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Biofilm Antimicrobial Susceptibility Increases With Antimicrobial Exposure Time

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Abstract

Background The antimicrobial concentration required to kill all the bacteria in a biofilm, known as the minimum biofilm eradication concentration (MBEC), is typically determined in vitro by exposing the biofilm to serial concentrations of antimicrobials for 24 hours or less. Local delivery is expected to cause high local levels for longer than 24 hours. It is unknown if longer antimicrobial exposures require the same concentration to eradicate bacteria in biofilm. Questions/purposes Does MBEC change with increased antimicrobial exposure time?

Methods Biofilms were grown for 24 hours using five pathogens (methicillin-sensitive *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, and *Pseudomonas aeruginosa*) and then exposed to four antimicrobials regimens: tobramycin, vancomycin, and tobramycin combined with vancomycin in 3:1 and 1:1 ratios by weight in concentrations of 62.5, 125, 250, 500, 1000, 2000, 4000, and 8000 μg/mL for three durations, 1, 3, and 5 days, in triplicate. MBEC was measured as the lowest concentration that killed all bacteria in the biofilm determined by 21-day subculture.

Results MBEC was lower when antimicrobial exposure time was longer. For the *staphylococcus* species, the MBEC was lower when exposure time was 5 days than 1 day in 11 of 12 antimicrobial/microorganism pairs. The MBEC range for these 11 pairs on Day 1 was 4000 to > 8000 μg/mL and on Day 5 was < 250 to 8000 μg/mL. MBEC for tobramycin/*P. aeruginosa* was 2000 μg/mL on Day 1 and ≤ 250 μg/mL on Day 5, and for *E. coli*, 125 μg/mL on Day 1 and ≤ 62.5 on Day 5.

Conclusions Although antimicrobial susceptibility was lower for longer exposure times in the microorganisms we studied, confirmation is required for other pathogens. Clinical Relevance One-day MBEC assays may overestimate the local antimicrobial levels needed to kill organisms in biofilm if local levels are sustained at MBEC or above for longer than 24 hours. Future studies are needed to confirm that antimicrobial levels achieved clinically from local delivery are above the MBEC at relevant time points and to confirm that MBEC for in vitro microorganisms accurately represents MBEC of in vivo organisms in an clinical infection.

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Introduction

For infections that do not involve biofilms, antimicrobial therapy is guided by the minimum inhibitory concentration (MIC), at which microorganism multiplication is prevented, relying on time and host mechanisms to kill the viable organisms. For antimicrobials to kill planktonic microorganisms, higher concentrations are required. The minimum bactericidal concentration (MBC) is defined as the concentration that will cause a 3-log reduction in microorganism numbers. Some viable microorganisms (0.1%) still remain for time and the host to eradicate. When the MBC is more than fourfold higher than MIC, the antimicrobial action is considered nonbactericidal. To kill the microorganisms in biofilm-related implant infections, much higher antimicrobial concentrations are needed. The minimum biofilm eradication concentration (MBEC), an in vitro estimate of the lowest antimicrobial concentration that will kill all the microorganisms in a biofilm, including the persister cells, can be hundreds- to thousands-times higher than the MIC for the same antimicrobial-microorganism pair [1, 5–7, 16–18]. The clinically important distinction between MBEC for bacteria in biofilm and MIC or MBC for bacteria in a planktonic (floating) state is that persister cells in biofilm are not accessible to killing by the host. For antimicrobials to achieve a cure for biofilm-related infections, antimicrobial levels need to be at MBEC or higher. MIC determination is standardized by exposing the bacteria to antimicrobials for 24 hours. Systemic therapy to prevent bacterial multiplication is continued at levels above MIC until cure is achieved. There is no critical need to know if the value for MIC would be different if longer antimicrobial exposure times were used for its determination. Treatment for biofilm-related implant infections usually includes local delivery to provide continuous levels that steadily decrease over time. It is critically important to know what level is needed to achieve total kill of all persister cells and how long it takes so that the local delivery can be formulated to achieve those parameters. MBEC determination is performed by exposing bacteria from the biofilm to antimicrobials, also for 24 hours or less. Local delivery is expected to achieve levels above MBEC for several days. In vivo pilot data from quantitative MRI and in vivo tissue levels are consistent with levels from local delivery of 100 to 2000 μg/mL lasting 5 days or more indicating the concentrations consistent with expected MBECs can be achieved from local delivery [12, 15]. Although MBEC is the concentration that potentially will eradicate biofilm in 24 hours, it is not known if treatment duration longer than 24 hours would require the same antimicrobial concentration. The relationship between antimicrobial exposure time and MBEC is not known. Consequently, we sought to determine whether increased antimicrobial exposure time affects the MBEC.

Materials and Methods

Five common orthopaedic pathogens, including three Gram-positive organisms, methicillin-sensitive *Staphylococcus aureus* (ATCC #49230) (MSSA), methicillin-resistant *Staphylococcus aureus* (ATCC #BAA-1556) (MRSA), *Staphylococcus epidermidis* (ATCC #35984), and two Gram-negative organisms, *Escherichia coli* (ATCC #25922), *Pseudomonas aeruginosa* (ATCC #27853), were used to grow biofilms. Tobramycin (X-GEN Pharmaceuticals, Inc, Big Flats, NY, USA) and vancomycin (Mylan Institutional, LLC, Rockford, IL, USA) were the antimicrobials selected because they are commonly used for local antimicrobial delivery in orthopaedic infections and they are effective for most orthopaedic pathogens; both are effective against Gram-positive microorganisms including MRSA, and tobramycin is effective against Gram-negative microorganisms (tobramycin and vancomycin are FDA-approved for systemic and topical administration; tobramycin has FDA approval for local delivery, vancomycin does not) [11]. Biofilms from Gram-positive microorganisms were exposed to four antimicrobial formulations, tobramycin sulfate and vancomycin hydrochloride as single agents and two tobramycin:vancomycin combinations prepared in six concentrations, 250, 500, 1000, 2000, 4000, and 8000 μg/mL with 0 μg/mL as a positive control. The tobramycin:-vancomycin combinations were prepared in ratios of 3:1 (3T:1V) and 1:1 (1T:1V) by weight of active drug (eg, for 4000 μg/mL, 3T:1V = tobramycin 3000 μg/mL and vancomycin 1000 μg/mL and 1T:1V = tobramycin 2000 μg/mL and vancomycin 2000 μg/mL). Gram-negative microorganisms were exposed to only tobramycin as a single agent in the same concentrations. Additionally, *E. coli* was also exposed at tobramycin concentrations of 62.5 μg/mL and 125 μg/mL.

Overnight cultures of each microorganism were grown in 3 mL trypticase soy broth (TSB) (BD, Sparks, MD, USA) by inoculating TSB with a single colony from an agar stock. Overnight inocula of *S. aureus,* MSSA and MRSA, *S. epidermidis,* and *P. aeruginosa* were diluted 1:50 in TSB supplemented to a total glucose concentration of 1.25 w/v% (TSB-1.25%). *E. coli* overnight inocula were diluted 1:50 in MOPS (3-[N-morpholino]propanesulfonic acid) minimal media (Teknova, Hollister, CA, USA). Two hundred microliters of each microorganism suspension were plated on a 96-well tissue culture plate (Celltreat, Shirley, MA, USA). Static cultures were grown for 24

hours at 37° C in a temperature-controlled incubator (Precision; Thermo Scientific, Waltham, MA, USA) without shaking before being exposed to antimicrobials. Biofilm production was confirmed by crystal violet assay as described by Kwasny and Opperman [9]. The initial biofilm bioburden was measured using broth microdilution on trypticase soy agar plates (Sigma-Aldrich, Co, St Louis, MO, USA) described by Miles et al. [14]. Biofilms were grown for 24 hours in triplicate, scraped from the respective wells in a 96-well plate, suspended in 0.5 mL of TSB, sonicated for 5 minutes at 42 kHz (Aquasonic 75D; VWR, Radnor, PA, USA), then serially diluted 1:9 with TSB, plated, and counted. Initial bioburdens (in colony-forming units/well) were *S. aureus*: 8.3 x 10^5, MRSA: 4.2 x 10^6, *S. epidermidis*: 3.9 x 10^6, *E. coli*: 9.3 x 10^7, and *P. aeruginosa*: 9.3 x 10^7.

The MICs of tobramycin, vancomycin, and both tobramycin:vancomycin combinations were determined for all five bacteria by broth microdilution in cation-adjusted Mueller-Hinton Broth (CAMHB) (Becton-Dickinson, Sparks, MD, USA) per CLSI methods (Table 1) [2]. Each microorganism was cultured overnight in TSB and then diluted in CAMHB to 10^6 colony-forming units/mL. Fifty microliters of each bacterial suspension were mixed 1:1 with 50 μL of CAMHB containing serial concentrations of each concentration of each antimicrobial formulation in a round-bottomed 96-well culture plate (Corning Inc, Corning, NY, USA), creating suspensions of 0.5 x 10^5 bacteria in media containing serially doubled antimicrobial concentrations from 0.125 to 128 μg/mL for all four antimicrobial formulations. The lowest concentration that inhibited growth after overnight incubation at 37° C was recorded as the MIC.

MBEC determinations were performed on biofilms grown in triplicate for 24 hours and rinsed once with 200 μL of TSB-1.25% to remove planktonic cells keeping the biofilm on the bottom of the wells intact. The growth media was replaced with TSB-1.25% containing each concentration of each antimicrobial formulation. The biofilms were exposed to antimicrobials for three durations: 1, 3, or 5 days. Media exchanges were performed every other day with media containing the respective antimicrobial concentration. The three Gram-positive microorganisms were exposed to serial concentrations from 250 to 8000 μg/mL for all three durations in triplicate; Gram-negative microorganisms were exposed to tobramycin only for concentrations of 250 to 8000 μg/mL for all three durations in triplicate. *E. coli* was also exposed to tobramycin in concentrations of 62.5 and 125 μg/mL for all three durations. At the end of the antimicrobial exposure period, biofilms were rinsed four times with 200 μL of antimicrobial-free TSB-1.25% to minimize residual antimicrobial in the biofilm. Biofilms were then removed from each well by scraping with sterile transfer pipettes (Karter Scientific, Lake Charles, LA, USA). The collected biofilm fragments were placed in 3 mL of antimicrobial-free TSB for subculturing. The subcultures were sonicated at 42 kHz for 5 minutes and then incubated at 37° C for 21 days. Aliquots of rinse media, sonicated and incubated similarly to biofilm subcultures, were used as bacteria-free negative controls. Each subculture was recorded as a nominal response (growth/no growth). It was not possible to identify small colony variants in the subculture broth although we expect that they were present when the staphylococcal subcultures were positive [8]. The MBEC was recorded as the lowest concentration that at least two of the three replicates were culture-negative after subculture incubation for 21 days, producing noncontinuous ordinal data with varying intervals. The replicates were not averaged. Differences between antimicrobial exposure times and between single and combination antimicrobial formulations were analyzed using the sign test.

Results

MBEC was lower when antimicrobial exposure time was longer (sign test, Day 1 vs Day 5 $p \leq 0.000488$). For the *Staphylococcus* species, the MBEC was lower in all antimicrobial/microorganism pairs from 5 days of exposure than one except for tobramycin/*S. epidermidis,* which was above the highest level tested at all exposure times (Table 1). Excluding the tobramycin/*S. epidermidis* combination, the MBEC range for Day 1 was 4000 to > 8000 μg/mL and the MBEC range for Day 5 was < 250 to 8000 μg/mL. MBEC for tobramycin/*P. aeruginosa* was less with 5 days of exposure than with 1 day, ≤ 250 μg/mL versus 2000 μg/mL, respectively, and for *E. coli*, ≤ 62.5 μg/mL versus 125 μg/mL, respectively, for 5 and 1 days. The MBEC for the *Staphylococcus* species was lower for exposure to antimicrobial combinations than single drug formulations (sign test, $p \leq 0.0386$).

Discussion

Biofilm-based infections are difficult to treat in part because bacterial persister cells are tolerant to systemic levels of antimicrobials and the glycopolysaccharide matrix is a protective substrate that prevents killing by immune cells [3, 10]. Surviving persisters can repopulate the biofilm matrix when systemic antimicrobials are discontinued. The antimicrobial level needed to kill persisters in biofilm, MBEC, is typically determined in vitro by exposing biofilm to antimicrobials for 24 hours or less [1]. It is unknown how duration of exposure to antimicrobials affects MBEC. We therefore explored how antimicrobial exposure time affects MBEC.

Table 1 Minimum inhibitory concentrations (MICs) and 1-, 3-, and 5-day minimum biofilm eradication concentration (MBEC) of *Staphylococcus aureus* (MSSA) (ATCC-49230), *Staphylococcus aureus* (MRSA) (ATCC-BAA-1556), *Staphylococcus epidermidis* (ATCC-35984), *Escherichia coli* (ATCC-25922), and *Pseudomonas aeruginosa* (ATCC- 27853) biofilms exposed to tobramycin, vancomycin, and two combinations of tobramycin and vancomycin by weight of active drug in 3:1 ratio (3T:1V) and 1:1 ratio (1T:1V)

Antimicrobial	MIC (μg/mL)	MBEC (μg/mL)		
		Day 1*	Day 3	Day 5*
S. aureus (MSSA)$^{ATCC-49230}$				
Tobramycin	1	>8000	4000	1000
3T:1V	0.5	**8000**	**500**	**500**
1T:1V	0.5	**>8000**	**4000**	**≤250**
Vancomycin	2	>8000	>8000	2000
S. aureus (MRSA)$^{ATCC-BAA1556}$				
Tobramycin	1	>8000	>8000	8000
3T:1V	0.5	**8000**	**500**	**500**
1T:1V	0.25	**8000**	**2000**	**≤250**
Vancomycin	2	>8000	8000	2000
S. epidermidis$^{ATCC-35984}$				
Tobramycin	32	>8000	>8000	>8000
3T:1V	8	8000	2000	4000
1T:1V	4	4000	2000	2000
Vancomycin	2	>8000	1000	1000
E. coli$^{ATCC-25922}$				
Tobramycin	2	125	≤62.5	≤62.5
P. aeruginosa$^{ATCC-27853}$				
Tobramycin	0.25	2000	≤250	≤250

MBECs are higher on Day 1 than Day 5; * sign test, $p < 0.000488$; for the *S. aureus*, MBECs for the antimicrobial combinations (bold values) are lower than for single antimicrobial therapy (sign test, $p < 0.0386$).

There are weaknesses in this study. First, the biofilms we tested were cultured under favorable conditions in nutrient-rich media without any apparent stressors such as unfavorable pH, O_2 tension, osmolality, nutrient availability, or host defenses (antibody and cellular). The environments in which biofilms develop in vivo can be vastly different with markedly unfavorable conditions that are expected to induce adaptive mechanisms, which modify the local environment and modify the microorganism phenotype (metabolic rate, protein production, cellular replication, and expression of surface proteins) so that local conditions in the biofilm are favorable for microorganism survival [13]. Biofilm susceptibility may be different on the flat, nonporous, hydrophobic plastic surfaces used in our assay compared with the various surfaces that may be colonized by biofilm in vivo. The antimicrobial exposure time-dependent decrease in MBEC that we report for microorganisms in a nonthreatening environment is expected to also occur in vivo, but the values for the MBECs associated with clinical infections may differ. Second, we did not investigate exposure times longer than 5 days. Given the large progressive decrease in MBEC over 5 days of exposure to antimicrobials, by as much as 32-fold, an unanswered question remains: How much further will MBEC decrease with longer durations of exposure to antimicrobials? An extended exposure study would answer this question. However, based on previously published quantitative MRI findings and in vivo tissue levels, concentrations in the range of 100 to 2000 μg/mL in a surgical wound are plausible for 5 days using high-dose delivery formulations. We consider 5 days a reasonable clinical goal for local antimicrobial delivery [12, 15]. Third, we used a *S. epidermidis* strain, which is resistant by MIC (32 μg/mL) and forms biofilms that are tobramycin-tolerant to > 8000 μg/mL; MBEC was measured in our laboratory outside the scope of this study to be approximately 40,000 μg/mL for tobramycin. We chose to study this microorganism because of its known robust biofilm production. Although it is a laboratory strain not obtained directly from a clinical infection, this finding indicates that microorganisms with extremely high MBECs for a single drug can be eradicated by dual therapy at levels plausibly achievable by local delivery [12, 15], in this case tobramycin and vancomycin. Based on the hundreds of strains that exist for

staphylococcal species and their ability to develop persister cells tolerant to antimicrobials, it seems possible that biofilm microorganisms with similar antimicrobial tolerance could be responsible for recalcitrant clinical infections, underscoring consideration for codelivery of antimicrobials with different mechanisms of action such as vancomycin and tobramycin. Fourth, we did not quantify the thickness of the biofilm or the bacterial density in the biofilm before initiating antimicrobial exposure. Bioburden in the biofilm is unlikely to affect total kill when the antimicrobial level is maintained at or above MBEC like in our study, although organism density may affect antimicrobial tolerance of the persister cells through quorum sensing. Thicker biofilm could limit transport of the antimicrobial leading to lower concentrations within the substance of the biofilm, requiring higher concentrations in the media to cause a total kill [13]. Our study measured MBEC. We did not determine the mechanisms that affect MBEC. If increased microorganism density and decreased antimicrobial transport did affect the MBEC values in our study, it is likely these factors would also affect MBEC in vivo. Fifth, all the microorganisms we studied were laboratory stock, which is commonly accepted for biofilm-related studies. The MBEC values for in vitro microorganisms propagated from laboratory stock may not represent the MBEC for in vivo microorganisms. MBEC determination for microorganisms taken directly from clinical infections is needed to address this concern.

We found that organisms in biofilm are more susceptible to antimicrobials by a meaningful amount, up to 32-fold in our study, when they are continuously exposed to antimicrobials for longer times. The relationship between MBEC and antimicrobial exposure time has not been reported previously; however, a relationship between concentration and microorganism killing with antimicrobial exposure time has been reported. Under constant antimicrobial concentration, time kill studies report increased kill with increasing time for biofilm-associated bacteria [20]. Increases in MIC over time reported in response to repeated sublethal antimicrobial exposures are likely due to progressive biofilm growth [19]. Our 5-day MBEC data are similar to or less than previously reported data from antimicrobial exposure of 24 hours [1]. There is one MBEC, 3T:1V/*S. epidermidis* on Day 5, that is not consistent with all the other values, increasing one dilution from the prior time period (Table 1). We attribute this to contamination of the 2000-μg/mL subcultures, falsely leading to the reading of 4000 μg/mL. The MBECs we measured at Day 5 are levels that could plausibly be sustained in vivo from local delivery for 5 days [12, 15]. For the *S. aureus* strains that we studied, MBECs for combined antimicrobial formulations were lower than for single drug exposures (sign test, $p < 0.0386$), which is consistent with synergy against planktonic *S. aureus* strains reported by other authors [21]. Although the data do not allow statistical analysis, the pattern of MBEC values for *S. epidermidis* appears to be more consistent with proportion of vancomycin in the formulation rather than synergy between tobramycin and vancomycin, consistent with the tolerance to tobramycin above 8000 μg/mL. Our data support previous reports that MBECs are very high compared with MICs [1, 4] but challenge reports that MBECs are immeasurable [1, 5, 17].

In conclusion, although the antimicrobial exposure time-dependent increase in antimicrobial susceptibility was found for all five biofilm-associated microorganisms that we studied, future studies are required to confirm that a similar increase in antimicrobial susceptibility occurs for other pathogens. One-day assays for MBEC may overestimate the local antimicrobial levels needed to kill organisms in biofilm if local levels are sustained for longer than 24 hours. Future studies are needed to confirm that the antimicrobial levels achieved clinically from local delivery are above the MBEC at relevant time points and to confirm that MBEC values for in vitro microorganisms represent the MBEC for in vivo microorganisms causing clinical infections.

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