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In Vitro Antimicrobial Susceptibility Testing of Biofilm-Growing Bacteria: Current and Emerging Methods

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Abstract

The antibiotic susceptibility of bacterial pathogens is typically determined based on planktonic cells, as recommended by several international guidelines. However, most of chronic infections – such as those established in wounds, cystic fibrosis lung, and onto indwelling devices – are associated to the formation of biofilms, communities of clustered bacteria attached onto a surface, abiotic or biotic, and embedded in an extracellular matrix produced by the bacteria and complexed with molecules from the host. Sessile microorganisms show significantly increased tolerance/resistance to antibiotics compared with planktonic counterparts. Consequently, antibiotic concentrations used in standard antimicrobial susceptibility tests, although effective against planktonic bacteria *in vitro*, are not predictive of the concentrations required to eradicate biofilm-related infections, thus leading to treatment failure, chronicization and removal of material in patients with indwelling medical devices.

Meeting the need for the *in vitro* evaluation of biofilm susceptibility to antibiotics, here we reviewed several methods proposed in literature highlighting their advantages and limitations to guide scientists towards an appropriate choice.

Keywords

Antibiotic resistance · Antibiotic therapy · Biofilm-related infections · Susceptibility tests · Treatment failure

Abbreviations

CLSI	Clinical and Laboratory Standards Institute
EUCAST	European Committee on Antimicrobial Susceptibility Testing
CF	Cystic fibrosis
AST	Antibiotic susceptibility testing
MTP	Microtiter plate
CBD	Calgary biofilm device
OD_{650}	Optical density at 650 nm
ASTM	American Society for Testing and Materials International
BRT	BioFilm® ring test
DFBR	Drip flow biofilm reactor

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MBIC	Minimum Biofilm Inhibitory Concentration
BPC	Biofilm-Prevention Concentration
BBC	Biofilm Bactericidal Concentration
MBEC	Minimum Biofilm Eradication Concentration
MIC	Minimum Inhibitory Concentration
PYO	Pyocyanin
IMC	Isothermal microcalorimetry
CVC	Central venous catheter

1 Background

The susceptibility of a bacterial pathogen to antibiotics is typically determined based on planktonic cells, as recommended by CLSI and EUCAST international guidelines. Nevertheless, successful treatment of chronic infections, such as a pulmonary infection in cystic fibrosis (CF) patients and those related to indwelling devices, usually requires the eradication of the pathogen growing in a biofilm (Hauser et al. 2011; Tande and Patel 2014).

Current definitions have described biofilms as a functional consortium of microorganisms attached to each other and onto biotic or abiotic surfaces, embedded in an extracellular matrix produced by the bacteria and complexed with other components derived from the host.

The treatment of biofilm-related infections is difficult, as sessile microorganisms are inherently tolerant/resistant to antibiotics compared with their planktonic counterparts (Stewart and William Costerton 2001; Caraher et al. 2007; Molina-Manso et al. 2013a, b; Otter et al. 2015; Luo et al. 2020). Because of this, the antibiotic concentrations used in standardized antibiotic susceptibility testing (AST), although effective against planktonic bacteria *in vitro*, are not predictive of those required to eradicate biofilms at the site of infection. This leads to multiple rounds of antibiotics, treatment failure, chronicization of infection and the removal of materials in patients with indwelling medical devices (Widmer et al. 1990; Smith et al. 2003; Hola et al. 2004; Kathju et al. 2014;). To make the picture even more complex, antibiotics at sub-inhibitory concentrations can also stimulate biofilm formation, further confusing the issue of appropriate treatment (Rachid et al. 2000; Wu et al. 2014).

There is, therefore, an urgent need for dedicated laboratory technologies to accurately assess, during diagnostic testing, the susceptibility of biofilms to antimicrobial agents. In this report, several models proposed over the last decade for the *in vitro* evaluation of the antimicrobial activity against biofilms are reviewed. Furthermore, the parameters that should be considered in the development of experimental protocols for the study of the efficacy of antibacterial agents against pathogenic bacteria in biofilms are also discussed.

2 Laboratory Models for *In Vitro* Biofilm Antibiotic Susceptibility Testing

The constant increase in the number of laboratory methods recently proposed for assessing the susceptibility of biofilms to antibiotics clearly indicates the demand for techniques alternative to the classic antibiotic susceptibility tests.

The biofilm-based growth models can be classified as closed (batch culture) or open (continuous culture) systems, based on nutrient delivery. The selection of the optimal model depends on the clinical setting there is need to mimic, considering the fact that this might require combining different approaches. The main features, along with the relative advantages and limitations, of each model proposed over the last decade for the *in vitro* evaluation of biofilm susceptibility to antibiotics are described below and summarised in Table 1.

Table 1 Main features of *in vitro* models recently proposed in the literature for assessing the *in vitro* susceptibility of biofilm to antibiotics

	Closed			Open			
Features	Microtiter plate	Calgary biofilm device	BioFilm® ring test	Flow cell	Microfluidic systems	CDC biofilm reactor	Drip flow biofilm reactor
High throughput testing	Yes	Yes	Yes	Not	Yes	Not	Not
Cost	+	++	++	+++	++++	++++	+++
Dedicated instruments	Not	Not	Yes	Yes	Yes	Yes	Yes
Exhaustion of nutrients	Yes	Yes	Yes	Not	Not	Not	Not
Single use	Yes	Yes	Yes	Not	Not	Not	Not
Reproducibility	+	++	++	++	++	++	+
Sensitivity	+	+	+++	++	++	++	++
Time to results	+	+	++	+	+	+	+
Aggregation	Possible	Not	Not	Not	Not	Not	Not
Endpoint measurement	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Microscopic observation	Yes	Yes	Not	Yes	Yes	Yes	Yes
Biofilm viability assessment	+	++	+	+	++	++	++
Combinatorial approach[a]	+++	+++	Not	+	+	+	+
Contamination	++	++	Not	+	++	+	+
Standardized	Yes	Yes	Not	Not	Not	Yes	Yes
Published biofilm-based AST studies	++++	+++	+	+++	+	++	+

[a]Combinatorial approach: challenge plate configurations can be set up to perform checkerboard assays to identify antimicrobial antagonism or synergy (Harrison et al. 2008), and to perform multiple combination susceptibility testing (Slinger et al. 2006)

2.1 Closed ("Batch Culture" Based) Models

In these models, the microorganisms with relatively fast growth rate are incubated in a closed vessel with a single batch of medium warranting a limited amount of nutrients (McBain 2009; Azeredo et al. 2017). The ease of use, reproducibility and applicability in high-throughput analysis make these models easily implemented in a microbiology laboratory's routine and useful for high-throughput analysis.

2.1.1 Microtiter Plate Method (MTP)

In this assay, bacteria are incubated in the "U"-bottom wells of a polystyrene 96-well microtiter plate containing sterile growth medium (Stepanović et al. 2007; Azeredo et al. 2017). Following incubation, planktonic bacteria are rinsed away, and the remaining adherent bacteria (biofilms) are quantified.

Biofilm measurement is generally performed by measuring the optical density after staining biofilm with crystal violet (Stepanović et al. 2007; Azeredo et al. 2017). Although rapid, easy to perform and reproducible, this technique is not informative for biofilm viability, but rather for biofilm biomass only. Indeed, the cationic dye stains the negatively charged biofilm constituents such as cells, in a non-specific way, regardless of their viability and extracellular matrix. The residual viability of a biofilm after exposure to an

antimicrobial agent can be accurately measured using a viable cell count assay, after detaching biofilm by scraping or sonication. Other less laborious but also less reliable approaches use "metabolic" stains directly on biofilm or the cells collected after its disruption: blue phenoxazin dye resazurin (Wannigama et al. 2020), tetrazolium salts (Sabaeifard et al. 2014), Alamar blue (Kim et al. 2010a, b), and fluorescent chromophores (e.g., Syto-9, propidium iodide) (Müsken et al. 2010).

MTP method was also used to combine the bacterial viability staining with automated confocal laser scanning microscopy, thus allowing easy qualitative and quantitative evaluation of biofilms after exposure to antibiotics (Müsken et al. 2010).

User-friendly, rapid and cost-saving (using small volumes of antimicrobials and minor media consumption) MTP is one of the most widely used models for testing biofilm susceptibility to both antibiotics and disinfectants. It allows to evaluate the efficacy of various concentrations of test compounds in preventing or eradicating biofilms (Pitts et al. 2003; Takahashi et al. 2007; Božić et al. 2018). Moreover, the possibility of testing multiple replicates with a low operating volume makes MTP commonly used for screening large drug libraries (Van den Driessche et al. 2017; Gilbert-Girard et al. 2020). However, a major drawback of this method is the nutrient depletion during the incubation period, with a significant impact on the biofilm susceptibility towards the antibiotics (Manner et al. 2017). Furthermore, portions of the biofilm biomass may stem from cells sedimented to the bottom of the wells, rather than being the result of a biofilm-forming process.

2.1.2 Calgary Biofilm Device (CBD)

In this method, the biofilm can form – under dynamic (rocking at 20 Hz) or static conditions – onto the surface of polystyrene pegs present on the lower surface of a coverlid that fit into the wells of the microtiter plate containing the growth medium and bacteria (MBEC™ Assay procedural manual, version 2.1.; Ceri et al. 1999). The peg lids are then rinsed and placed onto flat-bottomed microtiter plates, where they are exposed (18-20 h, 37 °C) to different antibiotic concentrations (MBEC™ Assay procedural manual, version 2.1.; Ceri et al. 1999). The peg lids are rinsed and placed into an antibiotic-free medium in a flat-bottomed microtiter plate where the biofilm is recovered after detaching it by light centrifugation/sonication (MBEC™ Assay procedural manual, version 2.1.; Ceri et al. 1999).

The biofilm viability residual after antibiotic exposure can be evaluated by (MBEC™ Assay procedural manual, version 2.1.; Ceri et al. 1999, Harrison et al. 2010): i) a visual check of wells for turbidity; ii) measuring optical density at 650 nm (OD_{650}) before and after 6 h-incubation at 37 °C considering a mean OD_{650} difference of $\geq$0.05 as adequate biofilm growth for the positive control wells; or iii) viable cell count.

CBD has been employed to perform AST of biofilm formed by enterococci (Sandoe et al. 2006), *P. aeruginosa* from CF patients (Hill et al. 2005; Høiby et al. 2019), *S. aureus* causing prosthetic infections (Molina-Manso et al. 2013a, b; Revest et al. 2016), *Burkholderia pseudomallei* (Anutrakunchai et al. 2015), and to compare efficacies of multiple antibiotic combinations against *P. aeruginosa* biofilm (Moskowitz et al. 2004; Tre-Hardy et al. 2008; Díez-Aguilar et al. 2017). In addition, CBD has been the first approved ASTM (American Society for Testing and Materials International) standardized biofilm disinfectant efficacy test method (Parker et al. 2014).

2.1.3 BioFilm® Ring Test (BRT)

Its functioning principle is based on the potential immobilization of magnetic microbeads by bacteria forming a biofilm in the well bottom of a modified 96-well polystyrene microplate (Chavant et al. 2007). Biofilm-associated adherence is determined when beads remain scattered after the application of a magnetic field; on the contrary, in the presence of planktonic cells the beads are immobilized in the centre of the well bottom (Liesse Iyamba et al. 2011; Puig et al. 2014).

BRT has been used to assess the ability to form biofilm by non-typeable *H. influenzae* strains

(chronic obstructive pulmonary disease, otitis media, pneumoniae), *S. aureus* and *S. epidermidis* (acute and chronic osteomyelitis, infectious arthritis), and *P. aeruginosa* CF strains (Valour et al. 2013; Valour et al. 2015; Olivares et al. 2016). An alternative protocol of the BRT, the clinical Biofilm Ring Test, can provide an accurate and rapid measurement of biofilm formation for the most common pathogenic bacteria seen in clinical practice (Di Domenico et al. 2016).

A recent, not yet standardized, extension of BRT is Antibiofilmogram®, which was tested for susceptibility profile testing of bone and joint infection-related *S. aureus* and *P. aeruginosa* CF biofilms, and could be of great interest after surgical operations on contaminated prostheses and after bacteremia to prevent the colonization of the device (Tasse et al. 2016).

The primary advantage of this methodology is the possibility to get results within a pair of hours; however, it allows for the evaluation of the adhesion, the initial step of biofilm, and requires a dedicated scanning plate reader.

2.2 Open ("Continuous Culture" Based) Models

In these models, microorganisms grow at a controlled rate and the nutrients are provided via continuous media flow (McBain 2009; Azeredo et al. 2017). The possibility of replicating *in vivo* conditions through the control of growth parameters and dynamics such as nutrient delivery, flow, and temperature, makes open models useful for the in-depth study of biofilm formation. However, they are hard to implement in a classic diagnostic workflow.

2.2.1 Flow Cell-Based Models

Biofilm formation is allowed in a capillary, onto coupon or glass slide. They enable a non-destructive, real-time, microscopic observation of the antibiotic effect against biofilm (Heydorn et al. 2000; Klausen et al. 2003; Pawley 2006; Haagensen et al. 2007). Biofilm viability can be assessed by fluorescence (e.g., Live/Dead viability kit tags live bacteria with green/yellow fluorescent proteins, whereas dead ones with propidium iodide). In addition, structural parameters such as biomass, average and maximum thickness and roughness coefficient can be measured by a dedicated software (e.g., COMSTAT) to assess the time course and spatial activity of antibiotics (Heydorn et al. 2000). Viable cells and antibiotic-resistant mutants can also be determined by cell viable count after detaching biofilms by washing the channels with glass beads in NaCl (Macià et al. 2011).

Completely autoclavable and therefore re-useable, flow cell-based models are particularly indicated to evaluate new approaches to control biofilm-associated wound infections. Indeed, "Gram-negative shift" – a well-known *in vivo* phenomenon in this kind of infections – occurs only under flow conditions (Alves et al. 2018).

It has been used for AST of biofilm formed by methicillin-resistant *S. aureus* (Smith et al. 2013), *P. aeruginosa* (Mettrick et al. 2020), and *P. gingivalis* (Asahi et al. 2012) as well to test the efficacy and the use of biocides to eliminate pathogens in the health care environment (El-Azizi et al. 2016). A flow-cell apparatus irrigated with an artificial CF sputum medium has been proposed for the mathematical modelling of *P. aeruginosa* biofilm treatment in CF lung (Miller et al. 2014).

2.2.2 Microfluidic Systems

The smaller volumes, inherent in microfluidic devices, along with the ability to produce multiple concentration gradients provided a faster alternative to current AST.

Quantification of viability after exposure to antibiotics is performed by measurement of fluorescence (using GFP-tagged bacteria or Live/Dead staining) (Kim et al. 2010a, b, 2012a). While these models are robust and promising, they require expensive optical equipment and genetically modified bacteria or selective labels (Richter et al. 2007; Holman et al. 2009; Yawata et al. 2010).

Microfluidics-based devices, including the relatively recent BioFlux™ device, are fully integrated platforms consisting of modified 96-well plates

with laminar flow chambers, a shear-flow control system, an imaging system, and advanced software for data collection and analysis; used to test the activity of antibiotics, alone and in combination, against biofilm formed by *P. aeruginosa, Staphylococcus pseudintermedius,* and *E. coli* (Webster et al. 2015; Kim et al. 2012b; Díez-Aguilar et al. 2017).

2.2.3 CDC Biofilm Reactor

Biofilms develop on coupons suspended from the lid and immersed in a growth medium. Antimicrobial agents can be added to the bulk fluid phase, simultaneously exposing all coupons. Sampling is achieved by removing the coupon holder at desired times, then coupons are sonicated, and finally vigorously vortexed to dislodge and disperse the cells from the biofilm. Biofilm measurement can be performed by viable cell plate counting or CLSM staining (Fjeld and Schuller 2013; Kim et al. 2008).

CDC biofilm reactor is a standardized device, particularly indicated for modelling prosthesis-related (allowing for high flexibility in choosing material) (Hall Snyder et al. 2014), and oral (operating under higher shear stress) (Siala et al. 2018) biofilm AST.

This model has been used to assess the efficacy of both antibiotic and antimicrobial dressings against mono- and polymicrobial *S. aureus*/*P. aeruginosa* biofilms (Miller et al. 2020; Suleman et al. 2020), as well as for assessing antibiotic susceptibility of biofilm by vancomycin-resistant *Enterococcus faecium* (Jahanbakhsh et al. 2020).

In addition, the CDC reactor has recently been used to assess the pharmacokinetics/pharmacodynamics of beta-lactams in continuous infusion for biofilm infections by *P. aeruginosa* (Gomez-Junyent et al. 2020).

2.2.4 Drip Flow Biofilm Reactor (DFBR)

It consists of several, separate, parallel channels, each one equipped with an individual lid to keep the aseptic conditions during the sampling process (Manner et al. 2017). The medium enters in each chamber, containing a coupon that may be made of a variety of materials, through a 21-gauge needle inserted in the lid septum. During operation, the reactor is maintained under low shear conditions, namely at an angle of 10° with the medium running down the length of the coupons (Manner et al. 2017).

DFBR is designed to assess the efficacy of disinfection strategies for biofilm control and removal (Curtin and Donlan 2006; Ledder et al. 2010; Ammons et al. 2011). Standardized, this model is suitable to mimic the biofilm growth occurring at the air/liquid interface under low shear stress situations, as is the case of CF lungs, teeth biofilms and wounds (Bird et al. 2002; Carlson et al. 2008; Brambilla et al. 2014). Furthermore, it allows for a generalized conceptual model of biofilm antimicrobial tolerance, with the establishment of concentration gradients in metabolic substrates and products, and biofilm cell dormancy (Stewart et al. 2019).

However, the main drawbacks of DFBR are laboriousness, the need for large volumes of media and test samples, and the limited number of antimicrobials (usually 4–6) that can be tested in parallel.

2.2.5 Robbins Device

This device consists of a pipe with several threaded holes where coupons are mounted on the end of screws placed into the liquid stream. The coupons are aligned parallel to the fluid flow and can be removed independently.

The main advantage is the possibility to sustain continued biofilm growth, for several weeks or more, without interruption (Teodosio et al. 2011; Teodosio et al. 2012). However, it relies on unidirectional shear, and requires intermittent sterilization, which limits throughput and risks contamination; in addition, artefacts due to the handling of the samples, and entry effects are common, thus requiring a stabilization length to allow for direct comparison of the biofilm formed onto different coupons.

The Robbins device was mainly used for testing the susceptibility to antibiotics of biofilms formed by several bacterial species in the oral cavity (Larsen and Fiehn 1995; Honraet and Nelis 2006; Blanc et al. 2014; Lasserre et al. 2018), on central venous catheters (Mekni et al. 2015), and voice prostheses (Free et al. 2003).

3 Quantifying the Antibiotic Activity Against Biofilms

3.1 Pharmacodynamic Parameters

Using both open and closed models, several pharmacodynamic endpoints can be obtained to finally evaluate the efficacy of an antibiotic against biofilm-growing bacteria (Macià et al. 2014). The ability of an antibiotic to affect biofilm formation is commonly evaluated by calculating either the Minimum Biofilm Inhibitory Concentration (MBIC) or the Biofilm-Prevention Concentration (BPC). MBIC can be measured both by spectrophotometry and cell viable counts. In the first case, MBIC is defined as the lowest concentration of an antibiotic causing an OD_{650} reduction of at least 10% compared to the unexposed control well readings (Moskowitz et al. 2004). 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. BPC is defined as the lowest concentration that prevents biofilm formation by reducing the cell density, and its measurement requires that biofilm be exposed to an antibiotic and tested at different concentrations, during its formation (Kolpen et al. 2010).

Both MBIC and BPC endpoints are particularly relevant in the case of CF patients where the early stage of colonization by *P. aeruginosa* can be effectively eradicated by adopting an appropriate antibiotic therapy (Cantón et al. 2005).

The effect of an antibiotic against preformed (mature) biofilms can be measured by calculating either the Biofilm Bactericidal Concentration (BBC) or the Minimum Biofilm Eradication Concentration (MBEC) (Girard et al. 2010; Macià et al. 2014; Brady et al. 2017). BBC is defined as the lowest concentration of an antibiotic that is able to cause a 99.9% reduction in the cell viable count of a biofilm culture as compared to the unexposed growth control. MBEC is defined as the lowest concentration of an antibiotic that is required to eradicate the biofilm, namely the lowest antibiotic concentration preventing visible growth in the recovery medium used to collect the biofilm sample.

3.2 What Does It Mean a "Significant Reduction" for Medically Relevant Biofilms?

There is need to define a "target" reference value to accurately ascertain the effectiveness of an anti-biofilm treatment. This would provide a useful guide to clinicians that generally manage infections choosing the most relevant and effective agent based on planktonic paradigms. Deciphering what may be a "target reference", there are two points of view to consider when posing questions around the performance standards of an agent that cites claims on "efficacy" or "effectiveness". First, there is a regulatory perspective that looks to determine a "target reference" based on standardized approaches using statistical attributes. Secondly, it is needed to understand how well results from *in vitro* studies could translate to clinical efficacy and if the adoption of the selected target references might lead to improvements in clinical symptoms until the resolution of chronic infections.

Despite the fact that the studies that compared clinical isolates using biofilm AST were focused on a small number of species (mainly *S. aureus, S. epidermidis, P. aeruginosa*, and *E. coli*), it has been suggested that treatment decisions should be based on MBIC or MBEC values (Brady et al. 2017).

Unfortunately, to date, there is no consensus on what a potential target value could be due to contrasting or insufficient scientific evidence. Indeed, two randomized controlled clinical trials that addressed the treatment of *P. aeruginosa* infections in CF patients have reported that MBEC value does not demonstrate the superiority of treatment based on biofilm AST over the conventional AST (Waters and Ratjen 2017). The authors suggested that biofilm-based AST may be more appropriate to define alternative, more

effective, formulations of drugs that can be tested in clinical trials.

In addition, MBIC value does not predict a successful clinical outcome for the treatment of catheter-related bloodstream enterococcal infections (Sandoe et al. 2006).

The absence of a "target" reference value required to ascertain the efficacy of an antibiofilm therapy suggests that a complete eradication is required. This conservative approach might over-simplify the real situation, since a target reference value may vary depending on the infecting strain, the type of infection, and the immune status of the patient.

4 When Is Biofilm AST «Justified»?

Alternatively, biofilm-based susceptibility testing should be justified only if the results cannot be predicted based on current microbiological characterization, and if their interpretation will provide clinical benefit.

The use of biofilm AST revealed that antimicrobial susceptibilities based on biofilm growth differ significantly from those based on planktonic growth. Several studies have reported BIC and MBEC values that were significantly higher (100 to 1000 times) than their corresponding Minimum Inhibitory Concentration (MIC) values (Smith 2005; Høiby et al. 2010; Kostakioti et al. 2013). Furthermore, Moskowitz et al. (2005) observed that the adoption of biofilm AST led to substantially different simulated regimens for *P. aeruginosa* CF airway infection when compared with conventional testing, with only 40 and 20% of chronic and acute regimens, respectively, consisting of drugs in the same two mechanistic classes by both methods.

Unfortunately, the current scientific evidence is insufficient to recommend the choosing of antibiotics based on biofilm AST rather than conventional AST. In this frame, a recent systematic review compared biofilm AST-driven therapy to conventional AST-driven therapy in the treatment of *P. aeruginosa* infection in CF patients (Waters and Ratjen 2017). The searches identified two multicenter, randomized, double-blind controlled clinical trials where BIC values were assessed by CBD. The authors found no difference in any of the selected outcomes (i.e., forced expiratory volume at one second, time to next exacerbation, adverse events, sputum density, quality of life) between the two groups (biofilm-based versus conventional AST) in either trial (Waters and Ratjen 2017).

5 Why Is *In Vitro* Biofilm AST Not Predictive of the *In Vivo* Situation?

Most of our knowledge regarding biofilm susceptibility to antibiotics are derived from *in vitro* assays, although they are often poor representatives of the "environment" observed at the infection site (Bjarnsholt et al. 2013). The lack in the clinical predictive value of biofilm AST might, therefore, be the consequence of an oversimplification of the bacterial growth conditions at the infection site.

Biological features should be considered in *in vitro* testing to mimicry the physicochemical conditions faced *in vivo* by an antibiotic are summarized in Table 2.

The antibiotic susceptibility of biofilms is typically performed under favourable conditions, namely in nutrient-rich media and without any apparent stressors such as adverse pH, O_2 tension, osmolarity or nutrient availability. Additionally, host defense mechanisms (cellular and antibody) are lacking in all proposed models since they are difficult to reproduce. The environments in which biofilms develop *in vivo* can be vastly different: several unfavourable conditions trigger adaptive mechanisms with consequent modification of both the local environment and the microorganism phenotype (metabolic rate, protein production, cellular replication, and expression of surface proteins) so that local conditions in the biofilm allow for microorganism survival (Koch and Hoiby 1993; Gibson et al. 2003; Jesaitis et al. 2003; Bjarnsholt et al. 2013; Campbell et al. 2014; McLaren and Shirtliff 2015).

Furthermore, biofilm structures observed *in vivo* often differ from those obtained in

Table 2 Biological and technical features responsible for the poor predictivity of biofilm AST

Biofilm features	***In vivo***	***In vitro***
Test medium	Sputum, saliva, urine, blood	Commonly used, rich, culture media
Flow type	Mostly dynamic	Mostly static
Exposure to nutrients	Not always exposed to a continuous flow of fresh media or suspended in static liquid	Continuous exposure
Exposure to antibiotics	Indirectly reached by antibiotics	Direct exposure
Adhesion to a surface	Not always; biofilm can be embedded in tissue or sited between implant and tissue	Mostly attached to a surface
Organization	Can be non-attached relatively small aggregates	"Mushroom-like" structure
Atmosphere	Mostly hypoxic or anoxic	Mostly aerobic
Etiology	Often polymicrobial	Mostly monomicrobial
Host response	Inflammatory response elicited	Not considered
Microbial diversity	Can be high (polymicrobial)	Mostly monomicrobial; underestimated (isolates selected due to prevalence or specific monotypes)
Technical features	***In vivo***	***In vitro***
Antibiotic neutralization		Mostly not performed
Cell recovery by viable count		Incomplete or underestimated (sonication; antibiotic carry-over)
Endpoint for treatment effectiveness	Possible time-dependent effect	Mostly 24 h
Adequate clinical outcome	Antibiotics might cause planktonically shed bacteria from biofilm, therefore not lead to a decreased bacterial density	Decrease in bacterial sputum is the most common outcome chosen to assess CF therapy effectiveness

in vitro studies, in terms of physical dimensions and microenvironments (Bjarnsholt et al. 2013; Roberts et al. 2015). Lastly, biofilm-based chronic infections have often polymicrobial aetiology and are characterized by high microbial diversity, whereas *in vitro* AST assays are commonly performed using monomicrobial biofilm formed by isolates selected due to the prevalence of specific monotypes (Wolcott et al. 2013).

On the other hand, technical variables could also account for the poor predictivity of biofilm AST (Melchior et al. 2007; VanDevanter et al. 2011) (Table 2).

An overestimation of the anti-biofilm activity of an antibiotic might be due to a "carry-over" effect, that is a continued antibiotic activity against biofilm during incubation and microbial recovery. This can be avoided by using a neutralizing agent or diluting the antibiotic to a sub-inhibitory level (e.g., by rinsing the biofilm prior to recovery) (Russel et al. 1979).

Sub-optimal biofilm cells recovery, during and/or after their detachment, could underestimate the efficacy of an antibiotic treatment. A highly conservative collection of adhered biofilm biomass without interfering with adhered cells viability can be achieved by using an ultrasonic cleaner, after standardization of the sonication time and power followed by a collection of microorganisms on the device by sampling the media after sonication, and finally by serial dilution and spread or spot plating to count the individual colonies plated (Harrison et al. 2010; ASTM International Standard 2012; Incani et al. 2015).

MBEC is typically determined *in vitro* by exposing biofilm to antibiotics for 24 h or less. However, biofilm-based infections are difficult to treat, especially because persister cells, tolerant to systemic levels of antimicrobials, might repopulate the biofilm when antibiotic therapy is discontinued (Lewis 2007). Confirming this,

several clinical trials have demonstrated that the chance of a positive therapy outcome increases with a longer duration of the therapy (Castaneda et al. 2016). Therefore, one-day assays for MBEC may overestimate the local antimicrobial levels needed to kill organisms in a biofilm if local levels are sustained for longer than 24 h, thus requiring the development of the extended MBEC assay.

Finally, both adequate clinical (VanDevanter et al. 2011) and technical (Melchior et al. 2007) endpoints should be also considered for a reliable assessment of the treatment efficacy.

6 Bridging the Gap Between *In Vitro* and *In Vivo* Biofilms: Beyond Commonplace AST Platforms

In trying to close the gap between *in vitro* and *in vivo* biofilm modelling, other more complex laboratory models have been recently proposed in the literature for studying biofilm formation and physiology. These models could represent new platforms for a reliable assessment of biofilm AST.

6.1 Poloxamer Thermo-Reversible Matrix

Poloxamers are nonionic triblock copolymers composed of a central hydrophobic chain of polyoxypropylene (poly(propylene oxide)) flanked by two hydrophilic chains of polyoxyethylene (poly(ethylene oxide)).

Microorganisms cultured in a semi-solid poloxamer matrix – such as the nontoxic and inert Poloxamer 407 – form microcolonies that exhibit a biofilm phenotype with increased tolerance to disinfectants, antimicrobials, and silver-containing wound dressings (Gilbert et al. 1998; Clutterbuck et al. 2007; Percival et al. 2007; Yamada et al. 2011; Taylor et al. 2016). Antimicrobial efficacy was measured by fluorescence (e.g., Syto-9 Live/Dead stain) or viable cell count.

The main advantages consist of an easy and improved biofilm recovery after poloxamer liquefaction by "flash cooling" (liquid at <15 °C, poloxamers become a semisolid gel at higher temperatures); in addition, the biofilm is not attached to a surface, as observed in CF lung infections, and chronic wounds (Bird et al. 2002; Carlson et al. 2008; Bjarnsholt et al. 2013; Brambilla et al. 2014).

6.2 Chip Calorimetry

This method enables the detection of microorganisms via their metabolic heat and can be applied for the real-time monitoring of biofilm activity. The main component is a silicon chip, equipped with a thermo-sensitive membrane containing 118 BiSb/Sb thermocouples to convert the heat generated by the bacteria into a voltage signal. A flow-through system is required to avoid bias due to the planktonic cells.

A chip-calorimeter has been validated for *Pseudomonas putida* biofilm AST, comparatively to ATP content measurement and cell viable count (Buchholz et al. 2010; Mariana et al. 2013).

This method has the potential for multichannel chip-calorimetry (the measurement of separated samples with one calorimeter) or calorimetric reading of microtiter plates. Furthermore, it does not require biofilm disruption or recultivation (long-term, real-time monitoring), has a small size (high flexibility, low medium consumption), and can be informative about the antibiotic mechanism of action.

6.3 Microfluidic-Electrochemical Coupled System

A cheaper and easier method of determining the relative number of live cells in a biofilm under exposure to antibiotics can be achieved by monitoring the electrochemical response of the system.

P. aeruginosa produces the blue electro-active molecule pyocyanin (PYO), a potential marker of cell viability and virulence (Usher et al. 2002; Allen et al. 2005). PYO undergoes reversible

redox (exchange of electrons) reactions, and its presence can be therefore measured with standard electrochemical techniques (Sismaet et al. 2014).

In this way, the decreased current response is directly related to a decrease in the measured PYO, indicating a correlation between the antibiotic concentration and PYO production.

The main advantage is that an electrochemical sensor might be used for monitoring the status of infections *in vivo* while carrying out antibiotic treatment.

6.4 Alginate Bead System

In this method, the biofilm can grow into alginate beads to obtain spatially structured aggregates like those seen in CF lungs and chronic wounds (Bjarnsholt et al. 2013; Kragh et al. 2014). Alginate-encapsulated bacteria are indeed metabolically less active – and, therefore, more tolerant to antibiotics – separated by a secondary matrix, and not attached to a surface. Moreover, a steep O_2 concentration gradient is generated within the alginate beads, with the possibility to incorporate the alternative electron acceptor NO_3- into the beads, to mimic the *P. aeruginosa* anaerobic growth.

The alginate bead model was mainly used to test *P. aeruginosa* biofilm resistance to disinfectants, such as hydrogen peroxide and monochloramine (Cochran et al. 2000), and antibiotics (Cao et al. 2015; Cao et al. 2016).

Recently, Dall et al. (2017) presented a dissolving alginate bead model utilizing a sodium alginate substratum for surface biofilm colony formation, which can be readily dissolved for an accurate evaluation of viable organisms after an antibiotic challenge.

6.5 Duckworth Biofilm Device

This device is a single part instrument, consisting of individual channels (Duckworth et al. 2018). Biofilms can be cultured on cellulose acetate/cellulose nitrate disks for recovery and enumeration, or on glass coverslips for microscopic analysis.

The main advantages are as follows: re-usable (sterilizable), technical expertise not required, no cross contamination, can be 3D-printed in a variety of materials, throughput, multi-sample analysis.

Particularly indicated for chronic wound biofilms, which are typically not submerged but grow at the air-liquid interface of the wound bed, being "fed" from beneath by wound exudate (Duckworth et al. 2018). This approach also allows for the application of wound dressings (Duckworth et al. 2018).

6.6 3D Collagen-Containing Matrix Wound Model

In this model, the biofilm grows as cell aggregates into a collagen gel matrix with serum protein mimicking the wound bed of chronic wounds (Werthén et al. 2010; Pompilio et al. 2017). The model comprised important hallmarks of biofilms including microcolonies embedded in a self-produced, extracellular polymeric matrix, increased antibiotic tolerance, and the host defence (Werthén et al. 2010). Furthermore, the developed bacterial aggregates in the collagen matrix resembled those observed in real chronic wounds thus indicating the relevance of the model (Werthén et al. 2010).

This model has been used to test the susceptibility to antibiotics of biofilm by common (*S. aureus* and *P. aeruginosa*) (Werthén et al. 2010), as well as infrequent (*Myroides odoratimimus*) (Pompilio et al. 2017) colonizers of chronic wounds.

6.7 Isothermal Microcalorimetry (IMC)

IMC allows for real-time monitoring of bacterial viability based on the metabolism-related heat production (Braissant et al. 2010). Biofilms are formed onto a substrate in a sealed glass ampoule placed in a microcalorimeter for real-time measurement of heat flow and heat.

IMC has been mainly used for testing the *in vitro* and *in vivo* activities of different antibiotic formulations on biofilms formed by Gram-positive (Gonzalez Moreno et al. 2017; Butini et al. 2019a; Butini et al. 2019b), Gram-negative (Wang et al. 2019) and mycobacteria (Solokhina et al. 2018). It also finds application for quantifying the antimicrobial efficacy of implant coatings and the study of the antibiotic eluting kinetics from different biomaterials (Butini et al. 2018; Santos Ferreira et al. 2018).

Although IMC does not allow for direct quantification of the non-replicating cells in the biofilm or the total biomass, the use of this technique shows several advantages. Samples are undisturbed and then can be reused for further analyses. Furthermore, it allows for a high-sensitive (detection limit 10^4–10^5 CFU/ml), fast (results are available within hours) and reliable investigation of biofilms. Indeed, although not yet standardized, IMC has been demonstrated to generate data in agreement with those obtained after performing standard conventional tests (Mihailescu et al. 2014; Gonzalez Moreno et al. 2017; Butini et al. 2018; Di Luca et al. 2018).

7 Choosing a Reliable *In Vitro* Model for Biofilm AST

Many laboratory biofilm models are available to assess the AST of biofilms, from "batch culture-based" models to "continuous culture-based" models. Their complexity varies considerably – increasing from "batch" to "continuous" models – making the choice very difficult.

Biofilm antibiotic efficacy studies should be assessed using one or more (combinatorial approach) models mimicking the conditions of its clinical application.

Indeed, the selection of the model system can have a profound influence on the results. Confirming this, biofilms grown under turbulent flow (e.g., using a CDC biofilm reactor) are less susceptible than when grown under laminar flow (e.g., using a DFBR) or a static (e.g., using a MTP method) biofilm system (Buckingham-Meyer et al. 2007; Nailis et al. 2010). Furthermore, the growth atmosphere can significantly affect the anti-biofilm activity. In this regard, CF *P. aeruginosa* biofilm grown under anaerobic conditions has shown to be more susceptible to colistin (Pompilio et al. 2015), and more resistant to tobramycin in an artificial sputum medium consisting of DNA from fish sperm, mucin from the porcine stomach, essential and non-essential L-amino acids, diethylenetriaminepentaacetic acid, NaCl, and KCl (Kirchner et al. 2012).

Models are often chosen based on their simplicity of use, their ability to reflect the growth and environmental survival conditions of the bacterial species tested, the preferences of the investigators, and the resources available.

However, a reliable *in vitro* model should take into consideration certain physiochemical and biological key elements to make the tests relevant for the intended clinical application to be considered (Table 3):

(i) biofilm should be grown under environmental conditions that resemble those observed at the infection site:
   - surface (in chronic infections, most biofilms form small aggregates of cells not attached to a surface, but embedded in host material) (Bjarnsholt et al. 2013); growth medium (e.g., artificial sputum medium, saliva, urine, blood) (Hill et al. 2010; Brackman and Coenye 2016; Pompilio et al. 2017; Kirchner et al. 2012); shear stress; pH; temperature; O_2 level (hypoxia until anaerobic niches were observed both in the wound and CF lung) (Sønderholm et al. 2017);
   - host immune response: knowledge of the interactions between biofilm bacteria and the immune system is critical to effectively address biofilm infections (Campoccia et al. 2019)

(ii) in the case of device-related infections, surface conditioning is needed when the device is expected to be exposed to a clinical environment prior to contacting microorganisms:
   - cardiovascular, as peripherally inserted central catheters, central venous catheters, and hemodialysis catheters (Rogers et al. 1996; Brooks and Keevil

Table 3 Criteria should be considered in choosing a reliable model for *in vitro* biofilm AST

Biofilm-related infection	*In vitro* models	Surface preconditioning[a]	Medium
Prostheses	CDC biofilm reactor	Urine (urinary catheteters)	Urine (urinary catheters)
	Robbins device	Blood components /fibrin, laminin, collagen), serum, plasma (CVC, hip prostheses)	Blood or serum (CVC[b], hip prostheses)
			Artificial saliva (ventilators)
Wound	Flow cell[c]		Collagen, fibronectin
	Duckworth biofilm model[d]		Serum, plasma or whole blood; saline
	Drip flow biofilm reactor		Simulated wound fluid
	Polaxamers		
	Alginate bead system		
	3D collagen model		
Cystic fibrosis lung	Flow cell	No surface	Artificial sputum medium
	Drip flow biofilm reactor		
	Polaxamers		
	Alginate beads		

[a]Preconditioning of the device is needed when it is expected to be exposed to a clinical environment prior to contacting microbes
[b]CVC, central venous catheter
[c]Flow cell allows for "Gram-negative shift"
[d]Duckworth model might be used as the test dressing can be applied directly on top of the biofilm, akin to the treatment of a real wound

1997; Al Akhrass et al. 2011): human or fetal bovine serum, plasma;
- respiratory, as endotracheal tubes, and ventilators (Leung and Darvell 1997): saline or artificial saliva;
- wound-related (e.g., wound dressings) (Hill et al. 2010; Poor et al. 2014): human or fetal bovine serum, plasma, whole blood, saline or water;

(iii) application of a dynamic, rather than static, environment to simulate the flow of biological fluids.

Furthermore, the method should allow for minimal sample handling to reduce contamination or to avoid alteration of biofilm structure during the testing.

8 Concluding Remarks

Recalcitrant and persistent biofilm-associated diseases require new, dedicated, therapeutic approaches. In this picture, *in vitro* methods for reliably culturing biofilms and evaluating their susceptibility to antibiotics are crucial in the assessment of therapeutic effectiveness. The choice of a biofilm model able to provide clinically relevant information is dependent upon several aspects in the *in vitro* design: selection of a suitable platform for generating biofilms with structural and physiological features resembling those observed at the site of infection, selection of physicochemical conditions mimicking the "environment" where the antibiotic will have to grapple with biofilm, and finally the use of clinically appropriate endpoints.

The complexity of this scenario makes the establishment of robust biofilm AST assays still a challenge for clinical entities. Furthermore, consideration and experimentation are, thus, warranted to:

- optimize or develop *in vitro* models where the conditions observed *in vivo* are accurately reproduced, also considering their

compatibility with routine clinical microbiology laboratory practice;
- standardize the procedures, parameters and breakpoints (MBIC, MBEC, BBC or BPC) to translate the obtained data to the clinical setting, including the comparative evaluation of different treatment strategies;
- perform *in vitro* studies comparing biofilm AST-driven therapy to conventional AST-driven therapy in the treatment of biofilm-related infections;
- carry out *in vivo* studies and clinical trials based on biofilm AST-driven therapy.

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