Protocol for Biofilm Evaluation on Explanted Orthopedic Hardware

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Of all hospital acquired infections, approximately twenty percent are surgical site infections (SSIs),[1] due to the use of metal implants. SSIs are common to the orthopedic specialty and are often associated with implanted hardware causing severe negative outcomes for both acute and long-term recovery. Complications include chronic/persistent infection and pain. A major factor that is thought to increase the risk of developing a SSI is the formation of bacterial biofilm in the wound. Biofilms are communities of bacteria that behave differently than single, individual (planktonic) bacteria. The study of biofilms is an emerging and diverse area of microbiology spanning both basic and applied sciences. Biofilms are especially difficult to treat, due to their nonresponse to antibiotics, and understanding biofilms role in SSIs is essential. Determining the degree to which biofilms cause SSIs will change advance both medicine and public health allowing health care providers to make better decisions regarding standard of care and ensuring better outcomes.

INTRODUCTION

Hospital acquired infections are a problem for both the practice of medicine and public health. Since surgical site infections (SSIs) account for twenty percent of these infections, the investigation of their cause and prevention is an important field of study.[1,2,3] This study focuses on SSIs following orthopedic surgery; however, it is important to mention that in addition to SSI there are other categories of chronic wounds affected by bacterial biofilms which are equally devastating to patients' quality of life, including Diabetic Foot Ulcers (DFUs), Venous Leg Ulcers (LVUs) and Pressure Ulcers (PUs).[3]

As with chronic wounds, SSIs do not follow the normal progression of wound healing, remaining in the inflammatory phase. These wounds are difficult to treat, especially when accompanied by antibiotic resistance from biofilm, which researchers believe play a significant role in SSIs.[1,2,3,4,5] Biofilm is a type of microbial growth that behaves differently from planktonic bacteria. They can contain heterogeneous or homogeneous collections of species. Biofilm is unique not only due to certain traits (i.e. antibiotic resistance), but in that they are surrounded by an protective matrix that assists in attachment and defense against physical and chemical treatments.[6] As part of the biofilm life cycle, a fully formed biofilm sheds planktonic species that spread to other areas of the wound bed, creating more biofilm. According to Edmistin et al. and Phillips, it has been found that up to sixty to eighty percent of SSIs may involve microbial biofilm. In addition, typically effective treatment regimens have little effect on these infections despite the fact that they are efficient at killing the same bacteria grown under normal (planktonic conditions).[4,6]

Treatment of these infections include disrupting the biofilm after it has already formed in the surgical site, antibacterial strategies, and targeting the orthopedic implants themselves through various alterations.[7] First, treatments aimed at disrupting the biofilm are used to disperse the bacteria and break the protective matrix, which allows for the bacteria to essentially function as planktonic once again and be susceptible to typical treatments. Methods used to disrupt microbial biofilm include electric pulses delivered to the biofilm or the use of cytotoxic agents, such as citric acid.[7] Secondly, since antibiotics are ineffective in treating these infections, other antibacterial strategies are being investigated, including the use of bacteriophages. Last, orthopedic implants themselves can be modified to hinder bacterial growth and biofilm attachment, a necessary step in formation. Titanium alloys and implant coatings that effect the metabolic pathways of microorganisms in the SSI are all current alterations being applied to implants.[7] In addition to the novel treatments focused on reduction and prevention of biofilm, the surgical standard of care varies with the type of implant and the length of time since surgery.[8] Once an orthopedic implant becomes infected and nonresponsive to treatment, it must often be removed and a spacer will be placed until there is sufficient healing to perform additional surgeries.

However, before any treatment decisions can be made by health care professionals, confirmation of infection and/or biofilm is essential. Pain at the implant site or acute signs of infection, such as erythema and/or drainage, are what cause the patient to return to their physician. This pain could also be due to other aseptic implant problems as well. If the implant is not infected, removing it and prescribing a regimen of antibiotics may be unnecessary and place

patients at risk. Therefore, the accurate diagnosis of SSI, specifically of biofilm, should not be undervalued.

Unfortunately, definitive diagnosis of biofilm-related infections proves challenging as well, due to the subtle symptomology of implant-associated infections and the fact that unlike other chronic wounds, they are entirely contained within the body. Biofilm diagnosis relies on molecular methods, physical culture, and imaging, which is due mainly to the fact that common laboratory tests, such as leukocyte count and erythrocyte sedimentation, are often inconclusive after the patient undergoes surgery.[5,7] Bone biopsy and swab of the presumed infected area can be cultured and sent for histopathology. Culture of biofilm samples is often difficult and provide false negatives if the transition of *in vitro* from *in vivo* growth is not straightforward. This process also relies on the location of where the sample was taken, which is not homogenous across the entire surgical site but can be improved by sonication of the implant if it is removed to dislodge possible bacteria and then culture the sonicate.[7] The three most common-cultured organisms resulting from SSIs are *S. aureus*, *S. epidermidis*, and *P. aeruginosa*, all biofilm forming species.[9] Histopathology of bone biopsy has a low sensitivity, reported at 43%.[7] Molecular methods can provide higher sensitivity. In a study conducted by Mauffrey et al.,[7] the usage of polymerase chain reaction (PCR) was able to positively identify 88% of samples while standard culture techniques identified only 23%.[7,10] Finally, imaging of bacterial biofilm on explanted hardware using scanning electron microscopy (SEM)[9] can be used to determine the presence of bacterial biofilm but shows only a small section of a much larger picture and may be missing important information regarding the route of entry and vulnerable sites of the implant.

While diagnosis methods continue to improve, the major flaw in identification of these infections is time. Standard culture methods allow for anywhere from 3-14 days for full growth of any possible microorganisms and PCR can take a similar time span.[5,8] For physicians attempting to make treatment decisions in the operating room, time is an important factor and waiting on these results is not an option. Therefore, methods that would allow for identification bacterial biofilm as a rapid point of care and without removing the impact unnecessarily would be extremely valuable in reducing the burden of SSIs. Drawbacks from previously used diagnostic methods have the potential to be solved using whole surface staining. In this publication, alkaline phosphatase immunohisto-chemistry is evaluated as a staining method for bacterial biofilms.

METHODS

Culture Methods

Culture of Mature Biofilm. For experimentation, mature three-day *S. aureus* and *P. aeruginosa* biofilm was grown using an ex vivo porcine skin model developed by Yang et al.[11] *S. aureus* strain ATCC3556 and *P. aeruginosa* PAO1 cultures were allowed to reach log phase (approx. 10^8 CFU/ml) and then loaded onto prepared 12-mm porcine skin biopsies. Prior to loading, porcine skins were prepared by creation of an artificial wound through a burr hole and sterilized with chlorine gas.[11] After sterilization, porcine skins were plated onto TSA soft agar plates and inoculated with log phase cultures. Mature biofilm formation is achieved after a three-day incubation at 37°C.

Staining Methods

Alkaline Phosphatase Immunohistochemistry. To validate the ability to detect biofilm, porcine skin biopsies inoculated with mature biofilm were stained using alkaline phosphatase immunohistochemistry. Biopsies were fixed with 10% neutral buffered formalin for 24 hours and stored or washed in 70% ethanol prior to staining. As part of standard immunohistochemistry protocol, the skins were first blocked using a 10% goat serum prepared using tris-buffered saline containing 0.1% Tween 20 (TBS-T) for 30 minutes. Without a washing step, the skins were immediately transferred to a primary antibody solution at a concentration of 1:1000 diluted in serum for one hour. Polyclonal rabbit antibodies for both S. aureus and P. aeruginosa were used as primary antibodies ordered from Abcam (ab20920, ab68538). All biopsies were washed with TBS-T three times before being transferred to a secondary antibody solution at a concentration of 1:5000 diluted in serum for 30 minutes. Anti-rabbit IgG polyclonal antibody raised in goat was used as a secondary antibody for both S. aureus and P. aeruginosa infected skins (Sigma-Aldrich, A3867). After three washes with TBS-T, all biopsies were transferred to an alkaline phosphatase substrate solution BCIP/NBT (Sigma-Aldrich, B5655) for no more than 30 minutes. Biopsies were carefully watched to prevent overdevelopment and removed from substrate after sufficient color change. Finally, biopsies were washed with deionized water and immediately imaged using macrophotography.

Biopsies infected with each species but not exposed to the primary antibody solution were used as controls and left with blocking serum for this step**.** In addition to the controls mentioned above (infected but not exposed to primary antibody) noninfected skins were also subjected to the entire protocol as a control. Last, a third control was created by exposing a noninfected skin to only the alkaline phosphatase substrate step of the protocol.

RESULTS

Mature biofilm was able to be positively detected using alkaline phosphatase immunohistochemical staining. For both *S. aureus* and *P. aeruginosa* infected biopsies a definitive color change (in comparison to control porcine

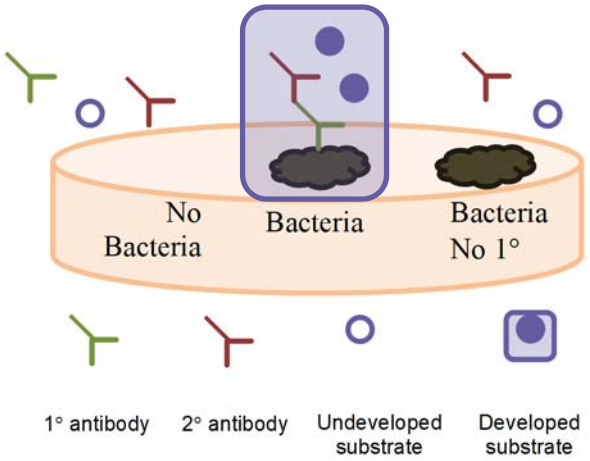

Figure 1. Schematic representation of alkaline phosphatase immunohistochemistry leading to different observable results on porcine explants. Substrate can not detect live vs. dead bacterial culture.

skins) was achieved after the substrate step of the protocol as seen in Fig. 2. To show the detection ability of staining protocol, *P. aeruginosa* porcine skin (Fig. 2e) was wiped prior to staining.

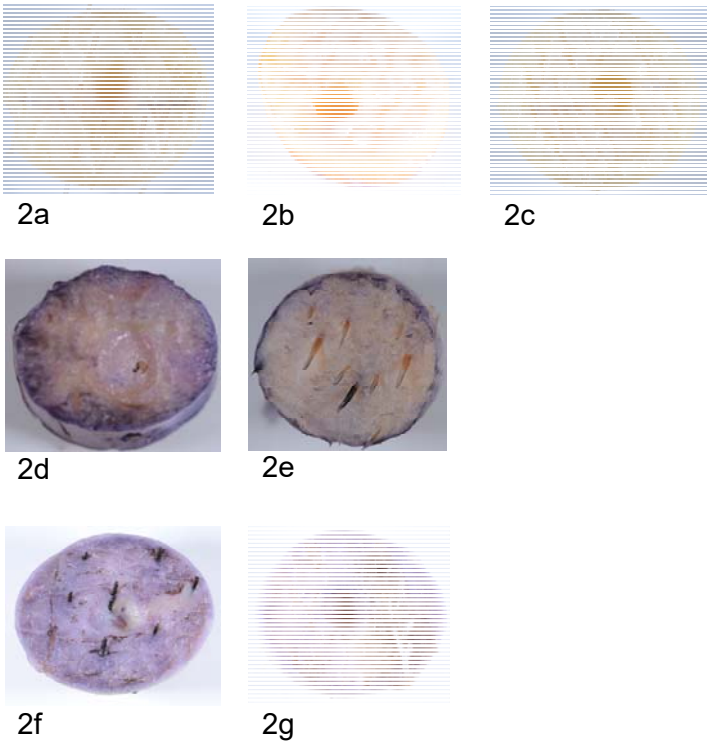

Figure 2. Images of biopsy staining. Image 2a-2c are controls for verification of staining and various checks of the protocol. Images 2d and 2e are images of positively identifed P. aeruginosa infected porcine biopsies. To show dilution effects, 2e was wiped prior to staining.

In comparison to normally prepared biopsy (i.e., no wiping), the depth of stain shows a contrast and demonstrates that the protocol can detect various levels of bacterial concentration.

Images 2f and 2g are positively identified *S. aureus* infected porcine biopsies.

Controls represent verification of not only staining but also staining protocol. First, the noninfected porcine skins subjected to entire protocol, Fig. 2a, demonstrate that positive identification, i.e. color change, is limited to infected porcine skins. Second, infected skins that were left with a serum block and were therefore not exposed to primary antibody, Fig. 2b, are provided to account for any nonspecific antibody binding or retention. Last, the control in Fig. 2c represents a noninfected porcine skin exposed only to the substrate step of the protocol. This controls for any bacterial derived alkaline phosphatase that might be present.

All controls show minimal, if any, staining. Staining observed in controls is limited to areas where porcine skin was bisected or original areas exposed by biopsy punch during preparation, which is most likely due to the high fat content in these areas of the porcine skin and is also presented in infected biopsies as seen in Figure 3.

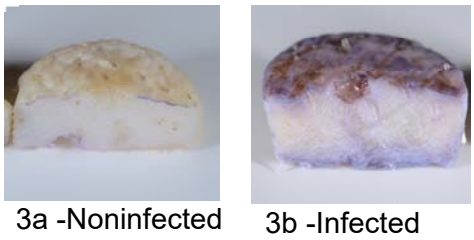

Figure 3. Comparison images of staining at compromised sites of biopsy

The protocol was verified for identification of both *S. aureus* and *P. aeruginosa* infected porcine skins. Finally, alkaline phosphatase immunohistochemistry stains based on presence of bacterial infection and can not discern live/dead bacteria. However when considering treatments, i.e. debridement, information about dead bacteria is equally important due to the fact that they are inflammatory, which is one of the main blockades to wound healing and a component of chronic wounds.[3]

DISCUSSION

Due to the impact that SSIs have on the health care system and patients, studies for ways to combat and more effectively treat these infections is necessary. In the United States, estimated monetary costs of chronic infections tops $25 billion annually and long term costs for patients often include numerous surgeries, complications and potential deaths[1,2]. The degree to which bacterial biofilm leads to chronicity and treatment failure of SSI has been shown previously.[1,2,3,4,5] Therefore, research concerning new treatments and diagnostic methods must consider biofilm.

A rapid staining method for biofilm holds the potential to impact the way physicians and patients experience and treat SSI. Identification of biofilm in the operating room would allow health care professionals to make informed decisions on a case-by-case basis for each patient depending on the

degree of infection or lack of infection. This study effectively demonstrates that biofilm can be stained and lends value to pursuing a non-toxic and rapidly detected method.

Future Directions

From the information gained through this study, it is evident that more research is necessary. First, this study also provides necessary background research and context into development of a non-toxic stain for usage in implant associated surgeries. Second, while the identification of biofilm on porcine skin has been verified it must now be demonstrated on orthopedic hardware explanted from patients with suspected infection. With the assistance of orthopedic surgeons, collected hardware will be stained using alkaline phosphatase immunohistochemistry to glean important information. This information includes areas of the implant that are more susceptible to infection and can therefore be targets for implant alterations. Secondly, staining of explanted hardware can also be used to show possible routes of infection, especially sutures, which have been suggested as a model for how bacteria enter the wound bed created during implant surgery.[4,13]

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