Optimization of an *In Vitro* Model of Staphylococcal Abscess Communities to Study Bactericidal Mechanisms

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Introduction: *Staphylococcus aureus*, the primary pathogen in bone infections, possesses unique capabilities to evade antibiotic therapy. For example, *S. aureus* forms abscesses within bone marrow or surrounding soft tissue, known as Staphylococcal abscess communities (SACs),[1] which protect the bacteria from antibiotics and host immunity via a fibrin pseudocapsule. While vancomycin, the standard of care (SOC) antibiotic for MRSA infections, is ineffective against SACs, previous studies demonstrate that sitafloxacin, a second-generation fluoroquinolone clinically used in Japan, can kill *S. aureus* inside SACs *in vivo* with degradation of the encasing fibrin ring.[2] To confirm sitafloxacin's ability to influence fibrin ring structure and elucidate sitafloxacin's bactericidal mechanism of action, we aimed to develop an *in vitro* model. Here, we demonstrate the adaptation of a previously described *in vitro* SAC model[3] to compare the efficacy of sitafloxacin vs. vancomycin, and to characterize the indirect effects on fibrin pseudocapsule synthesis and degradation.

Methods: *In vitro* SACs were grown using *S. aureus* JAR 06.01.31 as previously described.[3] Briefly, overnight cultures were diluted to ~14 colony-forming units (CFUs) in 25 µL of tryptic soy broth (TSB) and incubated between 2 layers of 1.78 mg/mL polymerized rat tail collagen in a 48-well plate. Gels were then overlaid with human serum, serum supplemented with fibrinogen, or plasma and incubated at 37°C. After 24 hours, gels containing mature SACs were fixed in 2.5% glutaraldehyde/4% paraformaldehyde, postfixed in buffered 1.0% osmium tetroxide, and embedded within 100% epoxy resin. One-micron sections were stained with Toluidine blue to identify the location of SACs within the gel for subsequent thin sectioning at 70 nm onto formvar/carbon copper slot grids and imaging with Hitachi 7650 TEM. In parallel, 5 µm fresh frozen sections were stained with H&E. For antibiotic treatments, the minimum inhibitory concentration (MIC) was calculated using broth microdilution, with the MIC being defined as the lowest concentration of antibiotic inhibiting visible bacterial growth. Mature SACs were then overlaid with sitafloxacin and vancomycin at 100x and 1000x their relative MIC, in addition to phosphate-buffered-saline (PBS) as a control. After 24 hours of incubation, collagen gels were either processed for TEM or homogenized, sonicated, and enumerated for CFUs. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons test, with $p<0.05$ considered significant.

Results: While overlaying the collagen gel with serum alone supported robust bacterial growth, SACs formed within the gel did not possess a fibrin pseudocapsule (**Figure 1**). In contrast, SACs grown with serum supplemented with 3 mg/mL fibrinogen had a dense fibrin ring that encased the bacterial community. This could be replicated by using heparinized plasma as the growth medium, although the pseudocapsule did not appear as dense under TEM. Importantly, collagen gels overlaid with plasma utilizing EDTA or citrate as an anticoagulant did not have visible SAC formation, with only plasma containing heparin proving sufficient for the model. To determine whether antibiotic efficacy against SACs could be compared within our model, the MIC of sitafloxacin and vancomycin against planktonic JAR 06.01.31 was calculated (0.00005 mg/mL and 0.00625 mg/mL, respectively). Plasma (heparin) overlying mature SACs was then replaced with sitafloxacin or vancomycin diluted in PBS to 100x or 1000x their relative MIC. After 24 hours of treatment, at 100x its MIC, vancomycin-treated SACs contained similar numbers of bacteria compared to the untreated control (**Figure 2A**). However, interrogating mature SACs with sitafloxacin at 100x its MIC caused a significant decrease in the total CFUs recovered from each gel. A comparable reduction in CFUs was only exhibited by vancomycin at 1000x its MIC. Morphologically, SACs treated with sitafloxacin at 1000x the MIC had notable degeneration of the pseudocapsule (*), in addition to antibiotic-killed bacteria (red arrows), characterized by cell wall remnants and vacuole formation (**Figure 2B**). In contrast, the pseudocapsule of vancomycin-treated SACs remained intact (#), with little evidence of antibiotic-induced death as the vast majority of the bacteria appeared as dense cocci with septal walls, corresponding to active bacterial replication (**Figure 2C**). These findings align with prior studies, which establish that sitafloxacin is potent against SACs *in vivo*, while vancomycin has limited bactericidal activity.

Discussion: Establishing a robust and easily reproducible *in vitro* model for SAC formation to study mechanisms of antimicrobial action creates the potential to improve treatment for bone infection. Here, we successfully adapt an *in vitro* SAC model to assess antibiotic tolerance and integrity of the fibrin pseudocapsule. With the current SOC, vancomycin, having limited efficacy against SACs both *in vivo* and *in vitro*, an understanding of how sitafloxacin kills bacteria within SACs and disrupts the encasing fibrin can aid the development of a successful treatment strategy. Since sitafloxacin itself cannot directly degrade the fibrin ring, sitafloxacin must influence the structure's disruption by acting on the encased bacteria. The accessory gene regulator (*agr*) quorum sensing system of *S. aureus* typically favors dissemination from the SAC once nutritional sources are depleted by inducing the secretion of staphylokinase. Staphylokinase can complex with plasminogen to cleave other plasminogen molecules to plasmin, leading to the degradation of the fibrin pseudocapsule. We believe that sitafloxacin uniquely acts on this pathway, leading to the digestion of the fibrin ring. In future studies, we will utilize our *in vitro* model to perform bulk RNA sequencing over the lethal time course of sitafloxacin to define how sitafloxacin activates *agr* and, in turn, disrupts the fibrin pseudocapsule. Moreover, replicating this model with the methicillin-resistant USA300 LAC strain will allow a more rigorous comparison to prior *in vivo* data.

Significance/Clinical Relevance: This work will delineate mechanisms by which antibiotics influence formation and degradation of the fibrin pseudocapsule that encases SACs, and serves as a rapid screening tool for novel antimicrobials that kill SACs to improve therapy for bone infection.

References: 1) Cheng et al. *FASEB J* 25:3393-404 (2009), 2) Ren et al. *Front Cell Infect Microbiol* 12:910970 (2022), 3) Hofstee et al. *Infect Immun*. 88(11):e00293-20 (2020).
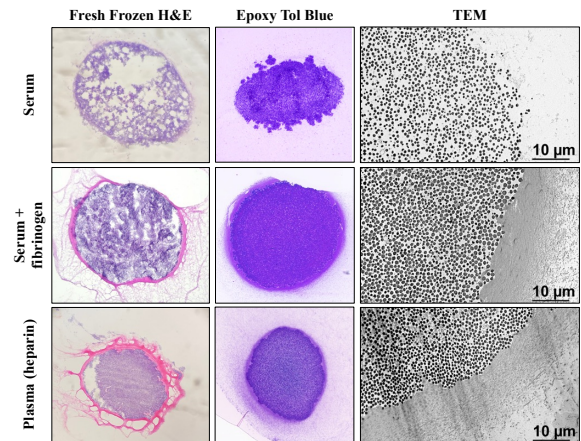
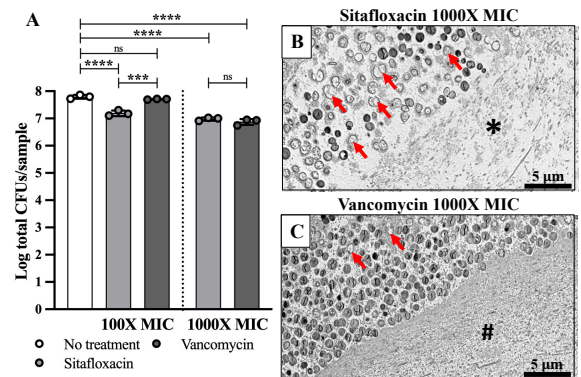**Figure 1. Fibrinogen promotes fibrin pseudocapsule formation**

Figure 2. Sitafloxacin is more effective than vancomycin against SACs *in vitro*