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Biofilms in periprosthetic orthopedic infections

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Abstract

As the number of total joint arthroplasty and internal fixation procedures continues to rise, the threat of infection following surgery has significant clinical implications. These infections may have highly morbid consequences to patients, who often endure additional surgeries and lengthy exposures to systemic antibiotics, neither of which are guaranteed to resolve the infection. Of particular concern is the threat of bacterial biofilm development, since biofilm-mediated infections are difficult to diagnose and effective treatments are lacking. Developing therapeutic strategies have targeted mechanisms of biofilm formation and the means by which these bacteria communicate with each other to take on specialized roles such as persister cells within the biofilm. In addition, prevention of infection through novel coatings for prostheses and the local delivery of high concentrations of antibiotics by absorbable carriers has shown promise in laboratory and animal studies. Biofilm development, especially in an arthroplasty environment, and future diagnostic and treatment options are discussed.

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Keywords

antibiotic resistance; arthroplasty; biofilm; coating; infection; planktonic; prosthesis; treatment

Infection in total joint arthroplasties

Rates of total joint arthroplasty placements & infection

Recently, a number of excellent articles have appeared detailing the pathogenesis, diagnosis, prevention and treatment of orthopedic infections and the growing evidence for the role of biofilm formation as a mechanism to explain issues in each of these areas [1–7]. Many of these studies have focused on the clinical perspective. This article discusses these important topics in relation to biofilm formation, not only on the foreign body, but also on adjacent soft tissues and bone in the context of a developmental process as a holistic integration with planktonic and invasive bacteria. We identify possible interventions arising from the consideration of specific tissue and cellular locations that might be considered ‘reservoirs’ of the infecting pathogens (e.g., joint fluid, intracellular areas and biofilms), with the concern that bacteria distributed among these various reservoirs could allow regrowth if all are not killed or removed.

Orthopedic infection continues to receive a high level of attention, due to the fact that nearly 1 million total hip and knee arthroplasties (Figure 1) are performed in the USA each year, and this number is expected to rise in the near term with the aging population [8]. Artificial joints can improve the quality of life for patients, but failure of the prosthetic can also inflict significant morbidity and suffering. The major causes of failure are loosening of the prosthesis from the bone, fracture of the bone or infection at the site of implantation. Although orthopedic infections overall include osteomyelitis and soft-tissue infections (e.g., diabetic foot infections – for a review, see [9]), this article will focus on infection following implantation, a serious complication that can often result in failure requiring revision [10–14].

In considering periprosthetic infection, there are two major routes and time scales associated with the entry of the pathogenic bacteria. The first is the perioperative period, most commonly via the surgical incision itself, with the source being the patient's endogenous flora or bacteria from the personnel or environment of the operating room (OR). The second is from hematogenous spread, which is generally thought to occur during the postoperative period [15]. The insertion of a foreign body that will reside in the patient for years increases the risk for infection that could ultimately mature into a biofilm. Infections can present in patients acutely and/or persist chronically for years following surgery, and the diagnosis of infection and the type of bacterial growth (i.e., planktonic vs biofilm) remains a difficult task for clinicians [16,17], often requiring highly specialized research equipment and time-consuming methods. Early infections are typically caused by high-virulence bacteria, while those that develop later tend to be caused by low-virulence bacteria [18]. Bacteria noted to cause periprosthetic infection include coagulase-negative staphylococci (30–43%), *Staphylococcus aureus* (12–23%), streptococci (9–10%), enterococci (3–7%), Gram-negative organisms (3–6%) or anaerobes (2–4%) [19]. However, the picture from culture

data is likely misleading, since species such as *Propionibacterium acnes*, which is generally considered to be nonpathogenic or ‘weakly’ pathogenic, may be inappropriately dismissed as a contamination. This species has now been found to be more common than previously thought when more robust sampling and detection methods are used, such as sonication and nucleic acid detection by PCR [20]. Another issue is the development of specific biofilm phenotypes such as ‘small-colony variants’, which are slow growing and easily missed in routine clinical cultures [21]. Regardless of the species, once bacteria have gained entry to the surgical site, they can persist within the body in three distinct phenotypic modes of growth (Figure 2).

Planktonic—The single-cell form of bacteria is most easily identified and cleared by the host's natural defenses or antibiotic therapy. This type of growth can also serve as a precursor for biofilm development if the bacteria are able to colonize a surface within the patient (either periprosthetic tissue or the prosthesis components themselves). Planktonic bacteria may grow in the joint fluid during an acute episode of infection.

Biofilm—A biofilm is a community of microorganisms in a structural matrix usually adherent to an underlying substratum; these microbes can take on unique phenotypic roles within the 3D biofilm structure in order to evade both antibiotic therapy as well as the natural defenses of the host. Biofilms are commonly associated with a foreign body such as when a prosthetic is implanted into the host, because these abiotic artificial surfaces offer a ready interface to which individual bacteria may attach and eventually form a biofilm. Biofilm bacteria can cause extreme modifications to the local environment, as for example in dental biofilms, which can shift normal physiological conditions to a highly acidic and anaerobic state within just a few tens of microns [22] through their metabolic activity and diffusion-limited mass transfer in the biofilm. There is mounting evidence that over 99% of bacteria reside in biofilms, with staphylococci (specifically *S. aureus* and *Staphylococcus epidermis*) being the most common biofilm-forming bacteria [10]. Together, *S. epidermis*, *S. aureus* and *Pseudomonas aeruginosa* make up almost 75% of the biofilms found in medical devices [11,12]. In the context of prosthetic joint infection (PJI), biofilm bacteria can attach to hardware components, cement, bone and fibrous tissue, and detached clumps of biofilm can also be found in the joint fluid [23,24].

Invasive & intracellular—Several species of bacteria are able to enter, survive or even proliferate within host tissue or the host's cells. This phenomenon is most often seen in epithelial and osteoblast cell types, and is used by the bacteria as a means to avoid immune cells and high concentrations of antibiotics present in the extracellular space. Interestingly, *S. aureus* small-colony variants, a slow-growing phenotype associated with biofilm formation, have been documented inside fibroblasts in PJI cases, thus making a potential link between surface-associated biofilms and invasion [21].

It is possible that each of these three anatomical locations – the overlying fluid, the foreign body or host surface and the subsurface tissue – may represent individual reservoirs of infection, each containing bacteria in different phenotypic states, from which pathogens could repopulate if they are not completely eradicated through means such as washing,

antibiotic therapy or surgical debridement. Figure 3 is a schematic showing how pathogenic bacteria might be distributed in a joint space, in this case using an example of a knee.

Formation of biofilms

Early stage

A biofilm begins when individual, planktonic bacteria adhere to a surface and begin to produce an extracellular polymeric slime (EPS) matrix composed of polysaccharides, DNA and proteins. This matrix can recruit and increase the adhesion of other microbes in the vicinity, which, through growth, can self-organize into a well-defined, highly structured collection of bacteria. The creation and maturation of biofilms are controlled by several factors in the matrix (e.g., exopolysaccharides) and mobility proteins on the bacteria (e.g., pili and flagella) [25]. Individual staphylococcal bacteria adhere to a surface and secrete positively charged homopolymers (polysaccharide intercellular adhesin) to aid in the aggregation of planktonic bacteria. These polysaccharides also create a physical and electrostatic barrier against the positively charged antimicrobial peptides and against phagocytosis by immune cells. Expression of polysaccharide intercellular adhesin has been positively correlated with the virulence of the bacteria [26]. In experiments conducted on *Escherichia coli*, the most motile strains in a constant flow system were also the best biofilm-forming strains, linking the phenomena of motility and biofilm development [27]. The flagella, which are used for swimming motility, can also act as points of initial adhesion. Once attached, the bacteria can transition from planktonic movement (swimming) to movement along a surface using type IV pilus motility (gliding or twitching). This surface motion can be part of a coordinated activity to form larger, mature biofilm structures. However, motility and motility appendages are not in every case required for the formation of biofilms, as evidenced by the profuse biofilms that can be formed by nonmotile pathogens, such as staphylococci and streptococci.

Maturation

Early in biofilm development, the structure can be less stable and more susceptible to antibiotics and host defenses [28]; however, as the biofilm rapidly matures, the bacteria within becoming increasingly tolerant of antibiotics. An important process in the maturation phase is the production of EPS, which binds the cells together and to the surface. The EPS forms a physical barrier, as well as causing diffusion-limited transport of chemicals into and out of the biofilm. The two main constituents of EPS are polysaccharides and extracellular DNA (eDNA). Recent evidence shows that eDNA can form net-like structures, similar in appearance to the nets used by neutrophils to trap planktonic bacteria [29]. In *S. aureus*, the release of eDNA is regulated by the *cidA* gene, which causes lysis of a subpopulation of cells within the biofilm [30]. *P. aeruginosa* also appears to have a regulated mechanism for the release of eDNA, which is coordinated with the development of biofilm structure [31]. There also appears to be an even higher level of extracellular ordering with respect to the extracellular matrix as shown in a study by Goodman *et al.*, which identified extracellular proteins that linked eDNA polymer strands to form networks in *Haemophilus influenzae* biofilms [32]. Targeting biofilm EPS components such as eDNA in order to dissipate the

biofilm with simultaneous application of antibiotics to kill the released cells is an area of active research.

The close proximity of cells, the high cell density and limited transport through the biofilm creates an environment conducive to cell-to-cell communication through quorum sensing (QS). QS in microbes is a regulatory mechanism that allows bacteria to modify gene transcription based on cell density. In staphylococcal biofilms, this is accomplished through a peptide (autoinducing peptide) that is secreted from the bacterial cells [33,34]. As bacteria aggregate, extracellular autoinducing peptide reaches a threshold concentration and begins binding to a membrane receptor, initiating a signaling cascade that results in the expression of the regulatory RNA, RNAIII. This effector activates the accessory gene regulator *agr* with the consequent expression of secreted virulence factors [35]. Mutations in *agr* also cause reduced dispersion, leading to increased biofilm formation [36]. Gram-negative biofilm-forming pathogens such as *P. aeruginosa* use a different QS system that is mediated by signaling molecules from the homoserine lactone family. Here, an accumulation of QS agents beyond a threshold concentration is also accompanied by an increase in biofilm formation, as well as an increase in secreted virulence factors [37]. The close proximity of cells within the biofilm is also conducive for horizontal gene transfer, which can allow for the transmission of virulence genes within the biofilm, as well as genes that are responsible for antibiotic resistance.

There are other consequences for cells within the biofilm as the biofilm grows and matures. The bacteria on the outside of the biofilm consume nutrients, such as oxygen and glucose, leaving the center of the biofilm relatively devoid of the same, such that centrally situated bacteria can become metabolically inactive. Nutrient deprivation and other stressors such as acidic conditions can cause bacteria within the biofilm to enter a dormant 'persister' state [38]. Both persister and small-colony variant populations in the biofilm can survive exposure to high levels of antibiotics due to their lack of metabolic activity.

Dispersal from the biofilm

In addition to its role in the developing biofilm, QS can also play a role in the detachment of bacteria from the mature biofilm in order to spread infection within the host, as previously discussed with respect to *agr*-mediated dispersal in staphylococcal biofilms [35]. *P. aeruginosa* has two dispersal mechanisms, one mediated by nitric oxide [39] and the other by *cis*-2-decanoic acid, a fatty acid messenger [40]. It is thought that coordinated dispersal is a mechanism used by bacteria to colonize new surfaces once nutrients and space have been depleted. Dispersal may therefore play a role in the dissemination of infection and can explain acute infectious episodes that appear as exacerbations of a chronic underlying process.

Clinical complications due to bacterial growth in a biofilm

Antibiotic resistance

Bacterial cells residing in the outermost aspects of the biofilm are most exposed to the host's defenses and antibiotics, but these microbes can themselves mount several protective defenses. The matrix and layers of cells within the biofilm create a physical barrier to slow

the diffusional penetration of antibiotics. Metabolic activity in the outer biofilm can create acidic or anoxic areas that might contribute to antibiotic degradation [41]. In addition, secreted polymers that make up the extracellular matrix of the biofilm can bind and deactivate antibiotics, forming an antibiotic ‘sink’ [42]. As previously discussed, the depletion of nutrients in the interior of the biofilm can lead to starvation-induced dormancy and the development of resistant persister cells. Moreover, diffusion-limited transport, binding to matrix components and uptake by bacteria can create a gradient of antibiotic concentration. This gradient exposes a subpopulation of the bacteria within the biofilm to a sublethal dose of antibiotics. Sublethal exposure has been shown to increase the development of antibiotic tolerance by increasing biofilm formation [43]. Because of these various defense strategies when bacteria are grown as a biofilm, the MIC of antibiotics is increased by as much as 1000-fold in *P. aeruginosa* [44–47] and in biofilm clusters from methicillin-sensitive *S. aureus*, which exhibited tolerance to antibiotics that is normally associated with methicillin-resistant *S. aureus* strains [48].

In addition to biofilm-specific mechanisms of tolerance, biofilm bacteria also utilize conventional resistance mechanisms; for example, β -lactamases are released from *P. aeruginosa* biofilms in order to breakdown extracellular β -lactams (e.g., penicillin and cephalosporin, among others), a common family of antibiotics used to treat orthopedic infection [49,50]. Biofilm bacteria can also upregulate the expression of efflux pumps in order to remove antibiotics that have successfully penetrated into the bacterial intracellular compartment [49].

Avoidance of the host's defenses

Biofilm structure also inhibits the adaptive and innate immune responses of the host [51]. The mere presence of a foreign body within the patient is already relatively inaccessible to the immune response, due to the lack of blood supply to the structure. This restricts the ability of immune cells and antibodies to reach the foreign surface and clear infections [52]. Moreover, the investing layer of scar tissue that typically forms around an embedded implant is also relatively hypovascular, and therefore similarly compromised. When biofilms do form on and about implants, polymorphonuclear leukocytes are unable to effectively phagocytose bacteria due to restricted access to the bacterial cells within, caused by the EPS matrix [53]. Granulocytes isolated from the implant in an animal model had poor bactericidal activity and reduced superoxide production when compared with polymorphonuclear leukocytes from the blood or exudates. This impairment is thought to be caused by the interaction of the granulocyte with the implant or wear particles in the environment [54]. These nonfunctioning immune cells have been referred to as ‘frustrated’ granulocytes [55]. This defective immune response is evidenced by the significant reduction (by at least 10,000-fold) in abscess-forming doses of *Staphylococcus* when a foreign body is present in animal models and humans [56,57]. Zimmerli *et al.* and Elek and Conen found similar results in a guinea pig model and showed that reduced opsonization might account for some of this effect [56,57].

Innate immune cells are also able to activate the adaptive immune response through lectin binding in order to produce antibodies [51]. Antibodies, similar to phagocytic immune cells,

are more effective early on against biofilm development, but these proteins are not able to penetrate the matrix or layers of bacterial cells once a biofilm has matured [58–60]. In certain situations, such as cystic fibrosis, this ineffective antibody response causes chronic inflammation, which can damage the host's tissues [61]. Similarly, in dental biofilms, chronic inflammation stimulated by the presence of a biofilm causes damage to the surrounding soft tissues, yet is ineffective in resolving the infection [62]. In PJI, there is a correlation with elevated inflammatory parameters, and the local tissue damage caused by inflammation might release nutrients that then become available to the biofilm bacteria, exacerbating the disease.

Role of biofilms in total joint arthroplasties

Diagnosis of infection

While the development of an infection can be accompanied by obvious signs (e.g., fever, swelling and purulent discharge at surgical site, among others), in the absence of a positive culture, differentiating between loosening due to wear reactions (aseptic loosening) and infection is very difficult. Ascertaining whether microbes are growing in a planktonic or biofilm state within the patient represents an even greater challenge. Even if bacteria can be recovered from the patient and identified through *in vitro* culturing, this technique is not necessarily reflective of the *in vivo* growth state, since most strains of bacteria can grow in both planktonic and biofilm modes (although recovered biofilm bacteria are frequently difficult to culture – see below). Acute implant infections have been defined as having “signs and symptoms lasting <14–28 days, an unambiguous diagnosis based on histopathology and microbiology, a stable implant, and susceptibility of the microorganism to an effective orally available antimicrobial agent” [63]. Acute infections are classically considered to be caused by rapidly growing planktonic bacteria, which are relatively easy to recover in sampling, since they are typically present in fluids and tissues in high concentrations. Because they are usually rapidly growing *in vivo*, they are also relatively easy to propagate on nutrient-rich clinical culture media. However, chronic subacute or indolent infections are harder to diagnose. This can be explained by the biofilm phenotype, in which many bacteria are in a metabolically quiescent state induced by nutrient limitation inside the biofilm. Biofilms are often patchy and might be missed in a fluid, swab or tissue biopsy. In multiple studies, simple *in vitro* culturing of the bacteria only detected biofilms 30% of the time compared with 80–90% using histology and microscopy [64]. Improvement in the methods used to detect the presence and phenotypic state of microbes is of critical importance, as this may help with determining the type and concentration of antibiotic(s) or surgical interventions needed to eliminate or more effectively control the infection.

The recognized difficulty in diagnosing periprosthetic joint infection (Figure 1) led to the formation of a working group of the Musculoskeletal Infection Society, and more recently a specific study group of the Consensus Meeting on Periprosthetic Joint Infection, with the task of defining consensus guidelines for diagnostic purposes [65]. The proposed guidelines consider three criteria, only one of which exclusively relies on direct culture (if a pathogen is cultured from two separate samples). Furthermore, culture of low-virulence pathogens, such as *P. acnes*, in the absence of other criteria is not considered a definitive diagnostic of

infection. It has also been suggested that even pathogens cultured from draining sinuses associated with total knee and hip arthroplasties do not correlate well with the pathogens involved in the PJI and might result in misguided treatment [66]. The other two criteria are: the presence of a sinus communicating directly with the prosthesis; and the presence of four out of six clinical and laboratory findings, including elevated erythrocyte sedimentation rate and C-reactive protein, elevated synovial leukocyte count or neutrophil percentage, presence of purulence in the affected joint, a single culture-positive sample and greater than five neutrophils per high-power field in five high-power fields of histological analysis of the explanted tissue. Since the level of C-reactive protein is often elevated postsurgery, levels prior to surgery should be established, with continued testing following surgery in order to ensure that levels return to baseline. Blood and synovial fluid should be cultured for the presence of both aerobic and anaerobic microbes. If the infection has already caused structural damage to the bone, a radiograph may visualize the subsequent weakness caused by the infection; however, radiographs are highly limited in their ability to confidently diagnose infection. Localized bone resorption caused by infection, appearing in radiographs as a focal lucency, often appears to be indistinguishable from granulomatosis [67] or periprosthetic osteolysis due to wear particles [68].

Molecular methods of diagnosis

Currently, diagnosis is based on standard microbiological cultures of recovered clinical samples, clinical signs and a consideration of various laboratory results, as described above [65]. However, due to the inadequacy of cultures for detecting biofilm bacteria, more recent studies have utilized molecular methods in order to assay for bacterial DNA and RNA directly. Many of these methods involve PCR amplification and sequencing of the nucleic acid mix isolated from clinical samples using either microbe-specific or broad-range primers, often derived from bacterial 16S rDNA sequences. This can allow for the simultaneous identification of multiple microbes that may be present in any given sample or patient. The use of PCR amplification and molecular analysis (in conjunction with cultures) has shown several advantages over cultures alone in the detection and identification of microbes:

- PCR in conjunction with culturing from biopsy or joint fluid has detected resident bacteria in 9% of culture-negative cases [69]. However, other studies report positive detection rates of between 70 and 80% [70];
- PCR has been used to improve the diagnosis of other infections, including port-related bloodstream infections, likely due to its ability to detect slow-growing microbes [71];
- A broader range of microbes have been identified as the putative cause of orthopedic infections due to greater sensitivity and the ability to identify bacteria without having to first 'target' specific suspected pathogens [72].

While PCR-based methods show promise for the improved diagnosis of infections in the clinic, several issues still need to be addressed. Due to the sensitivity of PCR, contamination remains a concern that requires specific controls/multiple samples. There is still some debate

as to whether the DNA from ‘unusual’ organisms detected by PCR is actually from contaminating bacteria, or even from the fluids and instruments used during surgery.

Investigation of the implant & surrounding tissue upon removal

On contemplating a potentially infected implant and its removal, a preoperative diagnosis and the identification of the organism and its method of growth would be ideal, but in many cases, the presence and identification of pathogens can only be resolved after the removal of the implant. During these procedures, surgeons should also collect periprosthetic tissue samples for pathological examination. Upon removal of the prosthetic, its surface can be tested using sensitive and accurate histopathic and microscopic examination in order to determine the presence and structure of adhered bacteria [73], although it is possible that the physical trauma of implant removal may mechanically dissociate the biofilm from the implant.

A technique that has proven useful in increasing the sensitivity of culturing for the detection of pathogens associated with failed orthopedic prostheses is sonication [74,75]. Briefly, the recovered implants are sonicated in a bath sonicator in a container containing buffered saline, and the sonicate is then cultured. In a 331-patient study looking at revised hips and knees, prior sonication increased the sensitivity of culturing for the detection of pathogens from 60.8% from homogenized periprosthetic tissue to 78.5% [74]. It is presumed that the reason for the increased sensitivity was that the bacteria were present as an adherent biofilm, which was more effectively dislodged, but not killed, by sonication. Interestingly, it was noted in the same study that presonation was particularly useful in increasing culture sensitivity from patients who had received antimicrobial therapy within 14 days before surgery. The authors hypothesized that this was due to the fact that “planktonic bacteria present in tissue are more susceptible to anti-infective agents than are biofilm bacteria.” However, our direct examination shows adherent biofilms on periprosthetic tissue as well as orthopedic hardware [23,24]; similar observations have been made for biofilms on periprosthetic tissue associated with other types of indwelling medical devices [76]. An alternative explanation is that the large volume of buffer required to immerse the implant for sonication might more effectively dilute residual antibiotics.

Dislodged biofilm bacteria in explant sonicate have also been detected using PCR and microscopic techniques. Tunney *et al.* used this technique as early as 1999 [77]. They reported that aggregates of bacteria (containing up to 300 cells) were only observed after sonication of the material. Furthermore, they reported that when using PCR targeting bacterial genes, they were able to identify infecting microbes at a higher rate (72%) than either immunofluorescence microscopy (63%) or cultures (22%) of the sonicate. Microscopic staining of the sonicate, although slightly less sensitive for detecting pathogens when compared with PCR, could discern whether bacteria were present in aggregates as biofilms. Culturing and PCR from the sonicate have also been shown to increase sensitivity for detecting pathogens when compared with simply vortexing of the removed prostheses in fluid. More recently, sonication of retrieved implants was combined with multiplex PCR, which enabled the detection of multiple bacterial and fungal pathogens [20]. In another

approach for the detection of pathogens following sonication, microcalorimetry was able to detect infection within 12 h [78].

Another approach is direct microscopic examination of the explanted prosthesis, cement or periprosthetic tissue in order to detect pathogens. This method has the advantage that it is arguably the only method that can directly confirm the presence of biofilms *in situ* going on the assumption that aggregates of bacteria firmly adhered to the surface represent a 'growth in place' process, that is, they were not merely casual contaminants which had attached during surgery and specimen collection but had actually grown on the surfaces over time. Microscopy can also distinguish surface-adhered populations from intracellular pathogens. Confocal microscopy is useful for the examination of periprosthetic tissue since it can penetrate approximately 100–200 μm into the tissue and thus identify invasive as well as superficially adhered pathogens.

This utility of combining microscopic methods with PCR was shown in a 2006 study by Hall-Stoodley *et al.* in which pathogens in biofilms were demonstrated in middle-ear infections [64]. This study successfully used a combination of live dead staining and FISH to determine the presence and viability of bacteria, and also to identify the species of bacteria (using 16S rRNA species specific FISH probes). A similar approach was successfully applied for the identification of pathogens in biofilms in periprosthetic orthopedic infections [23,24] and on implanted suture material in the abdominal wall [79]. A limitation to this technique, however, is that FISH requires significant washing steps that could dislodge possible biofilms. One way of overcoming this issue is that the samples can be cryoembedded in an embedding compound such as paraffin, thin sectioned and mounted on a slide before staining. However, in our experience, looking down directly at the surfaces in plain view provides a better representation of the distribution of bacteria and biofilms than would be found from thin sections. Thus, there is a compromise between embedding to preserve structure and being able to directly scan the exposed surface and the near-surface layers. While implant removal and inspection using nonroutine clinical methods improves the diagnosis of the type of bacterial growth, it also increases the likelihood of *in vitro* contamination due to the extra handling of the sample in a laboratory setting [80]. Moreover, although direct examination of the device components and intraoperative samples using a variety of research techniques improves our academic understanding of the infection from a failure analysis perspective, it is of limited use clinically, since the decision to explant has already been made without the benefit of this information.

Prevention & treatment of periprosthetic infections

Infection prevention

The first line of defense is containment in the OR environment. Measures to protect the sterile field include high efficiency particulate airfiltered air handling, limiting personnel and traffic, the use of spacesuit scrubs, disinfection of the skin and shielding of as much of the patient's skin as possible with surgical drapes. However, despite these methods, it is impossible to completely sterilize the 'sterile field', and it is likely unavoidable that some bacteria will gain access to the incision site. What percentage of such bacteria arrive via the external environment of the OR versus those originating from the patient's own body (e.g.,

the skin or blood) is not known, although nasal carriage of *S. aureus* has been shown to increase the risk of infection following orthopedic surgery [81]. In addition to containing pathogens by environmental controls, prophylactic antibiotics remain an effective method for reducing the likelihood of infection following surgery [82], although there is a narrow window of effectiveness for these antibiotics, with Widmer reporting that if antibiotics are given less than 30 min before incision, they are not beneficial [83].

As a possible means to slow the threat of antibiotic-resistant biofilms on prosthetic joints in patients, researchers are testing novel materials with which to construct prosthetics that will impede bacterial colonization on the artificial surface. For example, cobalt–chromium inhibited bacterial growth to a greater extent than titanium [84], while rough titanium has been reported to increase bacterial colonization when compared with smooth titanium in a rabbit model of orthopedic infection [85]. Part of this effect might be that the roughness increases the surface area and also provides protective depressions for the bacteria, but also rough surfaces increase the attachment of host matrix proteins such as fibronectin, which may in turn explain the increased attachment of bacteria such as staphylococci, which express matrix receptors [86]. It should be noted that while different materials have shown promise *in vitro*, to date, they have not shown efficacy *in vivo* [87].

Treating prosthetic infection

Once a patient develops a prosthetic infection that cannot be cleared by antibiotic therapy, often the only viable recourse is to remove the affected implant. For example, antibiotic administration without device removal was only found to be successful 22–37% of the time when treating catheter-associated bacteremia [88–92]. In cases of PJI, device removal is accomplished in one of two ways.

One stage—The prosthesis is removed and a new device is implanted in one surgery. This technique requires the surrounding tissue to be clear of infection and secondary damage, the microorganism to be susceptible to antibiotics and the patient not exhibiting any severe comorbidities [93–96].

Two stage—A two-stage procedure is used to try to ensure that all pathogens have been cleared before reimplantation because of the high risk of reinfection associated with placing hardware into a space that might contain retained pathogens. The period between stages allows for antibiotic therapy and surveillance before proceeding to the second stage. The implant is removed and intravenous antibiotics are typically administered for 2–8 weeks. A second prosthetic is then implanted after completion of antibiotic treatment [16,17,97–101].

Surgical spacer

During the two-stage surgical procedure (described above), an antibiotic-impregnated cement spacer is usually used to manage the dead space that results after explantation of the prosthesis while also providing localized delivery of antibiotics. The standard of care to date has involved the use of spacers made of poly(methyl methacrylate) (PMMA). Spacers are used to maintain the joint space during a two-stage revision and can also be formed to provide an articulate surface. PMMA is usually made up in the operating room by mixing

powder and liquid components together in order to initiate the polymerization process. At this point, antibiotics can be added so that the cement spacer has an eluting antimicrobial function as well as a mechanical function. In addition to its use as a spacer, antibiotic PMMA beads have also been used to pack into wounds as an antimicrobial delivery system.

Future directions in the prevention & treatment of biofilms in orthopedics

Due to the recognized difficulty in eradicating biofilms once they have formed, the prevention of infection following prosthetic or device implantation continues to be the focus of intense research in orthopedics [102] and other specialties. However, biofilms can also grow on periprosthetic tissue, so it is not only device protection that is important. Ideally, a surface or material would be able to project its antimicrobial effect to adjacent tissues and fluids, as well as to components that are not amenable to coating due to the type of material, size, geometry or manufacturing process. Incorporating conventional antibiotics or antimicrobial agents such as silver to kill cells on contact is one approach, while another is to disrupt the latter stages of biofilm formation by initiating dispersal, interrupting cell signaling or digesting the EPS matrix.

Prosthetic antimicrobial coatings

Biosurfactants—Various groups have begun testing compounds that feature both hydrophilic and hydrophobic moieties (glycolipids, lipopeptides, polysaccharide–protein complexes, phospholipids, fatty acids and neutral lipids) in an attempt to reduce the ability of microbes to bind to the surface of prosthetics (for a review, see [103]). The results thus far have been mixed, with studies suggesting that an initial cohort of microbes is able to adhere to these coated surfaces and subsequently modify the surface by creating a conditioning film that increases the binding of more microbes [104]. In addition, similar to antibiotics that only function against specific bacteria, many coatings only function against certain types of bacteria, prompting the notion of using multiple agents [105].

A recent report found that positively charged biomaterial surfaces were successful in slowing the adhering of Gram-negative but not Gram-positive bacteria [106]. In addition to coating prosthetic surfaces with charged molecules, the use of macromolecules (i.e., heparin and heparin and polypeptides) to form a hydrated layer on the artificial surface has also shown promise in reducing the colonization and formation of biofilms by interfering with the binding of the bacteria to the surface of multiple substrates [107–110].

Antimicrobial metal & metal oxide nanoparticles—Silver has a long history of antimicrobial activity, including medical device coating [111]. It has been reported to kill many different strains of bacteria, but the exact fatal mechanism remains unknown. Silver is known to bind to DNA, RNA and phosphoproteins [111], and it can also interfere with metabolism through the binding of thiol groups [112,113] and by causing the production of reactive oxygen species [114]. Unlike some other coatings that have been tested, silver nanoparticles can kill both Gram-negative and Gram-positive organisms [115]. These nanoparticles proved successful in a study of biofilm prevention on catheters exposed to *E. coli*, *Enterococcus* spp. and *S. aureus* [116]. Nanoparticle silver ion coatings also inhibited biofilm formation on titanium implants in a rabbit model [117]. While silver is currently the

most intensively studied metal in this context, other metals are also being considered for their potential antibiofilm efficacy. Among these are zinc, titanium, iron and copper. Zinc oxide is thought to disrupt the cell wall of both Gram-positive and Gram-negative bacteria, eventually leading to elevated membrane permeability and cell damage [118–120]. However, most work to date has been carried out with zinc oxide against planktonic bacteria; against dental pathogens, zinc oxide showed less of an ability to inhibit biofilms than silver [121,122]. Copper has shown promise as an antibiofilm agent for hospital and public surfaces; however, potential toxicity issues as a nanoparticle may limit its use for indwelling devices [123].

Antibiotic-coated metals—Direct tethering of antibiotics to metal orthopedic implants has promise to kill bacteria when they first come into contact with a prosthesis, and hence halt the progression of biofilm formation. Vancomycin and gentamycin were tethered to 316L-grade stainless steel by self-assembled monolayers and they retained their potency against *S. aureus* over periods of up to 48 h [124]. In addition, titanium implants with vancomycin covalently bound to the surface were found to reduce bacterial colonization and improve bone healing in an ovine model of implant infection [125]. While the reservoir of deliverable antibiotics is limited, using a tethering approach for the protection of the implant surface against colonizing bacteria immediately following implantation might be enough to significantly reduce the risk of infection, although such an effect is likely to be limited to the immediate vicinity of the implant surface.

While coatings offer the advantage of protecting a particular component, there are still issues regarding the longevity of the coating or possible reactions with the underlying material. Laboratory studies assessing surface protection of a coating tend to be of short duration and the influence of these coatings with respect to the long-term effects on wear or particle cytotoxicity remains to be evaluated.

Localized antibiotic delivery

Cements—Both absorbable and nonabsorbable cements can be utilized for drug delivery even though, in many cases, they are used for a different primary function, such as to provide mechanical stability, dead space preservation, stimulation of bone repair or osteoconductivity. Nonbiodegradable acrylic-based polymer cements (e.g., PMMA) and biodegradable mineral-based bone cements (e.g., calcium sulfate and/or phosphate) can be loaded with single or multiple antibiotics. These materials can deliver very high local concentrations of antimicrobial agents to the surgical site, which otherwise could not be achieved systemically due to toxicity. PMMA has the advantage of being mechanically very strong, while absorbable cements can offer better release characteristics, such that therapeutic levels are maintained over longer periods. The addition of antibiotics to commercially available cements and mixing procedures will affect the microstructure of the cement, which in turn influences the mechanical properties and elution characteristics. Both antibiotic-loaded polymer and mineral-based cements are routinely formed into beads specifically for drug delivery and dead space management. Release is generally controlled by diffusion and is difficult to control, and with a nonabsorbable material, there is often a rapid ‘burst’ release of antibiotic over the first few hours to days. One concern is that if all

bacteria in the vicinity are not killed during the initial burst, those remaining will be exposed to subinhibitory concentrations, with a possible consequence of increasing the likelihood of emerging antibiotic resistance. However, despite the fact that the high local concentration of antibiotic can be relatively short lived, eluting cements may reduce the risk of the establishment of a chronic infection in the initial postsurgical period by killing planktonic cells before they can establish a biofilm. Clinical evidence pointing to the optimal parameters required for infection control are difficult to interpret due to the variability in brand of cement, concentration and type of antibiotics, mixing techniques, anatomical location and shape and size parameters of the formed cement; however, the general consensus is that antibiotic-loaded acrylic cement is useful in the treatment and management of chronic orthopedic infections [126,127]. More recently, bioabsorbable materials such as calcium sulfate and calcium phosphate have regained the interest of researchers and clinicians. Calcium sulfate has been used as a bone graft substitute for over 100 years [128], but has more recently been utilized for additional functions in a pelletized form as a local antibiotic delivery mechanism [129], although adverse reactions to the pelletized form have been reported [128]. Synthetic calcium sulfate beads have been recently introduced in the hope of reducing the risk of adverse reaction and have been shown to clear osteomyelitis in a rabbit model [130]. In addition, the elution characteristics can be controlled by bead size and composition [131], and they do not provide a permanent artificial surface for bacterial colonization. While absorbable and nonabsorbable carriers have been found to be reasonably effective in the treatment of osteomyelitis [132], if no long-term mechanical function is required, absorbable carriers have the advantage of not providing a permanent surface for possible biofilm infection and would not require surgical removal in another procedure.

Nonantibiotic biofilm targets

In addition to the targeted killing of individual bacteria, as is performed with conventional antibiotics, our growing understanding of the chemical composition, mechanical properties and biological processes of biofilms has led to the development of strategies to target biofilm-specific components and processes.

Vaccines—In order to circumvent issues with antibiotic resistance, several groups have studied the use of biofilm-specific vaccines in order to help the immune system recognize the infecting microbes in patients. Unfortunately, there are several hurdles to overcome. First, the genetic expression in bacteria shifts as the bacteria aggregate from a planktonic form to a structured biofilm [36]; therefore, vaccines derived in the laboratory against epitopes expressed on the surface of planktonic bacteria may not be successful against biofilm bacteria. Protein-based vaccines targeting adhesive matrix molecules or cell wall proteins have also shown only limited success [133,134]. Another problem is masking, in which biofilm-specific cell surface epitopes might be ‘hidden’ from vaccine surveillance by the EPS. More recently, testing of a multicomponent vaccine has been conducted with better results. This vaccine combined four cell wall and membrane-associated proteins that have been shown to be immunogenic during the maturation of the biofilm and found that a combination of antibiotics and vaccination significantly increased the percentage of mice that could clear a methicillin-resistant *S. aureus* infection (87.5% clearance with vaccination and antibiotics, 55.6% with only antibiotics, 22.2% with only vaccination and

33.3% clearance in controls) [135]. This study suggests that while vaccines may not be sufficient to protect the host, they could function to improve clinical outcomes when coupled with antibiotic therapy.

Another approach is to direct the immune system to attack not the bacterial cells themselves, but components of the biofilm EPS that play a structural role. Goodman *et al.* used antisera against a DNA binding protein, DNABII, which connects strands of eDNA in nontypeable *H. influenzae* biofilms in order to significantly reduce biofilm formation by this organism [32]. The EPS of *S. aureus* biofilms is known to contain eDNA, which plays a structural role [136,137], and it is possible that a similar vaccination against EPS-connecting proteins (if they exist in *S. aureus*) might prevent biofilm formation by interfering with biofilm assembly.

Biofilm-disaggregating agents—Using agents that disrupt the EPS, which holds biofilm bacteria together and attaches them to a surface, is another antibiofilm strategy. An enzyme that has shown promise in this regard is the DNA-digesting enzyme, DNase I. This enzyme has been shown to break down eDNA *in vitro* [138]. Since eDNA is a major component of the EPS [139], this significantly disrupts the biofilm and enhances the efficacy of antimicrobials [140,141]. There are a number of advantages in targeting eDNA; it is common in biofilms formed by many types of pathogens, it is linear and it has only one basic linkage (phosphodiester bonds), which can be cleaved by nucleases.

The other major component of EPS is polysaccharides. However, unlike DNA, there are many types of biofilm polysaccharides produced by different pathogens. In addition, they are often branching, they can be homo- or co-polymers and there are different types of bonds holding the sugar monomers together. Thus, enzymes to digest polysaccharides need to be targeted to a particular species or genus. Staphylococci, which are the most common pathogens of orthopedic concern, produce poly-N-acetylglucosamine. Poly-N-acetylglucosamine can be digested by dispersin B, a glycoside hydrolase, which has been shown to function both independently and in conjunction with antibiotics to eliminate or inhibit biofilm development on polyurethanes and catheters [142,143].

While digestion of the EPS shows promise, current thinking suggests that it should be used in combination with conventional antibiotics, since breaking up biofilms will actually release live bacterial cells into the surrounding environment. Given the complexity of the chemical and physical structure of the EPS, combined with the fact that the host matrix can also be associated with the biofilm, it is likely that multiple components will need to be targeted in order to achieve complete digestion.

QS inhibitors—Because QS is important in the regulation of secreted virulence factors and in biofilm formation, many groups have tried to identify small-molecule inhibitors to disrupt this form of communication within the biofilm [37]. Many Gram-negative biofilm-forming bacteria use the acetylated homoserine lactone-based *las* quorum sensing signaling system, in which the signal molecule, an acetylated homoserine lactone, is secreted into the surrounding space, where it can be taken up by adjacent bacteria to bind with the LasR transcriptional regulator. This in turn can increase the expression of multiple virulence

factors, and also increase biofilm formation. Inhibitors that target the *las* quorum sensing system in *P. aeruginosa* accelerated bacterial clearance and reduced pathology in a mouse model of lung infection [144,145]. Importantly, these inhibitors also increased the survival time of mice against lethal *P. aeruginosa* infections [145]. In addition to receptor antagonists to disrupt the ability of bacteria to communicate through QS, enzymes that can breakdown QS signaling peptides have also been shown to be effective in a *Caenorhabditis elegans* model of infection [146]. Although proof-of-concept trials have been successfully carried out in the laboratory, these agents have yet to be tested in the clinical setting.

Biofilm-dispersal agents—Unlike disaggregation, which breaks apart a biofilm by digestion, dispersal signals are regulated by density-dependent QS, causing natural dispersal of bacteria from biofilms [40]. It is thought that natural dispersal can be induced by the exogenous administration of these signaling molecules and, as is the case with disaggregation, they would need to be used in combination with antibiotics in order to kill any released bacteria. In terms of dispersal, the two signals that show the greatest promise are nanomolar nitric oxide [39] and *cis*-2-decanoic acid, a fatty acid messenger [40]. Current research suggests that nitric oxide may be more effective at dispersing motile strains of bacteria and thus may be useful against a number of Gram-negative orthopedic pathogens, but it is not clear whether it would have activity against staphylococci or streptococci. There are also issues with the delivery of nitric oxide to deep tissues due to the fact that it is highly labile, and potential cytotoxicity is also a concern. *Cis*-2-decanoic acid has been shown to reduce biofilm formation by a number of pathogens, including *S. aureus* [147], and in combination with antibiotics, it has been shown to completely prevent biofilm formation. It is likely that delivery would have to be through local release from a carrier.

Attacking the persister/dormant state—Another potential biofilm target is the dormant or persister population within biofilms, which are thought to be responsible for the high levels of antibiotic tolerance. One possibility is adding factors to actually stimulate biofilm bacterial metabolism, such that metabolically active bacteria are rendered vulnerable to otherwise-ineffective antibiotics. It has been shown that a methicillin-sensitive strain of *S. aureus* was highly tolerant of oxacillin as a biofilm, and when the biofilm was dispersed in spent media or buffer, the bacteria were still tolerant [48]. However, in the presence of nutrients (fresh media) and antibiotics, the cells became sensitive again. In order to be used therapeutically, growth factors would likely need to be added in combination with a dispersal agent and an antibiotic, but the concept of activating dormant biofilm bacteria obviously has potential to worsen the infection.

A novel alternative approach is to attack the persister cells while in the dormant state. It has recently been shown that the cytoplasmic protease ClpP in *S. aureus* can be activated by the antibiotic acyldepsipeptide (ADEP4) in order to kill persister cells by causing them to digest themselves from within. In combination with rifampicin, ADEP4 was active against *in vitro* biofilms and was able to eradicate infection from a mouse thigh, even in the presence of a foreign body [148]. While these results are very promising, it was noted that not all cells have the ClpP protein, and the spread of ADEP4-resistant strains in the population was

rapid. It remains to be seen whether this approach has potential for the treatment of biofilm infections associated with orthopedic and other implants.

Conclusion & future perspective

Antimicrobial control of the OR environment due to the pioneering work of people like Joseph Lister and John Charney has dramatically reduced the rates of surgical site infection from the late-19th century to the current day. However, further significant reductions have remained elusive. The difficulty in the prevention and treatment of biofilms is a major obstacle in further reducing infection rates and the devastating impacts of infection. The ability of bacteria to overcome antibiotic therapy through the genetic acquisition of resistance, as well as from the phenotypic and barrier effects of biofilm formation, has led to diminishing returns in infection control using conventional strategies. As with any infection, prevention is the preferred control option, but when there is a risk of biofilm formation, prevention becomes even more critical. While the widespread overuse of antibiotics has led to selection for resistant strains, in implantation cases, where there is even a moderate risk of biofilm infection, the prophylactic application of high concentrations of antibiotics (and possibly combinations of antibiotics with multiple mechanisms of action) delivered directly to the surgical site in order to eradicate the pathogens before they can establish a biofilm could be used more aggressively. Optimizing release kinetics using carriers that are already used in orthopedic surgery, such as synthetic bone grafts and cements, is arguably the strategy that is closest to clinical deployment, and it is hoped that research in this area will translate to the clinic in the next 5 years.

Concurrently, improvements must continue to be made in the ability to diagnose and treat infections. In terms of diagnosis, PCR techniques are raising important – and difficult – questions as to what actually constitutes an infection and when and how to treat when a high number of ‘aseptic loosening’ cases appear to carry periprosthetic pathogens. Growing frustration with the ability of conventional clinical diagnostics to provide useful guidance on how to manage these difficult cases will push research beyond that of developing culture-free pathogen identification techniques to clinical studies correlating long-term outcomes with PCR-based results in order to provide a grounding for more useful clinical interpretations. As these data become available and results begin to emerge from the use of approved PCR-based techniques in other parts of the world, the US FDA will have clearer guidance in terms of the approval process for PCR-based diagnostic tests for orthopedic infections. It seems likely that PCR-based diagnostic platforms will be commonplace in larger clinical microbiology laboratories within the next 5–10 years. PCR (and other molecular diagnostic platforms) will not replace culturing (which is still essential for a providing a complete antibiogram or allowing the study of the pathogen in the laboratory), but will be used to provide rapid information that can be used for initial decision-making. Conventional culture techniques will be enhanced as orthopedic surgeons put growing pressure on clinical microbiology laboratories to perform cultures over longer periods in order to detect slower-growing strains, as well as for the more frequent use of anaerobic culturing. Diagnosis through sonication culturing or microscopic analysis is a useful intermediate step in helping to interpret PCR and conventional clinical culture results, but it is difficult to envisage that these tests will be performed routinely, since they are labor

intensive and costly. Medical imaging of biofilms is another area of great potential for clinical translation, but development is needed for biofilm-specific staining. Labeled antibodies have the potential for mapping periprosthetic biofilms (Figure 4). Imaging and rapid diagnostics of tissues will make it possible to better identify the infected margins intraoperatively for more effective debridement. Biofilm infections may come to be seen more like cancers, in that if aggressive steps are not taken to completely remove the biofilm nidus, the infection has a high likelihood of recurring.

Finally, with respect to the treatment of mature biofilms, while there are many exciting strategies being developed in the research environment, only a few of these have been assessed for cytotoxicity and progressed to animal trials. Those that have tend to be in rodent models and are assessed relatively short-term studies. It is unlikely that vaccinations will come to the market within a 10-year time frame. However, agents already in medical use for other indications, such as DNase I (the active ingredient of Pulmozyme® (Genentech Inc., CA, USA), a synthetic DNase I supplied as 1 mg ml⁻¹ solution, which is used to reduce the viscosity of sputum in cystic fibrosis patients by targeting the break-up of human DNA) and gaseous nitric oxide (used as a vasodilator), which have been shown to act as biofilm-dispersing agents, may make a more rapid clinical translation for the treatment of biofilm infections. While biofilms continue to reveal themselves as formidable foes, there has never been so much research directed towards their prevention, diagnosis and treatment. Currently, academic research has given little in the way of providing orthopedic surgeons with specific weapons to help in the management of biofilm infections. There is utility through knowledge, however, and the biofilm paradigm does provide the surgeon (and patient) with a science-based understanding of why an infection might be so difficult to manage. We expect that in due time, biofilm-specific therapies will begin to trickle from the bench to the clinic and enable a meaningful reduction in biofilm infections in orthopedics and other medical specialties.

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Executive Summary

Periprosthetic infection in total joint arthroplasties & fixation devices

- The number of arthroplasties and implanted fixation devices continues to rise, and while infection rates may decline, the absolute number of infections is expected to increase.
- These infections are now recognized to largely derive from bacterial biofilms residing on and around the implanted prostheses.
- Adequate prevention, diagnosis and treatment strategies for biofilm-based prosthetic joint infections (PJIs) and, more generally, periprosthetic infections are still lacking.

Formation of biofilms

- Planktonic bacteria attach to implanted prostheses as well as periprosthetic tissue.
- The bacteria produce an extracellular polymeric slime (EPS) matrix that holds the biofilm together and to the surface.
- Chemical gradients in the biofilm cause the generation of highly localized regions that facilitate the bacteria within to adopt specialized phenotypes, such as resistant slow-growing or dormant persister states.
- The close proximity of cells within the biofilm enables cell signaling communication mechanisms to coordinate behavior.
- Biofilms provide a reservoir from which bacteria can periodically disperse into surrounding tissues and joint fluid.
- The EPS limits the ability of antibodies and phagocytic cells to penetrate the biofilm.

Clinical complications due to bacterial growth in a biofilm

- Biofilm PJIs are often subclinical and culture negative, confounding diagnosis and treatment.
- The infection may only fully declare itself when the released cells revert to the rapidly growing planktonic phenotype and cause acute-on-chronic exacerbations of symptoms.
- Biofilm bacteria are more highly resistant to antibiotics than when rapidly growing in the planktonic phenotype, making prevention or early treatment paramount.
- Direct microscopic examination and indirect techniques (i.e., culturing following hardware sonication) support the hypothesis that biofilm formation is a major cause of PJIs.

- Guidelines for immunological, microbiological and clinical findings have been drawn up in order to construct a diagnostic decision tree for addressing issues regarding culture ambiguity.

Future directions in the prevention & treatment of biofilms in orthopedics

- Surface-tethered antibiotics and metal oxide nanoparticle prosthetic coatings show potential for the prevention of biofilm formation.
- Local antibiotic delivery from absorbable bone cements provides good release kinetics with no permanent surface for biofilm formation.
- Vaccines targeting biofilm-specific components, such as the EPS, have shown promise in laboratory studies.
- Dispersal agents as antibiotic adjuvants that attack the EPS matrix, breaking up biofilms and releasing bacterial cells, have shown promise in *in vitro* and animal studies.
- Quorum sensing inhibitors impede the ability of bacteria to communicate with each other, thereby interrupting biofilm development.
- Attacking the biofilm persister/dormant state by activating proteases inside dormant cells causes them to 'self-digest'.

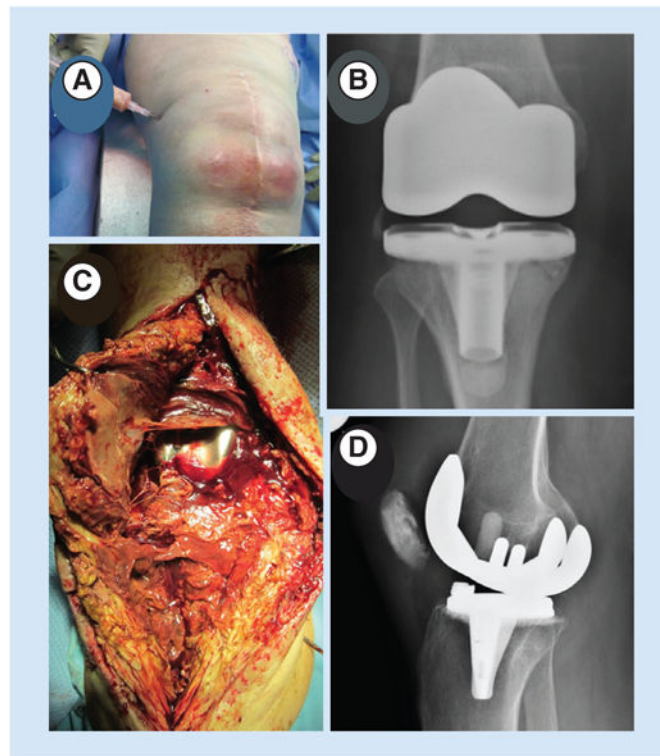


Figure 1. Diagnosis of infection can be challenging

(A) In this patient with a hot knee, diagnosis was straightforward from clinical signs and symptoms and a positive culture from aspirate. (B) Radiograph from a total knee arthroplasty patient for whom diagnosis was more difficult. The aspirate was culture negative and the radiograph revealed no indications of infection. (C) Intraoperative image from (B) showing purulence and slime in the joint space and an intraoperative positive culture. (D) Radiograph from a patient with only subtle signs of a TKA infection, a culture-negative aspirate, a normal erythrocyte sedimentation rate and slightly elevated C-reactive protein, but over 20,000 white blood cells with more than 90% polymorphonuclear leukocytes. Intraoperative cultures showed coagulase-negative *Staphylococcus*.

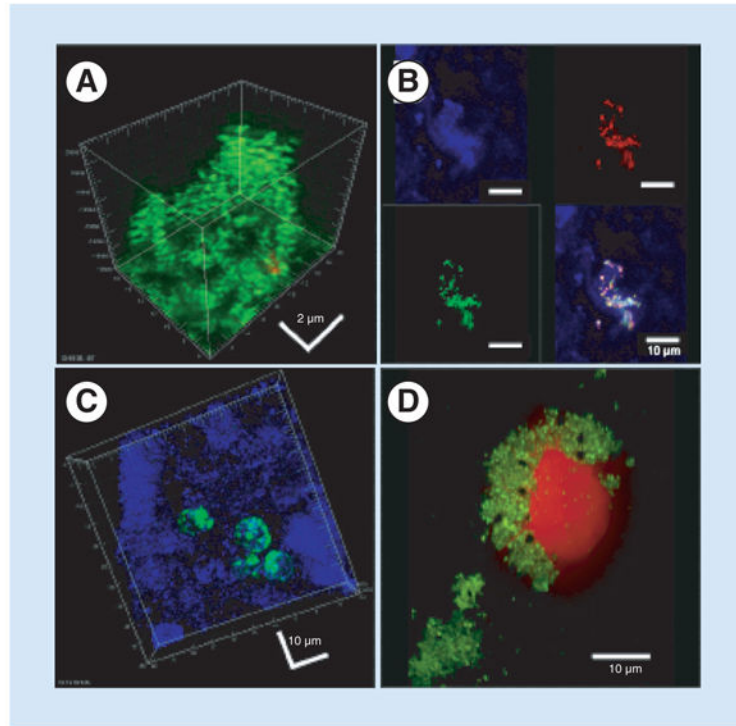


Figure 2. Various configurations of bacteria and biofilms in three different orthopedic patients
(A) Biofilm of live cocci (green) attached to a screw removed from a fixation device in a nonunion. The biofilm demonstrated classic 3D structure. **(B)** Patch of biofilm attached to periprosthetic tissue from a failed ankle arthroplasty. The upper left panel shows reflected light demonstrating the surface of the tissue (blue). The upper right panel shows a FISH ‘sau’ probe demonstrating *Staphylococcus aureus* bacteria (red). The lower left panel shows a FISH ‘Eub’ probe demonstrating all stained bacteria (green). The lower right panel shows an overlay demonstrating the *S. aureus* biofilm cluster attached to the tissue. **(C)** Periprosthetic tissue from the same patient as **(B)**, showing bacteria that appear to be intracellular. **(D)** Intraoperative fluid from a patient with a failed elbow showing clumps of live cocci (green). The large red object is a nucleolus from a host cell that appears to have been ‘attacked’ and damaged by the cocci.

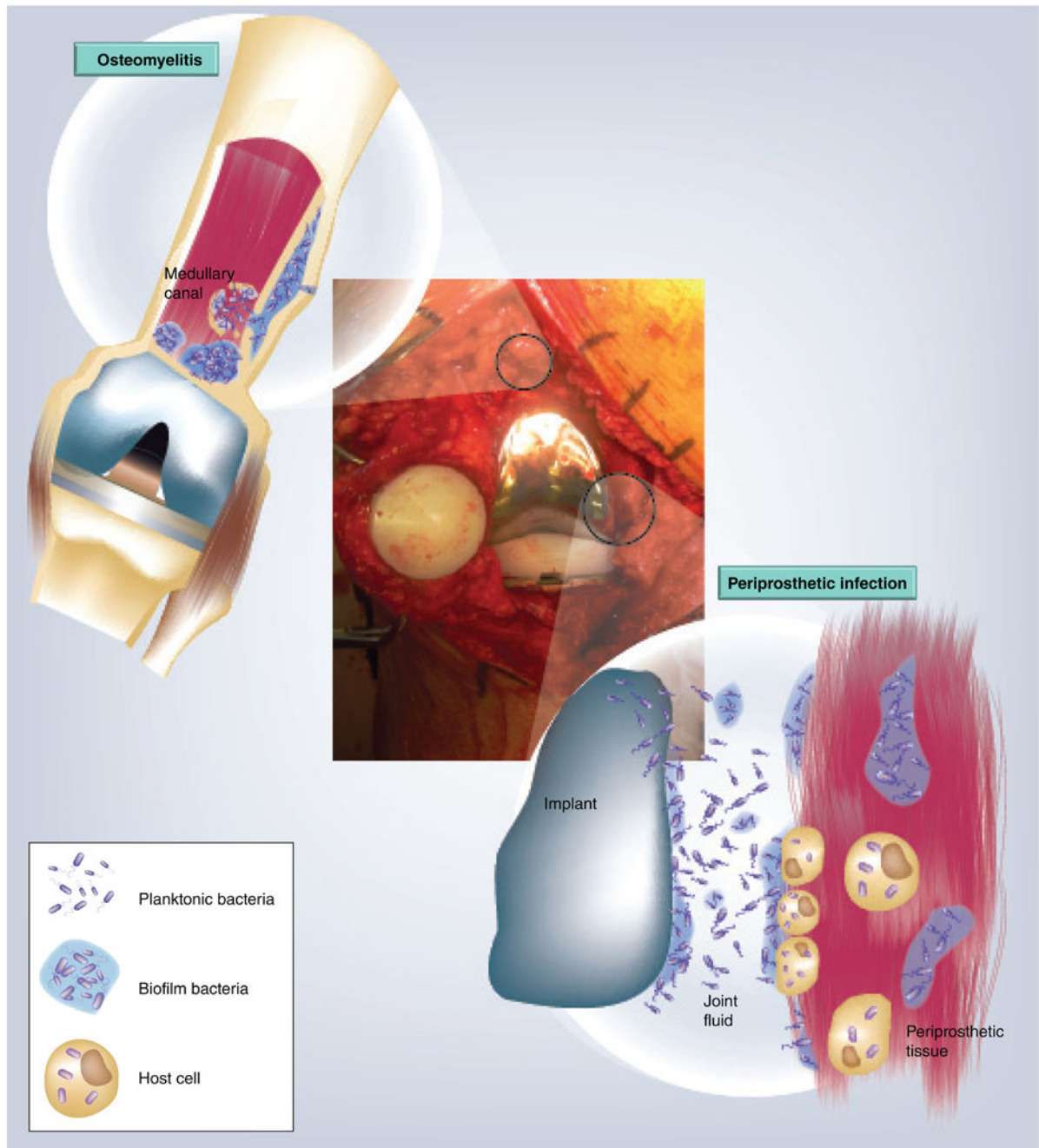


Figure 3. How bacteria and biofilms might be distributed in a periprosthetic joint infection using a knee as an example

Biofilms can grow on the prostheses components, the surrounding tissue and the fibrous sheath. Each of these can be considered separate, but communicating niches. The joint fluid might contain planktonic cells or clumps of detached biofilm. The periprosthetic tissue can be invaded by the infecting bacteria. There can be migration of bacteria between each of these locations (prosthesis, surface tissue and matrix, subsurface tissue and fluid), which could contribute to survival. If bacteria in one niche are eradicated by debridement or antibiotic therapy, there is potential for the repopulation of pathogens from the other niches.

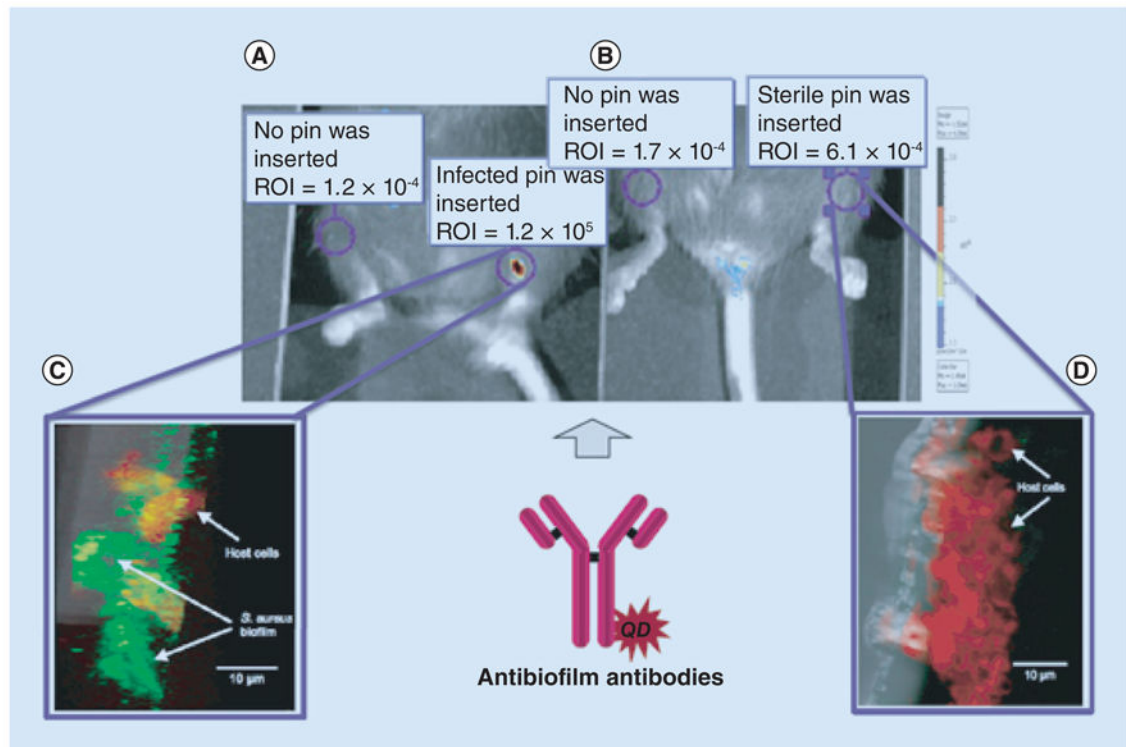


Figure 4. Imaging periprosthetic biofilms using quantum dot-labeled antibiofilm antibodies

The antibodies were injected via the intravenous route and allowed to localize in mice with (A) *Staphylococcus aureus*-infected or (B) sterile pins implanted into the left tibia. Live imaging was performed using an IVIS[®] Lumina Series III imager (PerkinElmer, MA, USA). (C) In order to confirm the results of the live imaging, the pins were extracted and probed with fluorescein isothiocyanate-labeled universal bacterial probe (green) and a rhodamine-labeled universal eukaryotic cell probe (red) that were specific for bacterial and eukaryotic rRNAs, showing well-developed biofilms. (D) No biofilms were evident in the sterile pins. The relative brightness which was proportional to the amount of biofilm is indicated in the ROI.

ROI: Region of interest.