In vitro investigations of Staphylococcus aureus biofilms in physiologic fluids suggest that current antibiotic delivery systems may be limited.

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ABSTRACT
Orthopaedic surgical site infections, especially when hardware is involved, are associated with biofilms. Clinical strategies for biofilm eradication still fall short. In this manuscript we use a novel animal model of long bone fixation with vancomycin or gentamicin controlled release and ask what levels of antibiotic are achieved at the site of release and in the surrounding tissue. We then ask, using fluids that contain serum proteins (synovial fluid or diluted serum), what levels of vancomycin or gentamicin are required to substantially reduce colonizing bacteria in a model representative of prophylaxis or of established biofilms. In the in vivo model, while levels in the first 24 h are up to 50X MIC immediately adjacent to the antibiotic release system, they quickly fall. At peripheral sites, values never achieve these levels. In the in vitro experiments, Staphylococcus aureus biofilms formed in serum or in synovial fluid show a 5-10 fold increase in antibiotic tolerance. Importantly, concentrations required are much greater than those achieved in the local delivery systems. Finally, we determine that the staged addition of vancomycin and gentamicin is not more efficacious than simultaneous vancomycin and gentamicin administration using planktonic bacteria, while for biofilms, the staged addition is suggestive of an improvement over adding the antibiotics simultaneously. Overall, our data show that amounts of antibiotics near an implant in an animal model fall short of the concentrations required to eradicate biofilms formed in either synovial fluid or in serum.
INTRODUCTION

To minimize surgical site infections (SSI), orthopaedic surgeons routinely administer antibiotic prophylaxis (Parvizi et al., 2017; Sweet et al., 2011), especially in revision surgeries (Jiranek et al., 2006). However, the challenge of local, perioperative, prophylactic antibiotics is the lack of consensus on the amount, the timing, the release profile, and the type of antibiotic that would be most beneficial, resulting in a great variability in antibiotic prophylaxis (Chen et al., 2018; Kamath et al., 2016). Utility of perioperative prophylaxis, such as systemic administration of cefazolin or vancomycin (VAN) is accepted (Parvizi et al., 2017). While the increased benefit of local antibiotics in the absence of clinical infection remains controversial (Jiranek et al., 2006; Parvizi et al., 2017), local antibiotic prophylaxis continues to be used, exemplified by the placement of VAN powder during wound closure in spinal implant surgery (Khan et al., 2014; Lin et al., 2021), and by use of drug-loaded local delivery systems such as gentamicin (GEN) sponges (Han et al., 2016). In all cases, antibiotic prophylaxis is designed to eradicate the perioperative contaminants that have the potential to progress to SSI.

The choice of antibiotics, especially for prophylaxis, is guided by principles set forth by the American Academy of Orthopaedic Surgeons enhancing the surveillance of three quality measures nationally related to infection prevention, namely that patients: 1) receive prophylactic antibiotics consistent with current recommendations, 2) receive prophylactic antibiotics within one to two hours prior to surgical incision (Bratzler et
al., 2013), and 3) have prophylactic antibiotics discontinued within 24 hours following the end of surgery (Berbari and Baddour, 2020; Hansen et al., 2013) These guidelines largely consider systemic and intravenous administration of antibiotics rather than locally administered and contained drugs. The selected antibiotics are dictated by the fact that *Staphylococcus aureus* (*S. aureus*) and the Coagulase-negative Staphylococci (CNS Staphylococci) are the most common causes of orthopaedic infection (Chirca and Marculescu, 2017; Rao et al., 2008). *In vitro* and *in vivo*, *S. aureus* rapidly forms biofilms and bacterial aggregates/floating biofilms in wound fluid and synovial fluid (SynF). These biofilms show increased antibiotic tolerance (Costerton et al., 1999), and in physiological fluid, tolerance may increase (Dastgheyb et al., 2015b; Gilbertie et al., 2019). Thus, increased local delivery/presence of antibiotics remain as possible solutions (Adams et al., 2009; Jiranek et al., 2006; Parvizi et al., 2017; von Plocki et al., 2012; Schwarz et al., 2021) for both prophylaxis as well as an established infection.

In patients presenting with clinical signs of prosthetic joint infection (PJI), debridement antibiotics and implant retention (DAIR) are often attempted to avoid more invasive interventions required with a one- or two-stage implant exchange (Cobo et al., 2011; Masters et al., 2019). Local antibiotic therapy is provided by antibiotic impregnated polymethyl methacrylate (PMMA) cement spacers or beads, biodegradable polymers or regional limb perfusions (Kanellakopoulou and Giamarellos-bourboulis, 2000; Zalavras et al., 2004). However, the rapid elution kinetics of carrier systems result in antibiotic
levels that often drop below the minimal inhibitory concentration (MIC) and allow the porous, non-degradable carrier matrices to become a substrate for bacterial adherence and biofilm formation (Neut et al., 2001). Antimicrobial tolerance and even fostering of resistance in the presence of sub-MIC antimicrobial concentrations may further complicate successful resolution of the infection (Smith, 2005). Importantly, even when *S. aureus* is added to synovial fluid containing many times the MIC of prophylactic antibiotics, bacterial eradication is attenuated (Dastgheyb et al., 2015b), suggesting that MIC is inadequate for predicting eradication; the situation is only worsened in established infections where biofilm is present. Some researchers have attempted to address this issue by investigating values such as minimum biofilm eradication concentration (MBEC) (Sepandj et al., 2004). Importantly, a higher concentration of antibiotics may be required not only to treat existing biofilms but to prevent bacterial adhesion in the first place.

In this report, we asked whether the concentrations of antibiotics that were eluted from local elution systems in vivo would be sufficient to markedly decrease, if not eradicate, *S. aureus* biofilms, in vitro. We chose systems that mimicked other elution systems, such as the TRYX Absorbable Antibacterial Envelope (Medtronic) (Tarakji et al., 2019), and bioglass-containing systems that are used to deliver drugs in bone (Baino et al., 2016; Soundrapandian et al., 2010). Specifically, we analyzed local extracellular fluid (ECF) data generated from two different antibiotic elution systems, one in an intramedullary
site and the other adjacent to the bone such as would be used in a fracture plate. From these ECF samples, we determined the concentrations and duration of antibiotic elution at the site of implantation, as well as in surrounding tissues. Using these antibiotic ranges as a guideline, we explored concentrations of VAN and GEN, separately and together, that would eradicate 24-hour biofilms or prevent bacterial colonization on the titanium alloy Ti6Al4V and poly lactic acid (PLA). Finally, we assessed the utility of staged combination delivery of VAN and GEN, as antibiotic combinations have been recommended to reduce the risk of resistance (Brooks and Brooks, 2014). Our findings raise questions about the requirements for effective local concentrations of antibiotics against biofilms.

METHODS:

**Ethics Statement:** Anonymized human synovial fluid samples from joint aspirations performed for therapy were retrieved and designated as “waste” and “not human research” by the Thomas Jefferson University Institutional Review Board (IRB), as per the revised Common Rule (2018). The Institutional Animal Care and Use Committee (IACUC) of the University of Pennsylvania approved the ovine study following Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines (du Sert et al., 2018).

*In Vivo Pilot Studies:*
Skeletally mature sheep were enrolled in pilot study of a clean long bone model (tibia) with implanted hardware to determine actual antibiotic concentrations at the site of the elution system (GEN=fractionation fixation plate, VAN=intramedullary (IM) Ti alloy rod) and at sites removed from the eluting antibiotics.

**Surgical Procedures:** Skeletally mature, female Dorset cross-bred sheep, free of clinical disease with unlimited access to exercise were acclimatized for 14 days prior to study enrollment. The day before surgery, based on a detailed physical examination, sheep free of signs of clinical disease were fasted and allocated to VANIM \( n = 4 \), GENPlasma \( n = 8 \), or GENECF \( n = 3 \) study cohorts. On the day of surgery, fasted (24 h) animals received a left jugular catheter using aseptic technique. Following sedation with Diazepam (0.5-1.5 mg/kg, IV), anesthesia was induced with Ketamine (2.2 – 4.0 mg/kg) and animals were endotracheally intubated and placed in dorsal recumbency. Anesthesia was maintained with Isoflurane (1.25 – 5 %) in oxygen and animals were monitored with standard anesthetic equipment supported by a jugular venous catheter, arterial line (blood pressure, arterial blood gas), pulse-oximetry, electrocardiogram (ECG), fraction of inspired oxygen (FiO\(_2\)), carbon dioxide (CO\(_2\)); if necessary animals were mechanically ventilated. No perioperative antimicrobial prophylaxis was provided. Analgesia was provided for three days perioperatively and consisted of transdermal fentanyl patches (2.5 µg/kg/h) placed at 12 hours prior to surgery and left in place for 72 hours and intravenous flunixin meglumine (1.1 mg/kg, every 12-24
hours). Analgesia was continued based on daily pain assessment by a veterinarian.

Using aseptic technique, eleven animals underwent a transverse mid-diaphyseal tibial osteotomy and unilateral plating (9-hole commercially pure Ti locking compression plate (LCP), DePuy Synthes, West Chester, USA); three animals received a non-structural intramedullary Ti alloy rod (Ti6Al4V) coated with VAN and placed in anterograde fashion via the tibial plateau. The IM rod was interlocked proximally with one 3.5 mm cortex screw. Animals in the VAN cohort did not receive a tibial osteotomy. In recovery, the operated limb was splinted with a bi-valve fiberglass splint to provide protection during recovery, during transport and until the animal settled in postoperatively (up to 48 h). After completion of surgery, each animal was observed until it was able to stand and walk to a stall. After complete recovery, it was returned to its housing. Perioperative analgesia (Fentanyl patches at a dose of 2.5 µg/kg/h) was administered for a period of 72 hours (longer if signs of pain were apparent). Clinical scores reflecting pain and animal welfare were recorded daily for two weeks following each surgery, then weekly until sacrifice.

**Drug Carrier Matrices: GEN antimicrobial sleeve:** The tightly adhering, perforated envelope covering the LCP was comprised of polyglytone 6211™, a bioabsorbable 87 lactide-glycolide-trimethylene carbonate-caprolactone polymer. This thin envelope was cast with approximately 43 mg of anhydrous GEN sulfate, which remained in a slurry (because of limited solubility) in the polymer solution and was fabricated so that it
slipped over the LCP, as previously described by von Plocki (von Plocki et al., 2012), (Synthes, West Chester, PA, USA).

**VAN**: the VAN coating of the intramedullary nail consisted of multiple coatings of bioactive glass using a nominal VAN concentration of 20% by weight (percent of drug weight to SiO2 weight) (Adams et al., 2009; Qu et al., 2016). Briefly, intramedullary nails comprised of the titanium alloy Ti6Al4V (length: 140mm; diameter: 6mm) were sandblasted, cleaned, and dried. The sandblasted and cleaned nail (substrate) was further sonicated in acetone for 30 min, and 2% detergent for 1 hour and finally rinsed with deionized (DI) water. A fresh oxide coating was then created by passivation in 35% nitric acid for 1 hour, followed by rinsing with DI water and drying in a laminar flow hood. For the coating procedure, a dipping device with controlled speed and mechanics was used, to ensure even deposition of layers. Each layer was comprised of the sol-gel film containing 20wt% VAN and was dried in a laminar flow hood for 2 hours before application of the next layer for a total of 10 layers. After application of the last layer, the films were dried overnight (Adams et al., 2009).

**Ultrafiltration Probes**: All animals were instrumented with customized ultrafiltration probes (30 KDa), supplied by BASInc and modified at our laboratory to improve durability. Probe distribution was: intramedullary (IM, mid-diaphysis), periosteal (PO, mid-diaphysis) and subcutaneous (SQ, mid-diaphysis) (Fig. 1). Probe tubing was tunneled subcutaneously towards the lateral aspect of the femur and externalized
collection vials were supported in a custom-made hind-body slinky. Collection vials were checked at 1, 2, 4, 6, 8, 10, 12- and 24-hours post-operative and then daily for the duration of the 30-day study. All tubes were replaced at each time point. Fluid was collected from tubes that contained the minimum volume (≥ 0.1 mL) for analysis of local tissue concentration of GEN or VAN.

Drug Analysis: Venous blood samples (K$_2$EDTA sheep plasma) were taken at time -0 (immediately prior to antibiotic doped carrier matrix placement), +0 (immediately following carrier matrix placement), and at probe sampling times for the first 10 days post-operative. After this time, venous blood samples were obtained every 7 days until sacrifice, and at endterm prior to euthanasia. For ECF collection, GEN or VAN samples were obtained following the same sampling schedule as described above.

LC/MS/MS Analysis of GEN and VAN: 0.100 mL of a GEN or VAN sample was mixed by vortexing with 0.200 mL of an internal standard solution (tobramycin at 250 ng/mL in methanol containing 0.1 % formic acid) in a capped 96-well Nunc polypropylene plate, followed by centrifugation. A 0.150 mL aliquot of the supernatant was then mixed by vortexing with 0.150 mL of water/0.1 % formic acid in a clean, capped 96-well Nunc polypropylene plate, followed by centrifugation. This extract was injected onto a high-performance liquid chromatography (HPLC) system equipped with a triple quadrupole tandem mass spectrometer (AB/MDS Sciex API-5000) detector operated in positive TurbolIonSpray® mode. GEN or VAN were separated from extracted matrix materials
using a Varian Pursuit C18 XRs column (50 x 2.0 mm, 3 µm particle size) at ambient temperature using a gradient mobile phase system of 0.2 % heptafluorobutyric acid in water (mobile phase A) and 0.1 % heptafluorobutyric acid in acetonitrile (mobile phase B) at a total flow rate of 0.300 mL/min. Calibration standards, prepared fresh daily at 10.0 to 2000 ng/mL, were used to construct standard curves for GEN or VAN.

*In Vitro Studies:*

**Materials:** Machined Ti6Al4V (10 mm x 2 mm, kind gift of Zimmer Biomet) and 3D-printed PLA (Ultimaker, 1.24 specific gravity, 10 mm x 2 mm) were used. Ti6Al4V was first cleaned with 4 N HNO3. Ti6Al4V and PLA were then rinsed with distilled, DI water, sonicated in 70 % ethanol, 15 min and sterilized under ultra violet (UV) light, 20 min. Samples were stored sterile and dry until inoculation.

**Bacterial strains and growth:** A single colony of methicillin-sensitive *S. aureus* (MSSA) ATCC®25923™ was grown in Trypticase Soy Broth (TSB, Becton-Dickinson) overnight, (ON) 37°C, 180 rpm, subcultured for 2-3 hours, and diluted by comparison to a 0.5 McFarland standard (~10⁶ colony forming units (CFU)/mL for MSSA). MSSA ATCC®25923™ was used as a well-established reference strain (Treangen et al., 2014) known for its biofilm forming capacity (Goggin et al., 2014). Strain integrity was achieved by using frozen subcultures from the commercially available ATCC strain, periodic culturing on blood agar plates to test for hemolysins (Wiseman, 1975), and on
selective maltose salt agar. Strain maintenance is ensured by periodically measuring the antibiotic sensitivity via Etest and MIC experiments (ATCC25923: VAN=2µg/mL; GEN=0.25 µg/mL by Etest).

**Bacterial Adhesion and Biofilm Antibiotic Treatments:** Biofilms were “pre-formed” in a 24 h static culture before antibiotic was added or bacteria and antibiotics were added in a “simultaneous” fashion. For pre-formed biofilm experiments, Ti6Al4V and PLA discs were submerged in 1.0 mL TSB or SynF (using 24 well tissue culture plates (Med Supply Partners), inoculated with 10^5 CFU/mL MSSA, and incubated for 24 h at 37°C. The resulting surfaces were then incubated with 0-500 µg/mL VAN (Athenex) for 24 h, 37 °C, in TSB or human synovial fluid. For simultaneous addition experiments, the Ti6Al4V and PLA discs were submerged and bacteria added using TSB, SynF, 50% serum (both human and fetal bovine: Sigma Aldrich)/TSB or 50% serum/phosphate buffered saline (PBS, MP Biomedicals). 0-100 µg/mL GEN (Alfa Aesar) or 0-100 µg/mL VAN were added at indicated concentrations and times (immediately after inoculation to up to 30 minutes after inoculation), and incubated for 24 h at 37°C. All experiments: surfaces were gently washed with PBS to remove planktonic bacteria and adherent bacteria were resuspended by bath sonication at 40 kHz in 0.3 % Tween20/PBS, 15 min. Suspended bacteria were serially diluted, plated on 3M™ Petrifilm™ (aerobic count, 3M Corporation), incubated, 37°C, 24 h, and counted (countable range, 30-300 CFU/spot).
Checkerboard Assay for Antibiotic Synergy: 96 well tissue culture plates (Med Supply Partners), containing $10^5$ CFU/mL MSSA in Mueller Hinton Broth (MHB) were tested using a matrix of VAN (0-8 µg/mL) and GEN (0-4 µg/mL), with antibiotics added together or separated by 20 min. Fractional Inhibitory Concentration (FIC) index was calculated using MIC, where:

$$\frac{A}{MIC_A} + \frac{B}{MIC_B} = \text{FIC index}$$

A and B are VAN and GEN concentrations in a single well. FIC index <0.5 denotes synergy, >4 antagonism, and 0.5-4 indicates additivity (Meletiadis et al., 2010).

Scanning Electron Microscopy (SEM): Samples were fixed with 4% paraformaldehyde (PFA), room temperature (RT), 15 min, and dehydrated by sequential incubation (RT, 10 min) with 10%, 30%, 50%, 70%, 90%, and 100% ethanol in DI water. Samples were dried overnight (ON), sputter-coated with gold or platinum/palladium and imaged using a Hitachi TM-1000 SEM.

Statistics: In vitro: ≥ 3 separate experiments, each containing 6 independent determinations. For simple comparisons between two populations, statistical significance was determined using the Student’s T-test or Mann-Whitney U test, based on the normality of data. For multiple comparisons, for normally distributed data, a one-way ANOVA with Bonferroni correction and Tukey’s multiple comparison post hoc test was used, with an Alpha value of 0.05; for nonparametric comparisons, a
Kruskal-Wallis test with Dunn’s multiple comparison correction was used (GraphPad Prism ver 8.4.0). In vivo: For the plasma GEN or VAN data (maximum concentration (Cmax), time to max concentration (Tmax) and area under the curve (AUC), and Max concentration)) were calculated. Linear weighted (1/x2) regression analysis of peak area ratio versus theoretical concentration was used to produce calibration curves.

RESULTS

In vivo sampling of ECF using ultrafiltration probes

The in vivo experiments measured the local tissue distribution of antibiotics over time when placed as a delivery system in a clinically relevant location. All sheep in this pilot study had uneventful recoveries from surgery and general anesthesia and completed the study. Vacutainers (Fig. 1) and probe tubing remained in place throughout the study and were well tolerated by all animals.

Local ECF to determine GEN and VAN concentration were collected at 1, 2, 4, 6, 12 and 24 h after probe placement then daily thereafter for up to 30 days. During the first 24 h, sample volumes were variable, ranging from 0.1 to 1.0 mL for the IM probes, from 0.2 to 1.7 mL for the Plate probes and from 0.1 to 1.3 mL for the SQ probes. At longer times, the mean volume per collection time point was 1.4mL for SQ probes, 1.0 mL for Plate probes and 0.2 mL for IM probes. In the VAN sheep cohort, ECF volumes sufficient for
analysis were unable to be reliably collected after day 14 due to tubing tortuosity. This was corrected for the GEN sheep cohort so that sufficient volumes were collected up to 28 days postoperatively.

Local VAN ECF and plasma concentration

When VAN elution from the coated Ti alloy rod was measured, the local VAN concentration rapidly increased in all 4 animals over the first 4 days. Two animals then exhibited a drop-off in VAN concentration, whereas the remaining two showed peak VAN elution at 10 d. The maximum VAN concentration achieved in the intramedullary canal was $C_{\text{max}}$ 15.50 µg/mL in sheep #2 at $T_{\text{max}}$, 10 d postoperatively (Fig. 2). In sheep #1, VAN IM concentration first plateaued at around 6 µg/mL followed by a second release of $C_{\text{max}}$ 8.98 µg/mL, $T_{\text{max}}$ 16 d. Sheep #3 and #4 both showed a $C_{\text{max}}$ around 5 mg/ml at 4 and 6 d, respectively. On the other hand, the plasma, SQ, and Plate VAN concentrations remained below the LC/MS/MS detection limit (0.050 µg/mL) in all sheep. Notably, even at peak concentrations, local VAN did not exceed 5-15X MIC for MSSA.

Local GEN ECF and plasma concentration

Using the GEN polymer system (Fig. 3; total GEN = 43 mg), the maximum mean GEN concentration at the plate was 80.50 µg/mL (0.5 d (12 h)); 13.1 µg/mL peak mean concentration was measured in the intramedullary cavity at 7 d (IM). The drug
concentration in the soft tissue envelope of 9.49 µg/mL was the peak mean concentration measured at 2 h (SQ). In these graphs, both the line resulting from plotting the mean of the concentrations and the individual values from the different animals are represented. Notably, IM and SQ trends reflect rapid elution in most animals during the first several days, whereas the Plate concentrations show variability.

The plasma GEN concentration-time curve follows the trend of the GEN levels for the plate probe but the C_{max} of 0.090 µg/mL at T_{max} 2 h is markedly lower (Fig. 3).

Specifically, C_{max(plasma)} is decreased by approximately factor 100 when compared to the drug concentration obtained from the SQ and IM probe samples and by factor 1000 when compared to the Plate probe samples.

Based on the elution data, at its maximum, the local concentration of GEN reached ~80 µg/mL within the first 24 hours. The maximum local concentration of VAN was ~15 µg/mL over the course of 10 days.

**Ti6Al4V and PLA surface and biofilm observations in SEM**

In order to determine effects of these ranges of concentrations on adherent bacteria, *in vitro*, we first determined the effects of different media and surfaces on biofilm formation using SEM. The bare Ti6Al4V disks showed machining lines, as well as some surface features (Figure 4A) whereas the PLA was 3D-printed so that the filament surface and melted interface were visible. When *S. aureus* was grown on either surface
in TSB, HS/TSB, or EqSynF, abundant bacterial colonization was apparent, with abundant 3D structures independent of media or surface (Fig. 4B). In TSB, colonies are visible in their entire spherical shape, with only a small percentage of the colonies embedded in mucinous extracellular matrix. In HS/TSB and EqSynF images, the mucinous nature of the biofilm is more visible. Specifically, in the PLA, HS/TSB image, small fibers that organize the structure are apparent, with areas covered by a matrix so that individual colonies are obscured. Bacteria grown in EqSynF (SynF is a filtrate of blood, (Felgenhauer and Hagedorn, 1980)) on both surfaces look similar to HS/TSB images, in terms of fibrous connectivity and mucinous matrix.

**VAN tolerance of preformed MSSA biofilms as a function of surface and media**

We first modeled active infection, characterized by surfaces covered with biofilm. VAN tolerance of preformed biofilms depended on the media (Fig. 5); in TSB, up to 500 µg/mL VAN showed no significant effect on MSSA in a 24 h biofilm formed on Ti6Al4V surface (Fig. 5A). For biofilms formed in TSB and on PLA surfaces, 10 µg/mL and 500 µg/mL VAN was statistically different than the control, while 100 µg/mL VAN was not; however, the numbers do not appear to be biologically significant despite the statistical significance, even at 500 µg/mL VAN. Bacterial killing in SynF biofilms showed small (< 2 log) decreases at the 100 and 500 µL concentrations (Fig. 5B) on both Ti6Al4V and PLA. These differences were statistically different from controls and each other at the 100 and 500 µg/mL concentrations.
Concomitant addition of VAN with MSSA

We next modeled bacterial contamination in the presence of antibiotics. In TSB (Fig. 6A), 10 µg/mL VAN significantly decreased MSSA number, with complete eradication by 100 µg/mL, independent of surface. In SynF (Fig. 6B), 10 µg/mL VAN significantly decreased bacterial colonization; at 100 µg/mL, this decrease was more marked on the Ti6Al4V surface compared to the PLA (both surfaces, p<0.0001 compared to the control) (Fig. 6B). Complete eradication only occurred at 500 µg/mL (p<0.0001 compared to the control for both surfaces).

SynF and Serum effects on antibiotic tolerance

Because both serum and SynF are rich in serum proteins, we compared MSSA adhesion on PLA in TSB, 50% human serum (HS) with TSB (HS/TSB), 50% HS with PBS (HS/PBS), and equine SynF (eqSynF), when the bacteria and 10 µg/mL VAN were added simultaneously (Fig. 7A). In the absence of antibiotics, similar average numbers of MSSA were adherent for all four media. When 10 µg/mL VAN was added, average numbers of adherent bacteria decreased by ~4 logs in TSB, HS/TSB and HS/PBS. However, VAN in EqSynF showed attenuated killing (1.5-2 logs). In the presence of antibiotics, numbers of adherent bacteria were highly variable in all media types, partially due to the averaging of 5 experiments to give 27 values/determination.
Overall, the trends show that VAN is less effective on average in EqSynF than in other media.

For measuring the dose dependence of GEN, we used TSB and HS/TSB. The choice of HS/TSB was to supply proteins present in wound fluid. Simultaneous addition of GEN with MSSA resulted in significant decreases in adherent MSSA at all doses compared to the control, both in TSB and HS/TSB ($p \leq 0.0001$) (Fig. 7B). However, increasing GEN doses did not cause greater decreases in bacterial numbers on average.

*Combined VAN and GEN against adherent and planktonic MSSA*

Addition of GEN or VAN alone (10 µg/mL each) or any combination of GEN + VAN caused a decrease in adherent bacteria compared to no antibiotics, and these comparisons reached statistical significance except for control vs VAN only or control vs simultaneous addition of GEN and VAN (G0, V0) (Fig. 8). Simultaneous addition of GEN + VAN did not cause a significant increase in the antibacterial activity over that of GEN or VAN alone. Trends for the staged additions suggested an increased activity, and staged additions were statistically significant compared to no antibiotic control as well as compared to G0, V0, with the exception of G0, V0 vs VAN added 10 minutes after GEN (G0, V10). To further investigate possible additivity of GEN + VAN, antibiotic synergy was determined with the checkerboard assay using planktonic
bacteria. GEN + VAN, independent of time of addition, showed some additivity, but not synergy.

DISCUSSION

Prevention of bacterial adhesion to implanted materials and subsequent biofilm formation are a major concern, especially when implanted in a site that has had a surgical site infection (Ricciardi et al., 2018). Development strategies for eradication rely on local drug delivery systems driven by strain MIC guidelines (Stebbins et al., 2014; Syal et al., 2017). In this report, we detailed a new probe system that allowed measurements of eluted drugs at the site of the implant and within adjacent tissues, in a sterile, long-bone model in sheep. Based on our measured elution rates of 5-50X MIC for VAN and 10-50X MIC for GEN (both determined for MSSA)(Leonard et al., 2013; Wang et al., 2006), we determined the effects of these ranges of VAN and GEN on reduction of bacterial adhesion in an in vitro model system. Based on the in vitro analyses, we concluded that eluted antibiotics in this range were only moderately effective at preventing bacterial colonization, especially when cultured in the presence of SynF or 50% HS.

While VAN or GEN loaded carrier systems are widely used to treat osteomyelitis associated with infected fracture sites and prosthetic joint infections (Schwarz et al.,
2021), there are limited in vivo studies vs. the many in vitro elution determinations (Henry and Galloway, 1995). These studies are further limited by questions about superior efficacy over parenteral antibiotic therapy (Diaz-Ledezma et al., 2014) and optimal dosing regime and duration of therapy for orthopedic infections (Schwarz et al., 2021). Antibiotic impregnated PMMA cements or biodegradable polymers are used for local antibiotic delivery (Garvin and Feschuk, 2005; Kanellakopoulou and Giamarellos-bourboulis, 2000; Rutledge et al., 2003; Stewart, 2002; Wininger and Fass, 1996; Zalavras et al., 2004) and the Musculoskeletal Infection Society consensus states that (1) antibiotic-impregnated cement reduces incidence of periprosthetic joint infections following elective revision joint arthroplasty and that (2) antibiotics should be added to cement in all patients undergoing cemented or hybrid fixation as part of revision arthroplasty (Diaz-Ledezma et al., 2014). Although antimicrobial impregnated PMMA is recommended by the East Practice Management Guidelines Workgroups, adequate tissue levels of antimicrobials may not be achieved without additional systemic antimicrobials (Luchette et al., 2000). An abiding concern in all elution systems is the elution kinetics where the rapid antibiotic release allows antibiotic levels to drop below MIC levels, raising the specter of antimicrobial resistance (Gullberg et al., 2011; Smith, 2005).

Animal models provide an elegant means to analyze local tissue concentrations of antibiotics over time (Orsini et al., 1992; Stolle et al., 2008; Thomassen et al., 2020).
Previous studies included a porcine model of local elution, which used IM microdialysis accompanied by serial bone samples (Stolle et al., 2008; Thomassen et al., 2020) and a canine model with serial aspiration of seroma from surgical sites for analysis of local tissue concentrations of antibiotic over time (Adams et al., 1992). As both studies required anesthesia for continued collection, neither allowed ambulation and the influence of biomechanical load, including gravity on fluid dynamics. Our pilot study that places collection probes at key tissue sites allows for the measurement of these levels without anesthesia and for long times. However, there was variability in our measurements, as not all samples obtained were of sufficient volume to allow antibiotic measurement.

As a priority, this study established a robust methodology to sample ECF from a region of interest (i.e. tibia and soft tissue envelope) in an ambulating model over a clinically relevant time. In our pilot study, plasma concentrations of VAN and GEN demonstrate levels consistent with literature values for VAN prophylaxis (Yusuf and Croughs, 2020). $C_{max}$ values measured for the IM canal and the fracture plate site are in the 10-100x MIC range for staphylococcal species, consistent with other reports (Gustafson et al., 2016; Khan et al., 2014; Liu et al., 2014; Selph and Carson, 2011), and consistent with the most common organisms responsible for orthopaedic SSIs (Hickok and Shapiro, 2012). However, based on our and others’ data in vitro, reduction in bacterial adhesion and biofilm formation requires high levels of antibiotics (Costerton et al., 1999)—these levels
become even higher when serum proteins are present (Gilbertie et al., 2019). We thus asked what effects antibiotic concentrations in these ranges would have on adherent bacteria cultured in fluids derived from physiological environments, i.e., SynF and serum.

We used pre-formed biofilms to model implant contamination such as would be observed in established infections. In keeping with many other studies (Dastgheyb et al., 2015a; Donlan and Costerton, 2002; Mandell et al., 2019), we showed that these biofilms are tolerant to antibiotics both in TSB and SynF. While the biofilm that we used was relatively immature (24 h), even these biofilms exhibited marked insensitivity, underlining the difficulty in decreasing numbers of already adherent bacteria. It has been suggested that a 48 h biofilm would better model \textit{in vivo} implant contamination and hence treatment strategies (Baeza et al., 2019). We would suggest that our limited success with antibiotics would disappear with these more mature biofilms which we would view as modeling established infection. Another consideration for our studies is the known time-dependent effects of VAN on bacterial eradication (Post et al., 2017). It is not clear how this property will impact bacteria exposed to the rapidly decreasing VAN measured in the sheep model, especially as the 100-200 mg/mL VAN was maintained for greater than 7 days in Post et al., 2017. It will be important to determine if this time-dependent killing can be exploited \textit{in vivo} to enhance eradication of adherent bacteria.
Faced with the difficulty in eradicating established biofilms, prevention of bacterial adhesion, as exemplified by the antibiotic prophylaxis that occurs concomitantly with perioperative contamination, becomes a focus. Of clinical importance is the fact that bacterial adhesion takes place within minutes to hours (Hall-Stoodley et al., 2004; Saeed et al., 2019), and longer antibiotic prophylaxis regimens may not significantly alter infection rates (Bondarenko et al., 2019). Our experiments are aimed at determining conditions where a biofilm-prevention concentration (BPC) (Macia et al., 2014) of antibiotics may be reached.

Our in vitro BPC model, in which bacteria are added simultaneously with antibiotics, showed that 5x MIC VAN decreased bacterial adherence in all media, but 50X MIC was required in TSB and 250X MIC in SynF (Fig 6). Using GEN, 40X MIC was sufficient to reach a 2-log decrease in average bacterial counts (Fig 7B), but even in the presence of 400x MIC GEN, bacterial adhesion was still measured. Importantly, the presence of serum proteins in the 50% HS or the SynF samples attenuated the effectiveness of the antibiotics. We note that we cannot determine the relative ratios of eradication of the planktonic bacteria or increased susceptibility of newly-adherent bacteria associated with increasing levels. While our in vitro investigations are limited in scope, VAN has been clinically demonstrated to be an effective local treatment while the data for GEN is less clear (Lin et al., 2021); our data may support reservations for the use of GEN as a solo treatment.
Finally, because GEN-elution systems are used to supplement perioperatively administered VAN, we asked the effect of staged addition of the two antibiotics. VAN and aminoglycosides like GEN have been combined and both no synergy (Streuli et al., 2006) and synergy (McGowan, 1998) reported against Staphylococcal species. There have been mixed results as to whether VAN and GEN, as well as other antibiotic combinations with VAN, show synergism, in particular against methicillin-resistant Staphylococcus aureus (MRSA) (Deresinski, 2009; Mulazimoglu et al., 1996). Interestingly, a staged addition of the aminoglycoside streptomycin with the cell-wall active penicillin enhanced efficacy against planktonic Escherichia coli (Davis, 1982). VAN and amikacin, another aminoglycoside, have demonstrated synergism against MSSA in planktonic form, but not when biofilm-embedded (Broussou et al., 2018). We thus asked whether the cell-wall targeted VAN would synergize with the aminoglycoside GEN against planktonic and adherent MSSA. In a checkerboard assay, we observed additive effects against planktonic bacteria, independent of order or timing of addition. When adherent bacteria were analyzed, concomitant addition of GEN + VAN was not additive and furthermore not statistically different from controls. Staged delivery showed trends towards enhanced activity, where 20 minute gaps consistently showed statistical significance against the control as well as concomitant addition of GEN + VAN. These staged addition studies, while only explored in TSB, suggest additional strategies, albeit translation to a clinically realistic protocol may be challenging.
There are limitations to our studies. We have used MSSA ATCC®25923™, a widely used, biofilm-forming reference strain (Treangen et al., 2014), for determination of the antibiotic effects. Our findings would be more generalizable with additional strains of MSSA as well as CNS Staphylococci. Another limitation arises in our use of different media. We used the ideal medium TSB as well as media rich in serum proteins, i.e., SynF aspirated from the joint (Felgenhauer and Hagedorn, 1980) or diluted HS as a surrogate for wound fluid (Buchan et al., 1981; Cutting, 2003; Katz et al., 1991). The commonality of serum proteins between the two fluids provides a framework for the proteinaceous bacterial matrix characteristic of in vivo bacteria aggregates and biofilms. We explicitly tested the impact of these fluids and while different, the antibiotic sensitivity of MSSA in HS and SynF was markedly reduced, although not equivalent. Based on the presence of serosanguinous fluid immediately post operatively, even in the joint, (whereas SynF would be more characteristic of longer times), and the clear increase in antibiotic resistance with the presence of serum proteins, we continued with the more easily sourced 50% serum. Of course, these fluids derived from tissue environments, while more faithful to in vivo conditions, do not replicate the native cells nor the immune cell-rich environment of the tissue (Spear, 2012). We have also not measured serum binding of drugs. Importantly, our in vivo results, demonstrate a dynamic antibiotic concentration profile, whereas our in vitro experiments maintain the designated antibiotic concentration for the duration of the experiment. In vitro biofilm
characteristics such as growth age and exposure time (Chen et al., 2020) as well as timeline of antibiotic therapy (Post et al., 2017) are important to consider when evaluating in vitro results (Chen et al., 2020). In vivo, Castaneda et al. have demonstrated that a five day course of antibiotic exposure lowered the MBEC compared to a 24 hour exposure, suggesting that a 24 hour model may overestimate minimum concentrations needed to eradicate biofilms in vivo (Castaneda et al., 2016). We suggest that in vitro experiments using constant, high antibiotic concentrations for 24 h may underestimate antibiotic requirements in vivo where falling concentrations confound the antibiotic effects.

Our findings raise questions about the effect of local concentrations of antibiotics against contaminating bacteria. Our data would suggest that even perioperative sterilization of surrounding tissue or implant surfaces would be quite difficult, suggesting that additional study of both in vitro and in vivo systems is needed, especially in the context of the applicability of MIC to therapeutic outcomes.

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LEGENDS

Figure 1: Probe Placement in sheep long-bone.

The radiograph shows the LCP with GEN delivery system (not visible in radiograph), with osteotomy, and the three UF probes in the three distinct tissue compartments: intramedullary canal (red), periosteal surface (green) and soft tissue envelope (yellow). The UF probes for the VAN coated Ti alloy rod were placed in the same fashion.

Figure 2: VAN concentrations over time at the site of antibiotic elution

**ECF VAN concentration** at each sampling interval from the IM ultrafiltration probe location: Sheep 1 $C_{\text{max}}$ 8.98 $\mu$g/mL, $T_{\text{max}}$ 16 d. Sheep 2 $C_{\text{max}}$ 15.5 $\mu$g/mL, $T_{\text{max}}$ 10 d. Sheep 3 $C_{\text{max}}$ 4.69 $\mu$g/mL, $T_{\text{max}}$ 4 d. Sheep 4 $C_{\text{max}}$ 5.3 $\mu$g/mL, $T_{\text{max}}$ 6 d.

Figure 3: GEN ECF and plasma concentrations over time
GEN ECF concentrations at each sampling interval from 3 ultrafiltration probe locations: intramedullary cavity, subcutaneous (SQ), periosteal/adjacent to plate (Plate). Each ECF graph shows data from individual sheep as well as the average plotted as a continuous black line. IM probe: maximum mean concentration ($C_{\text{max}}$) 13.1 $\mu$g/mL, time of $C_{\text{max}}$ ($T_{\text{max}}$) 7 d. Plate probe: $C_{\text{max}}$ 80.5 $\mu$g/mL, $T_{\text{max}}$ 12 h, SQ probe: $C_{\text{max}}$ 30.0 $\mu$g/mL, $T_{\text{max}}$ 0 h. B. Plasma GEN concentration at each sampling interval with a $C_{\text{max}}$ 90.5 ng/mL, $T_{\text{max}}$ 2 h. This plasma GEN concentration is below reported trough levels of less than 2 $\mu$g/mL. Values from 8 animals were used to determine the shape of the elution curves. The dotted line represents the limit of detection, 0.010 $\mu$g/mL and only data points that were above this detection limit were used in this analysis. The error bar is missing for 24 h time point as n < 3 samples due to the detection limit.

**Figure 4: Dry surface and biofilm morphology in different media**

A. Dry, clean Ti6Al4V and PLA surface structures as visualized by SEM. B. Biofilms (24 h) formed in TSB, HS/TSB, or EqSynF on Ti6Al4V or PLA surfaces, as visualized by SEM.

**Figure 5: VAN tolerance of biofilms in TSB, SynF**

Effects of increasing doses of VAN on bacterial number in 24 h biofilms as a function of medium (A. TSB, B. SynF) and surface n=18 independent observations per data point. Statistical determinations are tabulated below figures. Significance n.s. = not significant.
Figure 6: VAN dose response on surfaces.

Number of adherent bacteria 24 hours after concomitant addition of VAN and $10^6$ CFU/mL in TSB (A, n=18) or SynF (B, n=12). Statistical determinations are shown for the different media and surfaces below the histograms, with n.s.= not significant.

Figure 7: Effects of media and concentration on VAN and GEN tolerance.

A. Comparison of different medium on the number of adherent bacteria retrieved (24h) after concomitant addition of 10 $\mu$g/mL VAN and MSSA; n = 27 for each condition, Control and VAN comparison conducted only within each type of media. $d = p \leq 0.0001$.

B. Antibiotic efficacy of GEN when added simultaneously. Numbers of adherent bacteria are shown (n=24 for TSB, 18 for HS/TSB). Statistical determinations are shown for the different media and surfaces below the histogram, with n.s.= not significant.

Figure 8: Staged addition and effects on biofilm.

The x-axis labels denote the time of addition of each antibiotic, GEN = 10 $\mu$g/mL, VAN = 10 $\mu$g/mL; n=30. The FIC scores are presented for the same combinations on planktonic MSSA (n=8). Statistical determinations are shown below the FIC scores, with n.s.= not significant.
Figure 1: Probe Placement in sheep long-bone.
The radiograph shows the LCP with GEN delivery system (not visible in radiograph), with osteotomy, and the three UF probes in the three distinct tissue compartments: intramedullary canal (red), periosteal surface (green) and soft tissue envelope (yellow). The UF probes for the VAN coated Ti alloy rod were placed in the same fashion.
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134x73mm (600 x 600 DPI)
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100x136mm (600 x 600 DPI)
Figure 6: VAN dose response on surfaces.
Number of adherent bacteria 24 hours after concomitant addition of VAN and 105 CFU/mL in TSB (A, n=18) or SynF (B, n=12). Statistical determinations are shown for the different media and surfaces below the histograms, with n.s. = not significant.
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A. Comparison of different medium on the number of adherent bacteria retrieved (24h) after concomitant addition of 10 µg/mL VAN and MSSA; n = 27 for each condition, Control and VAN comparison conducted only within each type of media. d = p < 0.0001. B. Antibiotic efficacy of GEN when added simultaneously. Numbers of adherent bacteria are shown (n=24 for TSB, 18 for HS/TSB). Statistical determinations are shown for the different media and surfaces below the histogram, with n.s. = not significant.
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The x-axis labels denote the time of addition of each antibiotic, GEN = 10 µg/mL, VAN = 10 µg/mL; n=30. The FIC scores are presented for the same combinations on planktonic MSSA (n=8). Statistical determinations are shown below the FIC scores, with n.s. = not significant.

133x125mm (600 x 600 DPI)