PART XI

BIOFILM

Section 1: Formation

Section 2: Disruption

Formation

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QUESTION 1: What is the life cycle of biofilm and the mechanism of its maturation?

RESPONSE: A biofilm may be defined as a microbe-derived sessile community characterized by organisms that are attached to a substratum, interface or each other are embedded in a matrix of extracellular polymeric substance and exhibit an altered phenotype with respect to growth, gene expression and protein production. The biofilm infection life cycle generally follows the steps of attachment (interaction between bacteria and the implant), accumulation (interactions between bacterial cells), maturation (formation of a viable 3D structure) and dispersion/detachment (release from the biofilm). The life cycle of biofilm is variable depending on the organism involved. There are characteristics in the life cycle of biofilm formation. These include attachment, proliferation/accumulation/maturation and dispersal. Biofilm can either be found as adherent to a surface or as floating aggregates.

LEVEL OF EVIDENCE: Strong (this is a scientific review)

DELEGATE VOTE: Agree: 100%, Disagree: 0%, Abstain: 0% (Unanimous, Strongest Consensus)

PRE-MEETING RATIONALE

To answer this question the authors searched Pubmed and Google Scholar between January 1950 – August 2018. Search words included: biofilms, biofilm formation, bioflm life cycle, staphylococci biofilms, Gram positive organisms, pseudomonas aeruginosa biofilms, antibiotic resistance and prosthetic joint infections (PJIs). Relevant papers based on the above search words were reviewed.

Most studies found were animal studies, laboratory studies, in vivo studies and a few clinical studies. Due to time constraints, complete systematic review of the literature could not be performed.

A biofilm may be defined as a microbe-derived sessile community characterized by cells that are attached to a substratum, interface or each other are embedded in a matrix of extracellular polymeric substance and exhibit an altered phenotype with respect to growth, gene expression and protein production [1]. Biofilm thickness can vary between a single cell layer to a thick community of cells embedded within a polymeric matrix. Recent structural analyses have demonstrated that these biofilms possess a sophisticated architecture in which microcolonies can exist in discrete pillar or mushroom-shaped structures [2]. Between these structures, an intricate channel network provides access to environmental nutrients.

PJI can be initiated through hematogenous spread or by direct seeding via an overlying infection, penetrating trauma or contamination during surgical implantation of the prosthesis. Regardless of the seeding source or microbial species, the stepwise progression of the infection is dependent upon biofilm formation and maturation.

The biofilm infection life cycle generally follows the same steps of attachment (interaction between bacteria and the implant), accumulation (interactions between bacterial cells), maturation (formation of a viable 3D structure) and dispersion/detachment (release from the biofilm). This progression is mediated by the interplay of a number of microbial, host and environmental factors, and these are usually different in varying microbial species or even strains within species. A rapid stage progression can be seen with virulent, biofilm-forming pathogens in a susceptible host (e.g., a virulent *Staphylococcus aureus* (*S. aureus*) strain in a host with immunosuppression). In contrast, an infecting microbe with slow growth and low virulence (e.g., *Cutibacterium acnes* – formerly *Propionibacterium acnes*) in a healthy host capable of suppressing biofilm formation can produce an indolent infection with delayed progression.

By adopting this sessile mode of life, biofilm-embedded microbes enjoy a number of advantages over their planktonic counterparts. One advantage is the ability of the polymeric matrix to capture and concentrate a number of environmental nutrients, such as carbon, nitrogen and phosphate [3]. Another advantage to the biofilm mode of growth is it enables resistance to a number of removal strategies, such as antimicrobial and antifouling agent removal, shear stress, host phagocytic clearance and host oxygen radical and protease defenses. This inherent resistance to antimicrobial factors is mediated in part through very low metabolic levels and drastically down-regulated rates of cell division (e.g., small colony variants) of the deeply embedded microbes [4]. While low metabolic rates may explain a great deal of the antimicrobial resistance properties of biofilms, other factors may play a role as well. One such factor may be the ability of biofilms to act as a diffusion barrier to slow down the penetration of some antimicrobial agents [5]. For example, reactive oxidative species may be deactivated in the outer layers of the biofilm, faster than they can diffuse into the lower layers [6].

The last advantage of the biofilm mode of growth is the potential for dispersion via detachment. As mentioned, micro-colonies can exist in discrete, mushroom-shaped structures. These microcolonies may detach under the direction of mechanical fluid shear or through a genetically programmed response that mediates the detachment process [7]. Under the direction of fluid flow, this microcolony travels to other regions of the host to attach and promote biofilm formation on virgin areas. Therefore, this advantage allows a persistent bacterial source population that is resistant to antimicrobial agents and host immune clearance, while at the same time enabling continuous shedding to promote bacterial spread.

S. aureus Biofilm Formation

Although many bacterial pathogens are capable of forming biofilms in a range of clinical contexts, *S. aureus* is the main etiological agent associated with PJI.

The initial phase of biofilm formation is characterized by the attachment of planktonic cells to a surface. In a planktonic mode of growth, *S. aureus* up-regulates the expression of key mediators for immunoavoidance (e.g., Protein A) and the attachment to biotic surfaces. These mediators are a variety of proteins anchored in the

cell wall, the largest group of which are termed microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) [8]. Binding of MSCRAMMs to host components such as fibronectin, fibrinogen, collagen and cytokeratin are an important first step in the attachment of *S. aureus* to initiate biofilm formation [9]. Attachment to abiotic surfaces is also determined by properties and physicochemical characteristics of the abiotic surface as well as the bacterial surface, with hydrophobic and electrostatic interactions playing a major role [10].

However, it is worth noting that many abiotic surfaces, as is the case with many implanted medical devices, are rapidly coated in host matrix components upon implantation. Therefore, surfaces that have been engineered to be "biofilm-resistant" have failed in vivo since *S. aureus* mediates attachment to these conditioned surfaces [11]. The presence of a devitalized surface coated with host extracellular matrix proteins decreases the infectious dose required to cause infection to less than 100 viable *S. aureus* cells, thereby increasing the ability of *S. aureus* to cause biofilm infections by over 75,000 fold [12].

Following this initial attachment, bacteria proliferate and produce an extracellular matrix (ECM), often referred to as slime or glycocalyx, comprised of proteins (both host derived and bacterial), carbohydrates and extracellular DNA (eDNA). These serve as a scaffold for maturation and 3D structuring of the biofilm [11]. Ultimately, through coordinated degradation of ECM via proteases, nucleases, delta hemolysin and other factors (e.g., phenol soluble modulins), bacterial cells are released from the biofilm with the potential to seed secondary sites of infection [13]. Below is a brief discussion of the factors and mechanisms responsible for these stages of the *S. aureus* biofilm life cycle.

The next phase of biofilm formation entails the proliferation and accumulation of attached bacterial cells. During this early phase, intercellular attachment plays a key role in stabilizing the early biofilm before a significant amount of ECM can be produced to protect the attached cells from disruptive forces such as shear force [11]. One key contributor to intercellular adhesion is the polysaccharide intercellular adhesin (PIA), first studied in *Staphylococcus epidermidis* [14]. The MSCRAMMs (discussed above) and certain cytoplasmic proteins shown to bind to eDNA are also know to contribute [15–17]. Together, these factors not only play a role in early intercellular adhesion but also constitute major components of the ECM produced by biofilm-associated cells.

Recent studies utilizing technology allowing for nearly real-time evaluation of biofilm progression have suggested the addition of a stage of biofilm development following proliferation/accumulation referred to as an "exodus" phase [18]. This exodus phase is characterized by an early dispersal event with a reduction in total biomass from a biofilm. This is reportedly achieved through the coordinated bacterial expression of secreted nucleases by a subpopulation of bacterial cells resulting in degradation of eDNA and subsequent bacterial release [18]. The purpose of this phase and its necessity for the overall progression of the biofilm life cycle remain to be determined. However, given the timing of these observations within the overall progression of biofilm formation, it has been suggested that a dynamic shift occurs in which early events are largely protein-mediated and subsequent events are mediated by both protein and eDNA [11]. Although some literature would suggest that certain biofilms tend to be exclusively dependent upon PIA, protein or eDNA, these studies propose a more dynamic model of development with temporal and spatial changes in ECM components [11].

The maturation phase of the biofilm life cycle entails the 3D structuring of biofilms into classic architectural structures (towers and mushroom-like structures) and the development of microcolonies displaying some degree of phenotypic diversity [10,11]. This complex structuring is coordinated through the balance of adhesive and disruptive factors [10]. Adhesive factors include the ECM components discussed above such as PIA, proteins and eDNA. Disruptive factors include enzymes that degrade these components such as proteases and nucleases, as well as the surfactant-like molecules, phenol-soluble modulins (PSMs). These disruptive factors allow for the remodeling and maturation of biofilm structures. For example, studies have demonstrated that channels are created throughout a biofilm via the surfactant-like activity of PSMs, allowing nutrients to reach deeper layers of the biofilm [19]. Therefore, these studies describe biofilm maturation as a subtractive process. Alternatively, some studies suggest an additive process of maturation from observations of microcolonies emerging from slower growing basal layers of biofilms [20]. It is likely that both additive and subtractive processes contribute to the complex structuring observed during biofilm maturation.

The final step of the biofilm life cycle involves the dispersal of cells with the ability to travel to distal sites to disseminate infection. The mechanism by which *S. aureus* regulates this step is largely mediated by the accessory gene regulator (*agr*) quorum-sensing system [19,21]. The *agr* system responds to cell density through the accumulation of signal molecules, allowing for dispersal to occur once a threshold density is reached [22]. The *agr*-regulated factors that have been proposed to mediate dispersal include secreted proteases and resultant degradation of protein components of ECM [23]. Dispersal has also been proposed to be mediated by the *agr*-mediated production of PSMs, which act by disrupting molecular interactions within biofilms [19].

In addition to these staphylococcal factors responsible for PJI development, the complicit nature of the host towards biofilm formation also plays a role. In an early *S. aureus* biofilm infection, the intense inflammatory response is produced by the host. *S. aureus* is readily able to resist clearance from the host through a large number of virulence factors that specifically attack the host and promote immunoavoidance. The expression of *S. aureus* virulence factors, timed by the quorum sensing system, promotes the host to release T_H1 cytokines, including interleukin (IL)-12, interfenn gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), and IL-17 resulting in a shift of the adaptive immune system to an ineffective T_H17 and T_H1 cell-mediated immune response. This type of response is incapable of clearing a biofilm infection, thereby enabling *S. aureus* to form a fully mature biofilm and a persistent infection. The other branch of adaptive immunity, the T_H2 antibody-mediated response, is readily effective at clearing the infection in the early phase of biofilm formation before it progresses to a fully mature phenotype. However, this antibody-mediated response is shut down both by the host cytokines associated with the initial response to *S. aureus*, most notably IFN-γ, and by the *S. aureus* production of superantigens, capsule and other toxins. Additionally, *S. aureus* produces a number of highly immunogenic decoy antigens (e.g., lipase) that augments the ability of *S. aureus* to cause disease and reduces antibody production against more vital antigens [24]. By the time the antibody-mediated immune system recovers and mounts an effective response against the biofilm infection, the fully mature biofilm is able to resist clearance. Even if cleared through surgical intervention and infection resolution, this host immune response manipulation and variable antigen expression allows *S. aureus* to re-infect patients throughout their lifetime.

Once in this fully mature phase, the infection can remain quiescent for years or even decades, or more typically, will show remarkable signs of chronic inflammation [25]. This host response is often due to the metastasis of metabolically active and virulent planktonic subpopulations that have dispersed/detached from the localized biofilm aggregate. Antibiotic therapy is effective against these

active populations allowing for temporary suppression of clinical signs and symptoms of the underlying biofilm disease. However, upon antibiotic treatment cessation, exacerbation of the disease will necessarily result.

Biofilms Formed by Other Microbial Species

In addition to *S. aureus*, a number of other microbial species are able to form infectious biofilms in PJIs [26]. These include other facultative anaerobic, gram-positive, non-motile bacterial species, including coagulase negative staphylococci and *Streptococcus* and *Enterococcus* species. The stages of biofilm formation are similar, and these microbes use a number of homologs to the biofilm-associated virulence factors already described for *S. aureus*. Species other than these gram-positive microbes contribute towards PJI, particularly the facultative anaerobic gram-negative bacilli, including *Escherichia coli* and *Pseudomonas aeruginosa* and anaerobes to a lesser extent.

Gram-negative bacterial biofilms, especially *P. aeruginosa,* have long been studied in the biofilm research field due to their ubiquitous nature in the environment and disease, and their preponderance in chronic wounds and cystic fibrosis lung infections. Although the stages progress through early attachment, mature attachment, accumulation, maturation and dispersion/detachment, the mechanisms by which these steps are accomplished show important differences to gram-positive pathogens.

The motility provided through flagella allows *P. aeruginosa* to facilitate close association with surfaces, such as those in indwelling medical devices. The microbial cells will then proceed to irreversible attachment. Additionally, Type IV pili provide for differential virulence factor production associated with shear stress as well as allow subpopulations to migrate on the surface through twitching motility. As the biofilm accumulates, the formation of complex multicellular structures occurs that demonstrate heterogeneity of nutrients, pH and oxygenation. During maturation, the development of membrane blebs, nanofilaments, eDNA structural support and electrical coupling of the embedded bacterial cells also occurs. As the population swells, the homoserine lactone quorum sensing system induces the production of the surfactant and anti-leukocyte pseudomonal rhamnolipids to prevent clearance and add to the burgeoning inflammatory response. The microbes can then either disperse as single-celled planktonic populations or detach from the biofilm in large conglomerated flocs that allow for metastasis of the infection while enjoying the protective environment of the biofilm matrix.

Clinical Relevance: Treatment and Resolution

During the early acute stage of infection and inflammation, the biofilm is in an early accumulation phase. During this phase, the growing biofilm demonstrates higher susceptibility to antimicrobial therapy than the fully mature, quiescent and metabolically inactive biofilm phenotype. This increased susceptibility to antimicrobial therapy during the acute phase of PJIs translated into efficacious treatment without surgical intervention [28]. When effective combination antimicrobial therapy was used alone to treat PJIs with clinical signs of less than one month in duration, over 83% of patients were successfully treated without surgical intervention. However, once symptoms lasted for greater than six months, successful treatment of antibiotic therapy fell to just over 30%. Therefore, the potential for effective therapy of PJIs without surgical intervention may be a possibility if the infection is diagnosed early and targeted antibiotic therapy is quickly initiated with emphasis on adding Rifampin/Rifampicin when a *Staphylococcus* spp is the etiological agent. After this early therapeutic window, proper surgical debridement along with combination antibiotic therapy is necessary for optimal infection resolution.

Clinical Relevance: Diagnosis

Rapid, effective and sensitive discovery and identification and antibiotic sensitivity determination of the pathogenic bacterial species must be accomplished in order to effectively combat PJIs. Once identified, effective therapeutic counter-measures and treatment can be applied. Currently, pathogen identification requires microbial culture followed by diagnostic analyses that normally require additional rounds of replication in culture or purification of specific bacterial/fungal products. At best, microbial identification may require days to weeks, depending on the growth rate of a specific pathogen. These limitations of bacteria are dramatically exacerbated in diagnosing and speciation of the etiological agent in PJIs. Culture from tissue samples can be effective during the early stages of infection when the biofilm is in an accumulation phase and planktonic populations are present. However, all too often, patients have received antimicrobial therapy prior to proper tissue sampling, thereby eliminating the easily detected planktonic populations, leaving behind only small microbial aggregates that are often missed during biopsy. Also, as the biofilm matures, the host immune response walls off the infectious nidus to form these same hard-to-detect biofilm aggregates.

In conclusion, understanding the progression of biofilm life cycles and the mechanisms that pathogens use to regulate this progression is essential for the development of therapeutic approaches aimed at preventing, disrupting and eradicating biofilm-associated infections.

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QUESTION 2: What surface properties favor biofilm formation?

RESPONSE: The attachment of bacteria to implant and biological surfaces is a complex process, starting with the initial conditioning film. Roughness, hydrophobicity/hydrophilicity, porosity, pore topology and other surface conditions are the key factors for microbial adhesion. Because of the huge variety of these factors, most of the studies directed at bacterial attachment to the implant surface were limited to specific surface conditions since it is difficult to examine the plethora of parameters concomitantly. There are variable conclusions among the available basic science and animal studies relevant to this topic, many of which will be described in greater detail below. Bacteria can form biofilm on almost all prosthetic surfaces and biological surfaces. To date, this consensus group knows of no surface that is inimicable to the growth of biofilm in vivo.

LEVEL OF EVIDENCE: Strong

DELEGATE VOTE: Agree: 100%, Disagree: 0%, Abstain: 0% (Unanimous, Strongest Consensus)

PRE-MEETING RATIONALE

Bacterial biofilms are widely known to contribute to the etiology of chronic infections and implant-associated infections. Biofilm development commences upon formation of a conditioning layer conducive to bacterial attachment, the attachment itself and secretion of a slime-like substance [1]. It is this secretion that enables biofilm formation and ultimately introduces antibiotic resistance and resistance to the host immune system. Several surface properties have been identified that can influence biofilm formation, these include: surface chemistry and functional groups, surface free energy and level of hydrophilicity/hydrophobicity, surface charge, micro- and nano-topography and porosity. Surface chemical composition, micro-roughness and surface free energy would appear to prevail for importance [2].

There is strong evidence that the initial attachment of bacterial species to the surface of a biomaterial is influenced by the presence of adsorbed proteins [1,3]. Wagner et al. [1] found that titanium surfaces preconditioned through exposure to blood plasma enhanced bacterial adhesion for both *Pseudomonas aeruginosa* and *Staphylococcus aureus (S. aureus)*. Likewise, a study performed by Frade et al. presented similar findings with respect to surface adhesion of *Candida albicans* (*C. albicans*) and subsequent biofilm formation on multiple surfaces after serum coating, including polycarbonate, polystyrene, stainless steel, Teflon, polyvinyl chloride and hydroxyapatite [3].

Similarly, there is also strong evidence supporting the conclusion that bacterial adherence and biofilm formation increase with the roughness of the implant surface [4,5]. A study conducted by Karygianni et al. found that *Enterococcus faecalis, S. aureus,* and *C. albicans* adhered more to a rougher implant surface relative to a smoother surface [5]. Furthermore, Braem et al. demonstrated that a porous surface coating was more susceptible to biofilm formation than a smoother titanium-based surface after exposure to *S. aureus* and Staphylococcus epidermidis *Staphylococcus epidermidis* (*S. epidermidis)* [4].

A small number of studies have also examined the impact of the hydrophobicity/ hydrophilicity of implant materials on subsequent biofilm formation [2,3,6]. For example, a study performed by Koseki et al. using *S. epidermidis* showed decreased biofilm formation on cobalt-chromium-molybdenum alloy (Co-Cr-Mo) which was attributed to its increased hydrophobicity [2]. However, two other studies showed contrary results. For instance, *C. albicans* was shown to have less metabolic activity on polycarbonate and stainless steel (hydrophilic surfaces) relative to Teflon (hydrophobic surfaces) [3]. Similarly, some studies contend that hydrophilicity has only trace impact on biofilm formation, as shown by the fact that *S. epidermidis* biofilm formation was not significantly altered by differences in surface wettability [6]. With that, findings remain inconclusive as a whole concerning the impact of implant surface hydrophilicity/hydrophobicity on biofilm formation.

Finally, there are various surface properties that are given moderate recommendations here due to their high-quality evidence but low replication in the studies presented. The first is that surface

nanostructures, such as projections and recesses, reduce overall bacterial adhesion and biofilm formation compared to smooth surfaces [7]. The second is that low nanostructure stiffness inhibits biofilm accumulation, likely due to the susceptibility of these nanostructures to shear forces [8]. The third is that calcium-incorporated oxide coatings on a titanium surface reduces bacterial colonization when compared to non-calcium modified titanium. This is due to calcium drastically decreasing the contact angle [4].

Although there is little consensus in terms of which surface properties are most definitive in contributing to biofilm formation, there are certainly strides in examining the general impact of different properties when considered individually. Due to the complexity of biomaterial properties inherent to orthopaedic implant structure—and the lack of agreement among the literature concluding the impact of these properties—we conclude that biofilm formation is favored by combinations of surface parameters, and so should be assessed as such in the development of biofilm resistant implants. Furthermore, there are few studies examining the impact of surface properties in biofilm formation among human subjects postoperatively and further clinical studies are necessitated.

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QUESTION 3: Is the biofilm on orthopaedic implant surfaces permeable to neutrophils and macrophages in vivo? Are these innate immune cells (meaning any macrophages or neutrophils) capable of engulfing and killing bacteria?

RESPONSE: A mature bacterial biofilm has limited permeability to neutrophils and macrophages. Those that get through are clinically ineffective at eradicating biofilm bacteria. While neutrophils and macrophages are capable of engulfing and killing planktonic bacteria, they are not innately capable of effectively engulfing and killing sessile bacteria in biofilm.

LEVEL OF EVIDENCE: Strong

DELEGATE VOTE: Agree: 100%, Disagree: 0%, Abstain: 0% (Unanimous, Strongest Consensus)

PRE-MEETING RATIONALE

The most important pathogenic mechanism involved in implant-related infections is the ability of the microorganisms to form a biofilm [1], which leads to protection against environmental stress, host immune defense and antimicrobials [2]. The first cells arriving at the infection site are the neutrophils and macrophages [3]. The permeability and the phagocytosis ability of these immune cells have mainly been evaluated in two types of infection: cystic fibrosis [4–8] and device related infection, mainly catheter-related infection [9–17] and periprosthetic infection [18].

Neutrophils are innate immune cells capable of secreting an arsenal of toxic oxygen species, degrading enzymes, defensins and lipid inflammatory mediators to fight off infection [6]. These cells have shown the ability of sticking but not penetrating into a mature biofilm and phagocytizing biofilm encased microorganisms [4–8,10,11,14,19–23]. The exopolymeric substances of the biofilm matrix seem to be involved in the formation of neutrophil extracellular traps in biofilm of *Streptococcus suis* [21], *Candida albicans* [10] and *Candida glabrata* [11]. Data shows that neutrophils can destroy a two to six day old *Staphylococcus aureus* (*S. aureus*) biofilm, but a mature biofilm is capable of resisting penetration by these cells [24].

Guenther et al. studied the different behavior of polymorphonuclear neutrophils (PMNs) towards the biofilm formed by either *S. aureus* or *Staphylococcus epidermidis* (*S. epidermidis*). In the case of biofilm formed by *S. aureus*, the PMNs were observed to move across and scavenge bacteria along their path. Conversely, PMNs in contact with *S. epidermidis* biofilm were nearly immobile and phagocytized only bacteria in close proximity. Why biofilms of *S. aureus* appear more sensitive to a PMN attack compared to those produced by *S. epidermidis* is not well understood [19]. Insights on the behavior of biofilm formed by *S. epidermidis* have been offered by the in vitro and in vivo studies of Kristian et al. These authors found that *S. epidermidis* biofilms triggered higher levels of complement activation in terms of C3a formation than planktonic wild-type bacteria and isogenic ica-negative bacteria. On the other hand, a decreased deposition of immunoglobulin G (IgG) and C3b was observed in biofilm-embedded bacteria. This could possibly explain the evasion of PMNs killing [25].

Alhede et al. evaluated the role of immune system against biofilm formed by *Pseudomonas aeruginosa*. They demonstrated that both in vitro and in vivo biofilms of *Pseudomonas aeruginosa* produce

a shield of excreted rhamnolipids, which offers protection from the bactericidal activity of PMNs [26].

Arciola et al. did an extensive study of biofilm formed by Staphylococcus on an implant surface. Based on their work, PMNs were found to surround biofilm and become activated, but PMNs were not able to migrate into the biofilm, probably because of a lack of a chemotactic signal as well as by hindrance of migration into the "slimy" material. Thus, the inability of PMNs to penetrate biofilm results in progression of implant related infections. The activation of PMNs and their attempt to kill bacteria results in secretion of numerous cytotoxic and proteolytic enzymes that cannot act against bacteria but results in damaging and destroying the surrounding host tissues [27].

Macrophages become the prevailing cells and remain at the infection site a high concentration for several weeks and they are related to recognition, phagocytosis, secretion of enzymes, cytokines, chemokines and growth factors, to destroy and digest the phagocytized pathogens [3]. These cells can penetrate into a mature biofilm in a similar way as neutrophils, and phagocytize biofilm encased microorganisms, but not destroying them [9,12,13,18]. Moreover, these sessile phagocytized bacteria can even persist into peri-implant tissue inside macrophagic cells not only in experimental models, but also in the tissues of patients with intravenous catheters colonized by different bacteria [16,17]. *S. aureus* prosthetic infection in vivo model showed that limited bacterial macrophage uptake is due to inflammatory attenuation by *S. aureus* biofilm [13], which favor the transformation from M1 macrophages presents a high antimicrobial activity to M2 type inherently possesses less antimicrobial activity [13], and the cell death induction though leukocidin A/B [28] and human leukocyte antigen production [18]. At the site of staphylococcus biofilm infection, macrophages exhibit: down-regulation of interleukin (IL)-1β, tumor necrosis factor, CXCL2 and CCL2 expression, reduced bacterial uptake, minimal iNOS expression and consequent low efficiency in killing phagocytized bacteria and reduced induction of lymphocyte production of interferon-γ. These scavenging cells appear able to migrate into the biofilm but cannot clear the site from the pathogen causing the infection as their bactericidal activity appears compromised [27].

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QUESTION 4: Does the timescale of biofilm formation differ between bacterial species? If so, what is the timescale for common causative organisms?

RESPONSE: Currently, there is no clinical research available to answer whether the timescale in the development of biofilm formation differs between bacterial species. In vitro studies show high variability in biofilm formation based on bacterial strains and conditions. Animal studies have demonstrated rapid (minutes to hours) biofilm formation. The group notes that the timeline of biofilm formation may not correlate with the onset of infection symptoms.

LEVEL OF EVIDENCE: Strong

DELEGATE VOTE: Agree: 100%, Disagree: 0%, Abstain: 0% (Unanimous, Strongest Consensus)

PRE-MEETING RATIONALE

Biofilms are comprised of single or multiple species of microbial aggregates embedded in a self-produced matrix of extracellular polymeric substances. Regardless of the bacterial species, biofilm formation proceeds in known and well-defined steps. The first step or stage, adhesion, begins when bacteria sense and attach to surface of a material. The second stage is accumulation, where bacteria aggregate to form a mature biofilm. The last stage is dispersion or detachment [1]. The duration of each of these steps in biofilm formation varies from nanoseconds to hours to weeks, depending on various factors such as size of inoculum, mechanism of colonization (direct perioperative inoculation, later direct colonization due to break of barrier, bacteremic spread), surface properties of the foreign material, bacterial strain and virulence, bacterial species, host immunity, prior antibiotic usage and environmental factors, etc. [2–10].

For example, *Pseudomonas aeruginosa* (*P. aeruginosa*) contains several genes that are turned on within 15 minutes of its attachment to a surface that can be a starting point of biofilm formation [3]. Kanno et al. developed full thickness wounds on the backs of rats and inoculated them with *P. aeruginosa* carrying the green fluorescent protein gene; they found that biofilms could develop within eight hours [4]. When *Staphylococcus aureus* (*S. aureus*) was inoculated onto animal wounds, researchers found the development of clusters of cells (characteristic of a biofilm) after 6-24 hours post inoculation [11,12]. Oliveria et al. evaluated the time course evolution of biofilm in mastitis isolates and found no significant difference between *S. aureus* and *Staphylococcus epidermidis*. In their study biofilm forming ability increased with incubation period for both species [5]. Hoffman et al. researched adhesion patterns of single bacterium *Caulobacter crescentus* on a glass surface in a microfluidic device. They showed the importance of pili for hastening bacterial adhesion. In their study, irreversible adhesion events were more frequent in wild-type cells (3.3 events/min) compared to pilus-minus mutant cells (0.2 events/min) [13].

Koseki et al. [6] evaluated the difference in early biofilm formation of polysaccharide intercellular adhesin (PIA)-positive *Staphylococcus epidermidis* on five types of biomaterials and found no significant difference in biofilm coverage rate at two to four hour incubation, but at six hours post incubation cobalt-chromium-molybdenum alloy (Co-Cr-Mo) had a significantly lower biofilm coverage rate than other materials like titanium alloy (Ti-6Al-4V), commercially pure titanium and stainless steel. In this study authors point out a similar degree of smoothness across materials as a reason for no significant difference between them initially (two to four hours). In this study average roughness (Ra) was less than 10 nm [6]. This is corroborated by the previous reports that bacterial adhesion is influenced by the threshold of surface roughness at values more than 200 nm [14,15].

Some evidence suggests that bioactive substances such as hydroxyapatite may be more prone to bacterial adhesion than bioinert metals, such as titanium alloys and stainless steel [7]. Further studies have demonstrated that polymethyl methacrylate (PMMA) is capable of hosting biofilms that can cause acute, chronic and delayed-onset infections [8,9].

Biofilm adherence to biological or synthetic materials and foreign cells and resistance to antimicrobials are poorly understood. As biofilm formation can proceed through different pathways and time ranges, its detection may differ according to the time of observation. Investigational models to determine how environmental factors, such as surface geometry, physical and chemical characteristics, and local blood flow and immune system affect biofilm development on prosthetic joints are essential to further understand various bacterial biofilms and provide insight to therapeutic strategies.

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QUESTION 5: Do bacteria form biofilm on the surface of cement spacer in a similar fashion to a metallic implant?

RESPONSE: Yes. While the vast majority of studies have been in vitro, there is clinical evidence that majority of bacteria are able to form biofilm on the surface of cement spacer.

LEVEL OF EVIDENCE: Strong

DELEGATE VOTE: Agree: 100%, Disagree: 0%, Abstain: 0% (Unanimous, Strongest Consensus)

PRE-MEETING RATIONALE

The majority of data assessing biofilm growth on polymeric materials and smooth surfaces has been collected from in vitro experiments [1]. As a general outline, microbial adherence to materials occurs in the following order: latex > silicone > PVC > Teflon > polyurethane > stainless steel > titanium [1,2]. This hierarchy of materials to bacterial adherence suggests that biofilms may develop more readily on polymer-based versus metallic material surfaces. Roughness may play a role in this [3]. However, time is also an important factor to consider. Verran et al. showed that *Candida albicans* adhered to a greater degree on roughened surfaces compared to smooth [4]. In their experiment, polymeric samples were incubated for one hour, and then assessed for adhesion profiles. Similar work was performed by Taylor et al. on cobalt-chrome materials with the same conclusion [5]. Although a one-hour incubation period may be beneficial to determine initial adherence profiles, it would be difficult to compare test criteria such as these to clinical scenarios where implanted materials are present for days, weeks, months or years. Wolcott et al. have shown that time may play an important role in biofilm maturation and antibiotic tolerance [6]. Biofilms are well-known to condition surfaces and make them conducive to their growth requirements [3]. Perhaps one of the most well-known examples of this is *Streptococcus mutans*, which conditions the enamel surface that allows adherence for hundreds of other bacterial species [7]. Given enough time, biofilms may flourish on surfaces in many environments and on surfaces that may otherwise be considered less culturable [3,8,9]. In-house experiments that are in process of publication have shown that even amongst the same species, varying strains can differ in rates of biofilm formation on titanium surfaces, but over time degree of biofilm formation is similar in bench-top conditions.

The principles and problem of biofilm formation apply to bone cement and metallic surfaces used in orthopaedic applications. Biofilms have been shown to develop on both material types and adversely affect clinical outcomes [10–13]. A seminal paper published by Gristina et al. provided one of the first indications of biofilm growth on an implanted metallic implant that was found to contribute to biofilm-related infection [14]. More recently, Stoodley et al. directly observed biofilms on antibiotic-loaded bone cement associated with an infected total elbow arthroplasty [12]. McConoughey et al. have also identified bacterial biofilms on implanted components [15]. Shaw et al. observed biofilm, via methylene blue staining, that had developed on a tibial tray and other total joint components during revision surgery [16]. In multiple cases, biofilm has been observed directly on clinical samples. Due to the heterogeneous and at times difficult nature of collecting clinical samples, more highly controlled, albeit confirmatory outcomes of biofilm growth on metallic and cement materials have been obtained from in vitro and in vivo experiments.

Minelli et al. showed the ability of multiple staphylococcal bacterial strains to form biofilm on bone cement samples in all cases [17]. Neut et al. observed that slime-producing *Pseudomonas aeruginosa* can readily form biofilm on cement material, and in the biofilm phenotype it may be more tolerant to antibiotics loaded in cement than planktonic bacteria [18]. Ensing et al. assessed biofilm growth on cement material and the potential of ultrasound to remove its presence [19]. More recently in a study by Ma et al., polymethylmacrylate spacers that were removed at the time of reimplantation following treatment of infected total knee arthroplasty were shown to have high levels of bacterial DNA despite extended exposure to antibiotics [20]. Biofilm formation on metal surfaces is also well-documented [21–24]. Nishitani et al. have also observed growth of biofilms on metallic implants in mice [25]. Williams et al. have shown that over multiple days of growth in a CDC Biofilm Reactor, polymicrobial biofilms of methicillin-resistant *Staphylococcus aureus* and *Bacillus subtilis* grow similarly on smooth or rough titanium surfaces [26].

In summary, indications that biofilm forms on bone cement and metallic surfaces in a similar fashion are present from clinical samples as well as in vitro and in vivo animal studies. There are indications that bacterial cells may adhere to and form biofilms more quickly on rough/porous materials, but over time bacteria may condition material surfaces that are smoother in nature such as metal and allow biofilm to form to a similar degree.

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QUESTION 6: Does *Mycobacterium tuberculosis* (*M. tuberculosis*) form a biofilm on implants?

RESPONSE/RECOMMENDATION: Few data from experimental in vitro and in vivo studies and a limited number of case reports indicate that *M. tuberculosis* has a slow, albeit significant, ability to form biofilm on metal surfaces. The group suggests that management of *M. tuberculosis* implant-related infections should be treated using the same principles as that of other implant-related infections.

LEVEL OF EVIDENCE: Strong

DELEGATE VOTE: Agree: 100%, Disagree: 0%, Abstain: 0% (Unanimous, Strongest Consensus)

PRE-MEETING RATIONALE

Methods

A search of the English language literature on the question published during the period 1966–May 20, 2018 was conducted. The search strategy in PubMed used the terms *M. tuberculosis* and biofilm and identified 177 articles. All articles were reviewed for the response to the question. The vast majority of articles were categorized as basic sciences articles focusing on the components for tubercular biofilm formation in vitro. A systematic review to answer the provided question is not meaningful. Hence, the response of the question is answered as a summary of a narrative review.

Narrative Literature Review and Discussion

It is important to differentiate between *M. tuberculosis* and non-tuberculous mycobacterium. This review focusses only *M. tuberculosis*.

M. Tuberculosis Forms Biofilms

In the laboratory, *M. tuberculosis* shows peculiar aggregated growth, or in other words, can form organized pellicle-like structures [1]. The hallmark of biofilms is the self-production of the extracellular polymeric substance that holds the mycobacterial community together and confers phenotypic heterogeneity to

TABLE 1. Clinical data – PJI due to *Mycobacterium tuberculosis* treated antitubercular agents without surgery

Author/Year	Joint	Age/ Gender	Reported Risk Factors or Previous Clinical Hx	Preprosthetic Dx	Time Elapsed from Arthroplasty to Joint Infection	Time Elapsed from Joint Infection to Dx or Medical Therapy	Concomit-tant Infec-tions	Instrumental Examina-tions	Histological Examinations	Micro-biological Dx	Other Sites	Medical Therapy (Duration in Months)	Surgery	Time Elapsed from Start of Medical Therapy to Surgery	Follow-up from end of Therapy
Wray;1987	Knee	63/M	None	Osteoarthrosis	Postoperatively	NR	NR	Rx	Chronic granulomatous inflammation	NR	Lung	INH, RIF (12)	None	NR	18 months
Present work, case 2	Knee	62/M	None	Osteoarthrosis	Postoperatively	3 years	NR	Rx, Scint	Chronic granulomatous inflammation	Arthrocentesis cultures	None	INH, RIF (18), PZA (2)	None	NR	1 month
Tekin Koruk;2013	Knee	55/M	None	Osteoarthrosis	15 days	1 month	NR	Rx	Chronic granulomatous inflammation	Arthrocentesis microscopy and cultures	None	INH, RIF (12), PZA, EMB (2)	None	NR	NR
Kadakia; 2007	Knee	85/F	None	Traumatic fracture	1 month	3 months	Coagulase-negative Staphy-lococci	Rx	NR	Arthrocentesis cultures	Lung	INH, RIF, PZA, EMB (6)	None	NR	NR
Cansu;2011	Hip	46/F	None	Dislocation	4 months	NR	NR	Rx, CT	NR	Arthrocentesis cultures	None	INH, RIF, EMB (16), PZA (3)	None	NR	72 months
Marshall; 2007	Knee	48/M	AIDS	Osteoarthritis	6 months	3 months	NR	Rx, MRI	NR	Arthrocentesis microscopy, PCR and cultures	Lung, CNS	INH, PZA, EMB (1), MOX (1/2), RIF (1/2)	None	NR	Died during therapy
Present work, case 1	Knee	34/F	None	Rheumatoid arthritis	8 months	4 years	NR	Rx, MRI, LLS, PET	Chronic inflammation	Arthrocentesis cultures	None	INH, RIF (18), PZA, EMB (2)	None	NR	24 monhts
Jhoson;1979	Hip	51/F	Hip TB 41 yrs ago	Osteoarthritis consequent TB	13 months	NR	S. albus	Rx	NR	Arthrocentesis cultures	None	INH, RIF, EMB (on therapy)	None	NR	On therapy when published
Shanbhag; 2007	Hip	59/F	None	Osteoarthritis	14 months	2 months	Staphy-lococci	Rx, MRI	Chronic granulomatous inflammation	Arthrocentesis cultures	None	RIF, PZA, EMB (12)	None	NR	18 months
De Nardo;2012	Hip	67/F	None	NR	16 months	3 months	NR	CT, LLC	Chronic active inflammation	Arthrocentensis PCR	Psoas muscle, adrenals	INH, RIF (on therapy), PZA, EMB (3)	None	NR	On therapy when published
Lee;2012	Hip	62/M	None	Fracture	8 years	NR	NR	Rx, US, CT	Chronic active inflammation	Intraoperative microscopy and cultures	None	INH, RIF, PZA, EMB (6)	None	NR	24 months
Neogi;2009	Knee	73/F	None	Osteoarthritis	14 years	2 months	NR	Rx	Chronic granulomatous inflammation	Arthrocentesis PCR	None	INH, RIF (18), PZA (7), EMB (4)	None	NR	36 months
Egues Dubuc;2014	Knee	77/F	Anti-TNF therapy	Rheumatoid arthritis	NR	1 year	NR	US, Scint	NR	Arthrocentesis PCR	Lung, small intestine	INH, RIF, PZA (on therapy)	None	NR	On therapy when published

the genotypically identical cells [2]. Several studies have highlighted extracellular components within *M. tuberculosis* aggregation, including mycolic acids [3], complex sugars [4], cellulose, proteins, lipids and DNA [5,6]. In addition, *M. tuberculosis* residing within organized pellicle-like structures exhibits drug tolerance to antitubercular agents [3]. Thus, criteria of a structure to what is interpreted as biofilms are given.

M. Tuberculosis Biofilms in Humans

The clinical role of *M. tuberculosis* biofilms in humans is not fully understood. Basaraba and Ojha [7] provide convincing arguments that extracellular *M. tuberculosis* in necrotizing lesions likely grows as biofilms. Hence, mycobacterial biofilms may participate in the process of caseous necrosis and cavitation formation in lung tissue [5–7].

M. Tuberculosis Biofilms on Metal Surface

The vast majority of studies investigating *M. tuberculosis* biofilms uses polystyrene plates [8]. Ha et al. [9] compared the adherence and the biofilm formation of *Staphylococcus epidermidis* (*S. epidermidis*) with those of *M. tuberculosis* on four types of metal segments. In contrast to *S. epidermidis*, *M. tuberculosis* rarely adhered to metal surfaces and showed discrete biofilm formation. Similar results were reported by Chen et al. [10] who compared *S. aureus* and *M. tuberculosis* in vitro and in vivo. Adetunji et al. [11] analyzed *M. tuberculosis* biofilm formations on cement, ceramic or stainless steel coupons. The experimental settings in this study are difficult to transfer in an in-vivo implant model (e.g., more biofilms were formed when media containing 5% liver extract was used). However, more biofilms were formed on cement than on ceramic and stainless steel coupons [11]. Taken together, the few available data from in-vitro and in-vivo studies indicate that biofilm formation of *M. tuberculosis* on metal segments is poor in comparison to Staphylococcus spp.

Among the 66 cases reported by Veloci et al. [12], 13 (19.6%) were treated with antitubercular agents only. Hence, in these cases no surgical intervention was performed to reduce the mycobacterial load or to remove mechanically the biofilm adhering to the implant. One patient died because of far-advanced tuberculous meningitis, miliary tuberculosis of the lungs, femoral osteomyelitis and extended cold abscesses along the femoral shaft [13]. In the other cases, no failure was reported. Though only in 6 (50%) of 12 cases, follow-up results of ≥18 months after the end of therapy was available. Treatment duration ranged from 6 to 18 months. These data indicate that tubercular biofilm eradication is possible with chemotherapy only. Whether this is due to poor biofilm formation on metal implants or due to effective anti-biofilm activity of antitubercular agents cannot be assessed.

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QUESTION 7: What is the role of the microbial synergy in polymicrobial infections?

RESPONSE: In polymicrobial infections, a complex environment may be formed in which microbiological interactions exist between microorganisms. Scientific evidence exists to show that combinations of bacterial species may exist whereby these can protect each other from antibiotic action via the exchange of virulence and antibiotic resistance genes, and this may be evident in adverse outcomes for polymicrobial orthopaedic implant-related infections. It is also probable that polymicrobial infections may be more likely in patients with poor immunity and tissue healing.

LEVEL OF EVIDENCE: Strong

DELEGATE VOTE: Agree: 100%, Disagree: 0%, Abstain: 0% (Unanimous, Strongest Consensus)

PRE-MEETING RATIONALE

Varying incidences for polymicrobial infections have been reported with rates ranging from 6% to 37% [1–5]. The literature consistently demonstrates that patients with a polymicrobial infection demonstrate inferior treatment outcomes. Tan et al. reported that patients with polymicrobial periprosthetic joint infection (PJI) had a higher failure rate (50.5%) compared with monomicrobial PJI (31.5%) and a higher rate of amputation (odds ratio [OR] 3.80), arthrodesis (OR 11.06), and mortality (OR 7.88) [2]. Similarly, Wimmer et al. demon-

strated that the infection free rate after two years was 67.6% for polymicrobial infections vs. 87.5% for monomicrobial infections in a series of 77 polymicrobial PJIs [6]. In addition, Marculescu et al. demonstrated that the 2-year cumulative probability of success of polymicrobial PJIs was 63.8% compared to 72.8% for monomicrobial PJIs [7].

There are several explanations for the increased rate of failure in patients with polymicrobial PJI. Some explanations of polymicrobial infection include the following: the association with a sinus tract or a soft tissue defect; the frequent presence with difficult to treat organisms, such as Enterococcus spp and gram negatives [2,7,8]; increased comorbidities [2,7]; and microbial synergy.

Microbial synergy is defined as an interaction of two or more microbes in an infection site that results in enhanced disease by creating a more favorable condition for one another, compared to infections containing a single organism [9,10]. According to this definition, it can be appreciated that polymicrobial infection have less optimal outcome over that of monomicrobial infections because of the enhanced pathogen persistence in the infection site, increased disease severity and antimicrobial resistance [10,11]. While microbial synergy results in an enhancement of the disease, real experimental data supporting this phenomenon is still limited [12–14], which may be attributed to the complex and dynamic web of interactions that occur in natural systems [15].

Identified types of polymicrobial infections are due to: (1) changes in relative composition of individual species of microbiota [16]; (2) colonization of a pathogenic microbe of an infection site that already contains commensal microbes; and (3) colonization of a pathogenic microbe on a body they don't usually habit [17].

Several mechanisms of microbial synergy have been proposed in order to explain microorganisms interactions during polymicrobial infections: (1) metabolite cross-feeding: reported as the consumption of metabolic end-products by one of the microbial communities involved and optimization of local environment with the metabolic end-products [9,18,19]; (2) dedicated signaling systems: capacity of many microorganisms to communicate and coordinate activities as a group through low molecular weight signals, called "quorum sensing" [20]; (3) stimulation of resistance to the immune system: production of chemical substances that induce resistance to immune system like outer membrane proteins that inhibits immune pathways [9,18]; (4) suppression of the immune system by commensal bacteria: promotion of growth environment for commensal pathogens [9,21,22]; (5) direct contact: formation of biofilm by membrane-bound structures (adhesins) between microbes [23,24]; and (6) increased virulence of the organisms: production of substances that enhance the virulence of other bacteria [9].

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QUESTION 8: Is the mapping of biofilm to a particular component or anatomical location an important consideration in management of implant related infections?

RESPONSE: At present, mapping of biofilms is only possible in the laboratory, not in the clinical setting. Therefore, it is of unknown clinical importance in relation to management of implant-related infections.

LEVEL OF EVIDENCE: Consensus

DELEGATE VOTE: Agree: 100%, Disagree: 0%, Abstain: 0% (Unanimous, Strongest Consensus)

PRE-MEETING RATIONALE

Total joint replacement has become a vital tool for the treatment of end-stage osteoarthritis of the knee and hip and has the potential to substantially improve a patient's quality of life when successful. However, periprosthetic joint infection (PJI) is a dreaded complication of arthroplasty procedures that often results in expensive intravenous antibiotics, longer hospital stays and numerous negative effects related to patient morbidity [1]. Occurring at a rate of around 0.5-2% across all primary total joint procedures, these PJIs often involve bacteria growing in a composite of cellular and extracellular matrix material complex, known as biofilms [2,3]. The exact location or predilection of biofilm growth on specific prosthetic components or materials remains an important, albeit understudied, question. There is no evidence in the literature that has mapped biofilm formation to one specific material type or location or demonstrated mapping's importance in management of implant related infections.

Previous research examining the role of biofilms in PJI virulence is primarily focused on detection methods, imaging modalities and bacterial classification. While mapping to particular components is not commonly a primary focus, some work has examined patterns of bacterial formation that offer preliminary insight. Stoodley et al. [4] have shown that colored fluorescent proteins can be expressed to directly observe *Pseudomonas aeruginosa* biofilms on 316L stainless steel screws. Patchy development was noted on screw shafts and between the threads of several screws, with no significant pattern of development noted.

Confocal laser scanning microscopy has also been shown to aid in biofilm visualization on implant materials and surrounding tissue [5]; however, focused analysis does not exist regarding mapping or preferential formation of the biofilm on specific components or anatomic regions. Kobayashi et al. [6] and Nguyen et al. [7] have demonstrated the utility of ultra-sonication in detection of biofilms in PJI cases, showing that brief exposure of one to five minutes of infected components to ultra-sonication is effective in detecting bacterial adherence. However, few components were shown to harbor bacteria and those that did were not examined for anatomic or component-specific variability. Preliminary work by Gómez-Barrena et al. [8] showed no significant difference between hip and knee components in harboring bacterial biofilm formation. While this work focused primarily on the pathogenesis of various microorganisms and only classified components as "hip" or "knee," the finding that component type did not affect adherence shows primary indications that mapping biofilm formation may not be important to the management of PJIs. Existing research regarding biofilm mapping is not complete and cannot definitely define the importance of its practice. There is a need for additional work to replicate preliminary experiments and directly study the location of biofilm formation on orthopaedic components.

Another aspect of mapping to be considered is the material composition of orthopaedic components and the possible varying ability of such materials to harbor biofilm formation. Sheehan et al. compared stainless steel and titanium components using isolated strains of *Staphylococcus aureus* and *Staphylococcus epidermidis* in a femoral intramedullary implantation model in rabbits [9]. This study demonstrated higher levels of biofilm adherence to stainless steel components within the first 48 hours. Both strains showed this preferential growth, with higher levels of adherence reaching nearly 150% on stainless steel compared to titanium. Tuke et al. expanded the analysis of implant failure to analyze the potential role of metal-on-metal bearing surfaces [10]. A wear patch was noted to form on retrieved failed devices, indicating a potential loosening of the orthopaedic components and opportunity for colonization. These studies demonstrate the possibility of material-specific variation in biofilm formation that may allow for mapping. It appears possible that specific components, due to their composition or anatomical position, may be more susceptible to bacterial colonization with strains associated with PJI. However, there is a lack of evidence regarding materials commonly used in implant devices, with only preliminary and speculative data suggesting variation that may lead to improved surgical management.

Given the limited number of studies evaluating the location of biofilms on specific components isolated from PJI patients, either clinically or in the laboratory, we conclude that there is no strong evidence that biofilm formation favors either a specific location or material type in total joint arthroplasty. Anecdotally, it seems intuitive that knowledge of biofilm location would aid in surgical therapy, and a recent paper argues that an orthopaedic biofilm disclosing solution used intraoperatively would be a useful surgical tool [11]. However, the lack of evidence in the literature prevents the conclusion that mapping biofilms to a particular component is of clinical relevance.

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Disruption

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QUESTION 1: Is there evidence that interference with bacterial communication by blocking quorum sensing molecules can minimize biofilm formation in vivo?

RESPONSE: In vivo animal studies have demonstrated that interference with quorum sensing signals/molecules in some infections leads to decreased biofilm formation. There are contradictory results in *Staphylococcus* species. However, there are no clinical studies demonstrating this phenomenon.

LEVEL OF EVIDENCE: Limited

DELEGATE VOTE: Agree: 100%, Disagree: 0%, Abstain: 0% (Unanimous, Strongest Consensus)

PRE-MEETING RATIONALE

While there is extensive in vitro and in silica work being done and reported on quorum sensing and anti-quorum sensing molecules, otherwise known as quorum quenching, there are limited in vivo data and none of the anti-quorum sensing strategies are ready for widespread clinical application. Based on a search of the NCBI, Embase and Scopus databases, there are seven in vivo investigations that were reported during the last five years [1–7] (Table 1). In addition, there have been reports of quorum sensing inhibitor

TABLE 1. Seven in vivo studies over the last five years

Study #	Animal Model	Agent	Mechanism	Clinical Effect
1 [1]	Medaka fish peritoneal catheter infection	3-Phenyllactic Acid (PLA)	Antagonistically binds to quorum sensing receptors RhIR and RqsR, blocking initial attachment of Pseudomonas aeruginosa (PAo1) thereby delaying biofilm formation [1]	Decreased biofilm formation
2 [7]	Wistar rat pyelonephritis	phytol	Down regulate offimA, fimC, flhC, flhD, bsmB, pigP shlA genes in S. marcescens leading to decreased biofilm formation and virulence factor production	Decreased bacterial counts and virulence enzymes (lipase and protease) decreased inflammatory markers (MDA, NO, MPO) and histologically no acute inflammation
3 [2]	Mouse gingivitis	Quorum Sensing Inhibitors (furane compunds, d-ribose)	Interfere with AutoInducer-2	Decreased colony counts and alveolar bone loss
4 [4]	Round worm survival (Caenorhabditis elegans)	Sub-inhibitory concentration of ceftazidime	Inhibition of QS regulated virulence traits and biofilm formation; binds to the las and pqs QS receptors in P. aeruginosa	Increased survival
5 [5]		Acylase	Degrades Quorum sensing peptides	Delay biofilm formation for S. aureus and P. aeruginosa for up to 7 days
6 [6]	Larval oyster mortality	Phaeobacter gallaeciensis S4Sm	Down regulate pathogen virulence genes	Decreased mortality from V. tubiashii infection
7 [3]	Round worm survival (Caenorhabditis elegans)	Pyrrolo (1,2-a) pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl) from Alcaligenes faecalis	Modulate expression of quorum sensing (QS) regulators luxT and lafK	Increased survival from V. alginolyticus infection

and quorum quenching studies presented at scientific meetings utilizing multiple in vivo models [8].

The experimental strategy varies. In vitro data are relied upon to identify the molecular mechanism leading to interference with quorum sensing that causes decreased biofilm formation, whether it be blocking the signaling peptide production, blocking receptors or active initiation an antagonist signals by the agent. The in vivo data confirm that the agent decreases biofilm formation.

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QUESTION 2: Can a biomaterial surface be modified to dispel bacterial adherence and biofilms? What are the potential concerns in modifying implant surfaces to combat biofilms?

RESPONSE: The purpose of the surface modification is to decrease perioperative bacterial adherence and thus prevent biofilm formation. This has been shown in in vitro studies and in vivo animal models. There have been numerous strategies devised to alter surfaces. Such modified surfaces may interfere with the expected osseointegration, mechanical stability and long-term implant survivability. The duration of long-term anti-infective effects are unknown. To date, no positive in vitro effect has been translated into a clinical setting.

LEVEL OF EVIDENCE: Consensus

DELEGATE VOTE: Agree: 100%, Disagree: 0%, Abstain: 0% (Unanimous, Strongest Consensus)

PRE-MEETING RATIONALE

Periprosthetic joint infections (PJI) represent 1-20% of the failure mechanisms in total joint arthroplasty leading to significant morbidity and mortality [1–3]. The material surface used for implantation is a significant factor in bacterial colonization leading to PJI [4,5]. Some surfaces are more prone to bacterial adherence and formation of biofilms. A biofilm is an aggregate of microbial cells that are irreversibly associated with a surface and encapsulated in a complex polysaccharide "slime" extracellular matrix that may include enzymes, crystals and glycoproteins - together forming a living tissue [6,7]. The most common microorganisms residing in biofilms are *Staphylococcus S.* species [8,9]. The bacteria in biofilms take either sessile forms on metal, bone fragments and cement; or planktonic forms that can disperse as clumps within the joint fluid [10,11]. Due to such complexity of form, material and function, the question remains whether modified implant surfaces can play an anti-infective role and what are the main concerns with modifying biomedical devices.

Can a Biomaterial Surface Be Modified to Dispel Bacterial Adherence and Biofilm?

In 1987, Anthony Gristina [12] was the first to propose the concept of a race for the surface, wherein the fate of the biomaterial implant is dependent on a balance between tissue integration and microbial adhesion with biofilm formation. This concept sets the hypothesis that material modifications that improve osseointegraion while inhibiting bacterial adhesion would provide a theoretical advantage and eliminate the risk of infection [13]. As a result, there is a wide array of anti-infective surfaces proposed for utilization in orthopaedic implant applications.

Gallo et al. [14] summarized the available options as bactericidal, anti-adhesion surfaces, multifunctional/smart coatings and alternative materials.

Romanò et al. [15] propose a newer classification regime that describes antibacterial coating under three distinctive groups [1]:

1. *Passive surface finishing/modification* Surfaces that prevent adhesion without releasing anti-bacterial substances.
2. *Active surface finishing/modification* Surfaces that release anti-bacterial substances.
3. *Perioperative antibacterial carriers or coatings* Carriers or coatings applied during surgery that are antibacterial and either biodegradable or non-biodegradable.

Active surfaces and perioperative coatings provide only temporary solutions while they exhaust their antimicrobials in time. Passive surfaces may not provide the necessary bactericidal properties needed to eliminate the infection while their action is limited to the immediate peri-implant area. The ideal implant surface should have: (1) a strong anti-infective potential, (2) long duration of effect, (3) biocompatibility with mechanical construct and stability and (4) minimal host response and harm [16–18]. To achieve that, surfaces

TABLE 1. Proposed anti-infective surfaces for utilization in orthopaedic implant applications

Method	Type	Examples
Bactericidal	Inorganic	Ag, AgNP, AuNP, TiO2, Se, CuNP
	Organic	Coated or covalently linked antibiotics, chitosan derivatives
	Combined	Multilayer coating, positively charged polymers
	Other	Non-antibiotic (peptides, enzymes, oils)
Anti-adhesion		Anti-adhesive polymers
Multifunctional/smart coating	Passive	Nanostructured "smart" materials
	Active	Sensors conjoined to nanocontainers
Alternatives		Lytic bacteriophages

Ag, silver; NP, nanoparticles; TiO2, titanium oxide; Se, selenium; Cu, copper

can be physically and mechanically prepared and coated or chemically modified.

The early reversible adhesion stage of bacteria to titanium is largely influenced by the topographical features on the surface [19]. Several anti-adherent coatings on titanium have been created by surface modification with polymers, copolymers or proteins. Del Curto et al. [20] has shown that the crystalline phase of titanium oxide on the surface of biomaterials reduced bacterial attachment without adverse effects on the biocompatibility. Ferraris et al. [21] showed that mechanically produced nanogrooves (0.1-0.2 um) and keratin nanofibers can increase biocompatibility without increasing bacterial adhesion. Lorenzetti et al. [19] has applied hydrothermic treatment methods to similarly achieve decreased bacterial adhesion. This data is very encouraging and supports the concept that biomaterial surfaces can be modified to dispel bacterial adherence.

Silver (Ag) has been known throughout history not only for its jewelry applications but for its antimicrobial effects [22,23]. The mechanism of action is thought to be the formation of reactive oxygen species and biologically active ions that damage bacterial walls and bind to nucleic acids and interrupt bacterial replication [24]. An added advantage of Ag usage is the effect against surface-adhered bacteria without significant drug-resistance [25,26]. Harrasser et al. [27] studied the antimicrobial effects of Ag and has observed significant antimicrobial activity that was positively correlated with Ag concentrations. A recent study by Aurore et al. [28] indicated that Ag nanoparticles (AgNPs) enhanced the bactericidal activity in osteoclasts.

As such, AgNPs have gained attention for their application on implant surfaces due to their anti-biofilm potential, wide-spectrum antimicrobial properties and low cytotoxicity to human cells [18,22,29–33]. There is an abundance of literature that examine the anti-biofilm effect of AgNPs [18,25,34]. Kalishwaralal et al. [35] demonstrated that AgNPs at a concentration of 100 nM almost entirely inhibited biofilm formation (> 95%) from *S. epidermidis* and *Pseudomonas aeruginosa*. Slane et al. [33] found that bone cements impregnated with AgNPs significantly reduced biofilm formation compared to standard cement. Some studies have also mentioned the synergistic effect of AgNPs with antibiotics [36–38]. The most notable advantage of AgNP-coated surfaces is the ability to exhibit a continuously controlled-release of active agents to the periprosthetic region for a substantial period of time, thus working at both the surface layer but also in the immediate environment.

Recently, iodine has been shown to be a successful adjuvant for irrigation and debridement in cases of PJI [39]. Adapting this idea to implant surfacers, Tsuchiya et al. [40] report on a clinical study of more than 222 patients in whom iodine surface treated implants were very effective for preventing and treating infections after orthopaedic surgery. No clear cytotoxicity or adverse effects were observed. Shirai et al. [41] similarly demonstrated a significant reduction in pin tract infection rate by using iodine surface-treated insertion pins and external fixators. Kabata et al. [42] also show that iodine treated hip implants remained free of infection in 14 revision cases for infection and in 16 immunosuppressed primary total hip arthroplasties. No issues related to local and systemic toxicity or impaired osteoconductivity and bone bonding have been reported in any of these studies.

Similar to Ag and iodine, multiple studies have targeted incorporation of antibiotics into surface coatings directly deposited onto the implant [43–45]. Most of these applications build on the information learned from antibiotic-laden bone cements and provide an initial protective barrier for infection [46–48]. Current protocols include hydrogels, poly-D, L-lactide, calcium phosphate or carbonated hydroxyapatite antibiotic coatings. Other direct techniques attempt to physically modify the surface for antibiotic adsorption, or simply dip the implant in antibiotics producing a transient coating [48–50]. Recent scientific progress in biomolecular interactions and nanoscale engineering provides new inspiration for medical implant designs that may have the potential to deal with infection [51,52]. Antibiotics covalently linked to metallic surfaces have been shown to inhibit bacterial colonization both in vitro and in vivo [13,53,54]. Despite all progress, most systems are rudimentary and difficult to scale up to industry standards; further research and a smarter implant technology is necessary. Such technology should directly integrate biological defenses in the implant design, making protection feasible for the life of the replacement prosthesis.

What Are the Main Problems in Modifying Implant Surfaces in the Fight Against Biofilms?

One of the main concerns of antimicrobial biomaterials is the possible cytotoxic effect of the surface modification as related to osseointegration and implant survival in vivo. Based on a preliminary literature review, only four laboratory studies [55–58] and one clinical study [59] reported the side effects of surface modification. Ag surface modifications have shown higher lactate dehydrogenase (LDH) activity as a marker of cell death, as well as lower cell count and alkaline phosphatase (ALP) activity [55–58]. Nevertheless, such

effects are hard to correlate with clinical outcomes. Glehr et al. [59] performed the only clinical study that focused on Ag while examining its use in mega-prosthesis. They have documented the presence of heavy metal poisoning symptoms, even though no correlation with the blood Ag concentration was observed. Another two in vitro studies used zinc and farnesol (anti-fungi medicine) surface modifications respectively. The results showed lower ALP activity as well as pre-osteoblastic cell damage. Multiple studies thus agree that AgNPs have the potential to be toxic to many cell types in a dose- and time-dependent manner, especially when inhaled, injected or ingested [60–62]. Interestingly, Shen et al. [63] conducted a study which revealed that both cobalt chrome alloys and pure titanium had cytotoxic effects to osteogenic precursor cells and mesenchymal stem cells, while the incorporation of AgNPs reduced this cytotoxicity.

When working with modified surfaces, bacteria can ultimately adapt and develop resistance to the agent used. Antibiotic resistance is an everyday occurrence in clinical practice. Bacteria have also been shown to surmount resistance to the ionic form of Ag, and less commonly, to AgNPs [64,65]. This is because prolonged exposure to AgNPs, unlike Ag ions, is less likely to result in resistance genes, since AgNPs have broad-spectrum capabilities by targeting multiple sites on or within bacterial cells [66]. Nevertheless, resistance to silver seems to be a slow process and is a less of a problem compared to antibiotic resistance [67]. Concerning though, Kaweeteerawat et al. [68] suggest that AgNPs could potentially enhance bacterial resistance to antibiotics through promoting stress tolerance by induction of intracellular reactive oxygen species causing DNA mutations.

In conclusion, bacterial biofilms are difficult for antimicrobial agents to penetrate. Preventing biofilms and bacterial adherence is probably the only effective way to address the problem of PJI. AgNPs and iodine are gaining increasing popularity especially for their anti-adhesion, anti-infective, and minimal bacterial resistance properties. Nevertheless, further investigation of the long-term outcomes of patients with modified surfaced implants is warranted.

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QUESTION 3: What is the relevance of minimum inhibitory concentration (MIC) of infecting organisms in biofilm-mediated chronic infection?

RESPONSE: The use of MIC is limited to (1) defining antibiotics that the microorganism is susceptible to in its planktonic state but cannot be used to guide treatment of biofilm-based bacteria and (2) selecting long-term suppressive antibiotic regimens where eradication of infection is not anticipated. Alternative measures of antibiotic efficacy specifically in the context of biofilm-associated infection should be developed and validated.

LEVEL OF EVIDENCE: Strong

DELEGATE VOTE: Agree: 100%, Disagree: 0%, Abstain: 0% (Unanimous, Strongest Consensus)

PRE-MEETING RATIONALE

MICs are used to define an individual microorganism's (hereafter limited to bacteria) susceptibility to a distinct array of antibiotics. Established methodologies for determining MICs relate to the planktonic state of the bacteria but not to biofilm-indwelling bacteria [1].

The majority of information relating to susceptibility testing and biofilm-indwelling bacteria originates from research in Cystic Fibrosis [2]. In relation to implant-associated biofilm infections, central venous catheters and urinary tract catheters are often investigated, but little clinical research has been performed in orthopaedic implant-associated biofilm infections [2,3].

As early as 1990, Anwar and Costerton identified the need for an extreme increase in in vitro concentrations of antibiotics, to which the planktonic bacteria were fully susceptible, when treating biofilm-indwelling bacteria [4,5]. In a review by key-opinion leaders on the topic of antimicrobial susceptibility testing in biofilm-indwelling

bacteria, it was noted that MIC is not suitable in predicting the effect of an antibiotic for a biofilm infection [6]. In the 2014 European Society for Clinical Microbiology and Infectious Diseases guidelines for the diagnosis and treatment of biofilms infections, it is noted that antibiotic susceptibility determination by MIC offers no guide to clinicians in the treatment of biofilms [7]. Rather than MICs, clinicians may need to rely on other measures of antibiotic efficacy, such as minimum biofilm eradication concentration (MBEC), minimum biofilm bactericidal concentration (MBBC) or minimum biofilm inhibitory concentration (MBIC). These are likely to be 100-1000 times the MIC, but the associated breakpoints that would permit reliable prediction of treatment success have not yet been established.

Theoretical mechanisms driving the high-level of resistance to antibiotics in biofilm include both the mechanical exclusion of antibiotic molecules by the polysaccharide matrix and the presence of dormant persister organisms within the biofilm. The relative contribution of each of these mechanisms is uncertain, but emerging data suggest that persister organisms constitute up to 10% of biofilm. Due to the adapted phenotype, they are able to evade the antimicrobial action of a variety of conventional antibiotics that rely on disruption of cell processes for their efficacy. Post et al. showed that, although it was possible to eradicate biofilm caused by *Staphylococcus aureus* (*S. aureus*), the necessary time-concentration profile could not be achieved in vivo by systemic administration or by any local delivery vehicles currently available [8]. Urish et al. concluded that tolerance was primarily a phenotypic phenomenon as increasing cefazolin exposure did not result in changes in MIC [9].

In two studies, Antunes et al. identified that among biofilm-indwelling Staphylococcus species isolates, 89% were considered to be clinical resistant to vancomycin, even when the same isolates all presented MIC values categorizing the isolates as fully susceptible to vancomycin (MIC </= 2μg/mL) [10,11]. The authors concluded that this particular observation showed "that biofilm production results in an important barrier to antimicrobial diffusion into the biofilm" and that "antimicrobial susceptibility testing based on MIC values alone cannot accurately determine the exact susceptibility of bacterial biofilms."

Ray et al. tested ceftriaxone and gentamicin, both commonly used antibiotics in orthopaedic surgery, against *Serratia marcescens* biofilm in vitro at doses of 10, 100, 1,000 times that of the established MIC for the planktonic isolate and found that the antibiotic, even at these concentrations, did not reduce biofilm biomass [12].

Reiter et al. tested rifampicin and vancomycin against methicillin-resistant *S. aureus* planktonic and biofilm isolates in vitro and found 32-32,000 times increase in resistance for rifampicin and 8-512 times increase in resistance for vancomycin in biofilm isolates [13]. They subsequently concluded that the tested antibiotic were not able to eradicate mature biofilm at the concentrations needed for planktonic microbes (the MIC).

Ruppen et al. tested gentamicin as an adjuvant to penicillin in *Group B Streptococcus* biofilm in vitro, and found a 2,000-4,000 times increase in resistance for penicillin in the presence of biofilm and 1-4 times increase for gentamicin [14]. The authors noted that the gentamicin doses tested did not correlate with achievable in vivo concentrations. The authors concluded that the MIC did not correlate to the susceptibility to the tested biofilm strains.

Hajdu et al. tested an array of antibiotics against *Staphylococcus epidermidis* biofilm in vitro. The planktonic bacteria susceptibilities were tested to all antibiotics in the study. When biofilm-indwelling bacteria was tested, susceptibilities were up to 128-times the established MIC. Only ceftriaxone showed a minor reduction in total biofilm biomass. No eradication occurred for any antibiotics at any level above MIC; it was also noted that these levels were much higher than any clinical in vivo achievable concentration [15].

Ravn et al. tested dislodged biofilm from in vitro implant infections of *S. aureus*, *Staphylococcus epidermidis*, *Escherichia coli* and *Cutibacterium acnes* and found antimicrobial susceptibility to be identified at 4 times that of MIC (for *Escherichia coli* and ciprofloxacin) to 1.024 times that of MIC (for staphylococcus species + *Cutibacterium acnes* and vancomycin) [16]. The authors concluded that MIC correlation to in vivo values may not affect biofilm-indwelling bacteria.

Monzón et al. tested *Staphylococcus epidermidis* biofilm susceptibility on an array of antibiotics in vitro. All the isolates tested were fully susceptible to vancomycin in their planktonic form. The authors found that vancomycin, teicoplanin, clindamycin and oxifloxacin at MIC had a low killing rate in 24-hour mature biofilm. Rifampicin was not affected by the presence of mature biofilm and remained with a high killing rate at MIC [17]. The authors concluded that antibiotics may lose their killing ability in mature biofilm at clinical relevant in vivo levels, despite being fully susceptible at MIC.

Molina-Manso at el. tested susceptibility of Staphylococcus species biofilm in vitro and found that none of the tested antibiotics (including rifampicin, vancomycin, clindamycin, cloxacillin, ciprofloxacin) could eradicate the biofilm-indwelling bacteria, even at concentrations highly above the established MIC for the individual isolates [18].

Claessens et al. tested the effect of antibiotic concentration at up to 40 times the established MIC of the individual isolates in *Staphylococcus epidermidis* biofilm in vitro and found that only rifampicin could decrease but not eradicate the biofilm mass, whereas vancomycin, teicoplanin and oxacillin did not decrease the biofilm mass [19].

Given the plethora of evidence detailed above, there is a clear need to seek alternative approaches to the prevention and treatment of biofilm related infections. The use of local antibiotic delivery systems is widely regarded as a possible means to achieve sufficiently high concentrations of antibiotic to exceed the MBEC. However, there is little guidance on the optimal duration that MBEC should be exceeded to affect a cure. There is also concern that, although early elution of antibiotic from cement produces high local concentrations of antibiotics, late sub-MIC concentration may promote the development of antibiotic resistance, particularly amongst persister populations. Furthermore, the MBEC may well change with time of exposure to antimicrobials further complicating the determinants of optimal local dosage and carrier systems [20].

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QUESTION 4: What is the minimum biofilm eradication concentration (MBEC) of anti-infective agents?

RESPONSE/RECOMMENDATION: The MBEC of antimicrobial agents is a measure of in vitro antibiotic susceptibility of biofilm producing infective organisms. It is dependent on the surface, medium and the exposure period to an antimicrobial agent. There are no standardized measurement parameters for MBEC. MBEC is currently a research laboratory value and lacks clinical availability. In the group's opinion, there is value in developing a clinically-validated MBEC assay.

LEVEL OF EVIDENCE: Consensus

DELEGATE VOTE: Agree: 100%, Disagree: 0%, Abstain: 0% (Unanimous, Strongest Consensus)

PRE-MEETING RATIONALE

A Medline query on the item "minimum biofilm eradication concentration" retrieved 149 references. For the most part, these references relate to bacteria with little or no involvement in infection on orthopaedic devices. A query about "minimum biofilm eradication concentration of infective agents" retrieved 18 references; none of them clearly related to bone infection on material. The Medline request "minimum biofilm eradication concentration and implant associated infection" retrieves only three references [1–3].

The work of Coraça-Huber et al. [1] focuses on the evaluation of a study model of the minimum bacterial concentration (MBC) in infections on material, using strains of *Staphylococcus aureus (S. aureus)* and collection *Staphylococcus epidermidis (S. epidermids)*. Biofilm formation is supported by Innovotech, Inc.'s MBEC-HTP (high throughput plates) system (Edmonton, Alberta, Canada). The formation of biofilm is documented by electron microscopic study. The comparison of the minimum inhibitory concentration (MIC) and MBEC was made in this model for daptomycin, gentamicin, vancomycin, rifampicin, fosfomycin, clindamycin and linezolide. Biofilms generated by *S. epidermidis* show less resistance to antibiotics than those generated by *S. aureus*. The MBEC is much higher than the MIC of all antibiotics. Daptomycin and rifampicin are the most effective antibiotics against *S. aureus* embedded within a biofilm without obtaining their complete eradication.

Brady et al. [2] raised a question about the validity of the MBEC to replace the IJC in situations of infection on equipment. Twenty staphylococcal isolates from catheter infections were studied (17 CNS, 3 MSSA) and ten antibiotics were tested (penicillin, oxacillin, erythromycin, clindamycin, fucidine, tetracycline, gentamicin, vancomycin, teicoplanin and ciprofloxacin). The quantification of biofilm formation on microtiter plates and Tryptic Soy Broth (TSB) is obtained by crystal violet method. Detection of the biofilm formation mechanism (protein or polysaccharide) is obtained by treatment of sodium metaperiodate and protein kinase plates. The search for the ica operon (code in staphylococci for the production of enzymes necessary for adhesion) is done by polymerase chain reaction. Sixteen of the 20 strains (80%) tested produce biofilm; low for 8 strains, moderate for 2 strains, and high for 6 strains, all carriers of ica operons. The MBEC was 10 to 1,000 times higher than the MIC for bacteria producing biofilm. In practice, the MBC is > 256 µg/ml for all strains studied, whether or not biofilm production is proven by the techniques used, raising the question of strains forming a protein biofilm that cannot be quantified by the crystal violet method.

Zaborowska et al. [3] analyzed the sensitivity of staphylococci and enterococci from bone infections on material according to their biofilm production. The 13 strains studied were derived from infections on percutaneous bone anchoring material, on femoral amputation stumps for fitting. This technique involves a permanent protrusion of a titanium implant through the skin, a potential entry point for bacteria from the cutaneous and fecal flora. The bacteria studied were obtained from bone and material samples obtained from 11 infected patients. These are four strains of *S. aureus*, three strains of coagulase-negative staphylococci and six strains of *Enterococcus faecalis*. Ten antibiotics are tested in MIC and MBEC (clindamycin, gentamicin, vancomycin, linezolide, ciprofloxacin, oxacillin, fucidic acid, ampicillin, trimethoprim/sulfamethoxazole and rifampicin). The microtiter plate culture in TSB is used to evaluate the biofilm

production capacity of the bacteria analyzed. The total mass of the biofilm formed is measured by the crystal violet technique to determine a biofilm score (absent, low, moderate, high production). The production of exopolysaccharide (slime) is measured by the Congo red technique. The search for the ica operon for staphylococci is obtained by PCR test. The determination of the MBEC is obtained by the Calgary Biofilm Device (CBD). Eleven of the 13 strains studied produce biofilm, the quantity of biofilm is heterogeneous according to bacterial species. The MBEC is significantly higher than the MIC for the 10 antibiotics studied. The ratio MBEC/MIC is variable with marked differences between bacterial species. The MBEC is high and homogeneous for all strains of *Enterococcus faecalis*: MBEC/MIC from 64 to 2048, median 512, for vancomycin, ciprofloxacin, linezolide, ampicillin and rifampicin. In comparison, Staphylococcus strains show significant inter strain variability; for *S. aureus* MBEC/MIC ranges from 1 to 2048, median to 9, for the 10 antibiotics tested. For *S. epidermidis* the ratio ranges from 0.0038 to 64, median to 1. The ica operon is isolated for all staphylococci; however, two strains do not produce slime by referring to the Congo red technique, expressing variability in gene expression. For these two strains, the biofilm score assessed by the crystal violet method was strongly positive, indicating that this biofilm consisted mainly of aggregated cells without slime production.

The clinical follow-up of the 11 patients was correlated to the results expressed in MBEC. Failure was correlated with a high MBEC value without statistical evidence. Two patients did not present any complications (recurrence, reinfection or need for material removal). For one, the strain did not produce biofilm; for the other, biofilm production was low. For other strains with low to moderate biofilm production, patients experienced one or two complications. One patient developed all three complications and the infecting strain was highly biofilm producing.

Of these three studies, only Zaborowska et al.'s [3] corresponds to a clinical situation of infection on an orthopaedic device. As in the other two studies, the work presented here only tests antibiotics as monotherapy, whereas clinical use is readily with dual therapy, particularly when rifampicin is prescribed. The work of Saginur et al. [4] on 17 strains of *S. epidermidis*, 11 strains of methicillin-susceptible *Staphylococcus aureus* (MSSA) and 12 strains of methicillin-resistant *Staphylococcus aureus* (MRSA), isolated from infections on material tested in MIC and MBEC (CBD device) 9 antibiotics in monotherapy and 94 combinations of antibiotics in bi or tritherapy. The MBEC is significantly higher than the MIC, but a significant heterogeneity between strains is also found in monotherapy. Among the 94 antibiotic combinations tested, 11 are bactericidal on more than 90% of MSSA strains growing in biofilm and 9 are for *S. epidermidis*. Rifampicin is the antibiotic most often present in these combinations.

The efficacy of antibiotics against bacteria growing in a biofilm, is generally explored in vitro under standardized, brief conditions of exposure of the bacterial strain to the antibiotic tested. In clinical practice, exposure to antibiotics is prolonged [5]. In this work, bacterial strains (MSSA, MRSA, *S. epidermidis*, *E. coli*, *Pseudomonas aeruginosa*) are tested for growth in a biofilm at varying antibiotic concentrations for three antibiotic exposure durations of one, three and five days. For most strains and antibiotics tested, the MBEC is significantly lower after 5 days of exposure to antibiotics than that measured after 24 hours of exposure.

It is commonly accepted that bacterial adhesion and bacterial growth within a biofilm, are the determinants of infection on material. It is also commonly accepted that the effectiveness of antibiotics within a biofilm is greatly diminished. Measurement of in vitro antibiotic activity by the MIC determined on planktonic bacteria is not predictive of in vivo antibiotic activity on bacteria growing in a biofilm. The MBEC is the supposedly most appropriate parameter for predicting the efficacy of antibiotics in vivo. The literature review shows that this parameter is over the last few years increasingly studied and taken into account to test antibiotics or various molecules against multiple microorganisms.

While the in vitro MBEC determination method itself is not problematic, the measurement of biofilm production is more random. Biofilm is made up of both bacterial cells and a substance of either a polysaccharide (slime) or protein nature. Not all bacteria produce biofilm. For staphylococci, the production of biofilm is linked to the existence of an operon (ica), detectable by PCR but whose expression is variable, and the highlighting of the operon does not mean slime production. The measurement of the overall mass of biofilm, generally by the crystal violet technique, which potentially defines biofilm scores (absent, weak, moderate, strong), does not necessarily account for the composition of this biofilm, likely to modify the MBC of antibiotics.

The capacity to produce biofilm is heterogeneous depending on the bacterial species. On the available data, the capacity to produce biofilm is strong for *Enterococcus faecalis* without inter-strain variability. For staphylococci, the capacity to produce biofilm seems more marked in *Staphylococcus aureus* than in staphylococcus epidermidis, but inter-strain variability is important for staphylococci. Rifampicin appears to be a more active antibiotic in biofilm than average. However, the rule is by no means absolute. The efficacy of antibiotic combinations is significantly superior to that of monotherapy molecules.

In a clinical situation, for a given strain, the MBEC cannot be estimated a priori, at least for staphylococci. Of the few published data, the MBEC still appears to be higher than at least 64 times the MIC for antibiotics active against *Enterococcus fecalis* (ampicillin, vancomycin, linezolide, rifampicin). For other bacteria, the MBEC of active antibiotics is not known.

There is no antibiotic combination that guarantees bacterial eradication in the biofilm for a given strain of staphylococcus, although antibiotic combinations are generally more effective than monotherapy treatments. The in vitro measurement of the MBEC is not a routine use for the moment. The research field needs to define a standardized methodology for possible use in clinical practice. High biofilm production appears to correlate with a higher complication or failure rate than low or absent biofilm production without statistical demonstration at this time.

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QUESTION 5: Do bacteriophages have a role in treating multidrug-resistant periprosthetic joint infection (PJI)?

RESPONSE: Unknown. Although some preclinical and clinical studies have demonstrated a good safety profile as well as promising therapeutic effects using bacteriophages for treating bone and joint infections, further clinical research using bacteriophage therapy in patients with multidrug-resistant PJI is required.

There are known obstacles to bacteriophage therapy, including the fact that bacteriophages are neutralized in serum and relevant pathogens contain Clustered Regularly Interspaced Short Palindromic Repeats - associated protein-9 nuclease (CRISPR/cas9) immunity against bacteriophage. Phages are usually bacterial strain specific; thus, a cocktail of different bacteriophage lineages may be necessary to effectively treat biofilm-mediated infections.

LEVEL OF EVIDENCE: Consensus

DELEGATE VOTE: Agree: 100%, Disagree: 0%, Abstain: 0% (Unanimous, Strongest Consensus)

PRE-MEETING RATIONALE

PJI represent serious issues for patients worldwide. The surfaces of orthopaedic implants are all susceptible to colonization by biofilm-forming bacteria, such as methicillin-resistant *Staphylococcus aureus* (*S. aureus*) (MRSA), *Pseudomonas aeruginosa* (*P. aeruginosa*) and numerous other organisms, whose presence has been reported to play a key role in the occurrence of PJI, thus leading to antibiotic resistance [1–4]. To overcome these problems novel treatment strategies focusing on disrupting biofilms are being developed [5]. Utilization of lytic bacteriophages to eradicate bacteria causing biofilms is one of the promising emerging technologies [3,6].

Bacteriophages are natural viruses that infect bacteria. They are one of the most abundant organisms in the biosphere. Each bacteriophage is specific to a particular microbial species. Like all viruses, phages are only able to replicate inside their host cells. Lytic phages inject their genetic material into the host bacterial cell, cause bacterial cell lysis that liberates subsequent new phage particles. These new particles allow successive infection of additional bacteria in a rapid and exponential pattern, facilitating the complete eradication of the bacteria. The French microbiologist Felix d'Herelle first described bacteriophages in 1917 [7]. By their nature, bacteriophages are good candidates for antibacterial therapy. Indeed, they target a bacterium specifically, as long as the corresponding host bacteria is present. In comparison with antibiotics, this phenomenon is unique as it is exponential and self-sustained after a single or a few administrations. Moreover, lytic bacteriophages do not affect eukaryotic cells and not impact the gut microbiota when administered locally.

Bacteriophage technology is particularly promising in patients with multidrug-resistant PJI as: (i) multidrug-resistant PJI are becoming more and more frequent [8,9]; (ii) the rate of relapse is particularly high in patients with PJI caused by multidrug-resistant pathogen [9–11]; (iii) bacteriophages and antibiotics are synergistic [12,13]; (iv) there is no cross-resistance between antibiotic resistance and bacteriophage resistance [6–12]; (v) some in vitro and animal models demonstrated that bacteriophages could have an anti-biofilm activity [6,13,14]; and (vi) recent human and animal trials using phage therapy have not shown any local tissue toxicity or any adverse effects to the host [15–20].

Bacteriophages were used in the 1970s in France [21] and remained a popular treatment throughout the 20th century in Eastern Europe (Poland) and the former Soviet Union (Georgia, Russia) in patients with relapsing osteomyelitis. Few case series have been published in the literature, including patients with pyogenic native joint infection, chronic osteomyelitis, suppuration after bone fracture and diabetic foot osteomyelitis [22–26].

In preclinical studies using animal models for PJI bacteriophages were found to prevent bacterial adhesion and also effectively disrupt the formation of biofilm [13,27]. Animal studies also have proven synergism between antibiotics and bacteriophages [13]. In another animal study, Kishor et al. [26] studied the efficacy of several phages used in conjunction as a treatment modality for chronic osteomyelitis caused by MRSA in rabbits. The study showed that the combination of specific phages selected based on their virulence against various clinical MRSA strains was effective in eradicating the infection, thus suggesting that a "tailor-made cocktail" of phages can alone be effective in targeting specific bacteria in the setting of a chronic infection. Some of the issues with current PJI animal models are that they don't replicate mechanical stresses occurring in clinical settings and, therefore, may not be fully representative of clinical situations.

Wright et al. conducted a randomized, double-blind clinical trial using bacteriophages in humans [28]. They studied the effect of the combination cocktail of six phages targeting *P. aeruginosa* in the treatment of antibiotic-resistant chronic otitis media infection. The authors achieved measurable therapeutic effects with minimal dosing, thus suggesting a promising role for phage therapy in treating antibiotic-resistant infections.

No case series including patients with PJI has been published (we retrieved only two cases from a French series of bone and joint infection treated with bacteriophages) [6]. In the Georgian practice, specific phages mixtures are used, such as the "pyophage" cocktail that contains phages against *S. aureus*, *Streptococcus*, *Proteus*, *P. aeruginosa* and *Escherichia coli* (*E. coli*) or specific bacteriophages targeting specifically staphylococci, as the Sb-1 phage (that could be imported in the USA), the bacteriophage K or the bacteriophage ISP [22]. In Poland, phage(s) are selected from a bank based on their activity on the patient's strain to adapt the treatment (personal medicine) and to ensure antibacterial activity of phages used [23,24]. All these bacteriophages are classically prepared with a bacterial inoculum, in vitro infection with the bacteriophage and purification of the preparation in aliquots at 10^7 to 10^8 PFU/mL. These preparations are approved by local authorities but do not respect European "good manufacturing practice" (GMP) standards for conducting clinical trials and targeting Market Authorizations (MA). Indeed, the final product requires total elimination of bacterial components that are

generated during the production process, such as toxins, in order to limit pyrogenicity and adverse events that may arise during phage administration/use, especially when the phage is administered intravenously or directly in a joint cavity. As a consequence, bacteriophages are currently not injected directly into the joint in patients with PJI but locally throughout the fistula and/or orally in patients with chronic osteomyelitis [23–25].

Recently, an European multicentric clinical trial evaluating phage therapy of burn wound infections has been done using *P. aeruginosa* and *E. coli* bacteriophages from a GMP French bioproduction process that was implemented according to European Medicine Agency standards (ClinicalTrials.gov Identifier: NCT02116010). The French team from the Lyon bone joint infection (BJI) study group (also called CRIOAc Lyon, a regional reference center for the treatment of complex bone and joint infection in France; http://www.crioac-lyon.fr) has treated as salvage therapy, under the supervision of the French health authorities, three patients with chronic bone and joint infection (one osteomyelitis due to extensively drug-resistant *P. aeruginosa*; and two *S. aureus* PJI) with bacteriophages that follows the same process of production. For all the patients, the cocktail was personalized and selected based on the bacteriophage susceptibility of the clinical isolates (phagogram; similar principle as antibiogram but with bacteriophages) that was isolated after a joint puncture before the surgery. The two patients with PJI had chronic infection with purulent discharge and were treated with debridement antibiotics and implant retention (DAIR) supplemented with a direct administration of the bacteriophage *S. aureus* cocktail in the joint cavity at the end of the procedure. Both patients are doing well during the follow-up of 12 months and 3 months, respectively (unpublished data). A randomized clinical trial called PHAGOS will start soon in France, to evaluate the addition of *S. aureus* bacteriophage in patients with relapsing *S. aureus* PJI. The availability of *P. aeruginosa*, *E. coli* and *S. aureus* with GMP standard in France is a great opportunity to evaluate the phage therapy as an additive treatment in patients with PJI, especially in patients with multidrug-resistant PJI.

Although phage treatment looks promising and safe, further research is needed to understand immunogenicity and answer the remaining questions related to treatment by phage such as timing, duration, methods of delivery and route of administration. Limitations of present studies include the reduced spectrum of bacteria tested, which are limited to MRSA and *P. aeruginosa*, without considering coagulase-negative staphylococci (CoNS), which substantially contribute to PJI onset [29]. In addition to these there is a concern with regards to the immunogenicity of phages and resulting diminished therapeutic efficacy [30].

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