminoglycosides, are not active in anaerobic conditions; consequently, they affect only the outer part of the biofilm, where oxygen is present [40,41]. Additionally, most antibiotics show more effective killing of rapidly dividing cells, so slow growth contributes to tolerance [1,42].

Persister phenomenon

Persisters have been described as dormant variants arising within bacterial biofilms that are characteristically highly tolerant to antibiotics [43]. Whereas planktonic persisters are cleared by the host immune response, biofilm persisters are shielded from host defence [42], and may cause a relapse of infection.

Induction of resistance mechanisms

This type of resistance depends on the presence of antibiotic, and can be specific or non-specific for a particular antimicrobial. One of the most universal non-specific mechanism is the upregulation of efflux pumps. Indeed, various studies have revealed differential expression of several conventional and biofilm-specific antibiotic resistance genes in biofilms as compared with planktonic growth [44]. Moreover, the phenotypes produced by specific resistance mechanisms can significantly differ between biofilm and planktonic growth [45].

Mutational resistance

Although most of the hypotheses are based on biofilm physiological characteristics, classic mutational mechanisms play a major role in biofilm antibiotic resistance [46]. Actually, the increased antibiotic tolerance driven by the special biofilm physiology and architecture may favour gradual mutational resistance development during antimicrobial treatment, this being particularly favoured in mutator strains, which are highly prevalent in chronic respiratory infections [19,46,47]. In fact, recent findings have shown that mutagenesis is intrinsically increased in biofilms and that hypermutation plays an important role in development, adaptation and diversification processes [48–51].

Gene transfer

The biofilm structure allows effective horizontal gene transfer between bacteria, which plays an important role in the development of resistance to antibiotics [52].

In vitro Biofilm Models

Owing to the increasing interest in antimicrobial susceptibility testing in biofilms, several methods have been implemented in the last few years. Depending on nutrient delivery, biofilm growth models may be classified as closed systems (batch culture) and open systems (continuous culture) [53]. Closed models have the advantage of simplicity and applicability in high-throughput analysis, whereas open models allow better control of growth parameters and dynamics [54]. To choose the optimal experimental approach, it is necessary to consider which one is better suited for the assay to be performed. The most relevant systems are described below.

Closed systems

Microtitre plate method. The microtitre plate (e.g. 96-well plate) filled with sterile broth culture (depending on the type of microorganism) is inoculated with bacteria, and incubated for 24–48 h with an appropriate atmosphere and temperature. Biofilm formation takes place as a ring around the well. After rinsing of wells to remove planktonic cells, the biofilm can be stained with crystal violet and dissolved in acetone–ethanol for quantification of the biomass by measuring the optical density (OD) [55]. The main advantages are the ease, rapidity and reproducibility of the method. Conversely, one of the main disadvantages is the absence of a relationship between biomass and biofilm viability, as crystal violet stains dead and viable cells equally [56].

Calgary biofilm device. This device is a disposable 96-well microtitre plate with a lid that incorporates the same number of removable polystyrene pegs [57]. The bacteria are inoculated in the microtitre wells with broth culture, and the plate is incubated with or without [58] shaking to allow cells to attach to pegs. The biofilm is formed around the pegs, while planktonic bacteria remain in the broth. To facilitate the growth of bacteria, the pegs can be coated with a substance, such as L-lysine or hydroxyapatite. One of the disadvantages of this method is the possibility of contamination resulting from manipulation when pegs are removed for further analysis.

Open systems

Open systems try to replicate the *in vivo* conditions through the control of nutrient delivery, flow, and temperature. Moreover, these systems make possible the implementation of pharmacokinetic/pharmacodynamic (PK/PD) models, as well as allowing observation by microscopy. Another advantage is the study of biofilm dynamics in the absence of planktonic cells (eliminated by flow).

Flow cell. The flow cell system has been demonstrated to be the best approach for modelling biofilm formation, as real-time non-destructive confocal laser scanning microscopy (CLSM) analyses can be performed [59]. The system includes a vessel with sterile broth culture that provides medium through a multichannel peristaltic pump. The bacteria are directly inoculated into the flow cells by injection through silicone tubing. Cells are attached to a surface, where biofilm starts to develop. The most common attachment surfaces used are transparent and non-fluorescent microscope coverslips, in order to allow biofilm evolution to be observed. Another advantage is that a defined constant environment is provided by laminar flow [60]. In addition, biofilms formed in this model are thicker than those obtained with the Calgary biofilm device and the CDC Biofilm Reactor. Despite the many advantages, the process takes several days to prepare and is very time-consuming. Moreover, the coverslips are very fragile and can break easily [61]. Fig. 1 summarizes antimicrobial susceptibility testing with the flow cell biofilm model.

Suspended substratum reactor. CDC Biofilm Reactor. This system consists of a glass reactor connected to a flask with sterile broth culture, which is pumped through the system. Eight coupon holders, each one housing three coupons (diameter, 12.7 mm; surface area, 2.53 cm²), are suspended from a lid placed into the reactor filled with growth medium. The bacteria are inoculated into the reactor, and the biofilm is formed upon coupons while the broth is mixed with a stirring

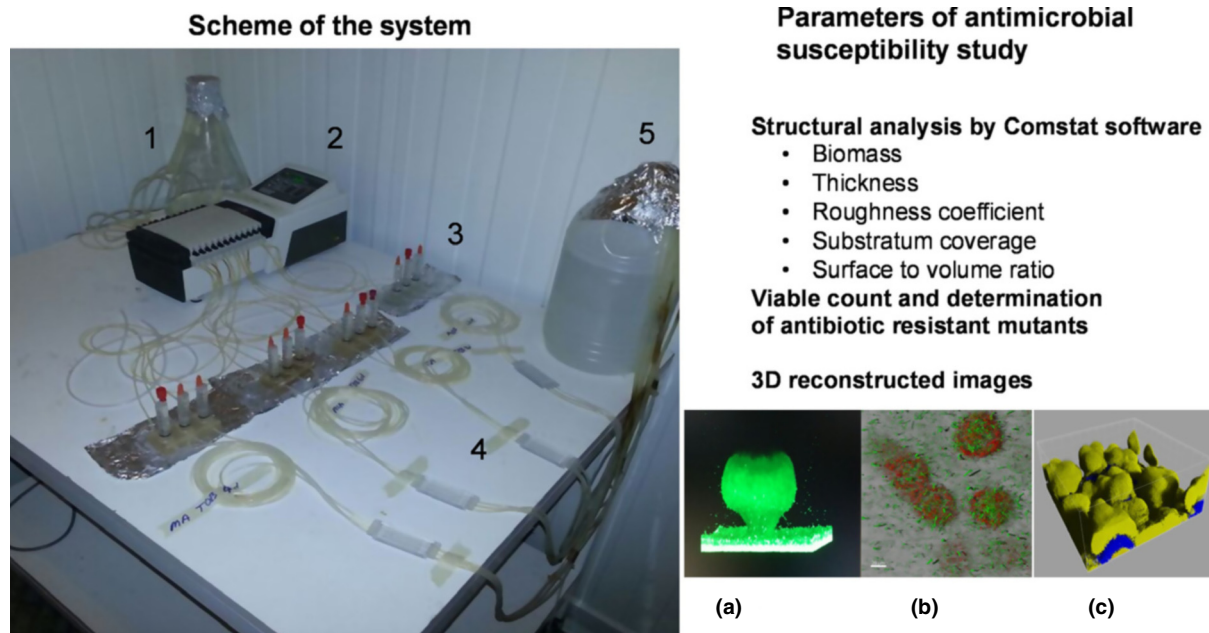


FIG. 1. Antimicrobial susceptibility testing with the flow cell biofilm model. (1) Medium bottle, where antibiotics are added and circulate through the flow cell for the required time. (2) Peristaltic pump, which provides laminar flow. (3) Bubble traps, which avoid destructive air bubbles forming on the flow cells. (4) Flow cells, where the biofilm develops. (5) Waste bottle. (a) Typical mushroom structure of *Pseudomonas aeruginosa* biofilm tagged with green fluorescent protein (GFP). (b) Red propidium iodide staining of dead cells/areas to observe and quantify the bactericidal effect during antibiotic treatment of GFP-tagged *P. aeruginosa* biofilm. (c) Different structural distribution of a mixed biofilm of two *P. aeruginosa* strains tagged with enhanced yellow fluorescent protein (outer part) and enhanced cyan fluorescent protein (internal part).

vane by magnetic rotation. Owing to the rotation, the biofilm grows under high-shear conditions. Coupons can be sampled by removing individual coupon holders and replacing them in the lid to continue the experiment in aseptic conditions. These coupons can be made from a large number of materials (polycarbonate, mild steel, stainless steel, PVC, vinyl, glass, etc.), according to the microorganism and assay. The conditions of the experiments can be controlled by modifying the flow speed, temperature, and residence times. This method allows the study of seeding planktonic cells by sampling the bulk fluid phase.

Application of Biofilm Models to Antimicrobial Susceptibility Testing

Classic antibiotic susceptibility tests (from disk diffusion to automatic broth microdilution methods) that provide the MIC used to define the susceptibility breakpoints and the PK/PD parameters that predict therapeutic success are performed with planktonically growing bacteria. As described before, biofilm-growing microorganisms are significantly more resistant to antibiotics than those growing planktonically, and

the corresponding breakpoints have not been established [62]. Therefore, the results of classic susceptibility tests cannot be used to predict therapeutic success for biofilm infections, and offer no guidelines for clinicians to treat such infections. Thus, there is growing interest in the development of susceptibility tests specific for biofilm-growing bacteria. As summarized in Table 1, several *in vitro* biofilm models have been implemented and tested in a number of different bacterial species. Nevertheless, the current lack of standardization of the methods, parameters and interpretation of results limits the application of the obtained data to the clinical setting, including the comparison of different treatment strategies.

Susceptibility assay on microtitre plates

In this simple quantitative assay, wells containing sterile growth medium are inoculated with bacteria and allowed to grow. The capacity of antibiotics to prevent or eliminate biofilms can be measured by adding various concentrations of test compounds to nascent or mature biofilms. Quantification of biofilm production is achieved, following removal of spent culture fluid from the wells and an optional wash in buffer to remove 'loosely adherent cells', by staining for crystal violet, which is a

TABLE 1. Overview of the *in vitro* biofilm models most commonly used for antimicrobial susceptibility testing

Biofilm model				
Parameters	Microtitre plates [63]	Calgary device [57,64]	Flow cell model [59,60,65]	Suspended substratum reactor [66]
Nutrient availability ^a Biofilm formation	Closed system (static) Adherence to the wells (polystyrene, polypropylene, or polycarbonate)	Closed system (static) Adherence to peg lids (polystyrene, polypropylene, or polycarbonate)	Open system (dynamic) Adherence to glass surface of coverslip	Open system (dynamic) Adherence to coupons (polycarbonate, silicone, stainless steel, etc.)
Antibiotic study	Wells incubated with antibiotic(s) Daily rinsing and medium renewal	Biofilms on pegs incubated with antibiotic(s) Daily rinsing and medium renewal	Antibiotics are added to the medium bottle and circulated through the flow cell for the required time	Antibiotics are added to the bulk fluid phase, simultaneously exposing all coupons
Biomass/CFU count	CV stain Ethanol dissolution	Biofilm transference by centrifugation or sonication OD _{650 nm} measurement (0–6 h at 37°C)	Biofilm detachment and collection by washing the flow cell channels with glass beads in saline	Biofilm transference by sonication and vigorous vortexing
Microscopic analysis	Imaging to determine viability (live/dead cell ratio)	Serial ten-fold dilutions plated SEM CLSM	Serial ten-fold dilutions plated CLSM Bacteria are previously tagged with FP	Serial ten-fold dilutions plated SEM CLSM Staining is needed
Structural analysis	Not described	Both require fixation and staining, which are destructive techniques for biofilms 3D imaging and viability studies (live/dead cell ratio) [67]	Analysis of structural parameters (biomass, thickness, roughness coefficient, etc.) with Comstat software Direct real-time non-destructive visualization and follow-up of biofilms over time	3D imaging and viability studies (live/dead cell ratio) [68]
Relevant characteristics	Feasible and reproducible Minimizes contamination risk Biofilm thickness of < 50 µm	Feasible and reproducible Minimizes contamination risk Biofilm thickness of < 50 µm	Pseudomonas aeruginosa [46] Staphylococcus spp. [78]	Allows simultaneous analysis of shedding planktonic cells and biofilm-embedded cells
Microorganisms tested	Staphylococcus aureus [69] Candida albicans [70] Pseudomonas aeruginosa [71] Streptococcus pneumoniae [72] Listeria monocytogenes [73]	Pseudomonas aeruginosa [58] Staphylococcus spp. [57] Streptococcus spp. [74] Mycobacterium spp. [75] Candida spp. [76] Burkholderia spp. [77]	Pseudomonas aeruginosa [46] Staphylococcus spp. [78]	Pseudomonas aeruginosa [66] Candida spp. [79] Staphylococcus spp. [80,81]

CLSM, confocal laser scanning microscopy; CV, crystal violet; FP, fluorescent protein; OD, optical density; SEM, scanning electron microscopy.

^aClosed system: batch culture. Open system: continuous culture.

cationic dye that non-specifically stains negatively charged biofilm constituents via ionic interactions [55]. Protocols may also be modified to incorporate different stains. The crystal violet is then dissolved by the addition of a standard volume of ethanol (or glacial acetic acid), and the absorbance is measured at 570 nm with a microplate spectrophotometer. Bacterial viability within the biofilm can be determined by use of the blue phenoxazin dye resazurin, which is reduced by viable bacteria to the pink, fluorescent compound resorufin [82,83]. After removal of the medium and washing of the wells with phosphate-buffered saline, biofilms are incubated with resazurin at room temperature in the dark, and fluorescence is then measured at a wavelength of 590 nm, with an excitation wavelength of 550 nm.

Susceptibility assay on the Calgary biofilm device

The biofilms are grown on pegs suspended from the lid of a microtitre plate by incubation at 37°C for 20 h with either rocking at 20 Hz or no movement [57]. The peg lids are then rinsed and placed onto flat-bottomed microtitre plates, where they are incubated (normally, 18–20 h at 37°C) in the presence of different concentrations of the antibiotics. After antibiotic exposure, the peg lids are again rinsed, and placed into antibiotic-free medium in a flat-bottomed microtitre plate (biofilm recovery plate). Light centrifugation (e.g. 805 g for 20 min) or 5 min of sonication at room temperature are used to transfer biofilms from pegs to wells. The OD_{650 nm} is then measured with a microtitre plate colorimeter before and after incubation at 37°C for 6 h. Adequate biofilm growth for the positive control wells is defined as a mean OD_{650 nm} difference (OD_{650 nm} at 6 h minus OD_{650 nm} at 0 h) of ≥ 0.05 . The biofilm inhibitory concentrations are defined as the lowest concentrations of drug that result in an OD_{650 nm} difference of $\leq 10\%$ of the mean of two positive control well readings [64]. The 10% cut-off represents a 1 log difference in growth after 6 h of incubation. This system allows incubation with antibiotics at different time-points, with daily rinsing and antibiotic renewal [64]. Modifications of this assay have also allowed determination of the number of viable cells and antibiotic-resistant mutants by simply plating serial ten-fold dilutions on medium and medium plus proper antibiotic concentrations, respectively, of the transferred biofilms [58]. The biofilm structure can be studied with scanning electron microscopy or CLSM after removal of the pegs [67]. Before microscopic observation, the biofilm has to be fixed to the surfaces of the pegs. Some of the available fixing techniques are destructive to biofilm, and only permit observation of the structure of underlying bacteria or observation of the extracellular polymeric matrix. For the use of CLSM, it is necessary to stain the biofilm with appropriated fluorophores.

Susceptibility assay on the flow cell model

Nascent or mature biofilms are challenged with antibiotics by adding these to the medium. Daily antibiotic renewal throughout the experiment is advisable. Confocal images acquired section by section generate useful three-dimensional images of biofilm communities after image processing [84] to visualize and monitor the effect of the antibiotic. For CLSM, bacteria can be fluorescently tagged with, for instance, green fluorescent protein, cyan fluorescent protein, or yellow fluorescent protein, as previously described [59]. Dead cells/areas may be stained red with propidium iodide to observe and quantify the bactericidal effect [85]. Structural parameters such as biomass, average and maximum thickness and roughness coefficient can be measured from a significant number of images (at least four pictures per channel per flow cell) by analysis with Comstat software [86]. Viable cells and antibiotic-resistant mutants can also be determined by washing the flow cell channels with a 1-mL glass bead suspension in 0.9% NaCl to detach and collect biofilms at the end of the experiments, and then plating serial ten-fold dilutions on medium and medium plus proper antibiotic concentrations, respectively [46].

Susceptibility assay on the CDC Biofilm Reactor

Biofilms develop on coupons suspended from the lid and immersed in growth medium. Antimicrobial agents can be added to the bulk fluid phase, simultaneously exposing all coupons. Sampling is achieved by removing coupon holders from the lid at various times during the experimental run. Coupons are placed in tubes with normal saline, sonicated for 5 min, and then vigorously vortexed for 60 s (three cycles) to dislodge and disperse the cells from the biofilm, to be used for plate counting and epifluorescence microscopy [87]. The biofilm structure can be observed by CLSM with staining of coupons [68,87].

Parameters of Antibiotic Activity on Biofilms

The pharmacodynamic parameters used to quantify antimicrobial activity in planktonic and biofilm-growing bacteria are summarized in Table 2. Similarly to the conventional MIC, the minimal biofilm inhibitory concentration (MBIC) was first defined, by Moskowitz *et al.*, using the Calgary device [64], as the lowest concentration of drug that resulted in an OD_{650 nm} difference of $\leq 10\%$ of the mean of two positive control well readings. Regarding cell counts, the MBIC is the lowest concentration of an antimicrobial at which there is no time-dependent increase in the mean number of biofilm viable cells when an early exposure time is compared with a later exposure time. Likewise, the planktonic minimal bactericidal

TABLE 2. Pharmacodynamic parameters of antimicrobial activity in planktonic and biofilm-growing bacteria

Parameter	Definition
MIC	The lowest concentration of an antibiotic that inhibits the visible growth of a planktonic culture after overnight incubation
MBIC	The lowest concentration of an antibiotic that resulted in an OD _{650 nm} difference of ≤10% (1 log difference in growth after 6 h of incubation) of the mean of two positive control well readings
MBC	The lowest concentration of an antibiotic producing a 99.9% CFU reduction of the initial inoculum of a planktonic culture
BBC	The lowest concentration of an antibiotic producing a 99.9% reduction of the CFUs recovered from a biofilm culture as compared to the growth control
MBEC	The lowest concentration of an antibiotic that prevents visible growth in the recovery medium used to collect biofilm cells
BPC	Same as the MBIC, but bacterial inoculation and antibiotic exposure occur simultaneously

BBC, biofilm bactericidal concentration; BPC, biofilm-prevention concentration; MBC, minimal bactericidal concentration; MBEC, minimal biofilm-eradication concentration; MBIC, minimal biofilm inhibitory concentration; OD, optical density.

concentration (MBC), the biofilm bactericidal concentration (BBC), defined as the lowest concentration that killed 99.9% of the cells recovered from a biofilm culture compared to growth control, has also been used to evaluate the efficacy of antibiotics on biofilm-growing bacteria [88,89]. Another parameter that is used is the minimal biofilm-eradication concentration (MBEC), defined as the lowest concentration of antibiotic required to eradicate the biofilm [57] or, in other words, the lowest concentration of antimicrobial agent that prevents visible growth in the recovery medium used to collect biofilm cells (0 CFU/peg on plate counts).

All of these parameters explore the activity of antibiotics on mature biofilms, which means that the biofilm has been established; however, in the case of CF patients, for instance, the early stage of colonization is when *P. aeruginosa* can be effectively eradicated with appropriate antibiotic therapy. In this sense, the biofilm-prevention concentration (BPC) is an interesting parameter that could be used with the aim of reducing the cell density to prevent biofilm formation. BPC determination involves a modification of the MBIC assay, consisting of incubating peg lids with the planktonic inoculum at the time of exposure to different antibiotic concentrations [89].

Interpretation and Application of Susceptibility Studies in Biofilms

Table 3 summarizes the available data comparing the pharmacodynamic parameters that quantify antimicrobial activity in planktonic and biofilm-growing bacteria, with *P. aeruginosa* as a model organism. It is important to highlight the fact that, in nearly all cases, these parameters are defined by use of the Calgary device or related systems. Most antibiotics show a more than one two-fold dilution increase in the MBIC vs. MIC or MBEC/BBC vs. MBC. Only the macrolide azithromycin, which is not active in standard *in vitro* susceptibility tests, showed bactericidal activity on biofilms (Table 3). However, in another *in vitro* study, using the flow cell model, it was found that, despite this good activity on biofilms, resistant mutants were readily selected, particularly hypermutable strains [58]. The resistance mechanism selected, overexpression of Mex-CD-OprJ, was found to confer resistance to unrelated antipseudomonal agents such as ciprofloxacin or cefepime, but, in contrast, made the strains hypersusceptible to other agents, such as aminoglycosides [58].

TABLE 3. Comparison of planktonic and biofilm growth pharmacodynamic parameters of antimicrobial activity described for *Pseudomonas aeruginosa*

Antibiotic	MIC (mg/L)	MBIC (mg/L)	MBC (mg/L)	MBEC (mg/L)	BBC (mg/L)	BPC (mg/L)	Biofilm model
AZT	4 ^a	>128 ^a	8	>1024 ^b	–	–	Calgary device
CAZ	2 ^a /1 ^c /2 ^d	128 ^a /128 ^d	2 ^c /4 ^d	>1024 ^b	16 ^c /1024 ^d	16 ^d	Calgary device
MER	≤1 ^a /0.5 ^c	4 ^a	1 ^c	–	8 ^c	–	Calgary device
IMP	2 ^d /1 ^e	64 ^d /32 ^e	4 ^d /4 ^e	1024 ^e /1024 ^b	256 ^d	32 ^d	Calgary device
CIP	0.5 ^a /0.125 ^c /1 ^d	1 ^a /1 ^d	0.25 ^c /1 ^d	4 ^b	2 ^c /64 ^d	1 ^d	Calgary device
TOB	2 ^a /2 ^d	4 ^a /8 ^d	2 ^d	2 ^b	64 ^d	4 ^d	Calgary device
COL	2 ^a /2 ^e	16 ^d /16 ^e	1 ^d /8 ^e	128 ^e	64 ^d	2 ^d	Calgary device
AZM	128 ^a /128 ^d	2 ^a /16 ^d	>128 ^d	–	512 ^d	8 ^d	Calgary device
CXA-101	0.5 ^c	–	0.5 ^c	–	0.5 ^c	–	Calgary device

AZM, azithromycin; AZT, aztreonam; BBC, biofilm bactericidal concentration; BPC, biofilm-prevention concentration; CAZ, ceftazidime; CIP, ciprofloxacin; COL, colistin; IMP, imipenem; MBC, minimal bactericidal concentration; MBEC, minimal biofilm-eradication concentration; MBIC, minimal biofilm inhibitory concentration; MER, meropenem; TOB, tobramycin.

^aObtained from [57]. Median values of a clinical collection.

^bObtained from [64]. *Pseudomonas aeruginosa* ATCC 27853 values.

^cObtained from [88]. PAOI values.

^dObtained from [89]. Fifty per cent value of a clinical collection.

^eObtained from [90]. PAOI values.

^fObtained from [58]. PAOI values.

Similarly, ciprofloxacin, which, as shown in Table 3, is one of the most active antibiotics on biofilms (MIC same as MBIC [89]), led to the selection and amplification of resistant mutants in a flow cell PK/PD model of *P. aeruginosa* biofilm treatment. In this model, a concentration of 2 mg/L ciprofloxacin, which correlated with the mutant-prevention concentration and provided an area under the curve/MIC ratio of 384, which should predict therapeutic success, was used, but demonstrated, nevertheless, that theoretically optimized PK/PD parameters failed to suppress resistance development on biofilms.

The results of other studies of PK/PD models of *P. aeruginosa* biofilm treatment showed time-dependent killing for β -lactam antibiotics and concentration-dependent or dose-dependent killing for ciprofloxacin, colistin, and tobramycin, which is similar to what has been shown for planktonic growth [90–92]. However, the concentrations of antibiotics needed were, in all cases, very much higher, even in the case of time-dependent killing, where, on β -lactamase-overproducing biofilms, the killing pattern of ceftazidime was changed to concentration-dependent killing for biofilm cells [93]. Moreover, analysis of antimicrobial activity on biofilms with the flow cell CLSM model reveals that some agents (such as ciprofloxacin) are effective only against the (metabolically active) outer layers, whereas others (such as colistin) kill only the (metabolically attenuated) inner layers, providing a rational approach for establishing combination therapy [94]. These results indicate the complexity of the interaction of biofilm mechanisms with antibiotic activity, and the need for deeper *in vitro* and *in vivo* studies before antibiotic strategies based on biofilm antimicrobial susceptibility testing can be recommended rather than conventional ones. Indeed, biofilm susceptibility testing has not yet resulted in reliable prediction of therapeutic success in the single clinical trial performed so far [95], and further data from future randomized clinical trials on this topic are therefore required to shed light on this question [62,96].

Future Directions

Alternative susceptibility tests that are useful for predicting therapeutic success for strains involved in biofilm infections are needed in the clinical microbiology laboratory. Therefore, an effort to implement biofilm-feasible antibiotic susceptibility testing assays [55,57,64] that supply endpoints such as the MBIC, MBEC, BBC or BPC, making them compatible with routine clinical microbiology laboratory practice is required. Nevertheless, standardization of the procedures, parameters and breakpoints, by official agencies such as the CLSI or the European Committee on Antimicrobial Susceptibility Testing,

is needed before they can be implemented in clinical microbiology laboratories for routine susceptibility testing. Moreover, a special effort should be made to establish the optimal growth conditions and media in an attempt to better reproduce the conditions *in vivo*. In this sense, the use of artificial sputum medium and an anaerobic atmosphere are among the measures thought to better mimic the *in vivo* conditions [97]. Other, more complex, biofilm models, such as the flow cell [60], are probably not feasible for routine testing, but should be very useful for establishing the dynamics of biofilm populations in the presence of existing and new antibiotics, including combinations of antibiotics, to elucidate biofilm resistance mechanisms and to determine optimal PK/PD antibiotic parameters on biofilms. In addition, more *in vivo* studies and clinical trials based on biofilm antimicrobial susceptibility testing-driven therapy are needed.

Transparency Declaration

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