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Experimental Model of Biofilm Implant-Related Osteomyelitis To Test Combination Biomaterials Using Biofilms as Initial Inocula

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Abstract

Currently, the majority of animal models that are used to study biofilm-related infections utilize planktonic bacterial cells as initial inocula to produce positive signals of infection in biomaterials studies. However, the use of planktonic cells has potentially led to inconsistent results in infection outcomes. In this study, well-established biofilms of methicillin-resistant *Staphylococcus aureus* (MRSA) were grown and used as initial inocula in an animal model of a Type IIIB open fracture. The goal of the work was to establish, for the first time, a repeatable model of biofilm implant-related osteomyelitis wherein biofilms were used as initial inocula to test combination biomaterials. Results showed that 100% of animals that were treated with biofilms developed osteomyelitis, whereas 0% of animals not treated with biofilm developed infection. The development of this experimental model may lead to an important shift in biofilm and biomaterials research by showing that when biofilms are used as initial inocula, they may provide additional insights into how biofilm-related infections in the clinic develop and how they can be treated with combination biomaterials to eradicate and/or prevent biofilm formation.

Keywords

Planktonic; Biofilm; Initial Inocula; Animal Model; Infection

Introduction

After a careful literature review, it appears that currently, the majority of animal models that are used to study biofilm-related infection utilize planktonic bacterial cells as initial inocula to produce positive signals of infection.^{1–24} The expectation has been that planktonic bacteria would, depending on the animal model, attach to host tissue or a medical device and subsequently form a biofilm. These animal models have been crucial in the development of novel therapeutic agents to treat and prevent biofilm-related and other infections. However, although the value of these animal models cannot be underestimated, the use of planktonic bacteria as initial inocula has provided inconsistent results in the repeatability of infection development. More specifically, results have shown that when planktonic cells are used as initial inocula, without any antimicrobial intervention, rates of infection are inconsistent between ~47% and 100%.^{1–24} In addition to these inconsistencies, these models have potentially limited biomaterials scientists, clinicians and other investigators from obtaining additional insights into the effect that bacteria might have if they contaminate a site while residing in well-established, mature biofilms.

In 1978, Costerton *et al.*²⁵ provided a general hypothesis that bacteria in nature reside predominantly in the biofilm phenotype. Since that time, data has indicated that 99.9% of bacteria in natural ecosystems reside in the biofilm phenotype.²⁶ Similar data has been derived from samples that have been collected from patients, retrieved medical devices and other sources.^{27–33} Moreover, the Centers for Disease Control has stated that 60% of all infections are biofilm-related.³⁴ A public announcement of The National Institutes of Health placed that estimate at 80% (see announcement PA-07-288). In addition to these estimates, chronic wounds are now considered to be the result of acute infection that begins with biofilm contamination as opposed to a non-healing wound that is later contaminated.^{35–38} A review article has been published discussing this concept of using biofilms as initial inocula.³⁹

Based on these observations and information, the authors hypothesized that a sizeable portion of biofilm-related infections, including those that accompany the use of medical devices, may be the result of contamination with bacteria residing in biofilms from natural ecosystems as opposed to planktonic bacteria contaminating a site. If supported, this hypothesis would suggest that using biofilms as initial inocula, as opposed to planktonic bacteria to model clinically relevant infection scenarios, may provide deeper insight into how biofilm-related infections may be treated and eradicated using current or novel therapies, such as antimicrobial eluting coatings.

In an attempt to address the sporadic nature of biofilm infection development in animal models, wherein planktonic cells are used as initial inocula, the goal of this work was to test the above hypothesis and establish a reproducible experimental model of biofilm implant-related osteomyelitis in sheep. More specifically, in this study, biofilms of methicillin-resistant *Staphylococcus aureus* (MRSA) were grown on the surface of polyetheretherketone (PEEK) membranes using a modified CDC biofilm reactor.^{40, 41} These biofilms were placed in apposition to the proximal medial aspect of a sheep tibia, on the bare cortical surface stripped of periosteum, and subsequently covered by a simulated fracture fixation plate. As such, this study modeled a massively contaminated Type IIIB Gustilo open fracture, wherein, in a worst case clinical scenario, biofilms from natural ecosystems may contaminate a wound site and be compressed between bone and a fracture fixation plate.

Materials and Methods

Biofilm Growth

For this study, a fresh, clinical isolate of MRSA was used. This isolate was collected from the knee of an infected patient and was passaged less than three times on Columbia blood agar. The isolate was confirmed to be one that formed biofilms as indicated by the presence of the *icaADBC* gene cluster, black colony growth on Congo Red agar and direct imaging of its growth on PEEK membranes using scanning electron microscopy (SEM). To grow the biofilms on PEEK membranes, a modified CDC biofilm reactor was used. The development and repeatability of growing biofilms in this reactor, as well as SEM images of the biofilms, have been published previously.^{40, 41}

In short, biofilms were grown by first inoculating the reactor with 500 mL of modified Brain Heart Infusion (BHI) broth that contained 1 mL of a 0.5 McFarland standard of the MRSA isolate. In this instance, a 0.5 McFarland standard equated to $\sim 10^7$ cells/mL. Following inoculation, the reactor was incubated on a hot plate set at 34° C for a 24 hour period. After the initial 24 hour growth period, a flow of dilute (10%) BHI broth was flowed through the reactor for an additional 24 hours using a peristaltic pump set at a rate of 6.94 mL/min. With the hot plate set at 34° C, the broth temperature was $30.2^\circ \pm 0.7^\circ$ during the first 24 hours and $30^\circ \pm 0.8^\circ$ C during the second 24 hours.

After the 48 hour growth period, eight PEEK membranes (the reactor held a total of eight membranes) containing MRSA biofilms were removed from the reactor, rinsed 3x in sterile PBS and transferred to 5 mL of BHI broth. The biofilms were then transported to the surgical suite for inoculation into animals. Two surgeries were performed on any given surgery day, which required the use of four PEEK membranes (two per animal). At the end of each surgery day, the remaining four PEEK membranes were used to quantify the number of bacteria that were present. Each PEEK membrane was found to have an average of $5.05 \times 10^9 \pm 2.07 \times 10^9$ (9.67 ± 0.18 when $\log_{10}$ transformed) colony forming units (CFU) of bacteria. These numbers were not significantly different from the previously published numbers of bacteria that grew on these PEEK membranes.

The quantification process to determine the number of bacteria was published previously. In brief, PEEK membranes were vortexed for 1 minute, sonicated for 10 minutes and plated on tryptic soy agar using a 10-fold dilution series to quantify the number of CFU per membrane.

Stainless Steel Plates

To model a clinically relevant material that is used in fracture fixation plates, 316L stainless steel (SS) plates were used as simulated fracture fixation plates. Each plate was machined to 2×2 cm with a height of 1.85 mm. The plates had a hole, measuring 2.7 mm in diameter, drilled in each corner. On the underside of each plate, a well was machined to a depth of 300 μ m with $1.2 \text{ cm} \times 1.2 \text{ cm}$ dimensions. The purpose of this well was to provide a contained area to place a PEEK membrane (Figure 1). Prior to implantation, plates were cleaned and passivated following American Society of Testing and Materials (ASTM) standard F86-04, then sterilized by autoclave.

Surgical Procedure and Post-Surgical Monitoring

All animal work was performed with approval from the Institutional Animal Care and Use Committee (IACUC) and the Environmental Health and Safety (EHS) department at the University of Utah. For this study, 2–3 year-old female Columbia Cross sheep, weighing 90 ± 20 kg, were selected. This species of sheep had a flat area of bone on the proximal medial aspect of the tibia with a surface area that was suitable for securing of the plates with trans-cortical screws. A total of $n=10$ sheep were used in this study with $n=5$ being treated with biofilm to serve as positive controls of infection (Group 1), and $n=5$ serving as negative controls of infection (Group 2). The 5 negative control sheep were treated with PEEK membranes that had been run through the modified CDC biofilm reactor without bacterial inoculation.

Prior to surgery, the sheep were fasted for >12 hours. Since the goal of this work was to develop a positive signal of infection, no antibiotics were administered. Sheep were initially anesthetized using an intravenous (IV) injection of either ketamine (5 mg/kg) and diazepam (0.5 mg/kg) or propofol (3–7 mg/kg) to allow for endotracheal tube intubation. Following intubation, the sheep were placed in the supine position and maintained under anesthesia with isoflurane to effect (ranged from $\sim 2\text{--}3\%$). An IV catheter was placed in the left forelimb and 0.9% saline was administered at a rate of 10ml/kg/hr. Veterinary surgical and equipment technicians monitored the sheep's heart rate, temperature, carbon dioxide and oxygen levels, and respiration throughout the procedure.

The right hind limb of each sheep was circumferentially clipped free of hair/wool, from immediately above the hoof to the groin, and then scrubbed and prepped with betadine and alcohol treatment. The hoof was isolated in a sterile rubber glove and wrapped with sterile VetWrap (Fisher Scientific). After sterile draping, the proximal medial aspect of the leg and

the region of the incision was treated with Chloraprep (Fisher Scientific) solution to further sterilize the skin.

An anterior midline sagittal incision was made from the region of the tibial tuberosity and extending distally, parallel to the anterior margin of the tibia. This incision was placed away from the plate and biofilm implantation site to avoid contamination of the site during wound healing. Dissection was carried medially and posteriorly, close to the bone, lifting the skin with the attached subcutaneous tissues from the surface of the medial tibial flare.

Since this study modeled a Type IIIB Gustilo open fracture—which may consist of periosteal stripping, bone exposure and massive contamination—a 2×5 cm area of periosteum was removed from the proximal medial aspect of each sheep tibia (Figure 2A). On the bare cortical surface, the positions of a proximal and distal plate were templated by sequentially drilling and placing transcortical screws through each plate. The screws were not tightened at this stage. This technique allowed compensating for any irregularity in the “flat surface” of the tibia, tapped each hole and prevented thread stripping in the thin bone. This prepositioning avoided spurious contamination of the site when placing the infectious biofilm.

A PEEK membrane (with or without biofilm, depending upon the animal group) was aseptically removed from the 5 mL of BHI broth using sterile forceps. The corner of the membrane was touched against a sterile towel, removing excess broth but not biofilm, and preventing broth from contaminating the surgical field. The membrane was placed into the well of a stainless steel plate and the fluid that remained on the membrane allowed it to adhere to the metal plate due to fluid cohesive forces.

Using careful, aseptic technique, the SS plate/PEEK membrane construct was placed in apposition to the tibia with the PEEK membrane residing between the bone and plate. Each plate was secured to the bone using 2.7 mm diameter×10 mm length cortical bone screws (self tapping; catalog #ST270.10, Veterinary Orthopaedic Implants). This process was performed twice in each sheep such that each sheep was treated with two PEEK membranes and two plates (Figure 2B). The rationale for using two plates was to have one available for microbiological analysis and one for histological analysis at the end of the study. To the best of the surgeons' ability, the two plates had a space of ~1 cm between them, however, anatomical variation existed amongst the sheep and not all were able to maintain exactly 1 cm of space between them.

After plate placement, a swab of the cortical bone surface was collected to determine if bacteria had already begun to dislodge away from the biofilm on the PEEK membrane. The surgical site was closed with interrupted 2-0 Vicryl (catalog #J339H, Ethicon) subcutaneous sutures and a running subcuticular 2-0 Prolene (catalog #8533H, Ethicon) suture. A swab of the incision site was taken to determine if bacteria were present on the skin. Prior to wrapping the surgical site, the leg was cleaned with saline and isopropyl alcohol to kill bacteria that may have been present on the skin. This helped to reduce the risk of cross contamination throughout the animal facility and between animals.

For post operative analgesia, each sheep was given an epidural dose of morphine (0.1 mg/kg) and two fentanyl patches (100 µg/hr) were placed in a shaved area on the left forelimb. An injection of flunixin (1.1 mg/kg) was administered to diminish inflammation. Post anesthesia monitoring extended until the animals could stand on their own, as well as eat and drink.

Surgical Follow Up

Throughout the course of the study, each sheep was monitored by the authors' team and a veterinarian in the animal quarters to assess any symptoms of pain and distress. Under veterinary supervision, animals that showed signs of pain or distress were treated with Buprenex (0.01 mg/kg) or additional fentanyl patches. If excessive inflammation was present, they were further treated with rimadyl (4.4 mg/kg).

Using a clinical grading system, based on the hallmarks of infection: calor (heat), rubor (redness), dolor (pain), and tumor (swelling), the sheep were monitored daily for a 12 week period for these signs of infection. A daily rectal temperature of each sheep was also taken. The animals were also monitored for limping, lethargy, irritability, and going "off feed" and/or water. Based on these criteria, a four tiered clinical grading system was established.

Grade I, or no infection, consisted of the signs of healing normally seen with surgical trauma and that resolved within one to two weeks of surgery. These signs included slight redness at the surgical site, mild warmth (to the touch), mild inflammation, a closed suture line, healed within 2 weeks, the sheep eating and drinking, a normal rectal temperature (for these sheep normal temperature was between 101.5° F and 102.8° F), and no signs of distress or limping. A Grade II infection included increased redness, a warmer surgical site with moderate inflammation, evidence of suture line dehiscence, irritable behavior, normal temperature and not limping. Sheep were euthanized if they displayed signs of a Grade III infection. This grade was characterized by significant redness and palpable heat at the surgical site, an open suture line with drainage, significant inflammation, tenderness, lethargy, fever, off feed and/or water, and positive bacterial growth on wound culture. A Grade IV infection was defined, but never allowed to develop in any of the animals. This included excessive heat, excessive inflammation, purulent drainage, implant exposure, excessive limping, local tenderness, off feed and/or water, lethargy, and fever.

Bone Labeling

As stated by Bloebaum *et al.*,⁴² "Fluorochrome labeling is a well established method of measuring the mineral apposition rate (MAR), at which osteoid matrix, produced by osteoblast cells, is deposited and mineralized to form new bone."

In this study, calcein fluorochrome (catalog #C-0875, Sigma-Aldrich) was used as a non-antibacterial agent to label bone and to calculate the mineral apposition rate (MAR), i.e. the growth rate of the sheep bone. The method by which this works is after the calcein is injected, it is taken up by osteoblast cells and released into the matrix of newly forming bone. After processing, calcein fluorochrome can be observed in tissue samples as they are imaged using an excitation wavelength of 495nm and emission of 515nm. The imaging that was performed in this study is described in the *MAR Analysis* section below.

Calcein was prepared in reverse osmosis water to a final concentration of 30mg/mL. The pH was adjusted to 7.2–7.4, filtered using a 0.22µm filter for sterility and the solution administered IV at 0.33mL/kg of body weight. Two separate injections were given: one 16 days and one 5 days prior to the established 12 week end point of each sheep to create a double label in the bone. The infected sheep that were euthanized prior to the 12 week end point did not receive calcein injection.

Euthanasia and Microbiological Sample Collection

Sheep were euthanized at the end of 12 weeks, or once a Grade III infection was determined to exist. To euthanize, animals were initially sedated with an IV injection of ketamine (5 mg/kg) and diazepam (0.5 mg/kg) in order to collect a 5 mL blood sample for microbiological

analysis. Euthanasia was then performed by IV injection of beuthenasia (1mL/4.5kg) solution.

A swab of the incision site (~1 cm² area) was taken and streaked onto Columbia blood agar for semi-quantitative analysis. More specifically, 1+ growth was defined as having growth in the first zone of streaking, 2+ having growth in the second zone and 3+ having growth in the third zone. The agar plate was incubated overnight at 37° C. Next, the skin at and surrounding the incision site was prepped using chlorhexidine/isopropyl alcohol antiseptic. A scalpel was used to aseptically re-open the incision site and a swab of the subdermal tissue was collected to determine if bacteria had penetrated into the soft tissues superficial to the plates and PEEK membranes. This swab was also cultured on Columbia blood agar.

One of the SS plates was randomly selected and the underlying PEEK membrane was removed and placed into 5 mL of 10% BHI broth. The sample was vortexed for 1 minute, sonicated for 10 minutes and allowed to recover in the broth at room temperature for 20 minutes (to allow the bacteria to convert from the biofilm to planktonic phenotype) before performing a 10-fold dilution series to quantify the number of CFU/PEEK membrane.

The SS plate that had been removed was then secured to the bone once again so that radiographs could be taken. The tibia was then disarticulated and used for gross photographic and radiographic analysis.

Gross Photography/Radiography

Gross photography of the soft tissues and bone were collected throughout the sampling/dissection process. Radiographs were obtained using a cabinet x-ray system (43855A Model, Faxitron X-Ray Corporation) set at 70 kV for 2 ½ minutes.

Tissue Embedment/Sectioning

After radiographic imaging, all of the soft tissue was dissected from the bone with the exception of the tissue that was directly over the undisturbed SS plate. The sample was then fixed in modified Karnovsky's fixative using 3×24 hour changes. Following fixation, the bone was rinsed in reverse osmosis water for 10 minutes and cut into two sections separating the two SS plates from one another. The plate that had been removed in order to access the PEEK membrane for microbiology was again removed and the biofilm on the underlying bone imaged with SEM. The remaining bone section, with plate and PEEK membrane intact, was used for histological analysis.

After cutting, both bone samples were placed in 70% ethanol for 24 hours to initially dehydrate them. The bone for SEM imaging was further dehydrated by hand using ascending concentrations (from 70%, to 95% to 100%) of ethanol, then coated with gold using a Hummer 6.2 sputter coater and imaged using SEM.

The bone sample for histology was also dehydrated using increasing concentrations of ethanol. However, this was performed in a Tissue-Tek VIP (Miles Scientific) instrument. It was then placed into a solution of 80% methylmethacrylate (MMA) and 20% N-butyl (the combination of these two solutions is referred to as Solution A), and mixed for 5 days to infuse the tissues. The Solution A was poured out and a fresh aliquot of Solution A, mixed with 2.5g/L of perkadox (the catalyst for polymerization), was added to the sample. The sample was kept in a dessicator at 4° C for 7 days. Finally, 5g/L of perkadox was added to another batch of fresh Solution A and exchanged for the used mixture in the container and the sample was kept in a desiccator at 4° C for an additional 9 days. Samples were then placed in a new container and Solution A with 5g/L of perkadox was added and polymerized in 2cm layers using ultraviolet light. Each layer required 48 hours to fully polymerize. The

final product resulted in a polymethylmethacrylate (PMMA) embedded sample containing the bone, PEEK membrane, stainless steel plate and soft tissue regions.

Once embedded, tissue samples were cut using a band saw to remove excess PMMA and isolate the area of interest. Samples were further sectioned into ~2 mm sections using a diamond blade water saw. Radiographs of the sections were obtained following the same procedure outlined above. One face of a section was then polished, and gold coated for SEM analysis.

SEM Analysis

SEM analysis was performed using a JEOL 6100 LaB₆ filament SEM to qualitatively examine bone and/or biofilm morphology in the region where a stainless steel plate/PEEK membrane construct was implanted. For those bone samples that had the SS plate removed, secondary electron images were collected of the cortical bone surface to examine the morphology of the bone and/or biofilm where the PEEK membrane had been present. For those samples that were sectioned in PMMA, backscatter electron (BSE) images were collected to examine the varying levels of mineralization, how the infection influenced the periosteal response in bone, and cortical bone activity.

MAR Analysis

The procedure for collecting MAR data was based on the published work of Bloebaum *et al.* In short, after sample sections had been imaged using SEM, the polished surfaces were glued to a slide and further ground to ~50–60 μm thickness for MAR analysis. Images were first collected using a mercury lamp Nikon Labophot microscope to detect the presence of calcein double-labeled osteons of the host bone. Three slides from each sheep were analyzed. From each slide, five osteons were randomly selected in the cortical/periosteal bone region beneath a stainless steel plate and a total of eight measurements were made along the span of each double label using ImagePro Plus software. The MAR of bone was calculated using the formula:

$$\text{MAR } (\mu\text{m}/\text{day}) = \square_x(e)(\pi/4)/nt$$

Where $\square_x$ is the sum of all the measurements between double labels, e is the micrometer calibration factor (μm), $(\pi/4)$ is the obliquity correction factor, n is the total number of measurements, and t is the time interval between calcein injections expressed in days.

Histology

For histological analysis, sample slides were further ground to a thickness of 40–50 μm and stained with H&E stain. For H&E staining, Mayer's solution was preheated to 50–55°C. Slides were placed in the Mayer's solution for 5 minutes, rinsed and dried. Slides were then placed in Clarifier for 4 minutes, rinsed, placed in Bluing Reagent for 4 minutes, rinsed again and dried with a Kimwipe. Finally, slides were counterstained by dripping Acid Fuschin/5% Acetic Acid solution for 35–45 seconds and dipped in 100% ethanol for 30 seconds.

Macroscopic images of slides were collected using a Nikon SMZ800 macroscope. Higher magnification images were collected using a Nikon Eclipse E600 microscope. Using a modified histopathologic grading scale of Smeltzer *et al.*⁴³, an outside observer, who was blinded to the samples in the study, examined the slides to determine what level of osteomyelitis was present in the bone and or surrounding tissue regions. Osteomyelitis was indicated by the presence of bacteria, as determined by the microbiological analysis, in

conjunction with chronic inflammation and bone necrosis. Cortical bone growth/response was not an indicator of infection as it was present in all five sheep from Group 1 and three from Group 2. Thus, it appeared to be a normal bone response to the surgical trauma and implantation. The modified Smeltzer *et al.* grading scale is provided in Table 1.

Statistical Analyses

From a Kaplan-Meier survival curve, a Log-Rank test was used to examine the statistical significance in survival times between those sheep treated with biofilm and those that were not. A separate Log-Rank test was used to compare the time it took for animals in both groups to become infected. Time to infection differed from survival time since some sheep in Group 1 survived the full 12 weeks of the study, but displayed signs of infection very early on.

Because the number of bacteria collected from PEEK membranes of those sheep in Group 1 were not normally distributed, the non-parametric Mann-Whitney U test was used, as opposed to a student's *t* test, to compare the number of bacteria that were collected on the PEEK membranes of Group 1 and Group 2 animals. In all instances, an alpha level of 0.05 was established to define statistical significance. All statistical data was analyzed using SPSS 17.0 software.

Results

Surgical Follow Up

A survival curve including each of the 10 sheep in this study was plotted using a Kaplan-Meier survival curve (Figure 3A). Two of the five sheep in the biofilm treated sheep (Group 1) were euthanized at 3 weeks due to a Grade III infection. The other three survived to the 12 week end point, but each of those three sheep displayed Grade II signs of infection during the 12 week monitoring period. Furthermore, each of the five sheep in Group 1 displayed signs of inflammation and abscess formation between day 4 and 11 after surgery. In contrast, all five of those sheep in Group 2 survived to the 12 week end point with minimal acute inflammation and no signs of infection at any point in the study. The Log-Rank test indicated that the difference in survival among the two groups was not significant ($p=0.184$). However, when time to infection was analyzed, there was a significant difference ($p=0.002$) between the groups (Figure 3B). This difference corroborated with the microbiological data.

Microbiology

Microbiological data showed that PEEK membranes collected from Group 1 sheep contained an overall log density of $5.32 \pm 5.41 \log_{10}$ CFU/PEEK membrane. No bacteria were detected on the PEEK membranes of Group 2 sheep. When compared using the Mann-Whitney U test, this difference was statistically significant ($p=0.008$).

All of the swabs taken from all animals showed between 1+ and 2+ growth of normal flora bacteria at the incision site. In three out of five of the Group 1 sheep, subdermal swabs detected 2+ growth of bacteria in the tissues surrounding the stainless steel plates, whereas swabs taken of the remaining two sheep detected no growth of bacteria. All of the culture swabs collected from Group 2 sheep were negative for growth.

These data indicated that the microbiological findings correlated with the clinical observations of the sheep, wherein those treated with biofilm (Group 1) suffered a Grade II or higher infection, and those not treated with biofilm (Group 2) did not suffer from infection.

Notably, when removing the SS plates for microbiological analysis, it was observed that in each of the five Group 1 sheep, the bone screws had become completely loose and in one animal the plates had even shifted position. This was due to bone resorption around the screws, which was a result of infection as indicated by SEM analysis and histological results (see below). No screw or plate loosening was observed in Group 2 sheep and it was also interesting to observe that in Group 2 sheep, the cortical bone began to grow on/attach to the PEEK membranes, which made it difficult to remove them for quantification. In contrast, the PEEK membranes in the infected sheep had no attachment of bone to them and were easily removed.

Gross Photography/Radiography

Gross photographs of the sheep limbs provided evidence that an abscess formed in the surgical area of Group 1 sheep, whereas no abscess formation was seen in sheep from Group 2 (Figure 4). Furthermore, signs of infected tissue, including pus and significant inflammatory and cortical bone response, could be seen in Group 1 sheep once the skin was resected (Figure 4B). In contrast, only a thin membrane of tissue grew over the plates in Group 2 sheep with minimal periosteal/cortical bone response.

Radiographic evidence also suggested that in Group 1 sheep, “moth eaten,” osteomyelitic bone was visible (Figure 5A). This result was particularly apparent in the microradiographs that were taken of bone sections after they had been embedded and cut (Figure 5B). From these sections a significant cortical bone response and an endosteal response indicative of responsive new bone formation could be seen in Group 1 sheep. No such response was seen in Group 2 (Figure 5C, D).

SEM Analysis

Due to the natural complexity of the cortical bone surface of sheep and components that resembled bacteria, it was difficult to confirm that there were biofilm dwelling bacteria on the cortical bone surface of Group 1 sheep using secondary electron SEM imaging. Although it did appear that the bone surfaces of Group 1 sheep had more degradation and trauma when compared to the bone surfaces of Group 2 sheep, the differences were not deemed substantial enough to support any conclusions. On the other hand, BSE images of the bone sections were much more indicative of bone trauma and infection.

More specifically, in Group 1 sheep, BSE images indicated that there was a considerable amount of bone resorption directly underneath the stainless steel plate (Figure 6A) and near the bone screws (Figure 6B), which supported the observation mentioned above that these screws were loose, whereas no signs of bone resorption were apparent in any sheep from Group 2 (Figure 6C, D). Furthermore, a much larger gap was seen between the cortical bone surface and plate of Group 1 sheep when compared to Group 2. More specifically, the distance from the bone to the plate surface in Group 2 was ~20–50 microns, whereas in Group 1 the gap was much larger and ranged from 200–800 microns. As will be seen in the histopathological results, this larger gap in Group 1 sheep was due to fibrous tissue formation and chronic inflammation—which the microbiology confirmed to be the result of infection.

MAR Analysis

MAR results indicated that in Group 1 sheep, the average bone remodeling rate was ~1.5 $\mu\text{m}/\text{day}$. In Group 2 sheep the average rate was ~1.2 $\mu\text{m}/\text{day}$. Images of double labels in the periosteal regions of Group 1 sheep showed that an intense remodeling response was present (Figure 7A). However, typical double labels of osteons were seen in the cortical bone regions (Figure 7B). No significant response was seen in the periosteal regions of sheep in

Group 2 and osteon structures were present in the cortical bone region indicative of remodeling (Figure 7C, D). Notably, calcein double labels further indicated bone viability.

Histology

Sections stained with H&E showed that there was an observable difference between the bone and soft tissues of Group 1 and Group 2 sheep. More specifically, when compared to the modified Smeltzer *et al.* histopathological grading scale, the three sheep in Group 1 that survived to the 12 week endpoint displayed signs of a Grade 4 osteomyelitis as indicated by moderate to severe chronic inflammation with significant intramedullary fibrosis and multiple foci of sequestra (Figure 8). These sheep also displayed Grade 3 cortical bone growth. The other two sheep in Group 1, which survived to 3 weeks, displayed Grade 3–4 osteomyelitis with Grade 2 cortical bone growth. None of the five sheep in Group 2 showed signs of osteomyelitis and were scored with a Grade 0 osteomyelitis. However, three of the five sheep in Group 2 did display Grade 2 cortical bone growth, whereas the other two were scored with Grade 0 cortical bone growth. Taken together, these results suggested that cortical bone growth/response could have been due to surgical trauma or the presence of infection, yet a notable difference in bone morphology was present in those that were infected versus those that had a response due to surgical trauma. More specifically, those that had infection showed signs of “moth eaten” bone that had jagged edges due to resorption/bacterial presence, whereas those with a cortical bone response due to surgical trauma had woven bone formation with little indication that resorption was occurring.

Discussion

Using biofilms as initial inocula in this study addressed at least three major limitations, which have been reviewed previously,³⁹ that may accompany the use of planktonic bacteria in animal models of infection, such as those that are designed to develop combination products of biomaterial coatings and other antimicrobials. 1) Planktonic cells may be cleared by the immune system more readily than cells residing in a biofilm. Thus, when planktonic cells are used in *in vivo* models, it may be that they are eradicated before they can form biofilms. As mentioned, this may contribute to the low reproducibility for the induction of osteomyelitis. 2) It is becoming ever more evident that planktonic bacterial cells are more susceptible to antibiotics than those residing in a biofilm. If antibiotics are administered prophylactically immediately following inoculation, they may affect planktonic bacteria more effectively and rapidly than they would biofilm bacteria. 3) When planktonic bacteria are added to the body, the possibility exists for them to be dispersed rapidly away from the site of initial inoculation due to the presence of flowing fluids in the body. This could dilute the concentration of bacteria per given area—potentially making it easier for the body to handle the bacterial load and prevent attachment to tissue or a medical device.

Notably, osteomyelitis developed in all five sheep from Group 1 of this study treated with biofilm, which strongly supported the hypothesis that using biofilms as initial inocula would cause infection. The hypothesis was further supported, and the data made significantly stronger, by the fact that none of the sheep from Group 2 became infected. This 100% vs. 0% rate of infection provided a promising outcome for future tests of combination biomaterials for device development to be performed in a repeatable fashion using this model.

The biofilms in this study appeared to have a gradual adverse clinical effect on the sheep. More specifically, osteomyelitis developed slowly, and persisted without significant signs of distress in 3 of 5 of the animals in Group 1. This may be similar to biofilm-related infections that are seen clinically. In patients, biofilm-related infections can take months or years to develop, and may persist without significant morbidity or mortality.³² This may be due to

the quiescent nature of biofilms and the fact that they have already established a community. Planktonic bacteria have yet to develop a community and it appears that their “goal” in nearly every ecosystem is to find a location to colonize and then begin to develop a biofilm community.⁴⁴

Importantly, there were no sclerosing agents used in this study to promote the development of osteomyelitis, whereas in previous studies these noxious agents have been commonly used to initially kill bone and/or tissue with the intent to make it more susceptible to infection.^{45–48} In addition, the model in this study also appeared to circumvent the problem of low reproducibility for the induction of osteomyelitis, which was cited by Gaudin *et al.*⁴⁹ as an important limitation of animal models of osteomyelitis.

In two of the sheep from Group 1, bacteria were not detected in the surrounding tissue regions of the stainless steel plate. This was likely due to the limited area of sampling with the swab culture technique. Bacteria may not have been in those tissue regions, and thus were not detected. However, bacteria were collected from the PEEK membranes of both of these sheep, which corroborated with the SEM, MAR and histopathological data—all of which supported the conclusion that infection had developed.

At least three limitations accompanied this study and will need to be addressed with future work. First, these results are based on the use of a single species of microorganism and although the outcomes are hypothesized to be similar with other biofilm forming organisms, different organisms may lead to different results. More specifically, although *S. aureus* is a common cause of metal, device-related infections,⁵⁰ a wide variety of other organisms can cause biofilm-related infections including *Pseudomonas aeruginosa*, *Enterococcus faecalis*, coagulase negative staphylococci, *Escherichia coli*, *Acinetobacter baumannii*, *Klebsiella pneumoniae* and others. Second, these results will need to be confirmed with other material types. For example, at least one study has shown that titanium implants have reduced infection outcomes when compared to stainless steel.⁵¹ Third, as this was a developmental model to test the ability of biofilms to cause infection, antibiotics were not used. However, if this were a clinical scenario, prophylactic antibiotics would have accompanied the implantation of the devices. Thus, future work will be needed to address these limitations.

In conclusion, to the authors’ knowledge, this is the first animal model utilizing biofilms as initial inocula to produce a positive signal of reproducible biofilm-related infection. As such, the model provides a promising outcome in that it may be used by future researchers and clinicians to utilize a reproducible model to examine the therapeutic potential of novel systemic antimicrobials and/or antimicrobial coatings on biomedical devices to treat and prevent biofilm-related osteomyelitis, as well as other biofilm-related infections. It may be that the use of this and similar animal models using biofilms as initial inocula will result in an important shift in the field of biofilm research that adds onto the work that has been done with planktonic bacteria. This shift may lead to the development of novel antimicrobial therapies, such as coatings on devices, that could help prevent biofilm-related infections in a more effective manner. In short, the development of this experimental model may have tremendous implications in the future of biofilm implant-related infection treatment strategies as well as other biofilm-related infections.

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Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Figure 6.

Figure 7.

Figure 8.

Table 1**Histological parameters and scoring system**

Intra- and peri-osseous chronic inflammation
0 Not present
1 Minimal to mild chronic inflammation with no significant intramedullary fibrosis
2 Moderate to severe chronic inflammation with no significant intramedullary fibrosis
3 Minimal to mild chronic inflammation with significant intramedullary fibrosis
4 Moderate to severe chronic inflammation with significant intramedullary fibrosis
Bone necrosis
0 No evidence of necrosis
1 Single focus of necrosis without sequestrum formation
2 Multiple foci of necrosis without sequestrum formation
3 Single focus of sequestrum
4 Multiple foci of sequestra
Cortical bone response
0 No cortical bone response
1 Cortical bone growth that does not extend beyond plate border
2 Cortical bone growth that begins to extend beyond plate border
3 Cortical bone growth that covers a plate part way
4 Cortical bone growth that covers a plate entirely