CAT
Critically Appraised Topic

Relevance of Biofilm Susceptibility Testing in the Clinical Microbiology Laboratory

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Date: 25-06-2024

CLINICAL BOTTOM LINE

Biofilm-growing bacteria resist the host's immune response and are significantly less susceptible to antibiotics than individual, planktonic bacteria. As a result, they often lead to chronic infections that persist despite adequate antibiotic therapy according to conventional susceptibility testing. Therefore, in this study, we aimed to investigate the value of biofilm-based susceptibility testing compared to conventional susceptibility testing in predicting therapeutic success.
The Calgary Biofilm Device (CBD), introduced in 1999, could be used for antibiotic susceptibility testing in biofilms. Biofilms are grown on pins in a microtiter plate, transferred to another plate containing various antibiotic concentrations, and the Minimum Biofilm Eradication Concentration (MBEC) is determined. Studies show MBEC values for biofilms are often several dilutions higher than MIC values for planktonic bacteria. New models like BiofilmChip and BioFlux provide advanced biofilm analysis under conditions resembling the in vivo environment. At present various parameters, including minimal biofilm inhibitory concentration (MBIC), biofilm bactericidal concentration (BBC), and biofilm prevention concentration (BPC), have been defined and they are all used to measure antibiotic efficacy on biofilms. Variability in procedures and parameters can affect results, emphasizing the need for standardized and reproducible methods. Biofilm susceptibility testing for antibiotics holds potential benefits but requires significant standardization. Biofilms exhibit different behaviors and antibiotic sensitivities compared to planktonic bacteria, necessitating specific testing methods. Developing a robust, standardized biofilm model and harmonizing testing protocols are critical steps. Key parameters like the MBIC and MBEC are essential for assessing biofilm susceptibility. Additionally, biofilm-specific breakpoints are needed to accurately interpret results. Despite advancements in standardization, large-scale clinical studies are necessary to validate the efficacy of biofilm susceptibility testing in clinical settings.

CLINICAL/DIAGNOSTIC SCENARIO

A biofilm is an accumulation of microorganisms surrounded by a self-produced matrix consisting of proteins, polysaccharides, extracellular DNA, and other macromolecules. Additionally, fibrin, platelets, and immunoglobulins can also be integrated into the biofilm.[1]
Pathogenic microorganisms use biofilm formation to adhere to artificial surfaces such as implants, prostheses, and catheters. Biofilm formation can also occur on biological surfaces, such as in the airways of patients with cystic fibrosis and in chronic wounds in patients with reduced immunity. *Escherichia coli, Pseudomonas aeruginosa*, and *Staphylococcus aureus* have been frequently studied for their biofilm development. Many other pathogens, like *Candida albicans* and *Mycobacterium tuberculosis* can also form biofilms.[2,3,4]

Bacteria that can form biofilms have a complex antibiotic resistance profile due to their unique properties and structure. This can partly be explained by the inefficient penetration of antibiotics through the polysaccharide layer of the biofilm. This mechanism temporarily protects the biofilm-growing bacteria and diminishes when antibiotics are administered long-term.
The penetration of an antibiotic into a biofilm also depends on the pharmacokinetic properties of the antibiotic, for example the molecular size and charge, lipophilicity, protein binding, water solubility and pH-dependent properties. Antibiotics with smaller molecular size, neutral charges and lipophilic antibiotics can more easily penetrate the extracellular matrix. Highly protein-bound antibiotics may have a reduced penetration into

biofilms. Additionally, antibiotics like quinolones and aminoglycosides that are substrates for efflux pumps, commonly upregulated in biofilm bacteria, may be actively expelled from the biofilm, reducing their efficacy.[5,3] Furthermore, biofilm-forming microorganisms are often in a sessile, slow-growing state induced by nutrient deprivation.[6] Such bacteria are referred to as "persisters" due to their reduced susceptibility to antibiotics in this stationary phase. Spatial heterogeneity in the physiological state of bacteria within model biofilms has also been demonstrated using various microslicing and microscopic techniques. Bacteria within biofilms exhibit distinct subpopulations and varying metabolic activity gradients, with outer layers typically growing faster than inner layers.
This heterogeneity of biofilms constitutes an important survival strategy because at least some of the "persisters," representing a wide range of metabolic states, are almost certain to survive after a metabolically targeted attack such as antibiotic treatment, thus enabling them to recolonize the biofilm.[5,7] Another hypothesis regarding "persisters" suggests that some cells in biofilms exhibit a distinct and protective biofilm phenotype that is biologically programmed and independent of nutrient deprivation. These bacteria have specific gene expression compared to their planktonic counterparts, which can vary their virulence. The altered gene expression is driven by the concentration of extracellular signaling molecules, activating quorum sensing signals among a group of bacteria. The process of quorum sensing (figure 1) is a complex communication mechanism regulating gene expression within bacterial populations in response to fluctuations in cell population density. It involves the production, release, and detection of small signaling molecules known as auto-inducers. As the bacterial population density increases, the concentration of these auto-inducers also rises. When the concentration of the auto-inducer reaches a certain threshold, the signaling molecules are detected by receptor proteins on the surface of the bacteria. This then activates a series of changes in gene expression within the bacteria, allowing them to coordinate their behavior and adapt to their environment. In Gram-positive bacteria signaling is mediated via small peptides while in Gram-negative bacteria derivatives of acyl-homoserine lactones (AHL) play a role. AHL is therefore a potential target to block quorum sensing and thereby biofilm formation.[8] The targeted genes encode virulence factors and proteins involved in antibiotic tolerance. This process is not only present in biofilms yet it is an important part of biofilm formation. Bacteria thus prepare for group behavior. Biofilm-forming strains can adapt to changed environmental factors much like a multicellular organism. In the presence of increased antibiotic concentrations, biofilms do not grow, further demonstrating that biofilm-growing bacteria do not develop traditional resistance mechanisms like planktonic cells.[3, 9]

Therefore, biofilm-growing microorganisms cause chronic infections that often persist despite adequate antibiotic therapy and the functioning of the host's innate and acquired immunity.[2,10, 11,12] Extending the duration of antibiotics treatment might result in biofilm-growing bacteria becoming less protected by this multitude of mechanisms. Firstly, the penetration of antibiotics through the polysaccharide layer of the biofilm may improve when administered long-term. Secondly, continuous exposure to antibiotics may lead to adaptive responses potentially reducing the ability to maintain the biofilm structure or upregulate resistance mechanisms. Thirdly, as antibiotics target the bacteria, nutrient levels within the biofilm may become depleted, leading to the death of some bacterial cells and reducing the overall protective capability of the biofilm. Fourthly, long-term antibiotic exposure might eventually lead to the selection of subpopulations within the biofilm that are more susceptible to the antibiotics, making the biofilm less resistant overall. These insights form the rationale for prolonged and combination antibiotic therapies to effectively manage biofilm associated infections.[2,13]

It is estimated that approximately 65 to 80% of hospital-acquired infections are associated with biofilm formation. Even though various studies do not always use the same criteria to define an infection as biofilm-related, we can still conclude that the impact on morbidity and mortality is significant.[2,14]
For instance, systemic infections such as bloodstream infections often arise from biofilms on intravenous catheters, urinary catheters, or orthopedic implants.[15] In patients with intravenous catheters, approximately 5 per 1000 catheter days in intensive care units will lead to a catheter-related infection. In patients with a prosthesis in situ, 0.5-2% of patients will develop a biofilm-related infection within the first two years postoperatively.[16,17,18]
Risk factors for developing a chronic device-related infection include immune-modulating therapy, diabetes, smoking, and renal insufficiency and hemodialysis.[19] These risk factors weaken the body's innate and adaptive immune response and thereby have the potential to shift the balance in favor of the infectious microorganism over the host. For example patients with renal insufficiency have an impaired immune response while other factors such as the frequent use of catheters and other indwelling devices, frequent hospitalization and chronic inflammation also play a part in favoring a biofilm-forming infectious microorganism when patients are dialyzed.[20,21] Locally, the innate immune response may be compromised due to lack of blood supply and altered

blood flow near the device due to surgical damage, local oxygen deficiency, impaired phagocyte function on foreign materials, inadequate immune signaling between the biomaterial and host cells, host protein deposition and protection of microorganisms from phagocytosis due to their attachment to the implant. Further research is necessary to uncover the specific molecular and cellular mechanisms.[19] Another commonly occurring biofilm infection is in patients with cystic fibrosis, where approximately 80% of these patients develop a chronic biofilm infection in the airways. Additionally, it has been shown that 60% of chronic wound infections in patients are biofilm-related.[15]
The persistent infection at the site of the biofilm causes local inflammation. Other clinical signs depend on the functional impairment of the organ or foreign body where the biofilm is located.[13,22]

The choice of antibiotic therapy is currently still based on the results of conventional susceptibility testing according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) or Clinical & Laboratory Standards Institute (CLSI) guidelines.[23] Therapy failure is frequently observed in biofilm infections, because conventional susceptibility testing relies on planktonic, individual microorganisms. Microorganisms in biofilms are clearly more tolerant to antibiotics due to their altered physiology and structure.[13] Currently, there are no established breakpoints for pathogens growing in biofilm, equivalent to MIC values that are established for planktonic bacteria.[24] Hence, in this literature review we attempted to determine the added value of antimicrobial susceptibility testing for bacteria growing in biofilms in the clinical microbiology laboratory.

QUESTION(S)

1) What techniques and guidelines are available for biofilm susceptibility testing?
2) Do the results of biofilm growth-based susceptibility testing correlate with clinical outcomes?
3) Is it feasible and relevant to implement biofilm susceptibility testing in daily practice in the clinical microbiology laboratory, and if not, how can we improve biofilm susceptibility testing to make it relevant?

SEARCH TERMS

1) MeSH Database (PubMed): MeSH term: "Biofilm" AND "Antimicrobial susceptibility breakpoint determination"
2) PubMed Clinical Queries (from 1966; http://www.ncbi.nlm.nih.gov/entrez/query.fcgi): Systematic Reviews; Clinical Queries using Research Methodology Filters (diagnosis + specific, diagnosis + sensitive, prognosis + specific)
3) Pubmed (Medline; from 1966), SUMSearch (https://sumsearch.org/), The National Institute for Clinical Excellence (https://www.nice.org.uk/), Cochrane (https://www.cochranelibrary.com/)
4) International organizations: e.g. European Society of Clinical Microbiology and Infectious Diseases (ESCMID), Clinical & Laboratory Standards Institute (CLSI)
5) UpToDate Online

RELEVANT EVIDENCE

1) Guidelines and Recommendations (most recent topics on top))
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- *Di Bonaventura et al. In Vitro Antimicrobial Susceptibility Testing of Biofilm-Growing Bacteria: Current and Emerging Methods. Advances in Microbiology, Infectious Diseases and Public Health 2022, 16: 33-51*
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4) Original Articles (RCT)

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APPRAISAL

1) What techniques and guidelines are available for biofilm susceptibility testing?

Ceri et al.[25] first described the Calgary Biofilm Device (CBD) (figure 2) or MBEC Assay System in 1999 for susceptibility testing of bacteria growing in biofilms. This device consists of a microtiter plate with a lid on which small plastic pins are attached. These pins serve as surfaces on which 96 equivalent biofilms form. To cultivate a biofilm, bacteria are inoculated onto the pins and then submerged in a liquid nutrient medium, trypticase soy broth (TSB), suitable for biofilm formation. After an incubation period of 20 hours at 37°C, the bacteria on the pins grow into mature biofilms. Biofilm growth curves demonstrated 3-5 x 10^7 bacteria per pin for *Escherichia coli* and *Pseudomonas aeruginosa*, while *Staphylococcus aureus* reached a maximum density of 1-2 x 10^5 colony-forming units (CFU)/pin. The growth and appearance of a biofilm on the artificial surface were confirmed using scanning electron microscopy.
To test the susceptibility of the biofilm-growing bacteria to antibiotics, the pins with formed biofilms are transferred to a new microtiter plate containing 96 wells filled with twofold dilutions of various antibiotics. After a new incubation period of 24 hours, the concentration of the antibiotic that is able to completely eradicate the biofilm is evaluated. This concentration is defined as the Minimum Biofilm Eradication Concentration (MBEC). Additionally, a MIC value was determined using the then-current CLSI protocol as well as with the CBD.
For *E. coli* ATCC 25922, the MBEC was 1000 times higher than the MIC values for ampicillin, ciprofloxacin, cefazolin, cefotaxime, and co-trimoxazole. For *P. aeruginosa* ATCC 27853, the MBEC values for ceftazidime, aztreonam, and piperacillin-tazobactam were each 1000 times higher than the MIC value. MBEC for *S. aureus* ATCC 29213 was 100-1000 times higher for the tested antibiotics.[25]
In a study by Saginur et al.[26] a modified CBD biofilm susceptibility assay was conducted to determine the susceptibility of *S. epidermidis* and *S. aureus*. Rifampicin, vancomycin, and fusidic acid were found to be effective against staphylococcal biofilms. MBEC results showed that 47% of *S. epidermidis* strains were killed by rifampicin. Rifampicin was suggested to reduce the adherence of biofilm organisms to the surfaces of the pins, as larger quantities of bacteria ended up in the biofilm broth. Furthermore, biofilm-growing isolates consistently showed higher antibiotic resistance, determined by MBEC values of several dilutions higher, compared to MIC values of planktonic bacteria.
One of the limitations of the CBD is the variability of results. An increase in tolerance and an elevation of the measured MBEC is demonstrated the longer *P. aeruginosa* biofilm growth is observed.[27] Additionally, the complex immune response and anatomy of the host are lacking in this setup.[28]
Several similar in vitro biofilm models, such as the BiofilmChip model and the BioFlux system, have already been developed and tested for various bacteria. The BioFlux system, in particular, allows for the evaluation of biofilm biomass and susceptibility testing without disruption by manipulation, similar to in vivo conditions. This system consists of a microfluidic chamber with multiple channels through which fluids containing bacteria flow. The system allows researchers to mimic physiological flow conditions such as blood flow to study how biofilms grow in response to environmental stimuli.[29,30]
The BiofilmChip system is structurally similar but it more focused on controlled, static conditions suitable for high-throughput screening purposes.[31] Unlike the MBEC Assay System, these systems are not commercially available.

Subsequently, several pharmacodynamic parameters (figure 3) for quantifying antimicrobial activity in biofilms were described using the Calgary Biofilm Device and derived methods. Moskowitz et al. defined the minimum biofilm inhibitory concentration (MBIC) as the lowest antibiotic concentration at which there is no time-dependent increase in the average number of viable biofilm bacterial cells. This parameter is equivalent to the minimum inhibitory concentration (MIC) used in determining antimicrobial activity of planktonic bacteria.[32]
Similar to the minimum bactericidal concentration (MBC), the biofilm bactericidal concentration (BBC) was defined as the lowest antibiotic concentration that kills 99.9% of the biofilm-growing bacteria in culture compared to the growth control. Another parameter used is the minimum biofilm eradication concentration (MBEC), defined as the lowest antibiotic concentration needed to eradicate the biofilm, preventing visible growth in a medium used to collect biofilm cells.[33]
All these parameters investigate the activity of antibiotics on mature biofilms, meaning that the biofilm is established. However, in the case of cystic fibrosis patients, the early colonization stage is crucial since *P.*

aeruginosa can be effectively eradicated at this stage with appropriate antibiotic therapy. In this context, the biofilm prevention concentration (BPC) is an interesting parameter that can be used to reduce sputum density to prevent biofilm formation. Unlike the MBIC, the BPC determines at which concentration of an antimicrobial agent the quantity of colonies in a planktonic culture has decreased sufficiently to prevent biofilm formation. The determination of BPC involves incubating pin lids with the planktonic inoculum immediately followed by exposition to different antibiotic concentrations. In other words, micro-organisms are exposed to the antimicrobial agent before biofilm formation has initiated. The concentration at which no biofilm formation is observed after a specific incubation period is then defined as the biofilm prevention concentration.[34]

The exact definition of these pharmacodynamic parameters varies between different studies and may also depend on the method used to quantify biofilms. Quantification of the number of (remaining) viable and/or culturable bacterial cells in treated and untreated biofilms is an important component of biofilm-based susceptibility testing. This can be performed with detached/dispersed cells from the biofilm, either immediately (i.e., plating detached cells and counting CFUs after suitable incubation) or after a second growth phase. In the latter case, the presence or absence of growth can be measured spectrophotometrically or by plating, or the length of the lag phase can be used to quantify the number of viable cells. The lag phase (figure 4) is the period of bacterial cell growth in which the population remains constant as it adjusts to the environmental conditions of the growth medium in which it was introduced. During this phase, bacterial cells are metabolically active but not rapidly dividing, instead they are synthesizing the necessary enzymes and other molecules for adaptation. The length can vary depending on factors such as the bacterial species, the composition of the growth medium and other environmental conditions.[35]
Alternatively, quantification can be performed directly on the biofilm, using methods such as resazurin-based viability staining, ATP measurements, crystal violet staining, microscopy, electrical impedance, or molecular methods. The Resazurin Assay uses resazurin, a blue dye metabolized to pink resorufin by metabolically active cells in a diluted biofilm sample to quantify biofilm growth. Fluorescence is measured every 10 minutes for 18 hours at 37°C and a standard curve is created by correlating the time to reach maximum fluorescence with CFU from a control biofilm's dilution series. This curve is then used to calculate the CFU from fluorescence data for treated and untreated biofilms, allowing for the determination of bactericidal effects by comparing these values. Another technique uses ATP measurements in order to quantify biofilms. This technique involves harvesting biofilms, lysing the cells to release ATP, and then mixing the ATP with a luciferase-luciferin reagent to produce light. The emitted light is measured using a luminometer, and the intensity is proportional to the ATP content. By comparing the luminescence readings to a standard curve, researchers can quantify the ATP, providing insights into biofilm viability and metabolic activity. These methods are highly sensitive and rapid, offering quantitative data useful in addressing biofilm viability. Thirdly, crystal violet staining for biofilm biomass involves fixing the biofilm on a surface, staining it with crystal violet dye, washing off excess dye, and solubilizing the bound dye to measure its absorbance spectrophotometrically. The intensity of the absorbance correlates with the amount of biofilm biomass, allowing for the quantification of biofilm formation and growth. This method is simple, cost-effective, and widely used to assess biofilm biomass in various studies.[35]

It is Important to notice that different quantification approaches often measure very different parameters, and small changes in procedures can lead to different results. Moreover, many studies do not investigate important characteristics such as reproducibility and responsiveness (i.e., the ability to distinguish between different antibiotic concentrations).[36]

Short answer

Given the complexity and variability of biofilms, it is difficult to develop a universal test that exactly mimics the architecture of an in vivo biofilm. The Calgary Biofilm Device also knows as the MBEC Assay System enables susceptibility testing of biofilm-forming bacteria, but variable results are observed, even with newer, adapted methods based on this approach to mimic in vitro biofilm. Additionally, pharmacodynamic parameters such as MBIC, BBC, and MBEC are essential for evaluating antibiotic activity on mature biofilms, although their definition and application may vary between studies.

2) Do the results of biofilm growth-based susceptibility testing correlate with clinical outcomes?

Cystic Fibrosis

In vivo and in vitro research in cystic fibrosis (CF) patients shows that *P. aeruginosa* frequently grows in biofilms in the airways. This pathogen causes chronic endobronchial infections and recurrent acute exacerbations, leading to progressive lung damage. Commonly used antibiotics for exacerbations are beta-lactam antibiotics. However, complete eradication is rare. Resistance development is a significant issue in these patients as they are frequently treated with antibiotics.[37]

Additionally, the EUCAST and CLSI do not take into account the ability of *P. aeruginosa* to form biofilms in the airways of CF patients and the differences in susceptibility between planktonic and biofilm-grown P. aeruginosa.[32,37,38]

The current guidelines recommend the use of antibiotics based on conventional susceptibility testing methods such as disk diffusion and broth microdilution.[39] However, it is often observed that the clinical outcome is inadequately predicted by these methods since biofilm infections often persist.[40,41,42] Testing the specific susceptibility pattern of these biofilm-growing strains could lead to better clinical outcomes.

Two multicenter, double-blind randomized controlled trials (RCTs) were identified, comprising a total of 78 participants.[43,44]

Moskowitz et al.[43] (figure 5) included clinically stable patients in the US, while Yau et al.[44] included patients with acute exacerbations in Canada.

Both RCTs prospectively assessed whether the use of antimicrobial therapy based on biofilm susceptibility testing improved microbiological and clinical outcomes after patients were infected with *P. aeruginosa*. The primary endpoint was the change in *P. aeruginosa* density in patients' sputum from the beginning to the end of antibiotic therapy. Sputum samples were collected prior to antibiotic therapy and cultured to verify the presence of *P. aeruginosa*. Spirometry was also performed before the start of antibiotic therapy to establish baseline lung function. Patients were randomized into one of two groups: those receiving antibiotic therapy based on conventional susceptibility testing (semi-automated broth microdilution; Sensititre) and the 'biofilm group' where antibiotics were chosen based on biofilm susceptibility testing. Biofilm susceptibility testing of P. aeruginosa was performed using a modified CBD.[32]

Antibiotics were administered for 14 days in both groups. The primary endpoint, microbiological response or change in sputum density, was calculated as the log10 of end-of-treatment density minus log10 of screening density in CFU/g. A secondary endpoint included pulmonary response, defined as the change in forced expiratory volume in 1 second (FEV1) as measured in spirometry. This parameter was calculated as end-of-treatment FEV1 minus baseline FEV1 and reported in liters. Additionally, a decrease in inflammatory parameters in blood and sputum was also monitored.

Although the intervention proved to be safe, data from both studies did not show a significant advantage of biofilm susceptibility testing over conventional susceptibility testing. Both studies demonstrated a decrease in *P. aeruginosa* density in sputum in both groups after discontinuation of antibiotic therapy. No difference was found in improvement of lung function between the two groups. In both studies, mild adverse effects like rash, diarrhea, fatigue and headache were reported. No serious adverse events were reported in either group. One of the studies also evaluated the risk and time to the next exacerbation, as well as measures to improve quality of life, but found no significant differences between the two groups. In the biofilm group, the time to next exacerbation was on average 162 days, while it was 185 days in the conventional susceptibility testing group. Both studies generally had a low risk of bias, and the quality of evidence assessed according to GRADE criteria ranged from moderate to high.[23]

The lack of differences in outcomes between the biofilm and conventional groups may be attributed to small sample size, clinical stability of participants, and variability in antibiotic susceptibility of *P. aeruginosa.* This highlights the necessity of further research on possible biofilm protective mechanisms in biofilm infections. Older age and frequent antibiotic use can lead to the selection of subclones with reduced sensitivity to all antibiotics, both for planktonic isolates and biofilm-growing bacteria.

It was concluded that biofilm susceptibility testing is not superior to conventional antimicrobial susceptibility testing for the treatment of respiratory infections with *P. aeruginosa* in patients with cystic fibrosis.[23]

Periprosthetic joint infections (PJI)

Periprosthetic joint infections arise from the formation of a biofilm on the abiotic surface of an implant, such as a hip or knee prosthesis. Even in these patients, prolonged antibiotic therapy alone often proves to be

insufficiently successful.[13] Extensive surgical debridement combined with prolonged antibiotic therapy remains the cornerstone of treating such chronic biofilm infections. A debridement and antibiotics with implant retention (DAIR) approach is an option reserved for patients presenting with bacteremia or PJI within 30 days of prosthesis implantation. Preserving the original hardware allows for a less invasive or extensive operation, while debridement is useful in reducing bacterial load and providing systemic antibiotics with a better chance of treating the infection. The rationale for selecting this option for early PJI lies in the development of a biofilm. Bacteria causing infection shortly after implantation theoretically have not had enough time to form a mature biofilm and may still be susceptible to debridement. However, in vitro studies suggest that biofilm maturation can occur in a matter of hours or days. Currently, there is a lack of *in vivo* data on the timing of biofilm formation.[45]

In recent years, guidelines for improved diagnosis of biofilm-associated (prosthetic) infections have been developed, leading to the identification of "biofilm-active" antibiotics such as rifampicin for staphylococci and fluoroquinolones for Gram-negative bacteria. These antibiotics, partly due of their lipophilic nature, have the ability to penetrate the biofilm and thereby destroy the bacteria residing within it.[28,36]
In a prospective cohort study by Gellert et al.[46], the infection and functional outcomes were compared among 131 patients with knee prosthesis infection who were treated with or without biofilm-active antibiotics. Of the 131 patients, 55 (42%) were treated with biofilm-active antibiotics, while 76 (58%) received non-biofilm-active antibiotics. The infection-free survival at 1 year was significantly better for patients receiving biofilm-active antibiotics compared to those who did not (83% vs. 70%; P = 0.040), and this superiority persisted at 2 years (67% vs. 48%; P = 0.038). Additionally, biofilm-active antibiotic treatment was associated with lower pain intensity (P = 0.006) and improved joint function post-treatment.[46]
In a study by Molina-Manso et al.[47], the minimal biofilm eradication concentration (MBEC) for various antibiotics against staphylococci in the context of PJI was determined using the CBD technique. The results for 32 clinical isolates showed that for antibiotics such as cloxacillin, vancomycin, clindamycin, co-trimoxazole, ciprofloxacin, and daptomycin, the median concentrations required to eradicate biofilms were consistently high according to MBEC measurements, while a significant number of tested isolates were susceptible to these antibiotics according to conventional MIC. Even with the most biofilm-active rifampicin, median MBEC values of 32 and 64 were observed for *S. epidermidis* and *S. aureus*, respectively, indicating that antibiotic concentrations in vivo would not be effective.
While these antibiotics show promise, the association between MBEC values and clinical outcomes does not always appear consistent, with mixed results reported in various studies.[47]

Specific biofilm-based susceptibility testing has not yet become a routine part of daily practice in clinical microbiology laboratories for these infections. The lack of standardization of methods, parameters, and interpretation of results limits its application in clinical practice.[28]

The available tests have not yet resulted in reliable prediction of therapeutic success for both types of infections, partly due to the limited amount of data correlating in vitro results with clinical success.[24,32,43,48] Furthermore, there is a lack of standardization in biofilm research, making comparisons between different studies challenging. Additionally, biofilm-specific breakpoints still do not exist, making interpretation of the aforementioned parameters challenging.[36]

Short answer

In the case of patients with cystic fibrosis, *Pseudomonas aeruginosa* often grows in biofilms in the airways. Antimicrobial treatment based on conventional susceptibility testing rarely leads to complete eradication as these strains are more resistant to antibiotics.
Susceptibility testing of biofilm-growing strains, unlike planktonic bacteria, may potentially result in better clinical outcomes for these patients. Currently, several double-blind randomized studies have not demonstrated a significant advantage of biofilm susceptibility testing compared to conventional susceptibility testing.
For PJI, studies indicate that biofilm-active antibiotics can improve infection-free survival, although standardization of testing methods and interpretation is still lacking. The lack of predictive power of biofilm-based tests for therapeutic success is due to the limited correlation between in vitro and clinical results. Moreover, standardization in biofilm research is lacking, making the comparability of studies challenging and complicating the interpretation of test results.
Predicting therapeutic success with biofilm susceptibility testing is therefore not yet possible at present.

3) Is it feasible and relevant to implement biofilm susceptibility testing in daily practice in the clinical microbiology laboratory, and if not, how can we improve biofilm susceptibility testing to make it relevant?

Although there are few large randomized clinical studies on the use of biofilm-active antibiotics in prosthetic infections, biofilm-active antibiotics are seen as an added value. This in turn suggests a potential benefit of biofilm-based susceptibility testing. However, in the context of biofilm-related respiratory infections in cystic fibrosis, two randomized clinical studies show no added value of biofilm-based susceptibility testing. While it cannot be ruled out that the different etiology of prosthetic infections and respiratory infections in cystic fibrosis is the cause of this discrepancy, it should be noted that various aspects of biofilm biology (including metabolism) can influence antimicrobial susceptibility. Biofilm susceptibility testing in an *in vitro* model that poorly represents the in vivo biofilm may yield susceptibility results that are not very representative of the activity of the antibiotic against *in vivo* biofilms.[36]

To establish biofilm susceptibility testing as a standard practice in clinical microbiology laboratories, significant standardization will be necessary. The recent establishment of organizations such as the ESCMID Study Group on Biofilms[49] and the International Biofilm Standards Task Group[50] is in line with the increased demand for this standardization.
A first major challenge is the development of an adequate, standardized biofilm model. This includes considerations such as the method of inoculum preparation and the incubation conditions of the biofilm. It has been demonstrated that a standardized protocol regarding inoculum preparation and biofilm growth is crucial to obtain reproducible results across different laboratories.[51]
Research into growth media that better represent the *in vivo* microenvironment *in vitro* is also needed for this purpose. Currently, the consensus is that there is no optimal method available. Biofilm-based tests are inherently more complex than tests based on planktonic bacteria, and even results from the (technically less demanding) conventional susceptibility tests are influenced by minor deviations from the reference methods.[36]

Prior to this, standardization of the known pharmacodynamic parameters is necessary. To date, comparing available study results is challenging due to variations in the definition and interpretation of these parameters across different studies, and there is currently only a limited number of studies available.[52]
When evaluating the biofilm susceptibility of an antibiotic agent, the focus can be on either inhibiting the formation of biofilm starting from a planktonic culture or from a young biofilm. If the goal is to prevent biofilm formation from a planktonic culture, the biofilm prevention concentration (BPC) should be prioritized to assess the anti-biofilm efficacy of an antibiotic. On the other hand, if inhibiting the progression of biofilm formation from a young biofilm is necessary, it is best to use the minimum biofilm inhibitory concentration (MBIC). It is still unclear whether biofilm infections are initiated by the introduction of planktonic cells, aggregates, or both on an artificial surface. However, since it cannot be assumed that antibiotic therapy will always be initiated early (either at the introduction of microorganisms or shortly thereafter), it can be argued that MBIC is preferable. Once a mature biofilm has formed and needs to be completely eliminated, the parameter minimum biofilm eradication concentration (MBEC) becomes more useful. Additionally, biofilm tolerance factors such as the biofilm tolerance factor (BTF) and biofilm tolerance factor for eradication (BTF-E) can be used to quantify the reduced sensitivity of biofilm-growing bacteria compared to planktonic bacteria.[36,53]
While some publications define MBEC as the lowest concentration of an antimicrobial agent that eradicates 99.9% of bacteria embedded in the biofilm (3 log10 reduction in CFU/mL) compared to growth controls, others define it as the BPC corresponding to the minimum bactericidal concentration (MBC) at planktonic level and refer to MBEC in the context of complete biofilm eradication.[36]

Thirdly, standardization of the method used for quantification of biofilms before and after antibiotic exposure is of paramount importance to obtain reproducible results. Methods that directly (number of colonies in culture) or indirectly (resazurin-based viability staining, ATP measurements) quantify the number of living bacteria present in the biofilm are preferred over methods that only measure a qualitative assessment of biofilm mass such as biofilm biomass staining with crystal violet.

A fourth challenge is the lack of biofilm-specific breakpoints defined by official bodies such as EUCAST and CLSI. For planktonic bacteria, these organizations have already established clinical breakpoints based on wild-type MIC distributions, PK/PD data, and large clinical studies. This allows the sensitivity of a microorganism to be

interpreted as ‘sensitive' or 'resistant' to the tested antibiotics. Since such data are lacking for biofilm-growing bacteria, we cannot interpret the sensitivity pattern in the same way.
An alternative is the use of epidemiological cut-off (ECOFF) values such as MBIC-ECOFF and MBEC-ECOFF to differentiate between wild-type strains and strains with acquired resistance mechanisms, as establishing clinical breakpoints is not straightforward. This could be a temporary solution in the absence of clinical biofilm breakpoints pending large clinical studies.[36]

Even if this substantial standardization were achieved, large clinical studies would still be needed to compare treating a patient group with antibiotics chosen based on biofilm sensitivity testing with a patient group whose therapy is based on conventional sensitivity testing.
In the Cochrane review by Waters et al., it was concluded that biofilm sensitivity testing was not superior to conventional sensitivity testing in the treatment of respiratory infections in cystic fibrosis patients. Furthermore, it is recommended to first use biofilm sensitivity testing for the development of new, more effective drugs such as anti-biofilm antibiotics, which can then be tested in clinical studies.[54]
Additionally, further research into anti-biofilm antibiotics and their pharmacokinetics and pharmacodynamics remains crucial in the coming years, independent of biofilm sensitivity testing.[15]
Other concepts leading to antibiotic tolerance in biofilm-growing microorganisms like persisters, genetic alterations, efflux pumps, quorum sensing and matrix production need to be further researched as well.
The introduction of biofilm sensitivity testing in the clinical microbiology laboratory, in whatever form it takes, will crucially rely on collaboration among researchers, microbiologists, and industry to develop the appropriate diagnostic tools and guidelines.

Short answer

Alternative antimicrobial susceptibility testing methods for bacteria growing in biofilms that can predict the therapeutic success of antimicrobial treatment for biofilm infections could be valuable in the clinical microbiology laboratory. Future research should focus on developing models for reliable biofilm sensitivity testing. However, standardization of procedures, relevant parameters such as MBIC, MBEC, BBC, and BPC, and clinical breakpoints by official organizations such as CLSI or EUCAST are necessary before implementation in clinical practice is feasible. Additionally, efforts should be made to establish the optimal growth conditions and media in an attempt to better replicate the in vivo conditions. Furthermore, more in vivo clinical studies are necessary comparing antimicrobial therapy based on sensitivity testing for biofilm-growing bacteria with conventional sensitivity testing.

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TO DO

Follow up literature on biofilm antimicrobial susceptibility testing and biofilm antimicrobial tolerance.

ATTACHMENTS

Figure 1: The basic quorum sensor

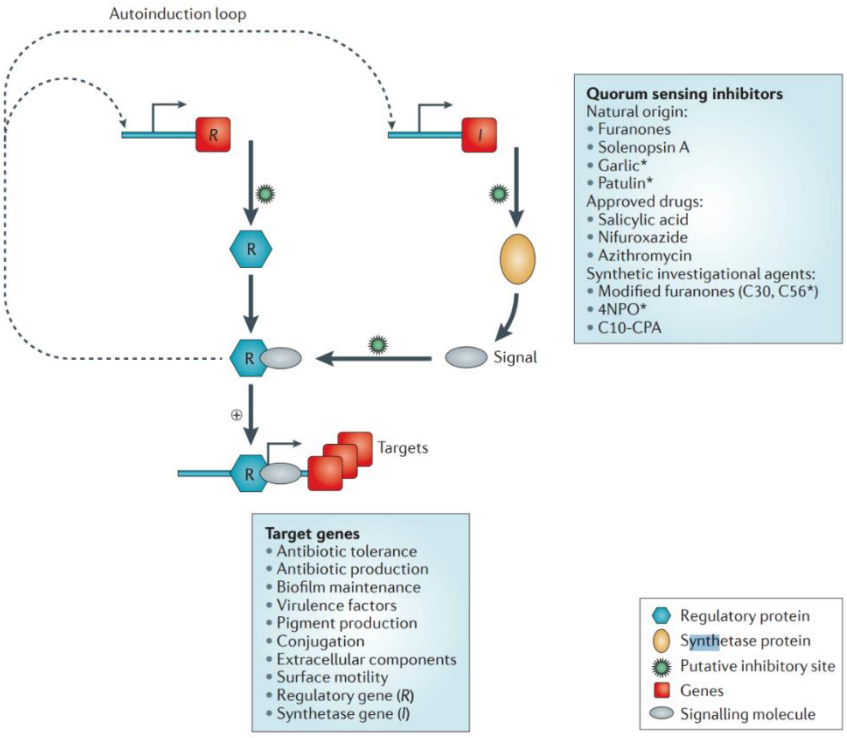


Figure 5 | **The basic quorum sensor.** The basic quorum sensor consists of an autoinduction loop. 'R' represents a gene coding for a regulatory protein and 'I' (inducer)represents a gene coding for a synthetase protein (examples for pairs of 'I' and 'R' genes in *Pseudomonas aeruginosa* are *lasI* and *lasR*, *rhlI* and *rhlR*). The synthetase protein catalyses enzymatic processes that generate signalling molecules. When a critical cell density is reached, a complex of the regulatory protein and a specific signalling molecule enables the autoinduction of the quorum sensor and the expression of the target genes. Processes that can be targeted by pharmacological intervention are marked by green stars. A list of quorum sensing inhibitors (for *P. aeruginosa*) is shown in the box on the top right; asterisks indicate quorum sensing inhibitors that have been shown to compete with the binding of the signalling molecule to the regulatory protein. The box on the bottom lists the variety of phenotypes that are regulated by quorum sensing[238].

Bjarnsholt T, Ciofu O, Molin S, Givskov M, Høiby N. Applying insights from biofilm biology to drug development—can a new approach be developed? Nat Rev Drug Discov 2013;12:791–808.

Figure 2: Calgary Biofilm Device

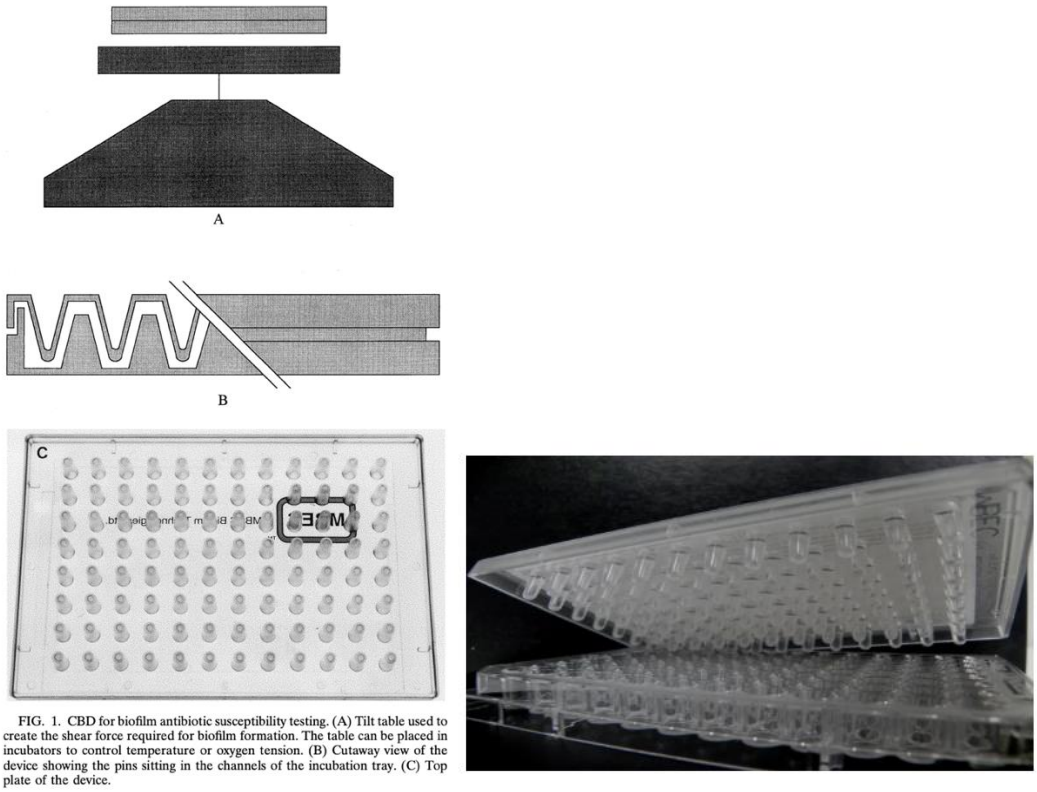


FIG. 1. CBD for biofilm antibiotic susceptibility testing. (A) Tilt table used to create the shear force required for biofilm formation. The table can be placed in incubators to control temperature or oxygen tension. (B) Cutaway view of the device showing the pins sitting in the channels of the incubation tray. (C) Top plate of the device.

Ceri, H., et al., The Calgary Biofilm Device: New Technology for Rapid Determination of Antibiotic Susceptibilities of Bacterial Biofilms. Journal of Clinical Microbiology, 1999. 37(6): p. 1771-1776.. & Innovotech.ca/MBEC Assay® Kit

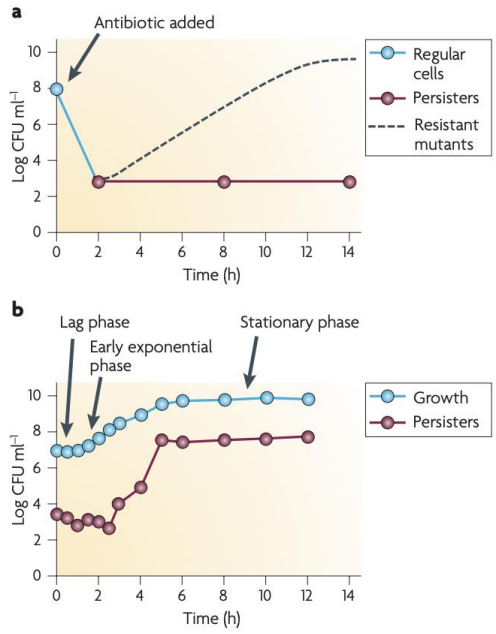
Figure 3: Proposed key pharmacodynamic parameters that could be use as measures for biofim susceptibility and their definition

	Parameter	Abbreviation	Proposed definition/comment[a]
Prevention	Biofilm prevention concentration	BPC	Lowest concentration of an antibiotic required to fully prevent formation of a biofilm (including biofilm aggregates) starting from planktonic cells
Inhibition	Minimal biofilm inhibitory concentration	MBIC	Lowest concentration of an antibiotic required to fully prevent the further development of a biofilm
Eradication	Minimal biofilm eradication concentration	MBEC	Lowest concentration of an antibiotic required to fully eradicate an established biofilm (i.e., resulting in a readout below the detection limit)
Killing	Minimum antibiotic concentration for biofilm killing to achieve x-log reduction[b]	MCBK-x	Lowest concentration of an antibiotic required to achieve x-log reduction in an established biofilm[c]
Relative parameters	Biofilm tolerance[d] factor-prevention	BTF-P	The ratio of the BPC and the MIC
	Biofilm tolerance factor-inhibition	BTF-I	The ratio of the MBIC and the MIC
	Biofilm tolerance factor-eradication	BTF-E	The ratio of the MBEC and the MIC
	Biofilm tolerance factor-x	BTF-x	The ratio of the MCBK-x and the MIC

[a]The definitions are proposed in general terms, i.e., independent of a specific quantification method.
[b]The word "biofilm" was added to the definition previously proposed (110) to avoid any confusion.
[c]The MCBK resulting in complete eradication is equal to the MBEC.
[d]For an in-depth discussion and definition of tolerance, see references (25, 117–121).
[e]Information in this table is partially based on (but not necessarily equal to) definitions proposed previously (107, 109–111, 113).

Coenye et al. Biofilm antimicrobial susceptibility testing: Where are we and where could we be going? Clinical microbiology reviews 2023.

Figure 4: Formation of persister cells



K. Lewis. Persister cells, dormancy and infectious disease. Nature V5 p48-, 2007. (doi: 10.1038/nrmicro1557)

Figure 5: Biofilm susceptibility assay by Moskowitz et al. (2004)

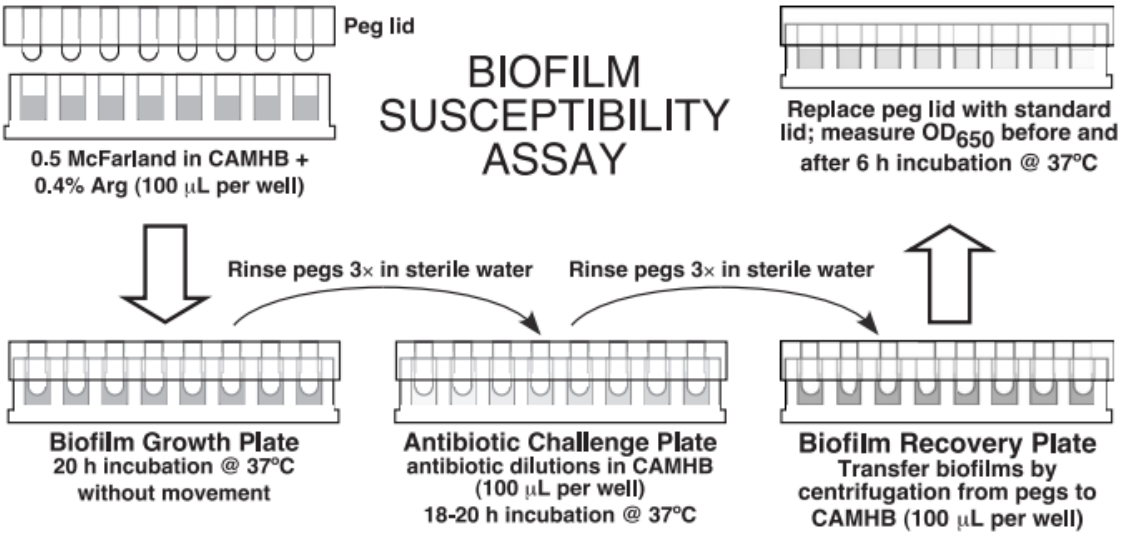


FIG. 1. Biofilm susceptibility assay. See the text for details.

Moskowitz SM, Foster JM, Emerson J, Burns JL. Clinically feasible biofilm susceptibility assay for isolates of Pseudomonas aeruginosa from patients with cystic fibrosis. J Clin Microbiol. 2004; 42:1915–1922.