Microbial biofilm: a "sticky" problem

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Summary

Bacteria can form, on virtually any surface, single- and multi-species biofilms intrinsically resistant/tolerant to antibiotics and elusive of the host immune response. The study of bacterial biofilm development has, therefore, received great interest over the past 20 years and is motivated by the well-recognized role of these multicellular communities in infectious diseases. In this review article, we provide a synopsis of (i) biofilm formation mechanisms; (ii) biofilm clinical significance and underlying mechanisms; (iii) the current methodologies for microbiological diagnosis of biofilm-related infections; and (iv) current and future therapeutic strategies to combat biofilm-associated infections.

Microbial biofilm: an "old acquaintance"

The first observation of aggregated microorganisms surrounded by a self-produced matrix adhering to a surface was described in the 17th century by Anthony van Leeuwenhoek. Using his primitive microscope on matter from his mouth, he saw aggregated microbes in the "scurf of the teeth" and from "particles scraped off his tongue" (22). Afterward, in 1864, Louis Pasteur observed and sketched aggregates of bacteria as the cause of wine becoming acetic (49). Since then, biofilm growing microorganisms were not of interest and unknown for medical microbiologists until the early 1970s when in patients with cystic fibrosis (CF), a link was observed between the chronic infection by mucoid *Pseudomonas aeruginosa* and the presence of aggregates of bacteria surrounded by abundant slime in sputum samples (28). The "biofilm" term was used for the first time in 1981 by J.W. Costerton in a technical microbiology report (46), and it is currently defined as "a structured consortium of microbial cells surrounded by a self-produced polymer matrix". Biofilms may adhere to surfaces or be found into tissue or secretions and may contain components from the host. In the last three decades, the perception of biofilms has changed considerably as a consequence of the technology development and the adaptation to biofilm science (5). Consequently, biofilm infections have been discovered to be widespread in medicine, and their importance is now generally accepted (17, 60).

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Biofilm formation: a complex multiphasic process

Biofilm formation is a highly complex and genetically regulated process in which microorganisms go through from the planktonic to the sessile mode of growth in order to adapt to diverse nutritional and environmental conditions (26). Although it has been observed in almost all bacterial species studied with large variations in the processes involved, there are a number of generalized distinct recognized steps (26, 30). Firstly, freely moving bacteria adhere to a surface whether it be tissue (*e.g.*, native cardiac valves, respiratory mucosa) or abiotic prosthetic material (*e.g.,* urinary or vascular catheters, orthopedic fixation devices). Bacterial adhesion occurs mainly by pili and flagella, although other factors are critical for strength's attachment such as physical forces (*e.g.,* van der Waal's forces, electrostatic interactions) and physical properties of both bacterial cell and the substratum (*e.g.,* hydrophobicity). Once they have adhered to a surface, cells begin to alter their physiology starting the production of large amounts of Extracellular Polymeric Substances (EPS), aimed at reinforcing their adhesion to the surface, and along with improved cell division leading to the formation of "microcolonies". EPS is the hallmark of a biofilm and mainly contains proteins, DNA, polysaccharides, and extracellular DNA. The establishment and maturation of biofilm architecture then occur with cell clusters interspersed with water channels forming three-dimensional "mushroom-like" structures. Microbial cells communicate with each other via a "Quorum-Sensing" system (QS) by the secretion of autoinducer signals attaining the required microbial cell density. The last

stage is characterized by the dispersal of individual or clustered cells from the external layers of the biofilm structure. Commonly regulated by QS, also in response to nutrients diminution and waste products accumulation, cell detachment can also occur due to mechanical stress. Released cells retain certain properties of biofilm, such as recalcitrance to antibiotics; they are free to disseminate, recolonize, and repeat the cycle of biofilm development or may return quickly to their normal planktonic phenotype.

Clinical significance of biofilms: biofilm-related infections and mechanisms of persistence

Biofilm-Associated Infections (BAIs) are usually persistent chronic infections intrinsically refractory to antibiotic therapy and, therefore, represent a significant health problem. It is estimated that biofilm formation accounts for nearly 80% of chronic microbial human infections, including both device-related infections and those established in the absence of a foreign body (17, 60).

Biofilms cause infections related to various indwelling medical devices, such as those described for ortho-dental prosthetics, contact lenses (8), central venous catheters (CVC) (74), prosthetic heart valves and pacemakers (10), peritoneal dialysis catheters (63), prosthetic joints (3), breast implants (2), urinary catheters (6) and voice prostheses (67). Biofilm can also be located on almost any tissue of the human body, causing infections such as chronic otitis media (35) and sinusitis (38), chronic lung infections in CF patients (14), chronic wounds (66), eye infections (8), urinary tract infections and prostatitis (16), as well as diabetic foot ulcers (44), and periodontitis (7).

Most of the clinically relevant microorganisms are able to form a biofilm, as a single species of bacteria or consortia of multispecies microbes (Figure 1) (72). Multi-kingdom biofilms were also reported, especially in chronic wounds (37) and CF lung (48) environments that promote multispecies biofilm formation between bacteria and fungi, with implications for pathogenicity, treatment, and outcomes. Biofilm forming capability has been reported in a large number of bacterial species, both Gram-positive (*e.g., Staphylococcus epidermidis, Staphylococcus aureus*) and Gram-negative (*e.g.*, *P. aeruginosa*, *Stenotrophomonas maltophilia*, *Escherichia coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae*) (11, 19, 40, 43, 53, 56). Among fungi, several *Candida* and *Aspergillus* species commonly colonize both living and implanted medical devices forming drug-resistant biofilms, along with *Fusarium* spp., *Trichosporon* spp., and *Malassezia pachydermatis* also (18, 20). Apart from these pathogenic species, also the microbiota colonizing the epithelial walls or exposed tissue surface forms biofilms inside the host body (*i.e.* wounds, urogenital system, respiratory tract or as dental plaques). Such biofilms, besides causing local infections also predispose patients to secondary long-term manifestations, as in the case of *Porphyromonas gingivalis* whose biofilm formation in the plaque or tongue was respectively associated with the progression of cardiovascular diseases (34) or with the clinical outcome in patients with rheumatoid arthritis (12).

BAIs have a great impact on public health considering they are related with higher healthcare costs due to the prolonged stay in hospitals as well as the administration of prolonged courses of antimicrobial therapy (30). Medical therapy of BAIs can be in fact exceptionally challenging with attempts at infection eradication that, in the case of indwelling medical device-related infections, often entailing complete removal or the substitution of the infected foreign body (30). This resistance is further improved when the biofilm is formed by polymicrobial communities, involving multi-drug resistant pathogens, which are generally more recalcitrant to antibiotic treatment than the corresponding single-species biofilms (25, 52).

The major reasons for the persistence of BAIs are recalcitrance to antibiotics and the evasion to immune responses.

Recalcitrance to antibiotics

Biofilm communities are inherently resistant and/or tolerant to antibiotics, at levels significantly higher (up to 1.000 times) as compared to those observed in the planktonic counterpart (40, 55). Inside the biofilm, several mechanisms confer the multi-factorial resistance to antibiotics (40, 54, 65): a) biofilm EPS: can act as a physical and chemical barrier thus limiting the diffusion of antibacterial through multi-layered biofilm communities; b) enzyme-mediated resistance: the transformation of bactericide to the nontoxic form of antibacterials can be mediated by enzymes; c) heterogeneity in bacterial metabolism and growth rate: due to the differences in nutrients and oxygen availability within biofilms, sessile cells enter into a dormant growth phase that is less susceptible to the antimicrobial agents; d) genetic adaptation: it is required within the biofilm to reduce susceptibility and to adopt the relatively protected and distinct phenotype; e) efflux pumps: the exposure of the bacterial biofilm to lower concentrations of antibiotics and to xenobiotic induces the expression of multi-drug resistance operons and efflux pumps; and f) persistence shown by cells: within biofilms, fraction of bacteria evolve as "persister" cells that are genetically similar but physiologically different compared to parent cells being metabolically inert, replicating slowly, upregulating DNA repair and anti-oxidative machinery and exhibiting unresponsiveness towards minimal inhibitory concentrations of antibiotics.

Elusion of the host immune response

The major immune evasion mechanisms underlying the maintenance of a chronic and indolent course of biofilm infection are the inhibition of opsonization, leukocyte phagocytosis, and Complement deposition. Adherent bacteria cannot be opsonized easily and affect signaling in PolyMorphoNuclear leukocytes (PMN) (50). The biofilm matrix provides a protective barrier against immune surveillance playing a significant role in biofilm resistance to phagocytosis. In CF patients, *P. aeruginosa* biofilm evades macrophages by the production of alginate (41) and causes killing of PMN through the production of rhamnolipids (70), whereas Polysaccharide Intercellular Adhesin (PIA) protects *S. epidermidis* against the killing by PMN and phagocytosis (71). Similarly, *S. aureus* escapes phagocytosis in blood by producing coagulase that finally stimulates the production of insoluble fibrin, a constituent of the biofilm matrix (41).

The contact with macrophages induces *S. aureus* biofilms to release lytic toxins that affect the differentiation of activated macrophages into M2 lineage, in this way activating collagen synthesis by increasing arginase 1 expression, and consequently leading to fibrosis and the evasion of recognition by Toll-like receptors (27).

Staphylococcus epidermidis biofilm affects both the activation of Complement proteins C3b or the deposition of IgG on the surface of the bacterium and provides resistance to phagocytosis by PMN (39). Extracellular DNA, generated by autolysis of bacteria, helps in the colonization of the sessile form, suppresses host innate immune response, increases tolerance to antibiotics and aids in virulence (61).

Laboratory diagnosis of BAIs

The diagnosis of BAIs is time-consuming and difficult, often resulting in false-negative results due both to the limited biofilm

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dimensions (ranging from 4 to 1200 µm) and the presence of commensal flora (9). This situation is further complicated by the evidence that commensal microorganisms can contribute to biofilm formation, as in the case of *S. epidermidis*. A synergistic relationship between the clinical microbiologist and the clinician is needed for a high diagnostic accuracy. Any clinician in the presence of clinical indications for biofilm infections (*e.g.*, medical history of implanted medical devices, persisting infection lasting > 7 days, failure in antibiotic treatment ad recurrence of the infection) should contact the clinical microbiology laboratory to collect an

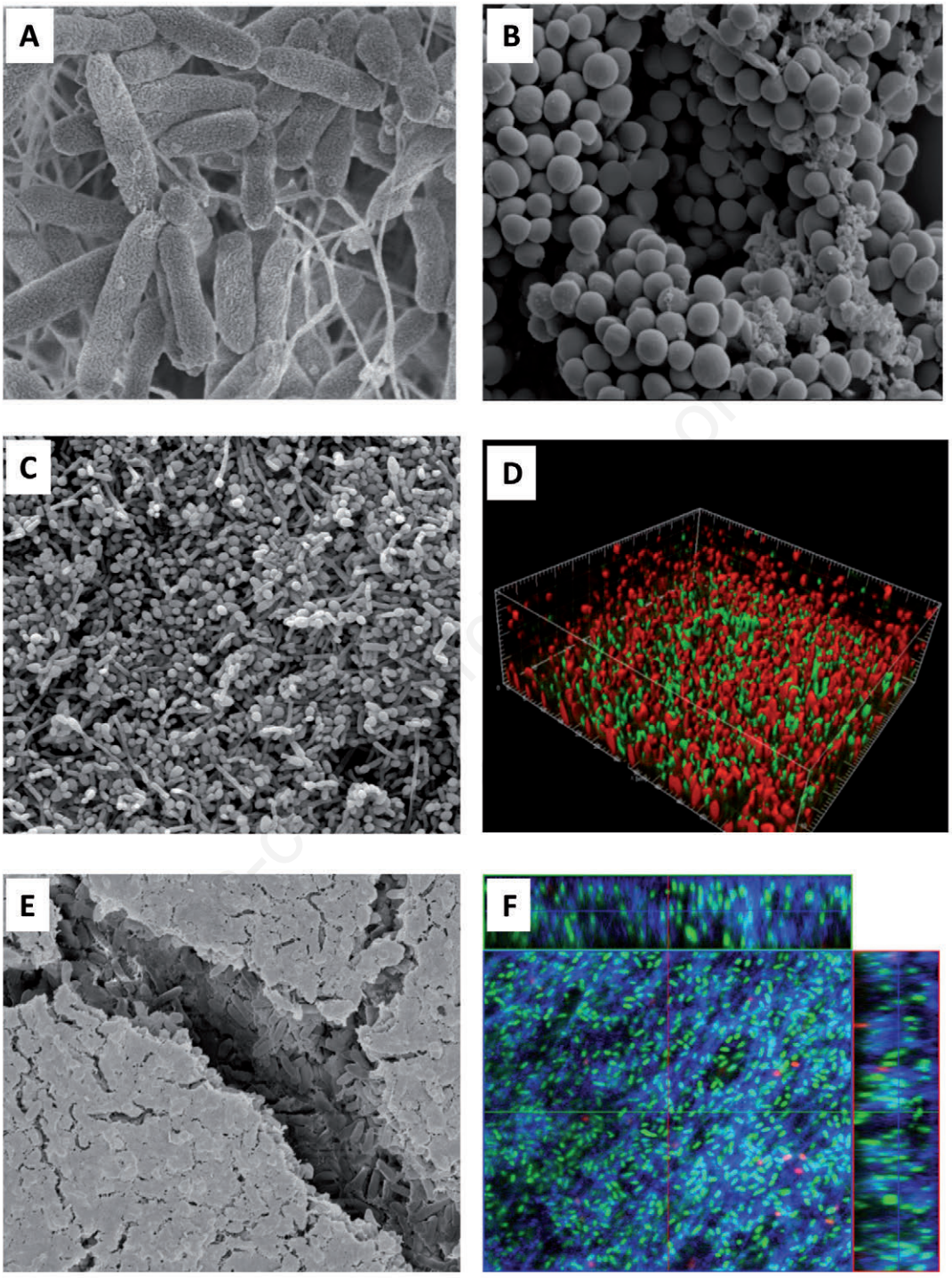

Figure 1. Biofilms formed by: A) *Myroides odoratimimus*, from chronic wound infection; B) *Staphylococcus aureus*, from CF patient; C) *Trychosporon asahii*, from neutropenic patient; D) *S. aureus/Stenotrophomonas maltophilia* mixed biofilm from CF patient; E,F) *Pseudomonas aeruginosa*, from CF patient. A,B,C,E) Scanning electron microscopy; D,F) Confocal laser scanning microscopy: D) *S. aureus* (red, hexidium iodide-stained); *S. maltophilia* (green, Syto-9-stained); F) Orthogonal images, collected within the biofilm as indicated by the green and red lines in the top view, show a multilayered structured biofilm (live cells in green, Syto-9-stained; dead cells in red, propidium iodide-stained) embedded in an abundant extracellular polymeric substance (blue, concanavalin A-stained). Magnification, ×100. All the photographs are unpublished (Di Bonaventura laboratory at the University of Chieti).

adequate sample (*i.e.* representative of the biofilm formed at the infection site) and to ensure that appropriate diagnostic methods are employed. Below are the main recommendations concerning the collection of appropriate clinical samples and the reliable methods to specifically detect biofilms reported in recent literature (31, 32, 43, 73).

Samples

The clinical specimen type depends on whether the infection is located onto a medical device or on living tissue.

i) In CF patients, samples from the lower respiratory tract (especially the sputum) are representative although a high probability of contamination with the commensal oropharyngeal microbiota exists. These samples commonly show high density and required pre-treatment with mucolytic agents or dithiothreitol.
ii) If a chronic wound infection is suspected, biopsy tissues are preferable to wound surface swab not only because biofilm is hardly adhered to the epithelium but also for the presence of skin contaminants.
iii) In the case of infections associated with an orthopedic device, debridement surgery must be driven by previous microbiological analysis of the synovial fluid. Useful intraoperative samples are biopsies (from 3 to 6 samples; each should be as large as possible) from peri-implant tissue and the removal of device or parts of it.
iv) Monitoring biofilm formation within the endotracheal tube in patients with ventilator-associated pneumonia (VAP) is very challenging due to the difficulty in evaluating if endotracheal tube could be the source of primary infection or just a concomitant colonized site. Respiratory samples can be obtained by endotracheal aspirate, bronchoalveolar lavage or protected brushing along with mucus collected into the inner lumen of the endotracheal tube.
v) If a CVC-related infection is suspected and catheter is removed, CVC tip (3-4 cm distal) and purulent fluid or necrotic skin surrounding the port or tunneled catheter (in case of local infection) are adequate samples. If CVC is still *in situ*, two blood samples (one each from peripheral and CVC) are collected for the time-to-positivity method.
vi) In patients with indwelling urinary catheters, the most practical sample is urine from the catheter although removal of the device ensures higher probability of biofilm detection.

Methods

Microscopic observation, culture-dependent and culture-independent molecular methods can be performed in the clinical microbiology laboratory to detect biofilms. It is advisable to use quantitative or semi-quantitative methods as well as identifying microorganisms at species level in order to discriminate between infecting microorganisms and contaminating flora. The observation of specific microbial phenotypes and the measurement of antibodies may be of value to detect biofilm infections in particular clinical settings (*i.e.* CF patients, alloplastic-related infections).

i) The gold standard for detecting biofilms in a sample is microscopic observation since it gives evidence of inflammatory cells (*i.e.* leukocytes, revealing an ongoing infectious process) co-localized with microorganisms organized in biofilm-like structures, that is cell aggregates embedded in a self-produced extracellular polymeric substance. With this aim, several samples (biopsies, fluid samples, swabs) can be stained with Giemsa or Gram techniques and observed using routine light microscopy. The polysaccharide matrix of the biofilms can be specifically stained by Alcian blue or Calcofluor. Confocal laser scanning microscopy and electron microscopy are the most appropriate methods to reveal biofilm in biopsies, however they are often unavailable. Fluorescence microscopy allows higher susceptibility and the identification of microorganisms using *in situ* hybridization probes (FISH), although dormant or slow-growing bacteria (such as those found within a biofilm) may show weak fluorescence given that the signal of the probe is dependent on the number of ribosomes in each cell.
ii) Contrarily to microscopic observation, the culture method requires biofilm cells being detached following physical procedures (scraping, mixing, vortexing, sonication). Biofilm cells release can also be obtained by imprinting (biopsy, catheter tip), rolling (urinary catheter), crushing (bone) or tissue homogenization (biopsy, cardiac valve). Although it cannot discriminate between biofilm-growing and planktonic microorganisms, microbiological culture is required to isolate microorganisms, the sine qua non to identify biofilm microorganisms at the species level and to assess their susceptibility to antibiotics.
In the case of a catheter tip, after being sonicated, the sample undergoes to quantitative (Brun-Buisson method; significant threshold: 10^3 CFU/mL) or semi-quantitative (Maki method; significant threshold: 15 CFU). In the case of catheter-related bloodstream infections, biofilm infection is indicated when the blood culture of CVC gets positive 2 hours earlier than the culture of peripheral blood or when a 3-fold greater colony count is observed in CVC sample. Regarding biofilm urinary tract infections in patients with an indwelling catheter, urine from bladder are processed as in non-catheterized patients, considering that more than 50% false-negativity rate occurs. Whenever catheter removal is possible, culturing after sonication of the catheter has been shown to be more sensitive than urine culture (33). Direct inoculation of homogenized biopsy samples can also be performed in conventional media for aerobic and anaerobic bacteria, Gram-negative bacilli and streptococci. Plates are incubated, at 35-37°C, for a time (ranging from 2 to 10 days) and under atmosphere depending on the presence of slow-growing microorganisms (*e.g.*, *Brucella* spp., *Neisseria gonorrhoeae*, mycobacteria). Multiple biopsies are cultured to increase the sensitivity, although the subsequent culture of the sonicates demonstrated increased sensitivity compared with tissue biopsy cultures alone (68).
iii) Culture-independent molecular methods (*i.e.* broad-range 16S rRNA gene amplification or ITS for fungi, real-time-PCR, multiplex PCR, next-generation sequencing, denaturant gradient gel electrophoresis) are particularly helpful for microorganisms identification when culture remains negative, when the patient had previous antibiotic treatment or, less commonly, in the presence of fastidious or viable-but-not-culturable organisms, such as *Coxiella burnetii* and *Bartonella* spp.. Therefore, although more sensitive than culturing and performable on most sample types, molecular methods complement it rather than replace it.
iv) In respiratory samples from CF patients, growth of small-colony variants (*S. aureus*, *P. aeruginosa*) (23, 36) or mucoid phenotype (*P. aeruginosa*) (57) is highly suggestive for the presence of biofilm infections. Both phenotypes are an expression of adaptation that favours bacterial survival within the lung of CF patients. They show an increased ability to form a biofilm and are frequently resistant to multiple antibiotics; their presence in the sputum of CF patients is associated with a worse clinical condition (57).
v) A significant increase in IgG titre against *P. aeruginosa* purified antigens (proteins, lipopolysaccharide, alginate) or crude extracts is diagnostic for biofilm infections in CF patients.

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ELISA-based tests were validated and are commercially available (58). Likewise, the antibody response against other biofilm growing CF pathogens (*e.g.*, *S. maltophilia, Burkholderia multivorans, Achromobacter xylosoxidans*) can be used diagnostically (29). Increased levels of IgM against *S. aureus* and *S. epidermidis* alloplastic-related infections have also been reported (4). Elevated levels of secretory IgA and IgG may be indicative, in the presence of negative cultures, for hidden foci (*i.e.* paranasal sinuses) (1).

Treatment strategies for combatting BAIs

The most important feature of biofilms is their increased tolerance to antimicrobial agents. Recently, Ciofu *et al*. (15) described some potential strategies for the antibiotic treatment of biofilm infections.

The first one ("topical") consists in delivering antibiotics directly to the site of infection to achieve high local concentrations with serum concentrations low enough (up to 1.000 times lower) to avoid systemic side effects (75). Topical administration may be used for the treatment of established biofilm-associated infections and even as prophylaxis to prevent infection in certain circumstances since it allows antibiotic concentration to remain well above the MIC. The administration of antibiotics by inhalation is the treatment of choice both in suppressive or maintenance therapy in CF patients where reduces *P. aeruginosa* load in the sputum and consequently improves pulmonary symptoms (64). Similarly, topical antibiotic delivery is achieved using coated catheters (*e.g.*, minocycline/rifampin vs. *S. aureus*) or the application of antibiotic lock technique (*e.g.,* minocycline-EDTA, linezolid, cotrimoxazole-heparin, and tigecycline plus rifampicin) for the prevention of biofilm formation on CVC (13, 15). Contrarily, the efficacy of this strategy for the treatment of VAP and chronic wound infections remains unclear due to conflicting results (62).

The second strategy ("combined") comes from the high structural and metabolic heterogeneity of biofilms that provides the rationale approach for a combination therapy where agents active against metabolically active layers (*e.g.*, tobramycin, ciprofloxacin, beta-lactams) are simultaneously administered with others (*e.g.,* colistin) instead of preferentially killing biofilm cells with low metabolic activity. In this sense, in CF patients the inhaled fosfomycin/tobramycin combination was found to be effective in Phase II clinical studies against the biofilm by Gram-positive and Gram-negative pathogens (69). Other combinations (ceftaroline plus daptomycin, vancomycin plus fosfomycin, clarithromycin plus daptomycin) were found to display potent activity against biofilm-producing staphylococci, thus providing a potential option for difficult-to-treat orthopedic device-related infections (15).

Another approach ("sequential") to prevent or delay the onset of resistance may be the use of sequential treatments based on antagonistic interactions. For example, treatment with efflux pump MexXY-OprM substrates (such as tobramycin) could theoretically lead to hyper-susceptibility to MexAB-OprM substrates (such as aztreonam). In this way, tobramycin followed by aztreonam would allow a clinical benefit by improving the therapeutic efficacy and diminishing the selection of resistant mutants (59). Furthermore, it has been recently observed that the sequential therapy is superior to individual treatments.

In spite of everything, BAIs remain a major challenge to human health, and current treatment regimens are not standardized or widely effective. Several therapeutic failures are still being observed: i) the cure rates never reach 100%; ii) the treatment failure can reach 50%, depending on host and pathogen factors; iii) a prolonged antibiotic treatment is frequently required, leading to increased selective pressure and the risk of antibiotic resistance, medical cost, and toxicity. For these reasons, alternative therapeutic strategies, used alone or in combination with antibiotics, to increase the likelihood of biofilm eradication or to reduce the length of treatment, are therefore viewed as modern "holy grails". Among these: antimicrobial peptides (21, 24, 45), natural compounds (*e.g.,* usnic acid and other secondary metabolites of lichens) (51), phages (47), enzymes degrading EPS (24), increase in O_2 tension (42), and QS inhibitors (24). The future use of these alternative strategies for the treatment of medical biofilms looks promising. Clinical trials, or even *in vivo* studies, are warranted to translate the results into the patient care setting.

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