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Interplay of antibiotics and bacterial inoculum on suture-associated biofilms☆

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ABSTRACT

Background: Biofilms are often antibiotic resistant, and it is unclear if prophylactic antibiotics can effectively prevent biofilm formation. Experiments were designed to test the ability of high (bactericidal) concentrations of ampicillin (AMP), vancomycin (VAN), and oxacillin (OXA) to prevent formation of suture-associated biofilms initiated with low (10^4) and high (10^7) numbers of *Staphylococcus aureus*.

Materials and methods: *S. aureus* biofilms were cultivated overnight on silk suture incubated in biofilm growth medium supplemented with bactericidal concentrations of AMP, VAN, or OXA. Standard microbiological methods were used to quantify total numbers of viable suture-associated *S. aureus*. Crystal violet staining followed by spectroscopy was used to quantify biofilm biomass, which includes bacterial cells plus matrix components. To observe the effects of antibiotics on the microscopic appearance of biofilm formation, biofilms were cultivated on glass slides, then stained with fluorescent dyes, and observed by confocal microscopy.

Results: In the presence of a relatively low inoculum (10^4) of *S. aureus* cells, bactericidal concentrations of AMP, VAN, or OXA were effective in preventing development of suture-associated biofilms. However, similar concentrations of these antibiotics were typically ineffective in preventing biofilm development on sutures inoculated with 10^7 *S. aureus*, a concentration relevant to contaminated skin. Confocal microscopy confirmed that bactericidal concentrations of AMP, VAN, or OXA inhibited, but did not prevent, development of *S. aureus* biofilms.

Conclusion: Bactericidal concentrations of AMP, VAN, or OXA inhibited formation of suture-associated biofilms initiated with low numbers (10^4), but not high numbers (10^7), of *S. aureus* cells.

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1. Introduction

Microbial biofilms are involved in a wide variety of infectious processes, such as periodontitis, otitis media, ventilator- and cystic fibrosis–related pneumonias, endocarditis, biliary tract infections, prostatitis, osteomyelitis, burn wound infections, device-related infections, and wound infections [1–3]. Biofilm infections are especially troubling in clinical medicine because microbes residing within a biofilm are generally more antibiotic resistant than their planktonic (free-living) counterparts [1,4]. In addition to increased antibiotic resistance, biofilms exhibit increased resistance to ultraviolet damage, desiccation, and pH gradients (reviewed in [5]). Using *Staphylococcus aureus* suture-associated biofilms [6], we have reported that bacteria residing in the biofilm are antibiotic resistant, even though mechanically dispersed biofilm cells have susceptibilities comparable with planktonic bacteria [7]. Lewis [8,9] has described a small percentage of biofilm cells, termed "persister" cells, thought to be responsible for the antibiotic resistance of biofilms, and persister cells are currently the topic of a considerable research effort. Because the antimicrobial recalcitrance of biofilm infections is a daunting problem, we designed experiments to clarify the effect of prophylactic antibiotics on the development of *S. aureus* suture-associated biofilms.

Although prophylactic antibiotics are frequently given to prevent infections, little is known about the ability of antibiotics to actually prevent biofilm development (and there is some evidence that antibiotics can actually enhance biofilm development [10]). Unfortunately, there are no universally accepted methods for studying the ability of antibiotics to prevent biofilm formation. Bacterial antibiotic susceptibilities are assessed in clinical microbiology laboratories by incubating various concentrations of a drug with low numbers of rapidly growing bacteria. The resulting antibiotic concentration that results in bacterial killing is defined as the minimum bactericidal concentration (MBC). If a drug was administered at a concentration resulting in a steady state MBC level in the patient's blood (often difficult to obtain), one would expect that this drug should prevent susceptible bacteria from initiating a biofilm infection. We sought to test whether ampicillin (AMP), vancomycin (VAN), and oxacillin (OXA) at concentrations relevant to the MBC could prevent biofilm formation using two strains of *S. aureus* and then tested if these drug concentrations would inhibit development of suture-associated biofilms initiated with a low and high (but clinically relevant) inoculum of viable *S. aureus*.

2. Materials and methods

2.1. Antibiotics and S. aureus strains

S. aureus RN6390 and ATCC 25923 are wild type strains known to produce biofilms [6,7,11,12]. Bacterial inocula were washed cells from overnight cultures incubated at 35°C in tryptic soy broth, and bacterial concentrations were confirmed by standard microbiological methods. The antibacterial agents used in this study were all cell wall active agents and included AMP, VAN, and OXA (Sigma-Aldrich, Inc, St. Louis, MO). Using methodology compatible with CLSI guidelines [13], the MBCs of these antibiotics for planktonic cells of these two *S. aureus* strains have been published. The MBCs of AMP/VAN/OXA for strains RN6390 and ATCC 25923 are 0.5/2.0/0.5 μg/mL and 0.25/2.0/0.5 μg/mL, respectively [7]. MBC is defined as the minimum antibiotic concentration that results in 99.9% killing of the cells in the bacterial inoculum.

2.2. Effect of antibiotics on developing suture-associated S. aureus biofilms

Suture-associated biofilms were cultivated as described [6,7] with minor modifications. Briefly, each well of a 24-well microtiter plate contained a 1-cm segment of black braided 3–0 silk suture (Ethicon, Inc, Somerville, NJ) suspended in 1 mL of biofilm growth medium, namely 66% tryptic soy broth supplemented with 0.2% glucose [12] and additionally supplemented with varying concentrations of AMP, VAN, or OXA. Control wells contained no antibiotic. Each well was inoculated with 10^4 or 10^7 *S. aureus* and incubated overnight at 37°C with gentle rotation (50 rpm). These inocula were chosen as low and high inocula, based on the fact that high concentrations of bacteria on normal skin flora are $10^{6-7}/cm^2$ [14]. After overnight incubation of bacteria with suture, suture-associated biofilms were analyzed for numbers of viable bacteria and biofilm biomass as described below.

To assess the numbers of viable bacteria, each suture was gently rinsed, transferred to 2 mL of sterile phosphate buffered saline, sonicated at ~50 J at 100% amplitude for 5 s using a sonicator at 20 kHz (Sonics and Materials, Newtown, CT). Sonication had no noticeable effect on bacterial viability, and microscopy confirmed that sonicated bacteria were single-cell suspensions. Bacterial concentrations in sonicates were determined by standard microbiological methods, and the lower detection limit was 1.7 $\log_{10}$ colony forming units (CFUs) per suture. Biofilm biomass was measured with crystal violet as described [15] with minor modifications. Crystal violet is a basic dye that binds negatively charged surface molecules, including those on live and dead bacteria, as well as on matrix polysaccharides. Biofilm-laden sutures were rinsed with phosphate buffered saline, fixed in 99% methanol for 15 min, air dried, incubated 20 min with 0.5% crystal violet (Fisher Chemical, Pittsburgh, PA), washed, and then incubated 20 to 30 min in 33% acetic acid to release the crystal violet, with absorbance read at 590 nm. Statistical differences were analyzed by unpaired Student's *t*-test. Bacterial numbers were converted to $\log_{10}$ before statistical analysis, and significance was set at $P < 0.05$.

2.2.1. Confocal microscopy

To facilitate microscopic observations, biofilms were cultivated overnight at 37°C on positively charged glass slides using biofilm growth medium inoculated with ~10^7/mL *S. aureus* and supplemented with either AMP (0.125 and 0.5 μg/mL), VAN (1 and 2 μg/mL), or OXA (0.125 and 0.5 μg/mL)