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Staphylococcal Persistence Due to Biofilm Formation in Synovial Fluid Containing Prophylactic Cefazolin

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Antibiotic prophylaxis is standard for patients undergoing surgical procedures, yet despite the wide use of antibiotics, breakthrough infections still occur. In the setting of total joint arthroplasty, such infections can be devastating. Recent findings have shown that synovial fluid causes marked staphylococcal aggregation, which can confer antibiotic insensitivity. We therefore asked in this study whether clinical samples of synovial fluid that contain preoperative prophylactic antibiotics can successfully eradicate a bacterial challenge by pertinent bacterial species. This study demonstrates that preoperative prophylaxis with cefazolin results in high antibiotic levels. Furthermore, we show that even with antibiotic concentrations that far exceed the expected bactericidal levels, *Staphylococcus aureus* bacteria added to the synovial fluid samples are not eradicated and are able to colonize model implant surfaces, i.e., titanium pins. Based on these studies, we suggest that current prophylactic antibiotic choices, despite high penetration into the synovial fluid, may need to be reexamined.

Infection remains a major concern with orthopedic procedures, although infection rates are generally <1% (1). Because of the high prophylactic antibiotic levels within synovial fluid, the use of minimally invasive techniques, and short surgical times, infection rates remain low. However, with increasing numbers of total knee arthroplasties, infections are projected to exceed 60,000 to 70,000 cases in the United States by 2020 (2, 3). When hardware, such as a prosthesis, is present, infection is recalcitrant to antibiotic treatment. Once established, infection can cause prolonged disability, multiple operations, and increased health care costs (3, 4). According to the WHO, patients with a surgical site infection have twice the mortality rate, are twice as likely to spend time in an intensive care unit, and are five times more likely to be readmitted to the hospital than uninfected patients (5).

To avoid the establishment of infection, antibiotic prophylaxis has become the standard of care (6, 7). For orthopedic procedures, cefazolin (CFZ), which is bactericidal for staphylococci, streptococci, and *Escherichia coli* (8, 9), is commonly used in the absence of a penicillin allergy (10). Alternative prophylactic antibiotics include fusidic acid, cloxacillin (11), cefixime (12), vancomycin, and gentamicin (13). Interestingly, synovial fluid has been suggested to possess antibacterial properties (14–16) that are attributed to hyaluronic acid within the synovial fluid (17), the induction of bactericidal/permeability-increasing protein (15), or unknown compounds, including antimicrobial peptides, within the synovial fluid (14). We recently reported that this ostensible decrease in bacterial numbers, which is perceived to be an "antibacterial" effect, is actually due to the clumping of bacteria within synovial fluid, which masks the true microbial load levels (18).

The recognition of this behavior also helps to elucidate the difficulties associated with detecting infections. Using standard procedures, the infection rates for revision knee arthroplasties are around 10%, with a 7 to 12% false-negative rate, even in cases in which gross infections are present (19). When more rigorous techniques are used to detect infection, >50% of the retrieved implants are found to have bacterial contaminants, suggesting that antibiotic prophylaxis has limited success (20).

In this study, we sought to determine if the prophylactic antibiotics, commonly CFZ, present in synovial fluid are able to prevent bacterial contamination. Based on our recent studies showing that bacterial clumping in synovial fluid dominates its phenotype, we hypothesized that even these high levels of the bactericidal antibiotic CFZ would not be able to eradicate a *Staphylococcus aureus* challenge. We used synovial fluid samples from patients who underwent preoperative prophylaxis and established the relative concentrations of active CFZ. We then asked if these concentrations affect *S. aureus* viability or colonization of a metal surface when bathed in synovial fluid containing preoperative CFZ. These findings have potential implications for the selection of the most effective means to attain antibacterial prophylaxis, as well as for the methodology to detect joint infections.

MATERIALS AND METHODS

Materials. Samples of synovial fluid were collected both in the clinic during knee aspirations and in the operating room during total knee arthroplasties, with the permission of the Thomas Jefferson University institutional review board. Cefazolin was obtained from AAP Pharmaceuticals. Kirby-Bauer disks were obtained from BBL. Wheat germ agglutinin (WGA) staining for polysaccharide intercellular adhesin (PIA) (also

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called poly-*N*-acetylglucosamine [PNAG]) was performed using Alexa Fluor 488-labeled WGA (Life Technologies). One-millimeter titanium alloy (Ti6Al4V) wires were obtained from Goodfellow Metals.

Bacterial strains. The following strains of *S. aureus* were used for the study: methicillin-susceptible *S. aureus* (MSSA) ATCC 25923, methicillin-resistant *S. aureus* (MRSA) clinical isolate TJU2, MRSA LAC (USA300), MSSA Xen 36 (Perkin-Elmer), and MSSA AH1710, a strain that constitutively expresses green fluorescent protein (GFP) that was used to visualize staphylococcal surface colonization (a kind gift from Alexander Horswill, University of Iowa [21]). In addition, *Staphylococcus epidermidis* strain ATCC 35984 was used in the disk diffusion assays.

Qualitative synovial fluid microscopy. Directly after the samples were collected, 1 ml of each sample was removed and imaged by light microscopy at 20× magnification in order to determine the overall cellularity and/or crystallinity of the sample.

Disk diffusion assays. Each sample of synovial fluid was tested for the presence of antibiotics by an analysis of the zone of inhibition (ZOI) of bacterial growth around a Kirby-Bauer disk, in which antibiotic concentration is proportional to the distance from the disk. In brief, 6-mm Kirby-Bauer disks were impregnated with 20-μl samples of synovial fluid, dried overnight, and placed on tryptic soy broth (TSB) agar plates streaked with lawns of *S. aureus* (200 μl, at a concentration of 10^7 CFU/ml).

Monitoring aggregate formation. An initial inoculum of 10^4 CFU/ml of MRSA USA300 was incubated in 5 ml of TSB or naive synovial fluid (no preoperative antibiotics) at 180 rpm and 37°C for 8 h. The resultant aggregates of USA300 were imaged using digital photography and scanning electron microscopy. In some cases, 10^8 CFU/ml of *S. aureus* ATCC 25923 was incubated in synovial fluid containing preoperative CFZ at 180 rpm and 37°C for 3 h and, after aggregate formation, with 10 μg/ml ethidium bromide for 20 min at 37°C; these were imaged using digital photography on a UV light box.

Scanning electron microscopy. Large aggregates of bacteria in the synovial fluid were removed using forceps and fixed for 1 h in 4% paraformaldehyde (PFA). The fixed aggregates were serially dehydrated for 10 min each in 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100% ethanol (% ethanol in double-distilled water [ddH_2O]). The samples were then affixed onto a coverslip (Nunc), sputter-coated with palladium, and visualized using a Hitachi TM-1000 scanning electron microscopy (SEM) (Ibaraki, Japan).

Wheat germ agglutinin staining. Wheat germ agglutinin staining for PIA was performed using Alexa Fluor 488-labeled WGA (Life Technologies) at a concentration of 0.1 mg/ml (Jefferson and Cerca [22]) for 30 min at 37°C.

Direct counting of *S. aureus* survival. The 200-μl samples of synovial fluid (SF) (samples SF4.1 to SF4.5) were challenged with 10^6 CFU/ml of a clinically isolated strain of *S. aureus* (TJU2), incubated under static conditions for 24 h, and serially diluted (with phosphate-buffered saline [PBS]). The entire 200-μl volume of each sample was plated on TSB agar to ensure that all surviving bacteria were detected.

The samples of synovial fluid (200 μl each) were challenged for 24 h at 37°C under static conditions with high inocula (between 10^7 and 10^9 CFU/ml) of *S. aureus* ATCC 25923, a laboratory-adapted strain of *S. aureus*, which is highly susceptible to CFZ in TSB, even at a high initial inoculum (18, 23). The samples were serially diluted in PBS, and the entirety (200 μl) of each sample was plated on TSB agar.

Monitoring *S. aureus* survival using Xen 36. A 10^8-CFU/ml inoculum of the luciferase-expressing strain Xen 36 was incubated with 200 μl of fresh samples of synovial fluid from patients given preoperative CFZ (samples SF4.1 to SF4.5) or with 200 μl of samples of TSB with or without 20 μg/ml CFZ for 24 h at 37°C in a standing incubator and then imaged for chemiluminescence (ImageQuant LAS 4000).

In other experiments, initial inocula of 10^3, 10^4, and 10^5 CFU/ml of Xen 36 were incubated for 12 h with naive synovial fluid or with different samples of synovial fluid from patients given preoperative CFZ (samples SF4.6 to SF4.8); luciferase expression was then measured by a luminometer.

***S. aureus* adherence to titanium pins.** A total of 10^7 CFU/ml of *S. aureus* AH1710 was added to 1 ml of synovial fluid samples containing preoperative CFZ and incubated 37°C for 24 h. With naive synovial fluid (SF), TSB, or PBS, the preincubation step was omitted. Three Ti alloy (Ti6Al4V) pins were then added and incubated at 37°C for 24 h. The retrieved pins were imaged by confocal laser scanning microscopy. The pins were also incubated with 10^7 CFU of *S. aureus* AH1710 in 100 μl of PBS plus 10% TSB or 100 μl of synovial fluid containing preoperative CFZ for 48 h. After imaging, the pin incubated in synovial fluid containing preoperative CFZ was washed 3× with PBS and incubated in 1 ml of TSB at 37°C for 24 h. After 3 PBS washes, the adherent bacteria were visualized by confocal laser scanning microscopy (CLSM) (Olympus FluoView 300).

To determine the number of adherent bacteria, three 2-mm Ti6Al4V pins were incubated under static conditions in 200 μl of synovial fluid containing preoperative CFZ and 10^7 CFU/ml of strain ATCC 25923 for 18 h at 37°C. The pins were removed with sterile forceps, gently washed 3× in PBS, sonicated for 10 min in 0.3% Tween 20-PBS, diluted, plated onto 3M Petrifilm plates, and counted.

Statistical analysis. Because of the size of the retrieved synovial fluid samples, some samples could only be tested in one set of 2 to 3 replicates. In those cases, multiple independent synovial fluid samples were always examined but not always averaged. Some evaluations were repeated at least in triplicate in ≥3 independent experiments. In those cases, statistical analyses were performed, and differences between the values and groups were tested using a paired *t* test. Significance was set at a P value of <0.05.

RESULTS

Preoperative cefazolin is present in synovial fluid. We collected synovial fluid samples from patients who were undergoing total knee arthroplasties (with preoperative CFZ) or who were undergoing knee aspirations in the office (no antibiotics [naïve]). The fluid had a golden color and tended to be either clear (Fig. 1A) or somewhat cloudy with crystals apparent. Under microscopy, the samples ranged from highly cellular (Fig. 1B) to few visible cells (Fig. 1C). Using a disk diffusion assay in which the clear zone (zone of inhibition [ZOI]) is proportional to the CFZ concentration, we tested the antibiotic content of the synovial fluid obtained from subjects who had been given 2 g of CFZ 1 h prior to surgical procedures (synovial fluid with preoperative CFZ) against different strains of staphylococci. As expected, a disk containing naive synovial fluid did not inhibit *S. aureus* ATCC 25923 growth (Fig. 1D*i*), whereas synovial fluid with 2 g of preoperative CFZ caused a ZOI due to CFZ diffusion from the disk (Fig. 1D*ii*). The ability of this synovial fluid with preoperative CFZ to cause a ZOI was also observed with *S. aureus* isolated from a patient with septic arthritis (Fig. 1D*iii*); with *S. aureus* AH1710, a genetically engineered isolate that expresses green fluorescent protein from a plasmid under chloramphenicol selection (Fig. 1D*iv*); with *S. epidermidis* ATCC 35984 (Fig. 1D*v*); and with a clinical MRSA isolate (from Jefferson Hospital; Fig. 1D*vi*). The zones of inhibition (ZOI) were measured digitally and compared to the ZOI measured from a control disk incubated with that strain (Fig. 1E). Based on these comparisons, we deduced that synovial fluid with preoperative CFZ had ~200 μg/ml CFZ (200× the MIC for *S. aureus* ATCC 25923 [23]).

***S. aureus* forms aggregates in synovial fluid.** We compared *S. aureus* growth in TSB to that in synovial fluid (Fig. 2A). The bacteria in TSB grew to a cloudy suspension, whereas in naive synovial fluid (nSF), the sample appeared largely clear, with a large

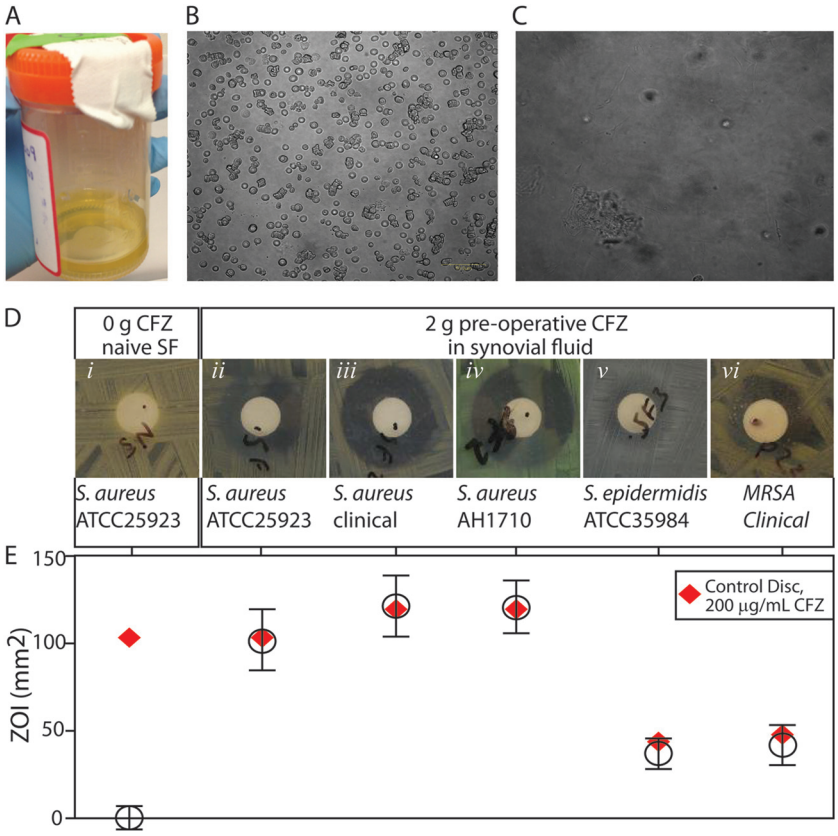

FIG 1 Synovial fluid containing preoperative antibiotics. (A) Representative image of a synovial fluid sample obtained during a total knee arthroplasty that contains preoperative CFZ. (B) Typical microscopic image of synovial fluid displaying high cellularity. (C) Typical microscopic image of synovial fluid displaying low cellularity. (D) Zones of inhibition (ZOI) obtained with synovial fluid containing preoperative antibiotics (2 g of CFZ). In all, at least eight different synovial fluid samples were tested on each strain in triplicate. The images shown are representative of the ZOI obtained across all samples. Shown within the panel are ZOI associated with synovial fluid from a knee aspiration that does not contain antibiotics (naive SF) (*i*), synovial fluid containing preoperative antibiotics in *S. aureus* ATCC 25923 (*ii*), synovial fluid containing preoperative antibiotics in an *S. aureus* clinical isolate (*iii*), synovial fluid containing preoperative antibiotics in *S. aureus* AH1710 (*iv*), synovial fluid containing preoperative antibiotics in *S. epidermidis* ATCC 35984 (*v*), and synovial fluid containing preoperative antibiotics in an *S. aureus* MRSA isolate (Thomas Jefferson University Hospital [TJU2]) (*vi*). (E) ZOIs measured digitally with ImageJ using the synovial fluid samples and strains shown in panel D. Each circle represents the average ZOI from at least three disks/strain. The red diamonds represent the ZOI obtained with 200 μg/ml CFZ/disk.

clump floating in the fluid (Fig. 2A*i*, red box) and a cloudy precipitate on the bottom of the tube. When the clump was examined more closely (Fig. 2A*ii*), it appeared to be a large slimy aggregate of bacteria; similar aggregates were observed with three different synovial fluid samples with 2 g of preoperative CFZ (samples SF6.1, SF6.2, and SF6.3; Fig. 2A*iii*), emphasizing the prevalence of aggregate formation. The very dense matrix of the bacterial aggregate in naive synovial fluid was visualized by scanning electron microscopy (Fig. 2A*iv*). Using confocal laser scanning microscopy, a three-dimensional reconstruction was compiled of aggregated *S. aureus* in synovial fluid with 2 g of preoperative CFZ (sample SF4.1) and showed a dense reticulated structure reminiscent of a biofilm (Fig. 2A*v*). We thus asked if aggregates in synovial fluid containing preoperative CFZ express a polysaccharide matrix characteristic of biofilms. When stained for polysaccharide intercellular adhesin (PIA) (also called poly-*N*-acetylglucosamine [PNAG]) with wheat germ agglutinin (WGA), *S. aureus* in synovial fluid with 2 g of preoperative CFZ (samples SF6.1, SF6.2, and SF6.3) clearly aggregated, and the aggregates showed green fluorescence, indicating the production of biofilm polysaccharide in the aggregated bacteria (Fig. 2B*i*). In three different trials with TSB, *S. aureus* did not aggregate, and staining for PIA was punctate (Fig. 2B*ii*).

Preoperative cefazolin in synovial fluid does not eradicate a bacterial challenge. Based on our previous studies and our findings in Fig. 2, *S. aureus* readily clumped in synovial fluid containing preoperative CFZ. We thus asked to what extent this CFZ-containing synovial fluid was able to eradicate a bacterial challenge. When a clinical isolate of *S. aureus* (from a patient with septic arthritis) was incubated with CFZ-containing synovial fluid, 4 out of 5 different synovial fluid samples (SF4.1 to SF4.5) showed no significant decrease in bacterial viability after a 24-h challenge. Only 1 sample, SF4.1, showed a marked decrease (~3 log) from the initial inoculum (Fig. 3A). Using the same five samples of synovial fluid, a visual analysis of *S. aureus* Xen 36 chemiluminescence revealed that luciferase activity appeared to be completely abolished in TSB containing CFZ. Conversely, luciferase activity persisted in samples SF4.1 to SF4.5, as seen in Fig. 3A, with noticeably lower activity in SF4.1 and robust activities in SF4.3 to SF4.5.

By a direct measurement of luminescence, naive synovial fluid samples challenged with 10^2, 10^3, and 10^4 CFU/ml of *S. aureus* Xen

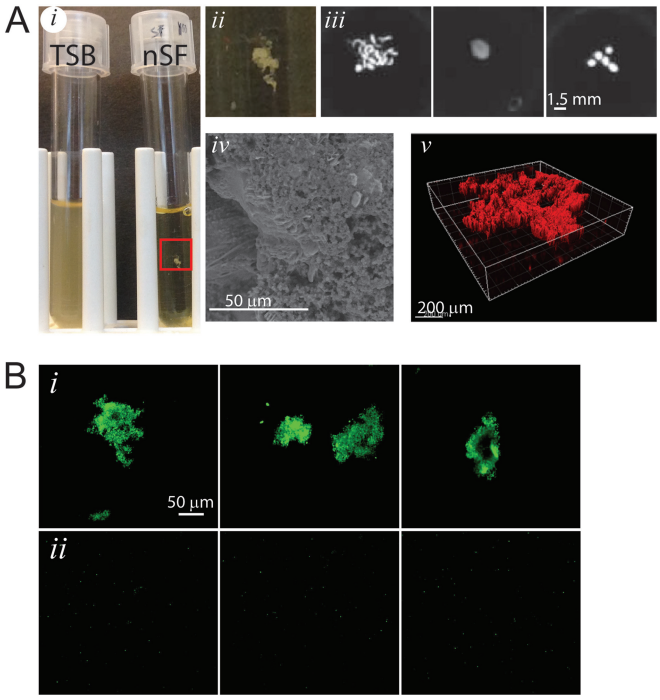

FIG 2 Bacterial growth and clumping in synovial fluid. (A) *i*, 5-h culture of *S. aureus* incubated in TSB or naive SF (nSF) (no antibiotics). These clumps have already been described by Dastgheyb et al. (18). Note the *S. aureus* aggregate that is boxed in red. *ii*, magnification at 10× of the aggregated *S. aureus* ATCC 25923 that was shown within the red box. *iii*, aggregated bacteria after incubation in SF6.1, SF6.2, or SF 6.3, each containing 2 g of CFZ from preoperative prophylaxis, visualized by ethidium bromide staining, and photographed. Scale bar = 1.5 mm. *iv*, scanning electron micrograph of an aggregate formed in naive synovial fluid. Scale bar = 50 μm. *v*, three-dimensional reconstruction of confocal laser scanning micrographs of an *S. aureus* ATCC 25923 aggregate formed in synovial fluid containing 2 g of preoperative CFZ. Similar images were obtained with other synovial fluid samples containing preoperative CFZ. (B) WGA staining for PIA/PNAG in *S. aureus* ATCC 25923 shown incubated in three synovial fluid samples (*i*) (left to right: SF3.1, SF3.2, and SF3.3) or three separate cultures of TSB without antibiotics (*ii*).

36 for 12 h showed a stepwise increase in luminescence. When the samples with preoperative CFZ were challenged with Xen 36, SF4.6 and SF4.7 showed levels of luminescence that were similar to those of the samples with 10^3 to 10^4 CFU in naive synovial fluid, independent of the size of the starting inoculum. Sample SF4.8, which had been dosed with 1 g of preoperative CFZ, showed a stepwise increase in luminescence with increasing inocula (Fig. 3C). In contrast, in TSB (Fig. 3D), luciferase activity was high with all three inocula; after 12 h, TSB containing 20 μg/ml CFZ resulted in markedly decreased luminescence, and these levels were ~10× lower than those measured in CFZ-containing synovial fluid. Of note, the CFZ concentrations in SF were ~10-fold greater than those in TSB.

***S. aureus* in synovial fluid containing antibiotics readily adheres to metal surfaces.** We reasoned that if viable bacteria were indeed present in synovial fluid containing preoperative CFZ, they should readily adhere to titanium alloy (Ti) pins, thus modeling biofilm initiation in the presence of an implant. After 24 h of incubation, ~10,000 CFU/ml of bacteria were recovered from Ti pins that had been inoculated with 10^7 CFU/ml of *S. aureus* in the presence of TSB (Fig. 4A). Importantly, no bacterial colonization was detected on Ti pins in which *S. aureus* was incubated in TSB+CFZ before the addition of the pin. In contrast, between 200 and 2,500 CFU of adherent *S. aureus* (average ± standard deviation, 1,156 ± 832 CFU) were recovered from Ti pins incubated in *S. aureus*-inoculated CFZ-containing synovial fluid.

When the pins were examined for presence of GFP-expressing bacteria, a Ti pin that was not challenged with bacteria showed no fluorescence (Fig. 4B*i*) and served as a negative control. Abundant fluorescence was apparent when a pin was incubated with *S. aureus* in PBS plus 10% TSB, a minimal growth medium (Fig. 4B*ii*). In synovial fluid containing preoperative CFZ, *S. aureus* colonization was not initially detectable (Fig. 4B*iii*) but became apparent when the colonized pin was removed from the synovial fluid and incubated in TSB for an additional 24 h. This additional incubation supported sufficient bacterial growth to be detected visually (Fig. 4B*iv*).

DISCUSSION

Antibiotic prophylaxis during orthopedic procedures is used to minimize the establishment of infection. In this paper, we show that synovial fluid containing CFZ due to a 2-g preoperative prophylactic protocol has attenuated efficacy against *S. aureus* in synovial fluid. Furthermore, we show that Ti pins in synovial fluid that contains CFZ due to preoperative prophylaxis can be colonized by added *S. aureus* at clinically significant numbers. In the presence of metal implants, it is now known that both *in vitro* (24, 25) and *in vivo*, as few as 100 CFU of *S. aureus* are sufficient to establish infection (26). Accompanying this diminished level of efficacy of antibiotics in synovial fluid, our *in vitro* findings show that in general, up to 90% of bacteria survive, suggesting that preoperative prophylaxis may be ineffective in the synovial fluid environment. Taken together, these *in vitro* studies suggest that the current standard for prophylaxis may be inadequate to combat *S. aureus* contamination.

Antibiotic penetration is thought to determine the effectiveness of prophylaxis (13, 27–29). Because CFZ is bactericidal, obtains high levels within the synovial fluid within 30 to 60 min, and has a broad spectrum of coverage, it is recommended for preoperative prophylaxis in procedures that breach the joint capsule (33). Based on our data, postprophylactic synovial fluid concentrations are ~100 to 200 μg/ml, which is ~400× the MIC for *S. aureus* (9). These concentrations were determined through the testing of 23 synovial fluid samples containing preoperative CFZ. Despite these CFZ concentrations, a large number of *S. aureus* bacteria persist and are seen to form clumps in the antibiotic-containing synovial fluid. These clumps are biofilm-like in that they are stained by WGA, which stains the polysaccharide coating of *S. aureus* biofilms containing PIA. Our recent work shows that the formation of these clumps depends on a proteinaceous component that is critical for clump formation (18). This aggregation causes an accompanying decrease in virulence and a biofilm-like phenotype (18; S. S. Dastgheyb, M. Otto, and N. J. Hickok, unpublished data). In addition to the protein content, the viscosity (150 ± 50 MPa [30]) of synovial fluid favors the formation of biofilms, whether anchored or floating. Therefore, while planktonic *S. aureus* in TSB might be effectively eradicated by 200 μg/ml CFZ, *S. aureus* readily survived in this same concentration of antibiotic in synovial fluid. This recalcitrance to the effects of CFZ was found in several strains, including a clinical strain of *S. aureus*, and with inocula ranging from 10^3 to 10^7 CFU/ml. It is worth

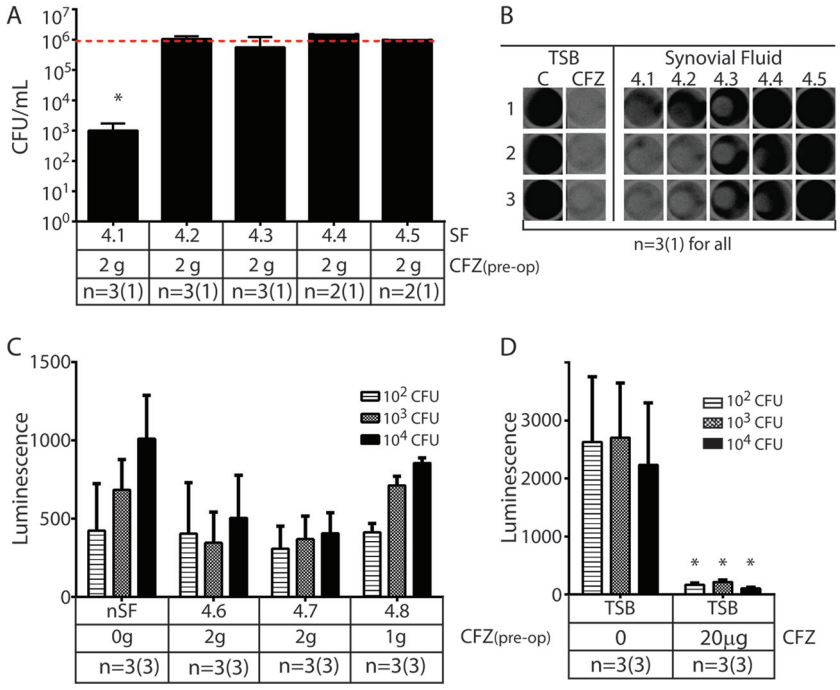

FIG 3 Bacterial survival in antibiotic-containing synovial fluid. (A) Survival of *S. aureus* incubated in synovial fluid from patients given 2 g of preoperative (pre-op) CFZ (samples SF4.1 to SF4.5). Red dashed line represents initial inoculum. (B) Xen-36 chemiluminescence after 24 h incubation in TSB (0 or 20 μg/ml CFZ), or 5 samples of synovial fluid containing 2 g of preoperative antibiotics (samples SF4.1 to SF4.5). (C) Intensity of Xen 36 luminescence after 12 h of incubation of 100, 1,000, or 10,000 CFU in 100-μl samples of naive synovial fluid or SF4.6, SF4.7, and SF4.8 from patients given 2, 2, and 1 g of preoperative CFZ, respectively. (D) Intensity of Xen 36 luminescence after 12 h of incubation with 100, 1,000, or 10,000 CFU in 100-μl samples of TSB containing 0 or 20 μg/ml CFZ. The numbers of repeats with the number of independent experiments in parentheses are indicated for each panel.

noting that throughout the study, the higher doses of bacteria have been used to more readily visualize the aggregation phenomenon that we are describing. However, as seen in the data in Fig. 3, the lower levels of bacterial contamination show similar behavior at higher levels. We would argue, therefore, that the high inocula used throughout will model the more clinically relevant lower levels of bacteria.

Bacterial survival in CFZ-containing synovial fluid is illustrated by the adherence of *S. aureus* to a titanium alloy pin. While a titanium alloy was used, the most common alloy used in knee hardware is cobalt-chrome-molybdenum. Data from others (31, 32) and our unpublished data (S. Dastgheyb, A. E. Villaruz, K. Y. Le, V. Y. Tan, A. C. Duong, S. S. Chatterjee, G. Y. C. Cheung, H.-S. Joo, N. J. Hickok, and M. Otto, submitted for publication) support the adherence of *S. aureus* to both materials. By direct counting, approximately 10^3 of the 10^4 bacteria inoculated into the sy-

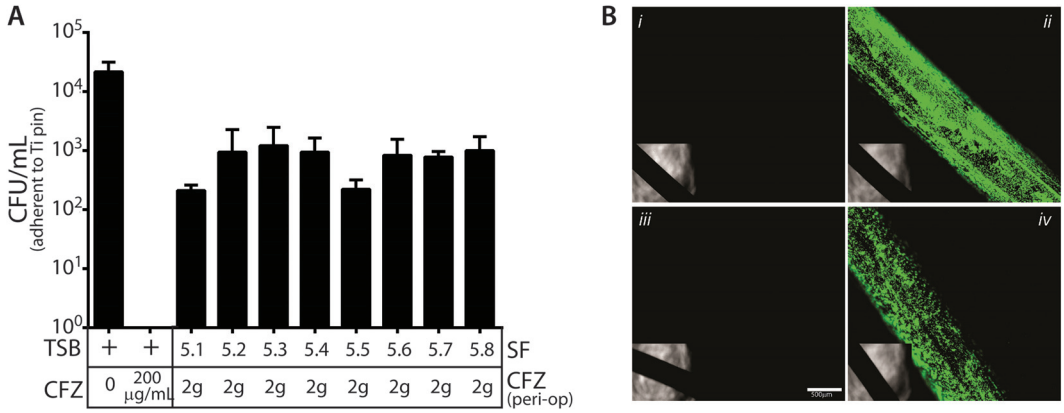

FIG 4 Bacterial survival and adherence in the presence of antibiotic-containing synovial fluid. (A) CFU recovered from Ti alloy pins incubated in TSB or in 8 synovial fluid samples containing 2 g of preoperative CFZ containing 10^7 CFU/ml of *S. aureus* for 24 h. All bacterial recoveries were performed independently three times in three independent experiments. (B) All experiments used three independent pins/experiment, and each experiment was repeated three times. *i*, control Ti alloy pin that has not been exposed to *S. aureus*. *ii*, adherence of *S. aureus* (green fluorescence) to Ti alloy pin in PBS plus 10% TSB. *iii*, adherence of *S. aureus* to Ti alloy pin in synovial fluid containing preoperative antibiotics. *iv*, pin in panel *iii* after removal from synovial fluid and 24 h of incubation in TSB. The green fluorescence is due to bacterial growth during this time frame. Scale bar = 500 μm.

novial fluid adhered to the pin. The recently published work of Dastgheyb et al. (18) established that despite early differences in proliferation rates, the rates of *S. aureus* proliferation in TSB and synovial fluid at 24 h are approximately equal. Thus, this difference in colonization is unlikely to be due to a simple difference in proliferation. Independent of surface adhesion, the nonadherent bacteria in synovial fluid exhibit a biofilm-like phenotype and demonstrate a biofilm-like recalcitrance to antibiotic treatment.

In synovial fluid, it is clear from these studies and our previous work that this aggregation alters antibiotic sensitivity and may have an impact on the numbers of bacteria that colonize an implant. Importantly, while performing these experiments, we relied on multiple synovial fluid samples to validate our results (1), as volumes were limited (2), and the use of multiple samples strengthened our observations. Interestingly, synovial fluid rapidly reforms after joint replacement so that the synovial environment is reestablished with a new more susceptible surface for bacterial colonization (i.e., the implant). It is important to realize that when infection has been established, an important part of the treatment is extensive lavage and removal of any fibrin clots; based on the data in this paper, this removal becomes critical, as this clot is an important structure in the maintenance of indolent bacteria.

In an arthroplasty setting, preoperative antibiotics are accompanied by the removal of synovial fluid during the arthrotomy, followed by blood clot formation, and an additional one to two doses of antibiotic are given postoperatively. These additional procedures and antibiotic treatments may be sufficient to sterilize the knee environment, but this scenario needs to be explicitly tested in light of our findings. Importantly, the results we report may be especially pertinent to clinical situations in which synovial fluid is aspirated without performance of joint replacements. A single preoperative dose of antibiotic is typically recommended, and the data here suggest that this is inadequate. Further exploration of clinical synovial fluid samples is warranted.

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We declare no conflicts of interest.

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