B23: Are there any methods to detect biofilms *in vivo*?

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Response/Recommendation:
No. There are no effective *in vivo* biofilm detection methods available for clinical practice. At this time, there are novel detection methods in development or in an experimental phase.

Level of Evidence: Moderate
Delegate Vote:

Rationale:
Periprosthetic joint infection (PJI) diagnosis is guided by identifying the microorganisms responsible, which are most often protected within the biofilm matrix. Biofilm formation on surfaces is an intrinsic defensive strategy that bacteria utilize to survive to shield them from environmental stresses, such as the host immune response and antibiotics (3). Therefore, developing reliable methods that can detect and characterize biofilm in vivo is essential to guide PJI management (1). There continues to be a knowledge gap in translating the pathogenesis of biofilm formation in PJI with the clinical signs and symptoms of infection, such as pain, loss of function, implant loosening, fever, and erythema. There is strong evidence to support the notion that biofilm formation poses numerous challenges in PJI management by contributing to culture negative infection, a subtherapeutic response to antibiotics, and overall treatment failure (2).

Many microbes in their natural habitats are found in biofilm ecosystems attached to surfaces, and not as free-floating (planktonic) organisms. Furthermore, it is estimated that nearly 80% of human infections are associated with biofilms. Biofilms are generally defined as three-dimensional, structured microbial communities that are attached to a surface and encased in a matrix of exopolymer material (4).

The current understanding of biofilm structure and composition arises from numerous preclinical studies that tested biofilm under various conditions and on different types of organic and inorganic surfaces. However, there are currently no standard biofilm detection methods available for clinical practice. Therefore, there is a need to develop clinical methods that can reliably detect biofilm formation to aid in the accurate diagnosis of PJI. In orthopedic implant associated biofilm infections, for example, current detection methods are based on nonspecific X-ray or radiolabeled white blood cell imaging, coupled with peri-prosthetic tissue or fluid samples taken invasively, and must be cultured (9).
The most widely accepted standard method for detecting and identifying the causative microorganism is cultures from patient derived samples, yet this method frequently fails to detect biofilm encased bacteria due to their resilient nature. These methods typically yield low sensitivity and specificity when biofilm is involved, causing clinicians to miss the underlying infection. Furthermore, biofilms can produce small colony variants and dormant cells that are particularly difficult to detect and may not grow under standard culture conditions (5).

Despite the correct implementation of special diagnostic culture techniques, such as tissue sample processing, prolonged incubation time, or sonication of removed implants, a considerable number of bone and joint infections (BJI) are either culture-negative or

misjudged as aseptic failure. Misinterpretation may lead to wrong or needless antimicrobial treatment, or even to unnecessary surgery (8).

The most frequently used methods to detect biofilms *in vivo*?

1.- Specific dye staining:

Dyes such as crystal violet or safranin red are used, which can adsorb to the biofilm and allow it to be viewed under an optical microscope.

2.- Scanning electron microscopy (SEM):

Provides detailed images of the biofilm and the prosthesis surface, allowing the three-dimensional structure of the biofilm to be observed.

3.- Molecular methods:

Non-culture techniques based on nucleic acid amplification, sequencing, and mass-spectrometry methods have been described. Polymerase chain reaction (PCR) is a frequently used technique in most microbiology laboratories for detection of a nucleic acid fragment by amplifying a sequence. PCR can help identify the presence of biofilm forming microorganisms.

3.1 Broad-Range PCR

The value of broad-range PCR has been extensively studied in the diagnosis of bone and joints infections, but extraction of pathogen DNA from bone, joint tissue, or implants remains challenging. Disruption of the biofilm is an essential step to release the DNA in order to improve the sensitivity of broad range bacterial PCR. Pathogens can be identified even if the genus or species are unknown using universal primers to amplify bacterial or fungal DNA, followed by identification of the species by sequencing, a technique which is also called universal PCR. This method is applied in isolated strains, or used directly from clinical samples, where the detection and identification of the pathogen by conventional techniques remains difficult or impossible (8).

Broad-range PCR consist of two steps: the amplification of the bacterial or fungal DNA within the sample, and the subsequent sequencing of the PCR fragment for the identification of the microorganism. The regions of the genome that are used must fulfill fundamental characteristics. First, they must be present in all bacterial or fungal species; second, they should contain highly conserved sequences to which the primers are directed; and finally, they have to include polymorphic sequences, in order to distinguish different species. After amplifying and sequencing the fragment, the obtained sequence is compared across public databases such as NCBI GenBank. For sequence alignment, programs such as BLAST are available, and allow online sequence comparison. In bacteria, species identification at the molecular level is based on analysis of the 16S rRNA gene sequence.

Broad-range PCR allows the identification of microorganisms previously not thought to cause infection, despite it being less sensitive than targeted or multiplex PCR. The main disadvantages of broad-range PCR are lack of sensitivity, false-positive results resulting from contamination, need of subsequent sequencing, and the challenge of result interpretation. During a prospective multicenter cross-sectional study, Bemer et al. (10) demonstrated a sensitivity of only 73.3% in the diagnosis of prosthetic joint infections (PJI). Although new PCR assays have been developed, the sensitivity of PCR for the diagnosis of PJI is variable in different studies, whereas its specificity is reliably high, facilitating the exclusion of PJI (11).

3.2 Targeted PCR

Targeted or specific PCR can be developed for any known microorganism, and can be designed to be extremely sensitive. The analysis is typically performed in real-time, because the amplification process and detection occur simultaneously in the same closed vial. Furthermore, it is possible to measure the amount of DNA synthesized at each moment during amplification, since the fluorescence emission produced in the reaction is proportional to the amount of formed DNA. Different companies have developed an automated, easy-to-use, fast, and accurate real-time *Staphylococcus aureus* identification PCR which can be combined with the search for methicillin resistance (*mec*A and/or *mec*C gene) to optimize the management and the appropriate treatment of the patient, especially for acute septic arthritis cases.

3.3 Multiplex PCR

Multiplex PCR is a technique in which more than two sets of primers are involved in the process of amplifying various target sequences, allowing the simultaneous detection and identification of different genes. The main advantage of these systems is the ability of grouping in a single process different targeted PCR, simplifying the process, saving time and cost, as well as shortening the diagnostic time. In a recent meta-analysis, it has been shown that multiplex PCR from sonication fluid of prosthetic implants is reliable and of great value for the diagnosis of BJI. Several groups have investigated different multiplex PCR panels. However, these tests have mainly been developed for bloodstream infections. Thus, their use for the rapid diagnosis of BJI is off-label. (12-13)

3.4 Next-Generation Sequencing Approach

The advent of next-generation sequencing (NGS) technologies, including 16S rRNA amplicon sequencing, shotgun metagenomics, and meta transcriptomics, is revolutionizing pathogen detection. Multiplex PCR and 16S rRNA sequencing have demonstrated improved sensitivity compared to traditional cultures (12), although the dependence of PCR on specific primers limits its range. NGS can detect a wide range of organisms, including non-culturable and non-viable ones, present in a joint, a bone biopsy, or a tissue in contact with the device. This is possible with metagenomic NGS providing detailed genomic data, and meta transcriptomic NGS offering insights into active infections (9-13). However, the primary challenge is of potential microbial DNA contamination, which may occur either during sampling, linked to the reagents, due to contaminated instrument surfaces, or in the environment. Therefore, the interpretation of results requires strict criteria coupled with clinical knowledge in order to detect fastidious microorganisms and to rule out contaminants.

However, there is also a risk of false-positive results, especially with *Cutibacterium acnes*, a bacterium from the skin microbiome, or with environmental bacteria linked to water. In addition, false-negative results may occur, due to the limited data-base. Therefore, each case should ideally be discussed in a multidisciplinary team. Read counts and depth of genome coverage are important to distinguish non-cultured potential microorganisms detected from uninfected case negative controls. This constitutes a major challenge, and the background reads should be analyzed carefully to determine their relevance.

Despite the lack of a generally recognized standard in all studies analyzed with NGS technology in BJI, it seems important to define a threshold depending on the reads detected. Moreover, to limit this bias between pathogens and contaminating microbial DNA, an

effective microbial enrichment and DNA isolation remains crucial to allow better analysis of the samples. Thus, using a strict protocol and prudent interpretation allows us to correctly recognize contaminants.

The NGS approach will be an additional diagnostic test when standard of care testing is uninformative. However, in the near future, it will still be limited to specialized laboratories, where a sequencing platform and bioinformatic pipeline are available, and the lab workers have the required ability, the costs for this technology will decrease, allowing a broader use of the NGS approach.

4.- Fluorescence microscopy:

Fluorescent dyes or immunofluorescence techniques highlight the biofilm, making it easier to observe using fluorescence microscopy. The following summarizes the current state of Bioluminescence and Fluorescent Imaging technologies (BLI and FLI) as applied to Biomaterial-Associated Infections (BAI). BLI offers the opportunity to observe the in vivo course of BAI in small animals without the need to sacrifice them at different time points after the onset of infection. BLI is highly dependent on the bacterial cell metabolism, which makes BLI a strong reporter of viable bacterial presence. Fluorescent sources are generally more stable than bioluminescent ones and specifically targeted, which renders the combination of BLI and FLI a promising tool for imaging BAI. The sensitivity and spatial resolution of both imaging tools are, dependent on the imaging system used and the tissue characteristics, which makes the interpretation of images, in terms of the location and shape of the illuminating source, difficult. Tomographic reconstruction of the luminescent source is possible in the most modern instruments, enabling exact localization of the colonized implant material, the spread of infecting organisms in surrounding tissue, and the immunological tissue reactions (14).

Bioluminescence imaging (BLI) is a valuable tool for longitudinally monitoring fungal biofilm formation and antifungal treatment in small animal models. Although detecting a quantifiable BLI signal from biofilms inside implanted catheters is challenging, BLI is a practical method for studying fungal biofilms. BLI can be used for in vitro and in vivo studies of device-related biofilm formation by C. albicans and C. glabrata, as well as for testing antifungal activity against these biofilms (15).

Bioluminescence imaging can detect Staphylococcus aureus biofilms on vascular prostheses in vivo. Silver coated prostheses exhibited the lowest number of viable bacteria, indicating superior bacterial resistance. The polytetrafluoroethylene and polyester prostheses had similar levels of bacterial density, with a slight, non-significant advantage for the polytetrafluoroethylene. Bioluminescence imaging was more accurate than standard bacterial enumeration for detecting the number of viable S. aureus bacteria in the biofilm (16).

5.- Other Methods

Optical coherence tomography (OCT) can detect and differentiate endotracheal tube biofilms in intubated critical care patients. Catheter-based 3D OCT can be used to detect and monitor the formation of biofilms in endotracheal tubes (ETTs) of intubated critical care patients in vivo. OCT-derived attenuation coefficient images can differentiate between mucus and biofilm in the ETTs. The OCT and attenuation coefficient image analysis results were correlated with microscopy and clinical data to verify the presence of bacteria and biofilm (17).

A point-of-care fluorescence imaging device can detect bacterial biofilms in vivo by detecting porphyrin-producing bacteria within the biofilm. The red fluorescence detected was not due to the host immune response, but rather from porphyrins produced by the bacteria. Bacteria within biofilms were able to take up ALA from the surrounding environment and produce porphyrins, leading to the observed red fluorescence (18).

Peptide-based probes have also been developed for in vivo diagnostic imaging of bacterial biofilm-associated infections. A peptide probe, 4Iphf-HN17, has been identified that is able to specifically and rapidly label bacterial biofilms without killing the bacteria. The 4Iphf-HN17 probe was able to accumulate in biofilm-infected wounds in animal models, indicating its potential for in vivo diagnostic imaging of bacterial biofilm infections (9).

Method for real-time, continuous monitoring of biofilm infections in a mouse model has been developed using bioluminescent bacteria. This provides a rapid, continuous method to monitor biofilm infections both in vitro and in a mouse model using bioluminescent bacteria on Teflon catheters. Bioluminescent S. aureus and P. aeruginosa were used to effectively assess the physiological state of biofilms in real-time. The mouse model with subcutaneously implanted catheters resulted in a reproducible, localized infection that persisted for at least 20 days (19).

Confocal laser scanning microscopy (CLSM) is a method for detecting and analyzing human oral biofilms in vivo. Different types of oral appliances and substrates can be used, and various microbiological and microscopic methods can also be applied in combination with CLSM. This introduces a new microscopic technique, confocal endomicroscopy, and discusses its potential for in vivo microscopic investigation in the field of dentistry. (20)

Dogs trained on in vitro S. aureus samples can identify the consistent VOC profile of PJI S. aureus biofilm infections, and this opens avenues for further investigations. These advances could revolutionize infectious disease diagnosis and treatment, potentially leading to better patient outcomes and perhaps indirectly addressing the global challenge of antimicrobial resistance. (21)

Microfluidics:
There is a new form of analysis of small volumes of synovial fluid in chip devices capable of detecting biofilm-associated pathogens. These microfluidic platforms are capable of detecting biofilm-specific markers, such as extracellular polysaccharides or microbial DNA, in real time with high sensitivity and specificity, making them a potentially valuable tool in clinical settings (22)

Conclusion: Biofilms create significant diagnostic challenges in the context of PJIs, and various efforts are underway to identify new candidates for preventing and treating biofilm forming bacteria. Chemical agents, along with nanotechnology based methods, have shown promise in antibiofilm activity. Nevertheless, there are currently no in vivo biofilm detection methods available for clinical practice.

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