BIOFILMS: A DIAGNOSTIC CHALLENGE IN PERSISTENT INFECTIONS

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ABSTRACT

Biofilm infections; by definition, are the communities of microorganisms which are attached to a surface and are not readily sampled by common diagnostic techniques. Biofilms are innately more resistant than their individual planktonic counterparts and microorganisms frequently exhibit increased antimicrobial resistance. Our expanding knowledge of biofilm-related infections has broadened our view of possible infectious agents and created a strong demand for new diagnostic attitudes and techniques that overcome the main limitation for detection of infections due to biofilms.

Keywords:

Biofilms, Biofilm infections, Coagulase Negative Staphylococcus spp., Laboratory diagnosis, Resistance.

1. INTRODUCTION

Biofilm is the formation of surface-attached cellular agglomerations; contributing significantly in increasing bacterial resistance against antibiotics and innate host defenses.1 Biofilm formation by pathogenic bacteria, have an enormous impact on the outcome of many bacterial infections. Infections associated with biofilms, as per the recent estimates, have raised the cost of public health systems enormously. Around 65% of clinical infections treated by physicians in the developed world are characterized by the involvement of biofilms.1 Among the biofilm-associated diseases in humans, predominant ones are Pseudomonas aeruginosa colonization of the lungs of cystic fibrosis patients, the colonization of indwelling medical devices by Staphylococci spp., and dental plaque formation by Oral Streptococci spp. and other bacteria in mixed-species biofilms. Neutrophils are the cornerstone of innate host defense during human infections and ingest and kill invading bacteria. Bacterial elimination by neutrophils involves a combination of mechanisms that include reactive oxygen species and antimicrobial peptides (AMPs). In addition, many epithelial cell types also secrete specific AMPs to kill colonizing bacteria without the need of neutrophil uptake.2 There is increasing evidence that biofilms protect from neutrophil killing and phagocytosis, although neutrophils can efficiently penetrate biofilms.3,4 However, the molecular basis of biofilm resistance is not yet entirely clear. Specifically, our knowledge about the interaction of AMPs with biofilms is relatively limited.
1.1 Physiology and structure of biofilms

Formation of biofilm is a sequential process of microbial attachment to a surface, cell proliferation, matrix production, and detachment. The complete process involves a coordinated series of molecular events, which are partially controlled by quorum sensing, an interbacterial communication mechanism dependent on population density. Mature biofilms demonstrate a complex 3-dimensional structure containing functionally heterogeneous bacterial communities. Embedded bacteria occupy numerous microenvironments differing in respect of osmolarity, nutritional supply, and cell density. A variety of phenotypes within one biofilm are produced by this heterogeneity – a single specific “biofilm phenotype” does not exist. Biofilm imaging using microsensors, fluorescent probes, and reporter gene technologies have allowed the correlation of the spatial distribution of nutrients with metabolic activity. Both oxygen and glucose were completely consumed on the surface layers of the biofilms, leading to anaerobic, nutrition-depleted niches in the depths. Areas of active protein synthesis were restricted to the surface layers with sufficient oxygen and nutrient availability.

1.2 Basis of biofilm resistance against antibiotics

The antibiotic resistance of bacteria in biofilms can reach levels that are approximately 10–1,000 times higher than during planktonic growth. The reasons may be:

a) Production of the biofilm-typical exopolysaccharide (EPS) structure - which is believed to decrease the penetration of antibacterial substances to their target. While this is the case for a series of antibiotics, e.g., ciprofloxacin, several other antibacterial substances have been shown to break through the exopolysaccharide layer. Rifampicin and Vancomycin, for example, can penetrate the extracellular matrix of Staphylococcus epidermidis.

b) Slow growth and low metabolic activity of biofilms- Genome-wide analysis of biofilm-specific gene expression in S. epidermidis has shown that basic cellular processes are down-regulated and aerobic energy production is shifted toward anaerobic fermentation.

c) Heterogeneity- It has been hypothesized that a small subpopulation of cells in a biofilm may survive increased concentrations of an antibacterial substance due to a specific physiological status. While most other cells are killed, the survival of such “persister” cells prevents the colony from being erased entirely.

Interplay of all these mechanisms probably leads to resistance to a variety of substances and contribute to the fact that eradication of biofilm-associated infections is very difficult.

1.3 Clinical infections caused by biofilms

Biofilm communities have been discovered in device-related and chronic infections using the microscopic technologies. A key report in 1982 documented large numbers of sessile, slime-embedded S. aureus on a pacemaker lead, which caused a systemic infection. Bacteremia secondary to an olecranon bursitis caused formation of the biofilm, and it drew considerable clinical attention because it showed resistance to weeks of high-dose antibiotic therapy. Since then, biofilms have been revealed in an increasing variety of diseases (Table 1). As many as 60% of bacterial infections currently treated by physicians in the developed world are considered...
to be related to biofilm formation. Biofilm infections are especially frequent in the presence of foreign-body materials. Biofilms on intracorporal devices mostly originate from perioperative contaminants; transcutaneous catheters become colonized by skin flora within days after catheter insertion. A fragile balance between colonization and infection is often maintained for months. Host defenses control the shedding of planktonic bacteria and toxins and thereby prevent clinical symptoms, but they are unable to clear the biofilm. Acute inflammatory episodes, caused by the breakthrough of planktonic cells, can be successfully treated with antibiotics. Flare-ups after treatment termination are frequent because short-term therapies usually fail to sterilize biofilms.

1.4 Diagnostic challenges associated with biofilms

The diagnosis of biofilm infections is difficult. The biofilm mode of growth can delay overt symptoms for months or years. Diagnostic aspirates or swabs are often falsely negative, possibly because the microorganisms persistently adhere to a surface, but not in planktonic form. Individual biofilm fragments with hundreds of slime-enclosed cells may yield only a single colony when plated on agar, or may fail to grow at all because of the dormant state (as explained below) of the embedded bacteria. Consistently, the sonication of removed implants and PCR amplification techniques have shown increased sensitivity in the detection of bacteria sequestered in biofilms. Furthermore, many biofilm pathogens are skin organisms that may be dismissed as contaminants. Culture-independent diagnostic techniques have revealed that several diseases associated with a presumably sterile inflammatory process are indeed bacterial infections that escape culture because of their biofilm mode of growth. For both culture-negative chronic otitis media with effusion and chronic prostatitis, a bacterial etiology has been evidenced by the detection of bacterial DNA and mRNA, as well as by electron and confocal scanning laser microscopy.

1.5 Pathogenic Mechanisms of Biofilms

Different pathogenic mechanisms of the biofilms have been proposed. These include:

- Attachment to a solid surface;
- "Division of labor" increases metabolic efficiency of the community;
- Evade host defenses such as phagocytosis;
- Obtain a high density of microorganisms;
- Exchange genes that can result in more virulent strains of microorganisms;
- Produce a large concentration of toxins;
- Protect from antimicrobial agents;
- Detachment of microbial aggregates transmits microorganisms to other sites.

Biofilms occur commonly on medical devices and fragments of dead tissue such as sequestra of dead bone; they can also form on living tissues, as in the case of endocarditis. Sessile bacterial cells release antigens and stimulate the production of antibodies, but the antibodies are not effective in killing bacteria within biofilms and may cause immune complex damage to surrounding tissues. Even in individuals with excellent cellular and humoral immune reactions, biofilm infections are rarely resolved by the host defense mechanisms.

More than half of the infectious diseases that affect mildly compromised individuals involve bacterial species that are commensals of the human body or are common in our environment. The surfaces of medical devices have been foci of device related infections showing the presence of large number of slime encased bacteria as evidenced by electron microscopy. Even the tissues taken from non device related chronic infections also show the presence of biofilm...
formation. These biofilm infections may be caused by a single species or by a mixture of species of bacteria or fungi.\textsuperscript{18}

1.6 Host Response in Soft Tissue Biofilm Infections

An inflammatory response with many of the cellular, molecular and histologic features of an acute response to infection is a prominent feature of skin and alveolar infections characterized by tissue biofilms. Histologic hallmarks of these superficial lesions include hydrolytic enzyme-mediated destruction of the cell junctions, premature keratinocyte detachment from each other and shedding (acantholysis), intercellular edema (spongiosis) and, finally, a large number of neutrophils traversing the epithelial layers and reaching into the biofilm pockets.\textsuperscript{19}

There is generally an abundance of neutrophils within catheter biofilms, as well as within human tissue biofilms \textit{in vivo}. Whether they become actively recruited by host or microbial proteins is currently unknown. In addition, their functional activation and phagocytic status within biofilms seems to vary, with active phagocytosis being detected within biofilms of certain bacterial species but not others.\textsuperscript{19}

1.7 Diagnosis of Biofilms

Currently, investigation of suspected infections is accomplished through a combination of diagnostic testing methods, including blood tests, microscopy, histology, optical studies, and culturing. Definitive diagnosis usually relies on several different tests with agreeable results as well as consistent repetition (i.e., two or three consecutive cultures identifying the same microorganism as the source).\textsuperscript{20}

Infections with biofilms on implanted medical devices can be divided into two groups: low-grade infections (indolent and subclinical) and those that are associated with clinical manifestations. The difference is related in part to the bacterial species involved. Highly virulent organisms (e.g., \textit{Staphylococcus aureus} and Gram-negative bacilli) are generally involved in infections characterized by the early appearance of symptoms, which is related to the production of toxins and virulence factors and to the tendency of these infections to spread to distant sites by means of septic metastasis. Patients with high-grade infections involving orthopedic prostheses generally experience fever, persistent local pain, erythema, edema, massive hematomas, and disturbances of the wound-repair process. The pain is often particularly severe when the infection is caused by \textit{Pseudomonas aeruginosa}, and this may be a reflection of the strikingly large number of proteases, leukocidins, and other virulence factors encoded in this pathogen’s extensive genome. Device-related infections in this category are usually suspected early and rapidly treated. Other bacteria that are less virulent (e.g., Coagulase-negative staphylococci) are usually the cause of low-grade, indolent infections. These infections can remain clinically silent for years while bacteria slowly accumulate on the device. Planktonic cells detach from its surface when the biofilm reaches maturity and spread throughout the body, inducing a complete inflammatory reaction related to sepsis. Late-onset infections (arbitrarily defined as those appearing six months or more after device implantation) can be caused by highly virulent bacterial species, such as \textit{S. aureus}. These infections are probably caused by hematogenous spreading from remote infection sites rather than by intraoperative contamination.\textsuperscript{20}

1.8 Nonspecific Blood Tests\textsuperscript{20,21}

Several laboratory tests are used to detect inflammation and possible infection by a bacterial pathogen. These tests, however, are not specific for the presence of bacteria and can be misleading when interpreted incorrectly. One of the most common tests used to indicate inflammation is the level of C-reactive protein (CRP). CRP is elevated during systemic inflammation and can be seen in post-operative patients. Shortly after
surgery, patient CRP levels rise but eventually return to normal. A second rise in CRP levels can indicate a postoperative infection, but again it is nonspecific for a pathogenic microorganism.

Complete blood counts (CBCs) are commonly used to monitor hematological homeostasis and inflammation and are more specific for an invading pathogen than other tests. Differential elevation of certain leukocytes can suggest the presence of bacteria or viruses but cannot accurately specify which pathogen, if any, is causing the inflammation. Another indicator of inflammation is an elevation in the erythrocyte sedimentation rate (ESR). These tests, while nonspecific, are useful indicators for further work-up and are performed frequently because of their fast processing times and comparatively inexpensive costs.

1.9 Diagnostic Imaging

The imaging modalities that have been used to detect and study device-related infections include:

- a) Plain-film radiology,
- b) Computed tomography (CT),
- c) Magnetic resonance imaging (MRI),
- d) Endoscopy,
- e) Angiography,
- f) Ultrasound, and
- g) Scintigraphy performed with labeled leukocytes or anti-neutrophil antibodies.

In terms of safety, cost, and availability, plain-film radiology and ultrasonography are the only two imaging approaches that might be used for routine screening purposes. The real problem, however, is that their success in the detection of infections related to medical implants depends on the presence of substantial morphological changes in the infected area, such as the formation of an abscess or the erosion of bone tissue. And, with the exception of scintigraphy, the same limitation applies to all of the other imaging modalities listed above, which are also more expensive and invasive than conventional radiology and ultrasound. Consequently, these approaches are useful only in the advanced phases of the infection, when the tissues have already been damaged by bacterial enzymes and by the inflammatory reaction.

The scintigraphic approach is considered the gold standard for imaging diagnosis of biofilm infections involving implanted medical devices. It is based on the acquisition of functional data related to accumulation of neutrophils at the site of infection. The use of radioactive tracers enhances visualization even of small lesions, making it an extremely sensitive method. Unfortunately, however, it cannot distinguish between septic and aseptic inflammatory processes. Apart from the problem of low specificity, the scintigraphic approach is far too invasive, toxic, and expensive for routine use.

In short, there is currently no imaging modality that meets the requisites for use as a routine tool for monitoring patients at risk for infections related to implanted medical devices. Additionally, none of the techniques described earlier actually help in identification of the genus or species of the microorganism causing the fouling of the device. Therefore, clinical treatment is at best, a guess, after such diagnostic approaches are employed.

1.10 Conventional Microbiology

A fundamental step in the laboratory diagnosis of any microbial infection is the collection of samples of biological fluids, tissues, or infected biomaterials for microbiologic analyses aimed at identifying and characterizing the causative organism at the species level. Intravenous catheters and endotracheal tubes can be removed easily for culture. For more complex devices (artificial heart valves, vascular grafts, artificial joints, etc.), removal is much more complex, invasive, and expensive. With conventional microbiological techniques, it is difficult to
culture bacteria from biofilm clusters contained in such material. New methods for detecting biomaterial colonization without withdrawal of the device are currently under investigation. An alternative to culture of the device itself involves the use of multiple blood cultures. This approach has several drawbacks. For one thing, false-negative results can emerge when antibiotic treatment has been administered prior to blood sampling. However, the main problem is that the bacterial species most commonly responsible for biofilm infections on medical implants are not pathogens, but are members of the human commensal flora. Consequently, it is difficult to the significance of their isolation from blood cultures may be difficult to determine.

1.11 Immunodiagnostics assays

A good alternative involves the use of immunodiagnostic assays for the detection of antibodies directed against antigens that are specific for the biofilm form of bacterial species, especially those that are traditionally considered as saprophytes. Since Staphylococci spp. are one of the main causes of biofilm infections involving implanted medical devices, the first attempts have been devoted to the diagnosis of these infections. Distinguishing pathogenic S. epidermidis and S. aureus strains from contaminant/commensal strains is one of the major challenges in clinical microbiology laboratories. The ideal immunodiagnostic test for diagnosis of staphylococcal biofilm infections should have as many of the following characteristics as possible:

− Broad-spectrum specificity for all Staphylococcal species
− Capacity to detect infections before the appearance of clinical signs and symptoms
− Ability to differentiate between active and past infections
− Suitability for use during the follow-up after replacement of infected graft
− Low cost
− Noninvasiveness
− Speed of obtaining results

Enzyme-linked immunosorbent assays (ELISA) can satisfy many of these prerequisites. They are simple, rapid, and repeatable, and they do not require removal of the implanted device. Furthermore, immunoenzymatic assays of antibodies to S. aureus antigens can identify cases of S. aureus bacteremia associated with false-negative blood cultures due to prior antibiotic treatment.

Another serological modality that fits in the criteria mentioned above is the lateral flow assay (LFA). An LFA has the potential to be as sensitive and specific as ELISAs and has a much shorter processing time. The disposable cassettes are easy to use, inexpensive, and require very little expertise. Like ELISA, a lateral flow immunoassay works on the principle of antibody detection using antigens that are specific for an infectious agent.

1.11.1 assays based on the use of whole bacterial cells

A sensitive technique for the serodiagnosis of infections sustained by planktonic forms of bacteria involves the titration of serum antibodies against whole bacterial cells. Since this approach does not require antigen extraction, it can be used to screen large number of samples.

This strategy was associated with two major problems. First of all, during ELISA, the wells were contaminated by planktonic cells that had been detached from biofilms during washing. In addition, neither the IgG nor the IgM titers obtained in these assays were capable of discriminating between the sera of patients harboring biofilm graft infection sustained by Staphylococci and sera of healthy controls.

1.11.2 assays based on discrete slime antigens

1.11.3 assays based on mixtures of S. epidermidis slime antigens
These assays use mixtures of slime antigens as probes for the detection of specific antibodies against *S. epidermidis* in biological samples. The SSPA ELISA has a number of advantages over other available methods used to diagnose biofilm-related infections.

- a) It is versatile: the SSPA ELISA is capable of detecting antibodies in late-onset device-related infections caused by different *Staphylococcus* species.
- b) It has been validated in a group of patients suffering from late-onset graft infections, which represent the best clinical model of the majority of chronic infections related to biofilm colonization of implanted medical devices.

A concise summary of current diagnostic techniques useful for diagnosis of biofilms in healthcare facilities has been provided in a tabulated form. (Table-2)

1.12 Novel approaches to prevent biofilm formation

Antiadhesive surfaces with altered physical, chemical and topographical properties that prevent adhesion and thereby biofilm formation are also being sought. Approaches to aid the dissolution of already established biofilms include:

- a) physical treatment of the biofilm,
- b) photodynamic therapy,
- c) targeting of the biofilm matrix for degradation,
- d) delivery of signal blockers,
- e) interference with biofilm regulation,
- f) induction of biofilm detachment and
- g) development of cytotoxic strategies to treat biofilm-forming bacteria.

Although numerous *in vitro* studies have demonstrated effective antibiofilm treatment, only a few *in vivo* (preclinical) or clinical studies have demonstrated improved treatment of biofilm infections. It is generally agreed that effective treatment of biofilms requires a combination therapy of an antibiofilm compound with an effective antibiotic, but no antibiofilm therapies are in current clinical use.

**CONCLUSION**

In the industrialized world, acute bacterial infections caused by rapidly proliferating planktonic cells (e.g., *Salmonella typhi*) have been gradually replaced by chronic infections due to environmental organisms (e.g., *Staphylococcus epidermidis*) growing in biofilms. Biofilm eradication requires the elimination of all bacteria, otherwise infection recurs and becomes chronic. Culture of specimen is the gold standard for diagnosing bacterial infections, but recent investigation of ability of this technique to grow bacteria from a biofilm has indicated that it is not reliable. Other testing modalities, such as PCR and serology assays, are either nonspecific for biofilm infections or they include the risk of contamination during sampling. Therefore, accurate diagnosis of an infection usually takes days and requires extensive test procedures, leading to increased healthcare costs and discomfort to the patient. In recent years, many attempts have been performed to design and set up new serology diagnostic assays to obtain early, noninvasive diagnosis of infections sustained by biofilms colonizing native tissues and medical implants. The new tools could also allow new medical and surgical approaches to monitor and treat biofilm infections.
LIST OF TABLES

Table 1. Partial list of human infections involving biofilms

<table>
<thead>
<tr>
<th>Infection or disease</th>
<th>Common bacterial species involved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dental caries</td>
<td>Acidogenic Gram-positive cocci (sp.)</td>
</tr>
<tr>
<td>Periodontitis</td>
<td>Gram-negative anaerobic oral bacteria</td>
</tr>
<tr>
<td>Otitis media</td>
<td>Nontypeable <em>Haemophilus influenzae</em></td>
</tr>
<tr>
<td>Chronic tonsillitis</td>
<td>Various species</td>
</tr>
<tr>
<td>Cystic fibrosis pneumonia</td>
<td><em>Pseudomonas aeruginosa, Burkholderia cepacia</em></td>
</tr>
<tr>
<td>Endocarditis</td>
<td><em>Strept. viridans, Staphylococci</em></td>
</tr>
<tr>
<td>Necrotizing fascitis</td>
<td>Group A streptococci</td>
</tr>
<tr>
<td>Musculoskeletal infections</td>
<td>Gram-positive cocci</td>
</tr>
<tr>
<td>Osteomyelitis</td>
<td>Various species</td>
</tr>
<tr>
<td>Biliary tract infection</td>
<td>Enteric bacteria</td>
</tr>
<tr>
<td>Infectious kidney stones</td>
<td>Gram-negative rods</td>
</tr>
<tr>
<td>Bacterial prostatitis</td>
<td>Escherichia coli and other Gram-negative bacteria</td>
</tr>
<tr>
<td>Infections associated with foreign body material</td>
<td></td>
</tr>
<tr>
<td>Contact lens</td>
<td>*P. aeruginosa, Gram-positive cocci</td>
</tr>
<tr>
<td>Sutures</td>
<td><em>Staphylococci</em></td>
</tr>
<tr>
<td>Ventilation-associated pneumonia</td>
<td>Gram-negative rods</td>
</tr>
<tr>
<td>Mechanical heart valves</td>
<td><em>Staphylococci</em></td>
</tr>
<tr>
<td>Vascular grafts</td>
<td>Gram-positive cocci</td>
</tr>
</tbody>
</table>

Arteriovenous shunts          Staphylococci
Endovascular catheter infections Staphylococci
Peritoneal dialysis peritonitis Various species
Urinary catheter infections E. coli, Gram-negative rods
IUDs Actinomyces israelii and others
Penile prostheses Staphylococci
Orthopedic prosthesis Staphylococci

Table 2. Summary of current diagnostic techniques used in healthcare facilities with their relative sensitivity, specificity, and processing time

<table>
<thead>
<tr>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Processing time</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC, CRP</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Culturing</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Molecular (PCR)</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Imaging (MRI, CT, Radiography)</td>
<td>Varies</td>
<td>Low</td>
</tr>
<tr>
<td>Microscopy (IFM, FISH)</td>
<td>High</td>
<td>Moderate</td>
</tr>
<tr>
<td>Serology (ELISA)</td>
<td>High</td>
<td>High</td>
</tr>
</tbody>
</table>

BIBLIOGRAPHY


