Original research article

Finding the Optimal Tobramycin and Vancomycin Exposure to Remove Biofilms on Muscle and Bone Tissue In Vitro

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Abstract

Background: Chronic orthopaedic infections are brought on by bacterial biofilms. Without adjuvant local antimicrobials, surgical debridement to remove biofilm may be inefficient because undiscovered biofilm pieces may stay in the site and reactivate the infection if left untreated. The levels and length of antibiotic exposure required to remove bacteria from clinical biofilms are still mostly unknown. For bacterial biofilms formed on bone and muscle in vitro, we calculated the minimal biofilm eradication concentration (MBEC) of tobramycin and vancomycin.

Method: CFU counts were used to characterizing the pathogen biofilms of S. aureus, S. epidermidis, E. faecalis, P. aeruginosa, and E. coli, which are commonly encountered in musculoskeletal illnesses. Serial $\log_2$ dilutions (4000-31.25 µg/mL) of tobramycin, vancomycin or a 1:1 mixture of both medicines were applied to tissue specimens covered in biofilm for 5, 25, or 70 hours. To test bacterial survival after antibiotic exposure, tissues were subcultured. For each pathogen-antimicrobial-exposure-time combination, the MBEC was determined as the concentration at which there were no surviving bacteria.

Results: On tissue, all infections that were tested developed biofilm. Using MBEC on muscle or bone, tobramycin/vancomycin (1:1) was the most effective antibacterial treatment, often in the range of 200-750 µg/mL with 25 or 70hr exposure. For 53.2% of biofilms between 5 and 25 hours, 53.2% of biofilms between 25 and 70 hours, and for 76.6% of biofilms between 5 and 70 hours, MBEC decreased with exposure duration. In comparison to equivalent MBECs in muscle tissue, MBECs on bone were substantially greater ($p < 0.04$). The majority of the time, tissue MBECs were lower than MBECs for the same pathogens on polystyrene tissue-culture plates that had previously been published.

Conclusion: While high-dose antimicrobial-loaded bone cement may be practically feasible, the majority of MBECs for orthopedic infections on bone and muscle is on the order of 200-650 **µ**g/mL of vancomycin+tobramycin when sustained for at least 25 hr (ALBC).

Keywords: *Biofilm susceptibility, antimicrobial susceptibility, local antimicrobial administration, surgical site infection, bone and joint infection, and minimum biofilm eradication concentration*

Introduction

The majority of surgical site infections, such as those affecting the bone, joints, and implants, are brought on by biofilms, which are bacterial communities in a polysaccharide matrix that form on their own as a result of bacterial adhesion to implant surfaces or damaged tissue

surfaces [1]. Because they are typically resistant to antimicrobials, biofilms pose a significant obstacle to effective clinical management [2; Figure 1]. Systemic antimicrobials that restrict or reduce bacterial growth in combination with a healthy host immune response can effectively treat planktonic infections [3]. Antimicrobial-tolerant bacteria, on the other hand, can withstand systemic antibiotics and avoid being killed by the human immune system in infections caused by biofilms [4].

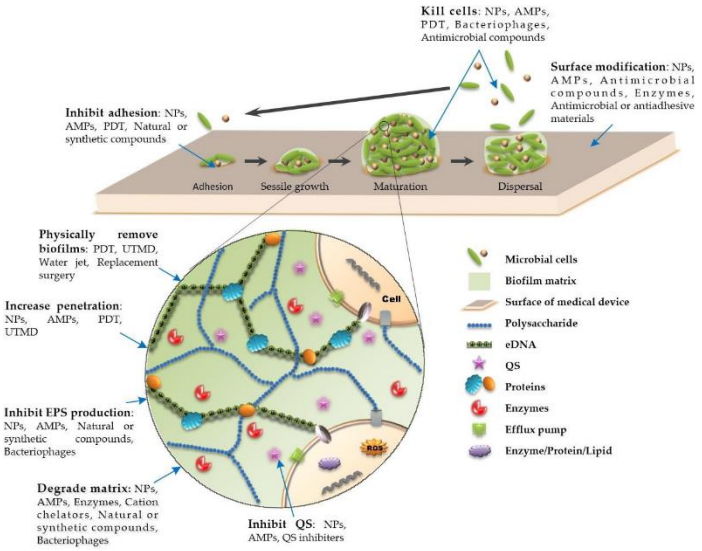

Figure 1: Approaches to overcome clinically associated biofilm

As an infection worsens, biofilm can develop on damaged host tissue surfaces, and it has recently come to light that floating bacterial clumps in synovial fluid can develop into biofilm [5]. Although surgical debridement of infected tissue is a standard treatment for established orthopaedic infections, these treatments could leave biofilm along the debridement margins or fragments in the surgical wound [4]. Preservation of biofilm bacteria in the site after surgery raises the possibility of recurrent infection [4]. Antimicrobials are frequently used locally to eliminate microorganisms, including any lingering biofilm, after debridement [6].

The minimal biofilm eradication concentration (MBEC), which is typically two or more orders of magnitude greater than the minimum inhibitory concentration (MIC) established for planktonic organisms [7], is the lowest level of an antibiotic that will kill all bacteria in a biofilm. Antimicrobials are given directly to the surgical site to maximise efficacy while lowering systemic toxicity because MBEC medication levels are higher than acceptable systemic levels. There are, however, few data MBEC reporting for particular infections in therapeutically pertinent situations. MBECs are often assessed in vitro, frequently utilising abiotic surfaces, with no standardised approach [9], despite the fact that biofilms might evolve differently depending on the surface on which they are grown.

Although biofilms grown on polystyrene may not be indicative of the clinical environment, previously determined MBECs for biofilms generated in polystyrene microtiter well plates that were generally non the order of 2,000-16,000 µg/mL with 25 hr exposure [10]. Another crucial point to remember is that antimicrobial activity takes time to take effect, and that as exposure time increases, less concentration is needed to completely kill all bacteria [11]. Once more, there aren't many data on MBECs during clinically significant antibiotic exposure intervals. The drug levels in surgical wounds in vivo peak around MBEC levels (>100 µg/mL) within the first 24 hours [12], and in some cases are sustained for up to 72

hours [13]. This information comes from earlier investigations on local antimicrobial administration.

The main goal of this research was to develop MBEC using a technique that preserves important elements of clinical infections, such as biofilms of orthopaedic pathogens growing on muscle and bone surfaces, medication exposure times up to 70 hours, and clinically important antimicrobials. Tobramycin and vancomycin were picked because they are widely used, clinically effective antibiotics for local delivery in orthopaedics, including in suggested formulations of antibiotic-loaded bone cement (ALBC).

METHODS:

Study Design: This was a prospective study carried out in SCB Medical College, Cuttack from January 2021 to March 2022.

Methodology: Standard in vitro growth experiments were modified to create biofilm on tissue [15]. Bacterial suspensions were made by overnight cultures in tryptic soy broth, which were subsequently diluted to 1.5 x 10^7 CFUs/mL in TSB supplemented with 1% glucose. Tissue samples were placed in the wells of sterile, flat-bottom 96-well tissue culture plates, then the tissue samples were immersed in 100 µL of bacterial suspension (one organism was used per plate) and incubated for 70 hours at 36°C to produce biofilms. To guarantee that tissues were exposed to a similar density of planktonic bacteria during the growth phase without disturbance and because significant evaporation did not take place, TSB was not exchanged. In bone and muscle, tissue-bound biofilms were identified by counting CFUs for each bacterial strain using a drop-plate approach [16]. Before creating serial dilutions for counting, tissue specimens were cleaned, put into 1 mL of sterile TSB, and bath sonicated for 10 minutes to loosen adhering bacteria. Biofilms were preserved in 100 mM phosphate buffer and 2.4% glutaraldehyde overnight at 4°C. Samples were critical point dried, sputter-coated with gold, and mounted after being dehydrated in a water-acetone series. Tobramycin alone, vancomycin alone, and a 1:1 mass combination of tobramycin and vancomycin were examined as three antibacterial regimens. By using a checkerboard assay with 64 concentration combinations, the fractional inhibitory concentration (FIC) index for tobramycin and vancomycin was calculated for each organism [17]. Using the checkerboard assay, which is comparable to the CLSI broth microdilution method, eight log2 serial dilutions of the respective antibiotic were applied in the control wells to estimate the minimum inhibitory concentration (MIC) of tobramycin and vancomycin.

Sample Size: Two S. aureus, three S. epidermidis, and two each of E. faecalis, P. aeruginosa, and E. coli were among the nine harmful bacterial strains that were examined. The strains of S. aureus and S. epidermidis are multidrug resistant.

Statistical Analysis: The two-tailed paired t-test was used to evaluate whether the differences in CFUs between the growth substrates were statistically significant ($\alpha = 0.05$). The sign test was used to assess differences in MBEC between growth substrates, antimicrobial exposure durations (5hr, 25 hr, and 70 hr), and against MIC values. The McNemar's test was used to examine the frequency of severe MBEC values between antimicrobial regimes and tissue types.

Ethical Considerations: SCB Medical College, Cuttack approved all research, which was carried out in accordance with all pertinent institutional and international norms.

RESULTS:

Determination of FIC AND MIC

S. epidermidis, a planktonic organism, was shown to exhibit a high MIC to tobramycin (15 µg/mL) but P. aeruginosa and E. coli, two Gram-negative microbes, were predictably unaffected by vancomycin at any quantity tested (MIC > 63 g/mL). The MIC ranged from 0.124 to 7 µg/mL for all other antimicrobial/microorganism pairings that were examined (Table 1). There was no antagonistic interaction between tobramycin and vancomycin for any of the organisms in the checkerboard assays, and tobramycin and vancomycin only synergistically affected S. epidermidis (FIC index 0.17).

Table 1: Musculoskeletal-associated biofilms: Minimum inhibitory concentration (MIC) and fractional inhibitory concentration (FIC) index. The FIC index values show medication interactions that are either antagonistic (≤3), neutral (<0.4–3), or synergistic (≤0.4).

Species / ATCC#	Vancomycin (µg/ml)	Tobramycin (µg/ml)	Tobramycin/Vancomycin 1:1 Combination
			FI C Index
S. aureus (1)	1	0.24	1.21
S. aureus (2)	1	0.4	1.33
S. epidermidis (1)	1	0.4	0.80
S. epidermidis (2)	6	15	0.55
S. epidermidis (3)	3	16	0.17
E. faecalis (1)	3	0.124	1.07
E. faecalis (2)	3	0.124	1.68
P. aeruginosa (1)	>63	1	0.88
P. aeruginosa (2)	>63	0.4	0.82

Characterization of Biofilm

Biofilm formation was confirmed on all specimens by CFU counts and SEM pictures. There were many different phenotypes of biofilms, and many of them featured broad EPS production on tissue surfaces. The filamentous, sheet-like, or granular extracellular structures that are seen on tissue surfaces between individual bacteria or those in which bacteria are embedded are thought to be EPS. On a qualitative level, muscle appeared to have more widespread biofilm covering than bone. The morphology of the study organism was consistent with the monomicrobial appearance of each biofilm. CFUs per specimen ranged from $8.34x10^2$ to $1.11x10^7$ on bone and from $1.18x10^3$ to $4.84x10^7$ on muscle; there was no appreciable difference in CFUs for biofilms on bone and muscle specimens ($p = 0.2741$); Pairwise two-tailed t-test.

Determination by MBEC

Tobramycin and vancomycin had MBECs that were larger than their respective MIC values against all 9 bacterial biofilms on muscle and bone ($p<0.0002$). The only organism present in all positive subcultures tested was the original organism. Throughout the first 4 to 8 days of subculture, 80% of positive subcultures appeared, and by day 15, all positive subcultures had turned turbid. MBEC reduced with increasing antimicrobial exposure time across all treatment combinations (i.e., for a specific microbe, drug dosage and exposure period, and tissue) ($p = 0.0002$, 5 vs. 25 hr; $p= 0.0123$, 25 hr. vs. 70 hr). 53.2% of cases between 5 and 25 hours, another 53.2% between 25 and 70 hours, and 76.6% of cases between 5 and 70 hours

saw a drop in MBEC. There were clear breakpoints in 88.2% of the cultural series. 2.1% of the total number of subcultures were found to be false negatives (negative subculture present in a concentration between consecutive positive subcultures), while 0.2% were found to be false positives (positive subculture present in a concentration between consecutive negative subcultures).

Severe MBEC

Tobramycin/vancomycin was found to be less commonly associated with extreme MBEC levels ($\geq$ 2,000 g/mL) than either tobramycin alone ($p = 0.0159$) or vancomycin alone ($p < 0.0002$). With the tobramycin/vancomycin combination, bone biofilms experienced extreme MBECs more frequently than muscle biofilms did throughout all exposure durations ($p < 0.0002$).

DISCUSSION:

This work's main objective was to determine the antimicrobial concentration and exposure duration required for two widely used antimicrobial agents to completely eliminate a variety of orthopaedic pathogens from biofilms produced on clinically significant tissue surfaces. There are no tests for the antimicrobial susceptibility of bacteria in biofilms that have received clinical validation. The most popular techniques involve applying antimicrobials for up to 24 hours after biofilm development, either on the pegs of the Calgary biofilm device [16] or in standard tissue culture plates [18]. These tests do not take into consideration the intricate surfaces of soft tissue and bone, such as the interior surfaces of the bone or the three-dimensional structures that biofilms develop on in infections of the musculoskeletal system. Muscle and bone tissue samples were used to test the antimicrobial susceptibility of biofilms because they replicate the physical and chemical characteristics of infected tissue fragments that can be found in a lesion after debridement. This research yielded a number of therapeutically significant findings: 1) MBECs vary depending on the growth substrate; 2) a clinically significant decrease in MBECs occurs with increasing antimicrobial exposure time, especially between 5 and 25 hr. MBECs of biofilms growing on muscle and bone using 1:1 tobramycin/vancomycin combination therapy were generally in the range of 200-650 µg/mL after 25 hr of antimicrobial exposure.

First, the maturity of the biofilm was not identified. Cell multiplication and metabolic activity decline as biofilms grow, which results in a commensurate decline in antimicrobial susceptibility [19]. Biofilm development was maintained constant at 70 hours to standardise the experiment, even though the antimicrobial susceptibility versus biofilm growth time will vary between organisms. Second, we made the decision not to change the liquid medium while the biofilm was growing or while it was exposed to an antimicrobial. Each time, there was little to no evaporation, and the tissue samples were kept completely buried. Because biofilms that would have been most vulnerable to nutrient deprivation (positive controls and biofilms exposed to low antimicrobial concentrations) consistently survived and grew after a 70-hour exposure, we do not think that nutrient deprivation is what causes the decrease in MBEC over exposure time. Third, for the purpose of determining MBEC, we employed tiny tissue samples of varying sizes. This research was not done to examine the effectiveness of antimicrobials in treating infections that had not been properly surgically debrided or to test their ability to penetrate tissue. To guarantee thorough antimicrobial penetration and to roughly estimate tissue fragments that might stay in a wound after intralesional debridement, small tissue specimens (20–50 mm^3) were employed. As previously stated, despite varying specimen sizes, 88.2% of the time, subculture results within each series were in perfect agreement. The overall number of bacteria is less significant because we examined the

presence or absence of bacteria rather than counting the number of surviving bacteria. Nonetheless, larger tissue specimens would have allowed for a greater number of bacteria to cling to the surface. Fourth, as previously documented in clinical practise [20], we employed bath sonication to help liberate bacteria attached to tissue in biofilm. Yet, this method has also been shown to have bactericidal effects [21]. To induce significant inaccuracy into the MBEC determination in this test, practically all bacteria would need to be eliminated by sonication. It might, however, have had a deleterious impact on CFU measurement. Finally, because we did not assess the antimicrobial concentration in the subculture media and because the approach involves immersing tissues for subculture that have been previously preserved in up to 3,000 µg/mL of antimicrobials, there is a chance that regrowth in subcultures may be hindered. Although we found positive cultures for samples incubated in 3,000 µg/mL after brief exposure times, showing that antimicrobials in these samples were reduced to sub-MIC levels, permitting regrowth, the washing procedure that we utilised seemed to be adequate.

Each MBEC value in the current investigation was calculated using a series of 9 distinct subcultures in addition to 3 controls to ensure bacterial viability and the absence of cross-contamination. Each subculture's binary results point to a distinct breakpoint within the series, and clean breakpoints were found in 88.2% of the subculture series. Moreover, just 2% of false negatives and 0.2% of false positives across all subcultures were found. In spite of the absence of replication, the high frequency of clean breakpoints within cultural series shows that sample-to-sample variability was minimal and the MBECs are fairly accurate. To determine MIC in a single experiment is typically conventional procedure, and in reference data these are frequently presented as a range rather than a single value. This illustrates that despite stringent controls, there is some intrinsic variability in microbiological investigations. Similar to MBEC values, which show ranges of antimicrobial susceptibility, these values are likely to change during an interval of one experiment to experiment. Second, the study's scope was constrained to five bacterial species, three of which had just one strain each. The chosen bacteria were initially obtained from clinical illnesses with the goal of providing a clinically representative spectrum of pathogens. Future research will likely focus on additional species, and we admit that testing biofilms recovered from infections may be more clinically applicable. Finally, the MBEC for biofilms developing on implant materials was not measured. The same process will need to be used to evaluate the surfaces of implant material. Fourth, because tobramycin and vancomycin have a long history of use in local delivery in orthopaedics and provide broad range protection against orthopaedic infections, we decided to investigate them both alone and in combination[20]. It would be interesting to explore additional antimicrobials for the removal of bacteria from biofilms in orthopaedic infections in the future. Due to the fact that tobramycin [21,22] and vancomycin [23,24] are both water- and heat-stable under comparable conditions and are not metabolised, we also did not test antimicrobial concentrations after exposure. Finally, we did not look into the possibility of local tissue damage, which has been linked to such high antimicrobial dosages in in vitro studies [25]. MBEC was lower after a 25-hour exposure for more than half of the pathogens examined than it was after a 5-hour exposure. Less pronouncedly, longer exposure times up to 70 hr were linked to decreased MBEC values. Just two of the 21 MBEC values across all conditions were classified as severe, with tissue MBEC for tobramycin/vancomycin often falling in the 200-650 g/mL total concentration range at a 25-hour exposure (all on bone). As a result, we predict that medication levels of 200–650 µg/mL sustained for 25 hr may be a suitable and achievable target for local delivery in the treatment of orthopaedic infection.

Our data show that MBEC changes depending on the growth substrate, despite earlier reports [11-15,18] indicating biofilm susceptibility on various surfaces.
In vitro biofilms created on bone tissue have been demonstrated to be more resistant to tobramycin and vancomycin than biofilms created on PMMA and PTFE biomaterials or planktonic cultures [14]. In this study, we discovered that the MBECs of biofilms on muscle are often lower than those on bones.

Except for S. epidermidis on muscle and E. coli on muscle and bone, MBECs on tissue were lower than on polystyrene. Due to a lack of statistical power, there were no statistically significant differences between polystyrene and either muscle or bone ($p = 0.0577$ for polystyrene vs. muscle; $p = 0.5270$ for polystyrene vs. bone). We believe that the MBEC levels measured here should not be compared to those needed for clinical cure because clinical local delivery may not sufficiently reach all bacteria and because biofilms are generated in our experiment under optimum growth conditions without immune system assault.

CONCLUSION:

As a result, MBECs identified using biofilms grown on tissue may be more indicative of MBECs of pathogens in clinical musculoskeletal illnesses than MBECs identified using previously described methods for identifying biofilm susceptibility. The MBECs for common musculoskeletal infections are far higher than the concentrations that can be reached through systemic injection, although the majority of them are probably reachable through local delivery methods if tissue levels of 200–650 µg/mL are sustained for at least 25 hours.

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