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MINIREVIEW

Towards diagnostic guidelines for biofilm-associated infections

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Abstract

Biofilms associated with the human body, particularly in typically sterile locations, are difficult to diagnose and treat effectively because of their recalcitrance to conventional antibiotic therapy and host immune responses. The study of biofilms in medicine today requires a translational approach, with examination of clinically relevant biofilms in the context of specific anatomic sites, host tissues, and diseases, focusing on what can be done to mitigate their pathologic consequences. This review, which grew out of a discussion session on clinical biofilms at the 5th ASM Biofilm Conference in Cancun, Mexico, is designed to give an overview of biofilm-associated infections (BAI) and to propose a platform for further discussion that includes clinicians, medical microbiologists, and biofilm researchers who are stakeholders in advancing the scientific pursuit of better diagnosis and treatment of BAI to mitigate their human and healthcare costs. It also highlights the need for better diagnostic markers, which exploit the difference between planktonic and biofilm cells.

Introduction

In the past few years, medical biofilm research has moved from *in vitro* culture systems where bacteria are clearly demonstrated to be in biofilms *ipso facto*, by direct visual or microscopic demonstration on clean flat surfaces to the increasing use of animal models (Jurcisek *et al.*, 2005; Jurcisek & Bakaletz, 2007; Weimer *et al.*, 2010; Byrd *et al.*, 2011; Nguyen *et al.*, 2011) and direct analysis of human clinical specimens where identification is more challenging (Hall-Stoodley *et al.*, 2006; Bjarnsholt *et al.*, 2009a, b; Nistico *et al.*, 2011). This has prompted the development of proposed criteria that can be used to demonstrate biofilm *in vivo* along with molecular methods that can distinguish specific microorganisms *in situ ex vivo*. Where *in vitro* biofilms are grown *de novo* from isolated cultures and the development and molecular components of extracellular polymeric substances (EPS) are known to be specifically of bacterial origin, host-derived components in experimental *in vivo* infections may be morphologically similar to microbial biofilms necessitating the distinction of microbial biofilms in complex host environments in an animal model. Clinical biofilm-associated infections (BAI) are even more challenging, because the infectious agents are often unknown, and pathologically significant biofilm infections need to be distinguished from microbial colonization with nonpathogenic organisms.

What are biofilm-associated infections?

A working definition of a biofilm

A core definition of a biofilm accommodating the diversity of BAI is needed. A biofilm is often defined as

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'an aggregate of microbial cells adherent to a living or nonliving surface, embedded within a matrix of EPS of microbial origin.' Biofilm EPS is an amalgam of *extracellular* macromolecules including nucleic acids, proteins, polysaccharides, and lipids (Flemming & Wingender, 2010). Within the biofilm, microbial cells are physiologically distinct from planktonic or single, free-floating cells of the same organism; however, at present, this crucial distinction is not a simple determination that can be evaluated by the tests and examinations usually employed in medical diagnostic work-ups. Classically, bacteria exhibit recalcitrance to antibiotics when they are in biofilms. *Pseudomonas aeruginosa* exhibits higher tolerance to tobramycin and colistin when it is surface-attached *in vitro* (Nickel *et al.*, 1985; Alhede *et al.*, 2011), compared with when it is planktonic. Although biofilms are typically described as being attached to a surface, they may also form at interfaces of spatially distinct microenvironments and as suspended aggregates. For example, an air–liquid interface can result in an aggregated mat of microbial cells just as well as those found on a solid surface-liquid interface. The notion that it is sufficient for a biofilm to be an aggregated mass of cells floating in liquid is supported by the observation that aggregates of a methicillin-sensitive strain of *Staphylococcus aureus* exhibit a much higher tolerance to the antibiotic oxacillin than single, planktonic, cells (Fux *et al.*, 2004), and aggregates of *P. aeruginosa* are also more tolerant to antibiotics than their planktonic counterparts (Alhede *et al.*, 2011). Clinically, in the chronic lung infection associated with cystic fibrosis (CF), the majority of aggregated *P. aeruginosa* are not found attached to pulmonary epithelial surfaces, but within the viscous mucus associated with larger airways (Worlitzsch *et al.*, 2002; Bjarnsholt *et al.*, 2009a). Therefore, although an elemental component of a biofilm is the aggregation of microbial cells, the necessity for attachment to a fixed substratum may be more elastic.

Biofilms differ from single cells, and in bacterial systems, research has focused on differences in structure, function, and behavior. Structurally, the amassing of microbial cells has been compared with multicellularity (Stoodley *et al.*, 2002) and constitutes a level of higher organization than single cells. As a strategy to help individual cells withstand diverse environmental conditions, phenotypic differentiation within a larger structure means functionally specialized cells to: (1) stick via different receptor–ligand interactions to a surface or to other cells (homotypic or heterotypic), (2) produce EPS, (3) metabolize slowly or rapidly grow, or (4) stay attached or disperse (Hall-Stoodley *et al.*, 2004).

Definitions of biofilms also include 'embedded in an extracellular polymeric matrix of microbial origin.' However, 'extramicrobial' host-derived components are particularly important in complex host environments such as dental plaques or intravenous catheter biofilms. Dental biofilms, for example, may use saliva proteins in the surface pellicle to attach to the tooth; bacteria may bind to fibronectin on medical implants; and microbial vegetations in infective endocarditis may be found enmeshed in a mass of fibrin, aggregated platelets, and other host proteins (Parsek & Singh, 2003; Diaz *et al.*, 2006; Moter *et al.*, 2010, Marsh *et al.*, 2011; Stoodley *et al.*, 2011). Restricting a definition of biofilm to 'microbial or bacterial origin' therefore ignores infections where bacteria interact with host molecules and receptors to attach, replicate, and aggregate. Therefore, a more comprehensive definition of a *clinically relevant* biofilm is: 'aggregated, microbial cells surrounded by a polymeric self-produced matrix, which may contain host components.'

Cells in microbial biofilms additionally differ from planktonic cells in two major ways: (1) they are usually more tolerant of antibiotics and antimicrobial treatment, and (2) they may persist in the host, often despite a heavy influx of inflammatory cells and effector functions of the adaptive immune response. This distinction cannot be demonstrated in a diagnostic sample by culture alone, illustrating why better diagnostic markers, which exploit the difference between planktonic and biofilm cells, are needed. The clinical importance is that biofilm infections are typically chronic infections. and the presence of chronic and recurrent infection in a patient should raise the clinician's suspicion of a biofilm infection.

Biofilm-associated infections

The notion that some infections are specifically mediated by bacteria in biofilms and distinct from those due to single-celled planktonic bacteria was first advanced by J.W. Costerton (Costerton *et al.*, 1981). Similarly, Niels Høiby had observed that the aggregation of *P. aeruginosa* in the sputum of chronically infected CF patients was relevant to CF-associated lung infection compared with single-celled bacteria (Høiby, 1977). In 1984, Costerton formally outlined the hypothesis that organisms like *P. aeruginosa* could behave similarly in human infections to the way they behaved in the environment. He further suggested that 'glycocalyx-enclosed biofilms of *P. aeruginosa* or other bacteria have been identified in experimental or clinical infections arising from contaminated prostheses and in chronic refractory infections, such as endocarditis, osteomyelitis, and *P. aeruginosa* pneumonia associated with cystic fibrosis.' (Costerton, 1984; Høiby *et al.*, 1986). Clinicians may be more familiar with foreign body (implant) infections because of microbial attachment to a nonliving surface distinguished from biofilms associated with host tissues, or 'native tissue infections' (Lynch &

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Table 1. Biofilm-associated infections (BAI)

Infection type	Reference example
Tissue-associated	
Dental Caries	Theilade & Theilade (1970), Diaz *et al.* (2006), Dige *et al.* (2007), Zijnge *et al.* (2010)
Periodontitis	Listgarten (1976), Berthold & Listgarten (1986), Wecke *et al.* (2000), Marsh *et al.* (2011)
Cystic fibrosis lung infections	Høiby (1977), Lam *et al.* (1980), Bjarnsholt *et al.* (2009a)
Chronic otitis media	Hall-Stoodley *et al.* (2006), Homoe *et al.* (2009)
Chronic Rhinosinusitis	Sanderson *et al.* (2006), Li *et al.* (2011)
Chronic tonsillitis	Chole & Faddis (2003)
Chronic wounds	Bjarnsholt *et al.* (2008), James *et al.* (2008)
Musculoskeletal infections: Osteomyelitis /	Gristina *et al.* (1985), Gristina & Costerton (1985), Marrie & Costerton (1985)
Endocarditis	Stewart *et al.* (1980), Moter (2010), Mallmann *et al.* (2009)
Urinary tract infection	Nickel & Costerton (1992), Reid *et al.* (2000)
Infectious kidney stones / biliary tract infections	Parsek & Singh (2003), Marcus *et al.* (2008), Scheithauer *et al.* (2009), Wang *et al.* (2010)
Implant/medical device associated	(see reviews) Donlan & Costerton (2002), Donlan (2002)
Cardiac devices	Marrie *et al.* (1982), Rohacek *et al.* (2010)
Catheter and shunts	Stoodley *et al.* (2010), Wang *et al.* (2010), Rolighed Thomsen *et al.* (2011),
Contact lenses	Stapleton & Dart (1995)
Dental Implants	Kumar *et al.* (2012)
Orthopedic prostheses	Stoodley *et al.* (2008, 2011)
Soft tissue fillers	Bjarnsholt *et al.* (2009b)
Sutures/surgical meshes	Kathju *et al.* (2009, 2010)
Stents	Waar *et al.* (2005)
Vascular grafts	Kaebnick *et al.* (1987), Makis & Stern (2010)
Ventilator-associated pneumonia	Hawe *et al.* (2009)

Robertson, 2008). These latter infections include chronic lung infections of CF patients, chronic otitis media (OM), native valve (infectious) endocarditis (IE), and chronic wounds (Table 1). More broadly, we propose that BAI are 'infections due to aggregated, pathogenic or opportunistic microorganisms encased in an exopolysaccharide matrix and recalcitrant to host defense mechanisms and antimicrobial treatment.' The pathogenesis of many biofilm infections also includes normal microbial flora of mucosal membranes or the skin, which gain access to an organ via foreign bodies and clinicians should suspect biofilm infections in such situations (Table 2).

Table 2. Natural and pathogenic biofilms on human tissue and foreign bodies

Organ/anatomic compartment A with normal flora	Connection (may be via foreign body)	Organ/anatomic compartment B without normal flora
Skin	→	Blood, peritoneum
Pharynx	→	Bronchi, lungs
Duodenum	→	Bile tract, pancreas
Urethra	→	Bladder
Vagina	→	Uterus
Air in operating room, skin flora*	→	Alloplastic, cerebrospinal shunt
No symptoms	→	Pathology

*Most frequently CoNS, which occur as biofilm on detached epidermal cells.

Criteria for BAI

BAI present significant challenges to current clinical practice guidelines because of the inherent difficulty in determining whether the infection is biofilm-related or is due to an acute infection with planktonic microorganisms. Therefore, functional, clinically relevant criteria would help to: (1) better distinguish BAI from acute planktonic infections, (2) obtain appropriate clinical samples, and (3) provide focus for the development of routine clinical tests. Criteria for biofilm infections have been previously proposed and modified, based on the initial Parsek–Singh criteria (Parsek & Singh, 2003; Hall-Stoodley & Stoodley, 2009) (Table 3). These criteria exemplify several characteristic features of BAI. The first two criteria include fundamental definitions of biofilms discussed earlier, such as association with a surface and aggregation. Whenever possible, sampling surfaces suspected of harboring biofilm microorganisms is preferred, even if fluid samples are also available. This is problematic, however, as it may involve invasive procedures such as biopsy, needle aspiration, or removal of an implant. In biofilms causing intravascular device-related bloodstream infection, however, methods have been developed that do not necessarily require device removal. These methods are based on qualitative or quantitative blood cultures through the device and paired quantitative blood cultures both through the device and percutaneously, with the number of bacteria greater in device-drawn cultures compared with peripherally drawn cultures, and the time to positive culture during continuous monitoring of growth, faster (Safdar *et al.*, 2005; Mermel *et al.*, 2009). Nevertheless, in many foreign body infections, bacteria may not be identified until removal of the prosthesis (Kathju *et al.*, 2009; Stoodley *et al.*, 2011) and this may also be the case with intravascular device-related bloodstream infection (Safdar *et al.*, 2005).

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Table 3. Previous suggested criteria for biofilm-associated infections (Adapted from: Hall-Stoodley & Stoodley, 2009)

1. Pathogenic bacteria are associated with a surface. This criterion queries where an infection is found in the patient (associated with various types of epithelium, as middle ear mucosa in chronic otitis media, bladder epithelium, in urinary tract infections, or skin in chronic wounds; with endocardium, in endocarditis; or associated with medical devices or implants such as catheters, shunts or prostheses).
2. Direct examination of infected tissue or materials demonstrate aggregated cells in cell clusters encased in a matrix, which may be of bacterial and host origin. For example, in endocarditis or in medical device-related infections, part of the matrix may be comprised of fibrin, collagen, fibronectin and other host proteins).
3. Infection is localized to a particular site in the host, (however there may be systemic signs which are secondary to the primary locus).
4. Recalcitrance to antibiotic treatment in spite of a demonstrated standard or routine susceptibility testing of the specific bacterium.
5. Culture-negative result despite a high suspicion of infection by the clinician (since localized bacteria in a biofilm infection may be missed due to incorrect sampling strategies and handling, or even in correctly obtained samples, conventional preparation and culturing may be inadequate for biofilm bacteria).
6. Evidence of ineffective host clearance with bacterial aggregates (microcolonies) demonstrated by the co-localization of host inflammatory cells with discrete areas of the host tissue.

Device-related bacteremia is thought to be due primarily to erosion or sloughing of biofilm cells because of mechanical shear when flushing the catheter, which detaches microbial cells from a biofilm (Donlan, 2002) and results in cells or cell aggregates entering the bloodstream and leading to the signs and symptoms of blood stream infection. Indwelling catheters are frequently colonized with biofilm shortly after insertion (Donlan & Costerton, 2002), and Kim *et al.* linked biofilm on a central venous catheter (CVC) to an outbreak of *Alcaligenes xylosoxidans* bloodstream infection (Kim *et al.*, 2008b). Many others, including Raad *et al.*, 1992, 1993, Yücel *et al.*, 2004, Lorente *et al.*, 2004, have noted that catheter colonization does not necessarily directly correlate with infection as measured by positive blood cultures. While blood cultures should of course be considered with other data, evidence that the presence of biofilms is not necessarily associated with clinical signs and symptoms reflects several challenges to diagnosing BAI discussed in this review including: (1) culture is not always reliable for determining BAI, (2) sampling methods do not always reflect where microorganisms are present and furthermore may not dislodge biofilm organisms, and (3) antibiotic treatment is often in place which decreases the likelihood of pathogen identification by blood culture.

Data from Larsen *et al.* and others suggest that molecular methods result, not only in the increased identification of pathogens compared with culture but also greater microbial diversity particularly in catheters with longer dwelling times (Donlan, 2002; Larsen *et al.*, 2008). A panel of molecular techniques including clone libraries based on broad range 16S rDNA gene amplification, denaturant gradient gel electrophoresis (DGGE) phylogeny, and fluorescent *in situ* hybridization (FISH) better resolved the diagnostic outcome in a study investigating biofilms on removed CVCs (Larsen *et al.*, 2008). The roll-tip method also influences the evidence for catheter-related infection, because this method only detects organisms on the external part of the catheter and may have limited sensitivity indicating that surface sampling needs careful evaluation (Donlan & Costerton, 2002). Raad *et al.* (1992) showed that sonication improved the efficiency of identifying catheter-related infections. A study by Yűcel *et al.* also suggests that biofilms on CVCs lead to catheter-related bloodstream infections, because antimicrobial-treated CVCs resulted in a reduction in these infections (Yűcel *et al.*, 2004). It is not yet clear whether specific catheters are less likely to lead to colonization and infection (Safdar & Maki, 2005), but further investigation of the link between biofilms and device-related infection is needed.

Recently dental implants have been a focus of study for oral biofilms that may eventually lead to peri-implantitis with loss of the supporting bone and ultimately failure of the implant. Organisms associated with peri-implantitis are similar to those found in periodontitis but also include etiological involvement of actinomycetes, *S. aureus*, coliforms, or *Candida* spp. (Pye *et al.*, 2009; Heitz-Mayfield & Lang, 2010). So far, only a few studies have used molecular techniques like checkerboard hybridization or pyrosequencing to study the microflora of failing implants, indicating distinct species associated with peri-implantitis (Shibli *et al.*, 2008; Kumar *et al.*, 2012). More systematic epidemiological studies are necessary for the development of standardized diagnostic and therapeutic strategies.

Criterion 3 indicates that BAI are localized and not systemic. Systemic signs and symptoms may occur, but they may also be a function of planktonic cells or microbial products being shed from the biofilm at the original focus of infection (Costerton *et al.*, 1999; Parsek & Singh, 2003). Immune complex-mediated inflammation leading to tissue damage around biofilms also dominates in some biofilm infections such as *P. aeruginosa* lung infection in CF patients (Høiby *et al.*, 1986; Bjarnsholt *et al.*, 2009a).

The fourth criterion addresses another tenet of biofilms: infections with planktonic bacteria are typically treated successfully with the appropriate antibiotics where the microorganism is found susceptible *in vitro*,

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whereas BAI are recalcitrant to antibiotic therapy or at least tolerant to higher antibiotic doses compared with planktonic cells of the same isolate. Although a BAI may show some response to conventional antibiotic therapies, it will not be eradicated and therefore recurs at a subsequent point. One example is the intermittent colonization of the lower respiratory tract with *P. aeruginosa* that sooner or later leads to chronic lung infection in CF. Intermittent colonization by *P. aeruginosa* can be eradicated by early aggressive antibiotic therapy in contrast to the chronic infection, which is treated by maintenance therapy (i.e. chronic suppressive antibiotic therapy). The chronic biofilm infection is not eradicated but rather is suppressed by daily inhalation of antibiotics and intravenous antibiotics, either regularly every 3 months, or during acute exacerbations leading to a much improved survival of the patients (Döring *et al.*, 2000; Döring & Høiby, 2004).

Another example of recalcitrance to antibiotic treatment is chronic OM, which is distinguished from acute OM. Two types of chronic infection profiles are described: OM with effusion (OME) where the effusion persists for > 3 months, or, a recurrent infection often referred to as recurrent acute OM or RAOM, where fluid resolves between recurrent events (Hall-Stoodley *et al.*, 2006; Post *et al.*, 2007). Both types are consistent with other BAI, exhibiting recurrent acute symptoms after repeated cycles of antibiotic therapy without eradication of the underlying infection. This is thought to be due to the release of planktonic bacterial cells from biofilms and their susceptibility to antibiotic treatment when microorganisms are not aggregated (Costerton *et al.*, 1999), while the biofilm causes a persistent infection that elicits a low grade inflammatory response. Evidence that recurrent OM, in addition to OME, is a BAI was shown using both immunofluorescent methods with pathogen-specific antibodies and FISH pathogen-specific 16S rRNA gene probes to demonstrate bacterial pathogens attached to the middle ear mucosa in children having tympanostomy tube placement for the treatment of recurrent OM in addition to OME (Hall-Stoodley *et al.*, 2006).

Criteria 4 and 5 illustrate that antimicrobial recalcitrance or evidence of greater tolerance is an important indication of BAI and may be linked to the failure of culture to identify a pathogen in fluid samples. Criterion 5 also suggests that other diagnostic guidelines are needed if BAI do not yield culture-positive results. In CF, three additional criteria are used to diagnose biofilm infection: (1) continued isolation of *P. aeruginosa* from sputum for at least 6 months, (2) detection of the alginate producing mucoid phenotype of *P. aeruginosa*, and (3) an increase in anti-*P. aeruginosa* antibodies (Pressler *et al.*, 2006, 2009; Proesmans *et al.*, 2006).

Toward better guidelines for the diagnosis of BAI

Culture as an exclusive diagnostic criterion is problematic

Reliance on culture as the 'gold standard' of medical microbiology *exclusively* for the identification of bacterial pathogens as a diagnostic criterion in clinical laboratories is not clear-cut with BAI. Numerous publications indicate a discrepancy between culture and molecular diagnostic methods. In OME, culture identifies a pathogen around 25–30% of the time, while culture-independent methods such as PCR and/or FISH identify pathogens 80–100% of the time (Post *et al.*, 1995; Hall-Stoodley *et al.*, 2006). This discrepancy was not because of the amplification of DNA from dead bacteria (Aul *et al.*, 1998; Dingman *et al.*, 1998) and contrasts with acute OM where culture successfully identifies a pathogen over 90% of the time (Post *et al.*, 1995; Rayner *et al.*, 1998).

Infectious endocarditis also has a proportion of cases (as much as one-third) that fail to grow bacteria in culture. In IE, blood culture-negative endocarditis (BCNE) is thought to be due to previous antibiotic usage or the presence of fastidious bacteria (Moter *et al.*, 2010). However, culture of the valve tissue itself was not necessarily more effective, whereas molecular methods *were* more successful at identifying a causative microorganism. The identification by broad range PCR and subsequent sequencing of heart valve material could be confirmed by FISH analysis showing extensive biofilms in culture-negative endocarditis cases (Mallmann *et al.*, 2009). As FISH targets ribosomal RNA, this molecular method also indicates recent metabolic activity of the bacteria. For subacute bacterial endocarditis, which may be present for weeks or even months before being diagnosed, an antibody response may be helpful (Kjerulf *et al.*, 1998a, b), whereas in acute bacterial endocarditis caused by *Streptococcus pneumonia* or *S. aureus*, an antibody response is not yet detectable (Kjerulf *et al.*, 1993, 1998a, b). Antibody response has also been useful for diagnosis of biofilm infections caused by other bacteria than *P. aeruginosa* (e.g. *Burkholderia*, *Achromobacter*, and *Stenotrophomonas*) in CF (Høiby & Pressler, 2006).

Diagnosis of prosthetic joint infection in orthopedics is another example where culture is suspected of producing a high rate of false negative results and suggests that infection might be commonly misdiagnosed as 'aseptic loosening' (Tunney *et al.*, 1998). Even in cases when the surface is sampled directly by swabbing, it has been shown that bacteria may be extremely hard to detach (Passerini *et al.*, 1992; Kobayashi *et al.*, 2007, 2009; Bjerkan *et al.*, 2009). Low intensity ultrasonication by ultrasonic bath

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with subsequent culturing of the sonicate has been shown to increase culture sensitivity. Data from 195 retrieved prostheses collated by Nelson (Nelson *et al.*, 2005) from multiple sources (Gristina *et al.*, 1985; Gristina & Costerton (1985); Dobbins *et al.*, 1988; Moussa *et al.*, 1997; Tunney *et al.*, 1998) and grouped here for statistical comparison of proportions (MedCalc®) showed that ultrasonication significantly increased positive culture rate from 14% to 35% ($P < 0.001$). A later study of 404 patients reported a similar trend: preultrasonication increased culture positivity relative to tissue culture from 61% to 79% (Trampuz *et al.*, 2007) but offered no statistically significant increase in sensitivity for synovial fluid. The interpretation is that sensitivity of culture is increased because ultrasonication breaks up attached biofilm and releases bacteria that would otherwise remain attached to the prosthesis. However, it is possible that sonication might also affect the physiology of released bacteria, converting them to the more readily culturable planktonic phenotype, in addition to a dilution effect on any residual antibiotics, because sonication is performed with the prosthesis immersed in a saline buffer. While presonication appears to increase the sensitivity of culture, the use of PCR to infer the presence of pathogens from detection of their nucleic acid (RNA or DNA) increases sensitivity even further (Tunney *et al.*, 1998). However, often the rate of positive samples is so high that suspicion has been raised that PCR might produce a high rate of false positive results by detecting contaminant bacteria or remnant bacterial DNA. Therefore, direct microscopic examination of recovered prosthesis components and associated tissue using viability stains and FISH to identify targeted pathogens has been used to corroborate PCR-based methods (Stoodley *et al.*, 2008, 2011; Gallo *et al.*, 2011). These studies have demonstrated that PCR and FISH show similar trends to presonication and culture and indicate a much higher proportion of orthopedic device failures may have an infectious etiology than currently considered (Costerton *et al.*, 2011).

Better guidance outlining sampling protocols for obtaining clinical samples for microbiological testing and how to treat the samples for releasing the biofilm bacteria may therefore improve culture outcomes, including sampling of multiple aspirate or effusion samples. Tissue biopsies that allow histological work-up or homogenization before culture are also more likely to detect biofilm bacteria than swabs, which may miss microorganisms in a niche, encased in a matrix, or within the tissue. Furthermore, multiple or successive biopsies might also reduce the sampling error, taking into account that BAI may be surface-associated or localized. The following samples are therefore recommended in BAI: (1) swabs (e.g. nasal, throat, and genital), (2) liquid samples (e.g. blood, sputum, ear effusion, purulent discharge—particularly from wounds, and synovial fluid), (3) solid samples (native tissue biopsies, e.g. bone fragments or heart valves), and (4) implant samples (e.g. sutures, meshes, catheters, stents, and prostheses). As discussed previously, in some cases, an ultrasonication step may increase sensitivity. Once the sample has been taken and processed, it remains to be seen from blinded clinical studies, which diagnostic samples are best for the determination of a course of treatment, culture, PCR, or a combination of the both.

Culture (plate counts with colony forming units (CFU) to determine viable bacteria) has been shown by many researchers to not necessarily accurately reflect viable bacteria. To assess antimicrobial effects, culture was directly compared *in vitro* with the bacterial Live/Dead kit, which uses membrane permeability/patency to assess *in situ* viability and a metabolic stain (CTC: 5-cyano-2,3,-ditolyl tetrazolium chloride) to measure bacterial respiratory activity in biofilms (Kim *et al.*, 2008a). This study found that although nearly half of cells within the biofilm were not cultured (compared with direct microscopic analysis), 90% retained respiratory activity and 70% demonstrated membrane patency. Several other studies have also demonstrated that CFUs do not always directly correlate with cell membrane permeability and enzyme activity, suggesting that bacteria in biofilms may be membrane compromised and nonculturable but still viable under stressful, nutrient limiting conditions (Shen *et al.* 2010). These *in vitro* studies also support the notion that culture of biofilm bacteria may reflect false negative results and should not be used as a stand-alone determination of the absence of a BAI. Taken together, the problem of *in situ* measurement of cell viability in biofilms is not unambiguous. FISH demonstrates ribosomes of cells, and fluorescence signal intensity is well correlated with ribosome content in most species, indicating recent metabolic activity (Poulsen *et al.*, 1993; Kemp *et al.*, 1993). However, it is also not proof of viability. Linking FISH detection of active metabolism through visualization of mRNA (Hodson *et al.*, 1995; Wagner *et al.*, 1998; Schmid *et al.*, 2001) or the 16S-23S internal transcribed spacer (Schmid *et al.*, 2001) would better indicate active microbial transcription. However, these techniques have not yet been routinely applied to clinical samples.

Finally, it is important to note that not all BAI are culture negative. Rather, culture-negative results do not necessarily *rule out* an infectious etiology, and more tests may be needed to eliminate this possibility. In addition, not every culture-negative infection is because of biofilms, because infection may be due to fastidious or yet uncultured microorganisms, like *Tropheryma whipplei*, *Borrelia*, or *Treponema pallidum*. Therefore, in addition

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to culture-negative results being due to inadequate sampling, the failure of laboratory culture to detect microorganisms may reflect inadequate incubation times, oxygen conditions, or insufficient nutrient composition in culture media to simulate the complex conditions of growth within the host for fastidious organisms (Moter *et al.*, 2010; Brook, 2011). However, in a clinical setting, the most likely explanation for culture-negative results may be that antibiotics have been used prior to sampling fluids, such as effusions, blood, or synovial fluid, which may be culture negative because planktonic cells in the fluid have been killed. In support of this, differential detection rates comparing pre- and post-antibiotic samples indicate that recovery of bacteria is reduced by 24% and 36% for staphylococci and streptococci, respectively (Grace *et al.*, 2001). It is also possible that culture is not accurate in polymicrobial biofilms, because the growth of some microorganisms may depend on the presence of metabolites of others within the localized microbial community. While this has been demonstrated in dental biofilms (Moter *et al.*, 1998; Brook, 2011; Marsh *et al.*, 2011), it remains to be shown for infections with more limited species diversity.

A common theme among BAI is that the *absence* of culture results has lead to an alternative explanation for the recurrent inflammatory signs and symptoms independent of an infectious agent. Therefore, the sixth criterion is important. Careful investigation of diseases where there is a strong suspicion of an infectious etiology using histological or *in situ* molecular methods to identify aggregated microorganisms often shows evidence of an adjacent influx of inflammatory cells such as polymorphonuclear cells (PMNs) or macrophages surrounding the microorganisms. As one of the concerns, even in the face of culture-positive infections, is that commensal bacteria, such as coagulase negative staphylococci (CoNS), may indicate contamination from the skin flora, the presence of inflammatory cells at the site of localized microorganisms more strongly supports evidence of an infection.

Fulfilling Koch's postulates for BAI

Criterion 6 also illustrates the difficulty of fulfilling Koch's postulates for BAI. Koch's postulates were designed to investigate the clinical consequences of infection with a specific pathogen. *Like many other complex infections with as yet poorly characterized pathogenicity, BAI are not easily subjected to Koch's postulates* (Evans, 1976). BAI are often site-specific, associated with a medical implant or foreign body such as sutures, or have a host-specific component such as immune-suppression or predisposing risk (i.e. CF). More problematically, BAI may also be polymicrobial or associated with fastidious microorganisms that are difficult to culture (Moter *et al.*, 2010; Brook, 2011). As Evans (1976), and later, Fredricks & Relman (1996) point out, there are numerous infections where failing to fulfill Koch's postulates did not eliminate the causative role of a putative infectious agent in disease but only delayed it until adequate molecular, microscopic, or serological evidence established the association of the causative agent in the disease. Indeed, in the case of cholera, Koch himself did not think that fulfillment of all postulates was sufficient (Evans, 1976; Fredricks & Relman, 1996). The failure to fulfill these postulates has frequently centered around two issues: the lack of appropriate culture methods for the putative infectious agent, and the technology available to demonstrate causation. The significance of previously unidentified microorganisms in a suspected biofilm infection provides additional problems for clinical interpretation and can, in many cases, only be hypothesis generating, even though treatment attempts may have to be carried out.

Supplementing Koch's postulates in the context of a specific host response and suitable animal models specific for biofilm infections may be helpful (Jurcisek *et al.*, 2005; Jurcisek & Bakaletz, 2007; Byrd *et al.*, 2011). Modified Koch's criteria have also been useful in CF where emerging pathogens also form biofilms (Høiby & Pressler, 2006; Hansen *et al.*, 2010; Dalbøge *et al.*, 2011). However, improved technology also offers several alternatives to culture, which are now used to detect and identify pathogens.

The importance of molecular diagnostic approaches

The development of molecular-based diagnostic approaches to BAI is central to improving the detection and identification of microorganisms and establishing their role in pathogenesis. This is consistent with molecular diagnostics increasingly being applied to microbial detection and identification in the microbiology laboratory for many putative infections that are either not able to be cultured (viruses) or are fastidious or slow-growing. Several molecular techniques are now used routinely to either augment existing culture results (for bacteria) or to detect and identify pathogens in the absence of culture (primarily for virus detection). The most widespread molecular methods are nucleic acid (NA) amplification techniques such as the polymerase chain reaction (PCR). Advantages of PCR include: high sensitivity that may detect very few microorganisms, availability of primer/probe sets for most common pathogens, routine extraction protocols for nucleic acid extraction, and the development of automated systems and readouts for higher throughput of

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samples. Quantitative PCR can also provide quantitative data on the relative abundance of microorganisms that are present. Disadvantages include: disassociation of the sample prevents microscopic evaluation of aggregated microorganisms, the detection sensitivity may not necessarily correspond to diagnostic sensitivity, potential sample contamination, complex samples containing inhibitors of PCR (such as eukaryotic DNA), and the potential amplification of DNA from nonviable microorganisms. Thus, PCR is a powerful approach that needs to be interpreted in the context of other diagnostic approaches and clinical data (Hall-Stoodley *et al.*, 2006; Larsen *et al.*, 2008; Rudkjøbing *et al.*, 2011; Wolff *et al.*, 2011).

FISH is another sensitive and specific approach, which is particularly well suited to the study of complex tissue samples and evaluation of the presence of microbial aggregates. FISH relies on hybridization of a fluorescently labeled probe to the 16S or 23S ribosomal RNA in bacteria or the 18S or 26S ribosomal subunits in eukaryotic microorganisms such as dimorphic fungal and protozoan pathogens. These molecular regions are specific to species level in microorganisms, and with careful optimization and use of controls, this approach can give robust *in situ* evidence of pathogens in a sample (Fig. 1a, c-f). Advantages of FISH include: culture-independent evidence of specific pathogens as spatially organized aggregates, *in situ* localization in the tissue and co-localization with other cell types (such as PMNs if used in conjunction with other NA probes or stains) (Fig. 2), or other microbial members of a biofilm (such as in polymicrobial communities in dental biofilms), and demonstration of rRNA content specific to microorganisms indicating recent metabolic activity. Disadvantages include: the dependence on laboratory expertise, requirement for fluorescence microscopy (or confocal laser scanning microscopy (CLSM) for research purposes), the need for fixation and permeabilization of the sample, few commercially available probes for diagnostic use coupled with the need for testing and of validating new probes, and cost. Furthermore, FISH is not a stand-alone technique in the diagnostic setting, as culture is still used for antibiotic susceptibility testing. While traditionally the probes for FISH were based on single stranded DNA, another set of probes increasingly used in diagnostics are based on a polyamide 'peptide' backbone (Egholm *et al.*, 1993; Bjarnsholt *et al.*, 2008). PNA FISH probes abide by Watson/Crick pairing but possess unique hybridization characteristics because of their uncharged chemical backbone, including rapid and stronger binding to complementary targets compared with traditional DNA probes. PNA probes can also be used with unfixed biological samples; however, only a limited number of probes are currently available, restricting the use of PNA FISH for the present.

CLSM and FISH emphasize that demonstrating biofilm spatial organization is extremely important to: (1) identify whether the bacteria present are aggregated, (2) indicate a polymicrobial nature of a biofilm, (3) indicate the extent of biofilm on a surface that CFU may vastly underestimate, and (4) to show biofilm EPS that may comprise a greater part of the biofilm than cells alone. On nonbiological, flat surfaces, biofilm spatial organization can best be measured by various parameters using image analysis software. The most common program is COMSTAT that yields a number of spatial parameters including thickness, biovolume, and roughness (Heydorn *et al.*, 2000).

Quantification of biofilm spatial organization is harder however in clinical specimens that usually have a complicated and convoluted surface geometry, and currently is largely descriptive or qualitative in these samples – that is, data showing cells or clusters per unit area without a good method to quantify spatial dimensions. As COMSTAT thresholding does not work well on tissue backgrounds, quantifying the biofilm involves a manual rendering of biofilm images in other software to resolve bacteria and laborious cell counting, particularly if NA probes are used because they stain host cell nuclei as well as bacterial DNA (Nistico *et al.*, 2011). Resolving biofilm spatial organization is also made more difficult because of the spatial scales involved. For example to be able to resolve individual bacteria in an image, the field of view needs to be on the order of 100 μm^2, while the specimen might be on the order of cm^2 (1 million fields) for tissue or even 100s of cm^2 (over 100 million fields) for large orthopedic implants making microscopic data from a small proportion of the sample often the only practical method to demonstrate biofilm *in situ*. Finally, because biofilms may also be extremely localized, it is difficult to quantify by averaging several images on the surface, because heterogeneity leads to extensive sample variability. For microscopy to routinely be used in a quantitative manner for clinical specimens, rather than the more corroborative way that it is currently used, will require significant methods development.

Nevertheless, bacterial biofilms can be detected as large 2D aggregates by Gram-stained slides as demonstrated in sputum or lung tissue of CF patients with chronic biofilm infections caused by *P. aeruginosa* (Fig. 3) (Hoffmann *et al.*, 2005; Bjarnsholt *et al.*, 2009a). The predominance of microscopy (Gram-stained smears) coupled with culture in the clinical microbiology lab, in addition to its role in fulfilling Koch's postulates, has mainly rested on its ostensible ability to detect and identify a broad range of different microorganisms with a single testing protocol. The Ibis T5000 Universal Biosensor (now called Abbott PlexID Bio-identification System®) is a promising technology that links multilocus PCR to electron spray ionization

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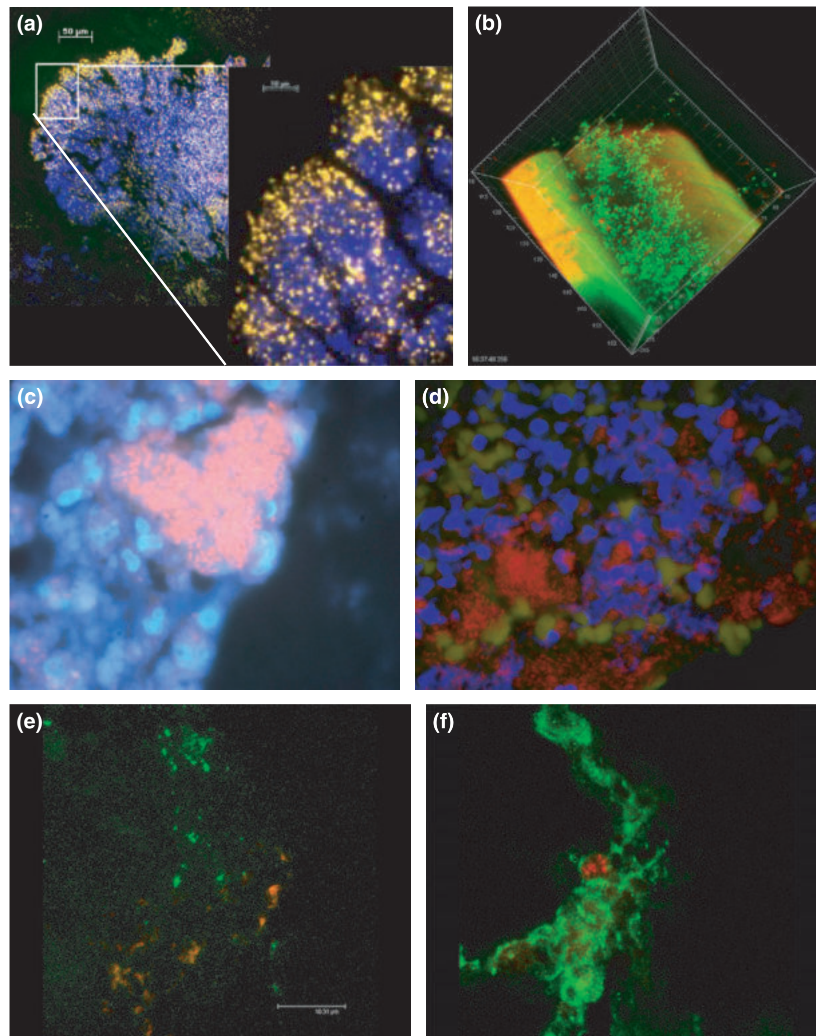

Fig. 1. Confocal laser scanning microscopic (CLSM) images of bacterial biofilm demonstrated by FISH and viability staining. (a) FISH of a heart valve section of a patient with *Streptococcus* endocarditis showing a mature biofilm. The overview shows a structured biofilm with bacteria detected by the *Streptococcus*-specific probe Strep1 (orange) alternating with layers of 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI)–positive cells. At higher magnification (inset), the discriminative fluorescence intensity is visible indicating differential ribosomal content of the FISH-positive cells among many bacteria stained with DAPI only (Gescher *et al.*, 2008). (b) Biofilm attached to the surface of an infected suture from a patient suffering from chronic surgical site infection as a complication of a Roux-en-Y gastric bypass. The bacteria were still predominantly viable despite nearly 1 year of antibiotic therapy and local wound care (stained with Molecular Probes BacLight viability Live (green)/Dead (red) kit). Individual monofilaments of the braid were autofluorescent. The infection only resolved after removal of the suture remnant (Kathju *et al.*, 2010). Scale: major grid divisions = 5 μm, minor divisions = 1 μm. (c) Lung tissue of a chronic *Pseudomonas aeruginosa* (red) infected CF patients. (d) The wound bed of a chronic *P. aeruginosa* (red) infected venous leg ulcer, bacteria in (c) and (d) were visualized by specific PNA FISH probes. As seen from the pictures, the biofilms are well protected from the surrounding leukocytes (DAPI, blue) (Bjarnsholt *et al.*, 2009a). (e) *Streptococcus pneumoniae* Cy3 (green) and *Haemophilus influenzae* Cy5 (red) on a middle ear mucosa (MEM) biopsy from a child undergoing tympanostomy tube placement for the treatment of chronic OM (Hall-Stoodley *et al.*, 2006). (f) *H. influenzae* Cy5 (red) and anti-FITC Pankeratin (green) showing *H. influenzae* biofilm associated with adenoid epithelium (Nistico *et al.*, 2011).

mass spectrometry (ESI-MS) (Ecker *et al.*, 2008). This approach uses a nested approach combining subsets of broad-based strategic primers such as 16S rRNA gene coupled with genera and species-specific housekeeping or antibiotic resistance genes to amplify NA sequences present in the sample without *a priori* targeting any given species. The ESI-MS then separates the amplicons and weighs them to yield microbial signatures with sufficient information to identify bacterial and fungal pathogens to species level. The technology is also capable of identifying viral and protozoan microorganisms as well as providing information on epidemiological surveillance

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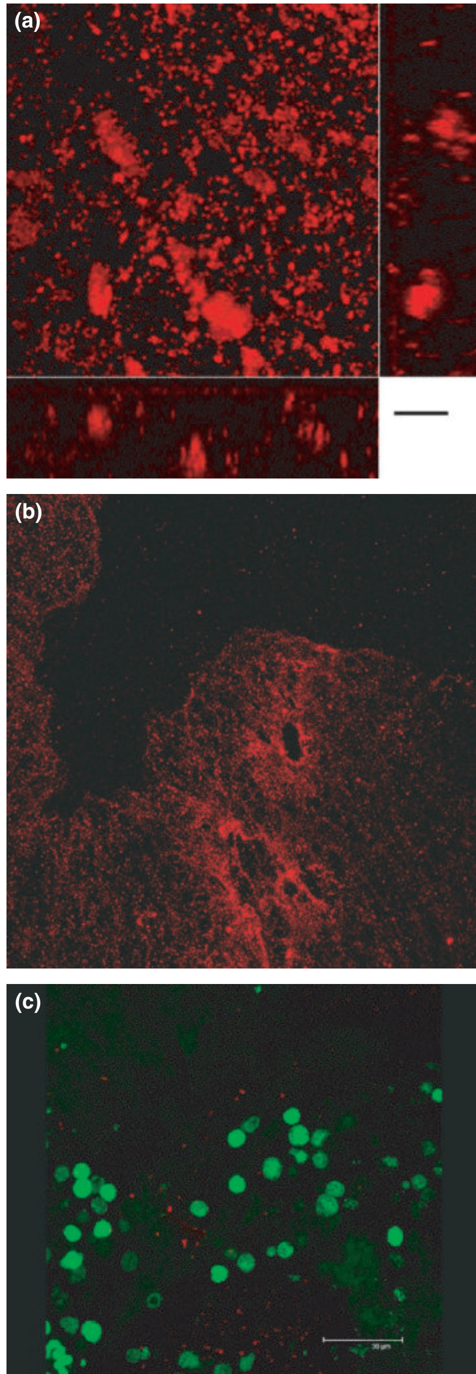

Fig. 2. (a) CLSM image of a MEM biopsy demonstrating *Streptococcus pneumoniae* biofilm by immunofluorescence. The pneumococci appear red because of binding by a Texas Red–conjugated antibody specific for *S. pneumoniae*. (b) Bacteria binding pneumococcal specific-antibody over the MEM surface taken from a patient with biopsy that was also FISH+ for *S. pneumoniae* and an ear effusion which was PCR+ for pneumococcus. Scale bar = 10 μm. (c) Pneumococcus bound with TR antibody surrounded by polymorphonuclear cells stained with Syto9 (green) on an MEM biopsy. (Images: L. Nistico and L. Hall-Stoodley.)

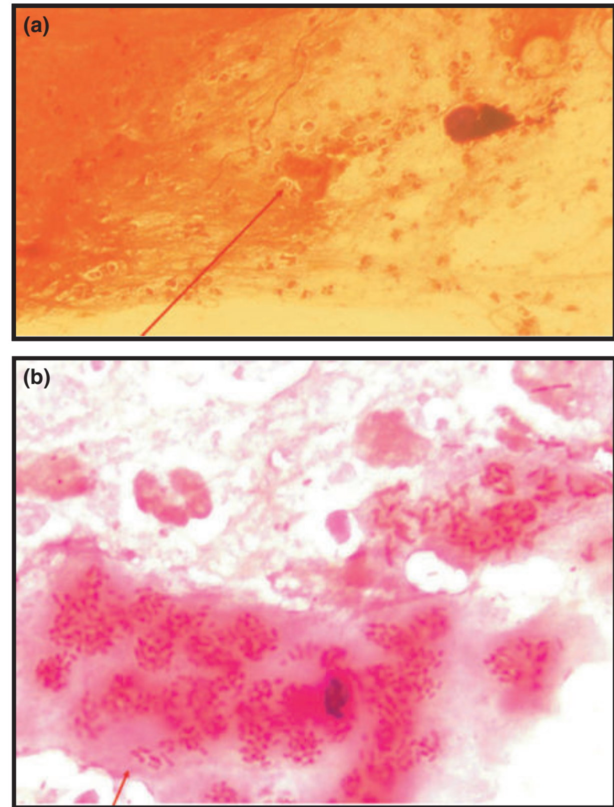

Fig. 3. *Pseudomonas aeruginosa* biofilms in sputum samples from patient with cystic fibrosis (CF). (a) Gram-stained sputum sample showing aggregated bacteria (100×). (b) Gram-stained sputum sample showing aggregated bacteria (1000×). The appearance of an alginate-containing *P. aeruginosa* biofilm in CF sputum caused by the mucoid phenotype is very characteristic and can hardly be mistaken for any other bacterial biofilm in humans, but formal identification *in situ* requires FISH technique (Hoffmann *et al.*, 2005; Bjarnsholt *et al.*, 2009a, b).

and antimicrobial resistance. Advantages of the Ibis/Plex-ID System for identifying BAI compared with culture are: speed (although not as fast as microscopy), and unlike culture and light microscopy, this technique is more likely to detect and identify a pathogen in a single step to species level. For validation, the sample can then be interrogated further using *in situ* methods such as FISH or PNA FISH and CLSM to show microbial aggregates associated with a specific tissue or implant/foreign body (Kathju *et al.*, 2010; Costerton *et al.*, 2011; Nistico *et al.*, 2011).

Phylogenetic sequencing is another high-throughput approach for nonculture, nontargeted PCR-based detection of bacteria utilizing the massive sequencing capacity of instruments such as the 454 pyrosequencer to sequence bacterial 16S rRNA genes from multiple species and multiple samples in a single run. It has been utilized to characterize bacterial communities in environmental

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(Lozupone & Knight, 2005), animal (McKenna *et al.*, 2008), and human specimens (Dowd *et al.*, 2008a, b; Dewhirst *et al.*, 2010; Bielecki *et al.*, 2011). Pyrosequencing analysis of microbial communities in chronic wounds reveals a much wider diversity of microorganisms than by culture alone. Examination of venous leg ulcer samples with pyrosequencing identified 29 distinct genera present, including three with no matching sequences in the database (potentially representing as yet unrecognized microbes) (Dowd *et al.*, 2008a). Culture of these patients (taken from medical history) was positive for a total of only eight genera. Similarly, pyrosequencing analysis of microbes resident in diabetic foot ulcers identified 38 distinct genera and again yielded a subset of sequences unmatched to any recognized microbial sequences (Dowd *et al.*, 2008b). The microbiome of the healthy oral cavity when examined by cloning and sequencing comprises more than 1000 distinct taxa with over half of them yet to be cultured (Dewhirst *et al.*, 2010). This heretofore unappreciated microbial diversity raises significant questions about the relative importance of the component organisms, individually and in communities, to health and disease.

Much progress has also been made in the examination of bacterial gene expression patterns associated with biofilm formation, including whole transcriptomic studies on multiple microbial species. The vast majority of these studies have been on *in vitro* biofilms and employ a range of technologies. DNA microarray analysis of microbial transcriptomes has now been accomplished for a variety of organisms associated with human disease, including *Escherichia coli* (Reshamwala & Noronha, 2011), *Streptococcus mutans* (Shemesh *et al.*, 2010), *Streptococcus pyogenes* (Kreth *et al.*, 2011), and *Candida* (Sellam *et al.*, 2009). Direct RNA sequencing (RNA Seq) has also been undertaken to distinguish biofilm-specific patterns of gene expression. Dotsch *et al.* used RNA Seq to compare planktonic cultures of *P. aeruginosa* with stationary phase cultures and bacteria grown as a biofilm. They found that although there was substantial similarity in the gene expression profiles of stationary phase and biofilm cells, there were also significant differences, indicating that the physiology of biofilm bacteria was not simply surface-attached stationary phase cells. Some studies have begun to examine the transcriptomes of bacteria *in vivo*. Bielecki *et al.* (2011) investigated the expression profiles of three distinct clonal isolates of *P. aeruginosa* from burn wounds in five different conditions: directly from a burn wound sample, in a plant infection, in a murine tumor infection, and as planktonic and biofilm cultures. They found distinct patterns of gene expression in each condition, indicating distinct adaptive responses of *P. aeruginosa* to different environments.

Immunohistochemical or immunofluorescent techniques represent another targeted approach to identifying pathogens in host tissue. Polyclonal or monoclonal sera specific to pathogens are routinely used to detect encapsulated pathogens in fluids such as *S. pneumoniae*, *Neisseria meningiditis*, and *Haemophilus influenzae*. These antibodies have not been consistently applied for the detection of bacteria in biofilms often because it is thought the matrix may bind antibodies nonspecifically. However, antibodies can be used by performing parallel controls and careful testing of sera, as well as using blocking steps to reduce nonspecific interactions (Fig. 2) (Hall-Stoodley *et al.*, 2006). A major obstacle, however, is the lack of commercially available antibodies specific for many pathogens, particularly for unencapsulated bacteria, such as nontypeable *H. influenzae* or *Moraxella catarrhalis*, and for fastidious organisms. There is therefore a need to develop antibody-based diagnostics that detect specific microbial antigens in a fluid or aspirate. For serological-based assays, ELISA is used in CF patients with *P. aeruginosa* biofilm infection to detect antibodies specific to *P. aeruginosa* in general (e.g. water-soluble antigens obtained by sonication of bacterial cells from 17 different serotypes of *P. aeruginosa* (Høiby, 1977), or to specific toxins such as *P. aeruginosa* elastase, alkaline protease or exotoxin A, or alginate to diagnose *P. aeruginosa* in serum from CF patients (Pedersen *et al.*, 1990; Pressler *et al.*, 2006, 2009; Proesmans *et al.*, 2006; Ratjen *et al.*, 2007). The exploration of serological tests for circulating antibodies specific for other BAI organisms would also add a useful method to the biofilm diagnostic toolbox (Selan *et al.*, 2002; Brady *et al.*, 2006).

Clinical history and signs and symptoms

What clinical information may inform the diagnosis of BAI? Chronic or recurrent infection itself has been suggested as a diagnostic criterion along with recalcitrance of the infection to antibiotic treatment (Høiby *et al.*, 2010a). For example, the BAI in CF is characterized by progressive chronic lung infection in response to multiple respiratory pathogens, which are eventually dominated by *P. aeruginosa*. This organism then may adopt a mucoid phenotype that is highly resistant to clearance by antibiotic or host immune responses. CF illustrates several aspects of biofilm-associated disease (Høiby *et al.*, 2010b) and contrasts with acute pneumonias that are resolved with antibiotic therapy. This parallels chronic OM that is recalcitrant to antibiotic treatment and distinct from acute OM that responds well to antibiotic treatment. Thus, both recalcitrance to antibiotic treatment and long-term duration of the infection are important indicators of BAI.

A more detailed diagnostic algorithm will be more likely to result in a more accurate diagnostic tool. At a discussion session regarding clinical biofilms at the 5th ASM Biofilm Conference in Cancun, Mexico (Biofilms 2009

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Proceedings, 2010), several images from clinical cases were shown and discussants were asked whether the case was biofilm associated. Consensus was reached primarily by showing microscopic images of aggregated bacteria associated with host tissue. Interestingly, most of the images were considered by the discussants to show biofilms with no knowledge of the specific bacterial etiology or details of the case, indicating that a key attribute was the visual demonstration of aggregated bacteria (by FISH) attached to host tissue, demonstrating evidence of microbial organization as well as a microbial–host interaction. Line sepsis, though a rapidly progressing infection associated with catheters, is considered a BAI because of the presence of a foreign body (catheter), and the diagnosis of biofilm infection in such cases has been discussed earlier.

Diagnostic guidelines should also depend on the medical history of the patient, the anatomic site of infection, and even the primary organism. For example, *P. aeruginosa* may occur deeper in the tissues than staphylococci (Kirketerp-Møller *et al.*, 2008; Fazli *et al.*, 2009), and diagnostic criteria for wound infections are also specific to the type of wound (Cutting & White, 2004). IE also illustrates that determining the anatomic site is important, because in this infection, biofilm bacteria colonizing the endocardium are localized on the heart valves (Parsek & Singh, 2003; Mallmann *et al.*, 2009; Moter *et al.*, 2010). Characteristically, IE, although frequently associated with bacteria that exhibit antibiotic susceptibility in the microbiology lab, requires prolonged (2–6 weeks) antibiotic treatment. Thus, chronicity or recurrence and documentation of antibiotic recalcitrance are important clues for BAI (Hall-Stoodley & Stoodley, 2009).

As specific biofilm markers along with definitive signs and symptom criteria for occult or suspected deep biofilm infections are currently lacking, detection at the site of infection may include advanced imaging techniques such as whole body {^{18}F} fluorodeoxyglucose positron emission tomography (PET/CT) (Makis & Stern, 2010;

Table 4. Proposed guidelines for the differential diagnosis of biofilm and planktonic infections

Microbiological evidence of localized chronic or foreign body-associated infection		
Positive culture of a microbe (bacteria, fungus), which is known to cause biofilm infections from one or preferably several or repeated relevant specimens: • Fluid • Swab • Tissue sample	Molecular/nonculture-based identification of microbial pathogen	
	NAT positive results for microbes associated with biofilm infections: • CoNS or *S. aureus* with implants, • *P. aeruginosa* with CF, • *H. influenzae* with COM • oral streptococci with endocarditis)	FISH positive results for known biofilm-associated microbes showing aggregated microorganisms (in association with microscopic evidence – see microscopic evidence below)
Microscopic evidence of aggregated microorganisms		
Microscopy revealing the presence of microbial aggregates and biofilm structure (smear or fluid sample, but from tissue sample if possible)	Microscopy revealing evidence of microbial aggregates co-localized with inflammatory cells	
Medical history of biofilm predisposing condition (implanted medical device, CF, IE, chronic OM) (see Table 1)		
Recurrence of the infection (particularly if evidence is provided that the same organism is responsible at multiple time points)		
Documented evidence/history of antibiotic failure or persistent infection despite adequate choice of antibiotic agent		
Evidence of local or systemic signs and symptoms of infection that resolve with antibiotic therapy, only to recur after therapy has ceased such as:		
Fever, localized classical signs of infection: • Rubor, redness • Tumor, swelling • Calor, heat • Dolor, pain • Functio laesa, impaired function	Laboratory infection parameters*: • Increased white blood cell (WBC) count • CRP, LDH • Procalcitonin • Sedimentation ratio	
Evidence of specific immune response to identified microorganism –for example, antibodies to specific pathogens (to alginate or other P. aeruginosa antigens in CF patients)		

NAT, nucleic acid amplification techniques, CoNS, coagulase-negative staphylococci, CRP, C-reactive protein, LDH, lactate dehydrogenase.
*Although inflammatory markers are not specific and not necessarily present in an infection, when they are present they are helpful in supporting the clinical significance of the pathogen and monitoring the response to treatment even though there is often a prolonged return to normalization of inflammatory markers.

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Bensimhon *et al.*, 2011). If such imaging techniques or other signs of occult or foreign body-associated biofilm infection are convincing, then guided (ultrasound or X-ray or surgery), aseptically obtained diagnostic biopsies are, in most cases, necessary unless bacteria are released from the biofilm to the blood (endocarditis) or secretions such as sputum. Microscopy (indicating microbial aggregates), culture (aerobic and anaerobic on differential media and for 1–2 weeks), and culture-independent broad spectrum methods (PCR) should then be used to detect any bacteria or fungi. Contaminants such as CoNS from skin may also cause biofilm infections on foreign bodies such as intravenous catheters and other implantable devices. Ultimately, indirect methods such as antibody detection can only be used, if their predictive diagnostic value has been proven in clinical studies (Pressler *et al.*, 2009).

Similar problems in diagnosing and classifying patients with IE lead to the Duke criteria (Durack *et al.*, 1994) and later modified Duke criteria (Fournier *et al.*, 1996; Li *et al.*, 2000), which have been developed to facilitate and standardize the diagnostic process. A combination of major and minor criteria including echocardiography, microbiological, clinical, and histological findings results in a score, which indicates the probability of IE. However, although the Duke criteria may be helpful and provide a starting point for a BAI algorithm, it must be noted that they are used for one disease, in one organ, whereas biofilm infections are much more diverse. Second, although these criteria are often used in daily practice to help decide whether a patient has IE or not, cardiologists mainly use them as an epidemiological tool in retrospective studies. Finally, even these established criteria are having problems accommodating new molecular technologies and how to implement them.

Although a useful adjunct suggests that the biofilm paradigm better explains the clinical realities of certain infections, this falls short of specific guidelines that are necessary to satisfy evidence-based clinical medicine. The biofilm research community must also address that conventional Koch's postulates using culture may not provide the best evidence for BAI. Therefore, notwithstanding future developments such as the discovery of a universal biofilm marker, the biofilm and medical community needs to provide guidance to the clinician using existing techniques.

Toward new diagnostic guidelines

Ultimately, the goal is to agree on a set of guidelines that lead to what Fredricks and Relman call 'scientific concordance of evidence' in the absence of the absolute fulfillment of Koch's Postulates (Fredricks & Relman, 1996). Therefore, we propose a set of guidelines for the differential diagnosis of biofilm and planktonic infections (see Table 4). These guidelines combine both research criteria for biofilms and clinical criteria for infection and are proposed as a diagnostic algorithm. A combination of positive results from Table 4 should be agreed upon by clinicians and researchers working with BAI, leading to a score that correlates with the probability of BAI that could be evaluated epidemiologically.

Future work

Table 4 represents a systematic, substantive set of guidelines by which to diagnose BAI that is evidence-based rather than anecdotal. Much research remains to be carried out, however. First, the development of imaging-based diagnostic approaches to BAI is important, because a primary feature of BAI is currently the presence of aggregated microorganisms. One of the most convincing diagnostic approaches demonstrating the presence of microbial aggregates is FISH, accompanied by CSLM that provides the ability to spatially resolve microorganisms three dimensionally and show that they are aggregated. Unfortunately, this approach is expensive and time consuming and not useful for all diagnostic laboratories, although Gram-stained smears that show the aggregates, but do not directly identify the species, can also demonstrate biofilm (Fig. 3). Future development may facilitate the diagnostic use of CSLM, particularly at large diagnostic labs.

All those involved in the diagnostic process should collaborate in differentially diagnosing these complex infections accompanied by a robust diagnostic algorithm and good communication. Problematically, in our experience, H&E staining of thin sections is ill-suited to showing biofilm aggregates (Fig. 4). Differential staining with carbohydrate stains such as alcian blue (Hoffmann *et al.*, 2005) or ruthenium red or calcofluor (Yang *et al.*, 2008) or specific antibodies against alginate (Bjarnsholt *et al.*, 2009a), however, might indicate the presence of a biofilm matrix in conventionally stained sections. Moreover, the investigation of novel stains specific for microbial biofilms is needed. Biofilm-specific biomarkers, such as antibodies, would also be desirable as a diagnostic tool; however, this is likely to be pathogen, not biofilm specific and possibly limited to certain anatomic or surgically accessible sites. To date, no biofilm-specific antibodies are marketed. While there are some promising diagnostic technologies in development, it may be years until these diagnostics are certified for use in clinical laboratories (van Belkum *et al.*, 2007).

Summary

The guidelines presented in Table 4 are designed to provide a useful starting point for scientists and clinicians in

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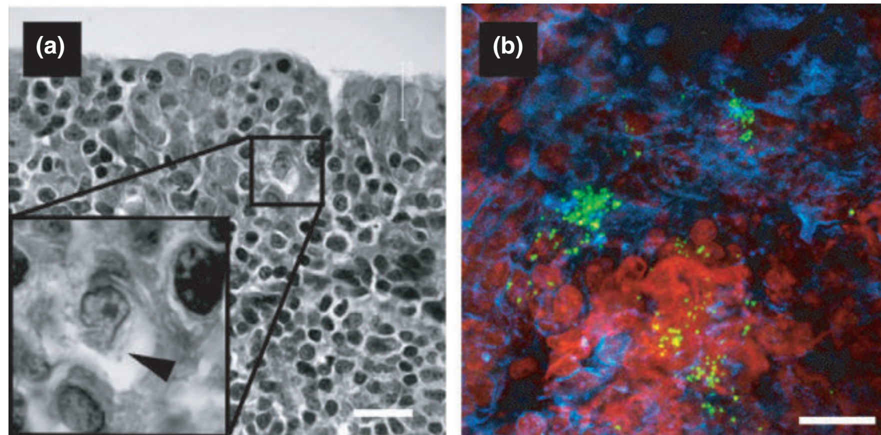

Fig. 4. Pediatric adenoid removed for treatment of chronic OM with effusion. (a) Conventional H&E stained histological sagittal thin section showed occasional invasive bacterial cocci (black arrow). No bacteria were observed on the surface of the two thin sections that were prepared. (b) Confocal plan view image of the surface of a portion of fresh tissue from the same adenoid. The adenoid was stained with the staphylococcal FISH probe (green), propidium iodide (red) that stained the host nuclei, and phalloidin-Alexa488 (blue) that stained F-actin in the cytoskeleton. Live staphylococci as single cells and biofilm clusters were distributed over the adenoid surface. It is interesting to note that a thin section would have a high probability of missing a biofilm cluster entirely, and if it did cut through, it might only capture a few bacterial cells. Scale bars = 20 μm. (Images: Y. Liu and L. Nistico).

distinguishing biofilm infections and a framework for discussion for further refinement and improvement by the larger biofilm and clinical community. Although providing evidence from molecular markers that specific organisms are present, and microscopic evidence that a biofilm may be present, these may not be sufficient to demonstrate that the patient has a biofilm-associated disease without clinical signs and symptoms. Nonetheless, diagnostic guidelines are necessary to distinguish and verify a BAI as soon as possible, because evidence from CF suggests that biofilm infections that are left untreated are more recalcitrant to resolution (Döring *et al.*, 2000; Döring & Høiby, 2004). Additionally, diagnostic guidelines are essential for the evaluation of treatment regimes aimed at resolving BAI, because efficacy of antibiofilm treatment must indicate a significant reduction in bacteria as an outcome measure. BAI are difficult to diagnose because culture, although generally sufficient in acute disease, is not necessarily an accurate indicator of BAI. Thus, to investigate biofilms *in vivo*, identify an infectious etiology, or evaluate treatment, clear clinical signs and symptoms of BAI are also necessary. We have therefore combined criteria that biofilm microbiologists use to distinguish microbial biofilm from planktonic modes of growth, with guidelines that clinicians use to evaluate laboratory results and clinical signs and symptoms of infections. These guidelines are useful not only for the clinician sampling the infection but also for clinical microbiologists handling these samples and emphasize that when there is a high clinical suspicion of infection, molecular tests should be ordered if possible in the face of culture-negative results to assess the possibility of BAI.

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