Characterization of Antimicrobial Susceptibility of Bacterial Biofilms on Biological Tissues

by

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ABSTRACT

Prosthetic joint infection (PJI) is a devastating complication associated with total joint arthroplasty that results in high cost and patient morbidity. There are approximately 50,000 PJIs per year in the US, imposing a burden of about $5 billion on the healthcare system. PJI is especially difficult to treat because of the presence of bacteria in biofilm, often highly tolerant to antimicrobials. Treatment of PJI requires surgical debridement of infected tissues, and local, sustained delivery of antimicrobials at high concentrations to eradicate residual biofilm bacteria. However, the antimicrobial concentrations required to eradicate biofilm bacteria grown *in vivo* or on tissue surfaces have not been measured. In this study, an experimental rabbit femur infection model was established by introducing a variety of pathogens representative of those found in PJIs [*Staphylococcus Aureus* (ATCC 49230, ATCC BAA-1556, ATCC BAA-1680), *Staphylococcus Epidermidis* (ATCC 35984, ATCC 12228), *Enterococcus Faecalis* (ATCC 29212), *Pseudomonas Aeruginosa* (ATCC 27853), *Escherichia Coli* (ATCC 25922)]. Biofilms of the same pathogens were grown *in vitro* on biologic surfaces (bone and muscle). The ex vivo and in vitro tissue minimum biofilm eradication concentration (MBEC; the level required to eradicate biofilm bacteria) and minimum inhibitory concentration (MIC; the level required to inhibit planktonic, non-biofilm bacteria) were measured using microbiological susceptibility assays against tobramycin (TOB) and vancomycin (VANC) alone or in 1:1 weight combination of both (TOB+VANC) over three exposure durations (6 hour, 24 hour, 72 hour). MBECs for all treatment combinations (pathogen, antimicrobial used, exposure time, and tissue) were compared against the corresponding MIC values to compare the relative susceptibility increase due to biofilm formation. Our data showed median *in vitro* MBEC to be 100-1000 times greater than the *median* MIC demonstrating the administration of local antimicrobial doses at MIC level would not kill the persisting bacteria in biofilm. Also, administering dual agent (TOB+VANC) showed median MBEC values to be comparable or lower than the single agents (TOB or VANC)

DEDICATION

"To my parents, Sujatha Venugopal"

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CHAPTER 1 – INTRODUCTION

1.1 Prosthetic Joint Infection (PJI)

Prosthetic joint infection is usually caused by bacteria adhering to an implant and adjacent biological surfaces and formation of an exopolysaccharide matrix (biofilm). It leads to the complete destruction of the joint with potential to spread to other sites causing life-threatening conditions if not diagnosed and treated properly. With the presence of a prosthetic device, the probability of infection in the joint rises. In the United States alone, more than one million arthroplasties are conducted each year and it is estimated to rise to four million by 2030.[1] With more than 50,000 PJIs each year in the United States,[2-3] and treatment of each case costing over $100,000,[4-6] PJIs impose a burden of more than $5 billion on the healthcare system.

In implant-related infections, the biofilm matrix can establish and enhance tolerance to antimicrobials by limiting the diffusion of antimicrobials to the bacterial cells or by producing enzymes to counteract the antimicrobial effects.[7-11] The most important characteristic of bacterial biofilms is that they host a subpopulation of bacteria called persister cells. Persister cells are dormant, non-dividing, and often multi-drug resistant.[12-13] Consequently, compared to their planktonic counterparts, bacteria in biofilms exhibit increased tolerance to antimicrobials. Adequate treatment of a biofilm-based infection requires a combination of surgical removal of the vast majority (ideally all) of the infection, and multi-day antimicrobial exposure levels capable of killing persister cells as the host response is often not effective.[12-13] The antimicrobial levels should be sufficiently maintained to eradicate any remaining bacteria. The minimum biofilm eradication concentration (MBEC), which is the antimicrobial concentration required to sterilize a biofilm, is reported to be 10 – 1000x greater[14-17] than the minimum inhibitory concentration (MIC), which is the concentration of antimicrobials required to inhibit the overnight growth of planktonic, free floating bacteria.[18-23] Unlike the MIC for a typical antimicrobial-bacterium pair (which is on the order of 0.5-8 μg/mL), MBEC values are often much higher than concentration of antimicrobials that can be provided safely by systemic administration.

The figure below represents typical barriers and mechanisms involved in the increased tolerance of biofilms against antimicrobial agents.

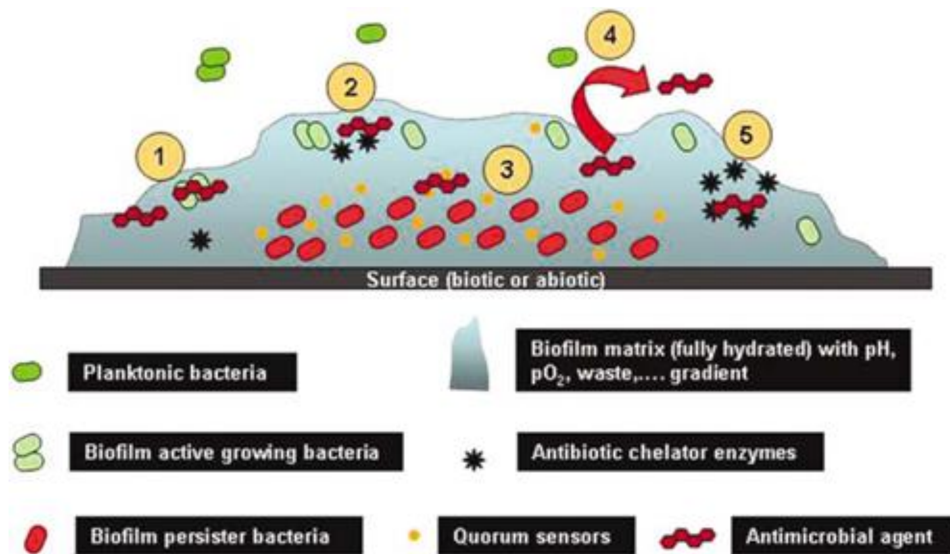


Fig 1.1 Potential mechanisms of Biofilm Tolerance[24]
(1) Failure of antimicrobial agents to penetrate the biofilm surface layers; (2) Antimicrobial agents may be trapped and destroyed by enzymes in the biofilm matrix; (3) Antimicrobial agents may not be active against non-growing persister cells;. (4) Possible expression of biofilm specific resistance genes; (5) Stress response to hostile environment conditions i.e. overexpression of antimicrobial destroying enzymes.

Current treatment of PJI involves a two-stage approach. The first stage includes thorough debridement of all foreign bodies, infected material and necrotic tissue. It is followed by extensive irrigation of the infected site to remove residual debris, biofilm and free floating bacteria. This is followed by the implantation of a medium-high dosage antimicrobial-loaded bone cement temporary spacer for local antimicrobial delivery. After about 6 weeks to allow for inflammation from the first stage to resolve, a second surgical procedure is performed, during which the spacer is removed and the joint is reconstructed with a permanent joint prosthesis. The total cost of the two-stage revision approach can be five times or more than that of the initial arthroplasty.[4,5,25]

Benefits of a one-stage treatment include reduced morbidity, improved functional outcomes, and reduced cost.[26] However, one-stage treatment is currently not recommended for most patients with PJIs in part because there is no opportunity for sustained local antimicrobial delivery in these

procedures.[27] A new approach capable of providing the necessary antimicrobial concentrations locally over a prolonged period of time could enable one-stage treatment if compatible with a permanent implant. Our laboratory is developing a sustained-release formulation of antimicrobials for local delivery as a part of a one-stage approach to treat PJIs.

There is a lack of biofilm-related animal models and studies of biofilms grown on tissue and material surfaces which reflect the pathogens and tissue and material surfaces involved in PJIs. Previously reported evaluation of biofilm susceptibility to antimicrobials has been conducted on *in vitro* grown biofilms on synthetic surfaces.[28,29] The information of target antimicrobial concentration to prevent the recalcitrance of infection on biological tissues is required. Therefore, generating this MBEC data will help establish goals for local drug delivery approaches that can improve biofilm associated infection treatment by providing realistic target drug concentrations on tissues relevant to PJIs. Therefore, a model of chronic, implant-associated infection of the femur in rabbits was developed using bacteria thought to represent common orthopaedic pathogens, with the goal of producing infected tissues suitable for MBEC determination *ex vivo*. Antimicrobial susceptibility studies of bacterial growth on the infected tissues were performed after recovery from euthanized animals. Simultaneously biofilms were grown *in vitro* on aseptically harvested tissues and their antimicrobial susceptibilities were studied. The studies involved combinations of microorganisms and antimicrobials over exposure durations from 6 hr to 72 hr through quantitative microbiological broth dilution assays.

CHAPTER 2 – RABBIT IMPLANT INFECTION MODEL

2.1 Materials and Methods

2.1.1 Microorganisms and Antimicrobials

Orthopaedic infections can be caused by a diverse group of bacteria including 50-75% gram-positives, and 10-20% each gram-negative, polymicrobial, and culture-negative.[30-33] Recognizing that about 70% of orthopaedic infections are gram-positive staphylococci[34-36], a majority of the strains we evaluated were staphylococci. The remaining strains represent some of more common species seen in non-staphylococcal orthopaedic infections, including Pseudomonas (gram-negative), Enterococcus, and Streptococcus species. The full list of organisms studied consisted of the following: methicillin-resistant *Staphylococcus aureus* (ATCC BAA 1556 and methicillin resistant *Staphylococcus aureus* (ATCC BAA 1680 – BSL 2), methicillin sensitive *Staphylococcus aureus* (ATCC 49230 – BSL 2), *Staphylococcus epidermidis* (ATCC 35984 – BSL 1), *Staphylococcus epidermidis*: ATCC (12228 – BSL 1), *Streptococcus agalactiae* (ATCC 13813 – BSL 2), *Enterococcus faecalis* (ATCC 29212 – BSL 2), and *Pseudomonas aeruginosa* (ATCC 27853 – BSL 2). *P.aeruginosa* ATCC 27853 is gram-negative, while the rest of the pathogens are gram-positive.

Table 2.1 Pathogens and Properties

Microorganism	**ATCC#**
S. aureus	BAA 1556
S. aureus	BAA 1680
S. aureus	49230
S. epidermidis	35984
S. epidermidis	12228
S. agalactiae	13813
E. faecalis	29212
P. aeruginosa	27853

The antimicrobials tobramycin and vancomycin were chosen as they are the most common drugs utilized in local delivery in orthopaedics.[37] They are also off-patent, easily available, inexpensive

and have minimal local tissue toxicity.[38-40] Tobramycin is effective against large number of gram-negatives and selected gram-positives including staphylcocci,[41-43] whereas vancomycin is effective against most gram-positive organisms, and resistance to vancomycin is relatively rare.[44] Thus, the two agents together have activity against the vast majority of bacteria found in orthopaedic infections, with dual coverage against some of the most common species.

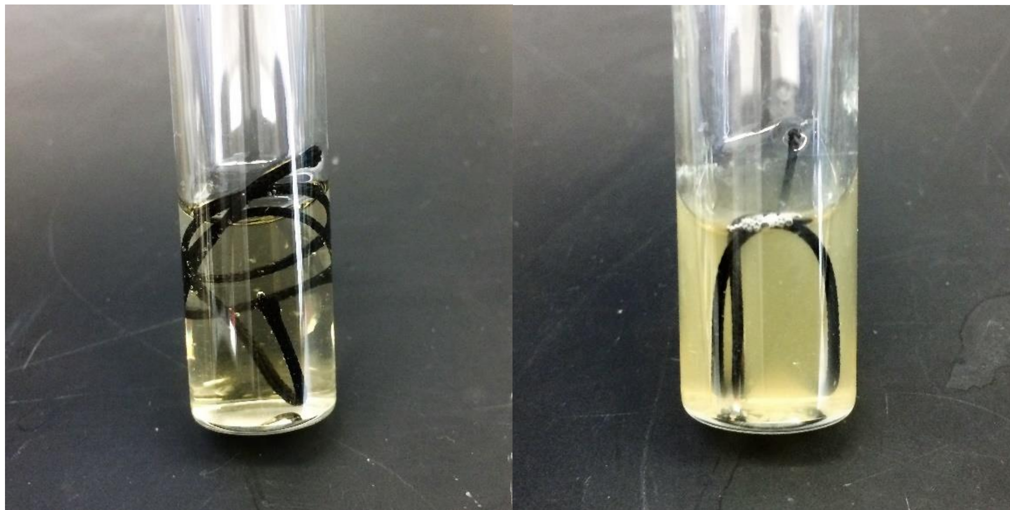
2.1.2 Animal Model

The animal model involved performing inoculation surgery on New Zealand White female rabbit in which the inoculum of bacteria was introduced into the femur to cause an infection. In addition, foreign materials (stainless steel wire and braided silk suture) simulating foreign bodies were also implanted.

2.1.3 Preparation of Inoculum

Two days prior to the inoculation surgery, a liquid culture of the microorganism of interest was prepared by inoculating a colony of the microorganism from an agar plate using a sterile loop into a 20 mL screw cap culture tube (Tube A) containing 3 mL sterile growth medium for the microorganism as per the American Type Culture Collection recommendation.

The tube was sonicated for 30 seconds and incubated overnight at 37°C. The day prior to the surgery, the incubated overnight culture was diluted to match the turbidity of a 0.5 McFarland standard [1.5x10^8 colony forming units (CFUs)/mL] into a new 20 mL sterile screw cap culture tube (Tube B) containing 3 mL sterile growth medium. In addition, a sterile, braided silk suture (size 4 measuring ~10 cm in length) was placed in the culture tube and incubated for 24 hours at 37°C as shown in the figure below.



(A) (B)

Fig. 2.1 Preparation of biofilm contaminated suture

(A) Start of incubation containing growth medium and suture (B) After 24 hour incubation with suture containing biofilm

Two hours prior to the surgery, the microorganism liquid culture soaked braided suture from Tube B was transferred into a sterile 2 mL screw top vial. To this 2 mL screw top vial, 600 μL liquid culture was added from Tube B and the vial was closed.

2.2 Femur Model

2.2.1 Introduction

As a part femur infection model, 31 surgeries were performed on 26 rabbits (rabbit 1-24 and 26-27) among which 5 rabbits (1, 2, 10, 11, and 12) underwent a second inoculation procedure as they did not establish an infection after the first. Rabbit 25 was used for a different study. Eight rabbits were inoculated with *S.aureus* (ATCC# 49230), four rabbits were inoculated with *S.aureus* (BAA 1556), three rabbits were inoculated with *S.aureus* (BAA 1680), four rabbits were inoculated with *P.aeruginosa* (27853), five rabbits were inoculated with *S. epidermidis* (35984), one rabbit each with *S.epidermidis* (12228), *S.agalactiae* (13813), and *E.faecalis* (29212).

2.2.2 Surgery and Inoculation

All animal procedures were approved by the Institutional Animal Care and Use Committee at St. Joseph's Hospital and Medical Center (Phoenix, AZ). The left hind legs of New Zealand White rabbits (Female, ~3 kg) were shaved from the hip to below the knee on the day before surgery. Rabbits were operated on under general anesthesia (2-3% isoflurane in oxygen) using standard aseptic techniques.

The lateral portion of one femur of each rabbit was accessed via skin incision, and 3 holes of 2 mm diameter were drilled through the lateral cortex of the femur. The holes were inoculated with approximately with inoculum ranging from 75 µL to 225 µL. Different densities of the inoculum were chosen resulting in final count of 1.125x10^7 to 6.75x10^7 colony forming units (CFU). Inoculation started with 75 µL and the volume of inoculum was increased in response to failure of infections in the animal. A 10-cm long stainless steel Kirschner wire segment was inserted through the hole to contact the opposite cortex and protrude slightly from the drill hole. In, addition a 10-cm long section of biofilm-containing silk suture was introduced into the wound before closing. The silk suture method is expected to be more effective than inoculating with the planktonic bacterial suspension alone because it will allow bacteria to form biofilm prior to introduction *in vivo* and protects the bacteria against the host response in the initial stages of infection development.

The surgical site was closed using 4-0 polypropylene suture. The animals were administered sustained-release buprenorphine as post-operative analgesia and were monitored to detect clinical symptoms of infection (formation of abscess, draining of pus, necrotic tissue) or general symptoms that could be indicative of infection (food intake, fatigue, stool and urine output, and general behavior). The time for the development infection was generally expected to be 2-2.5 weeks.

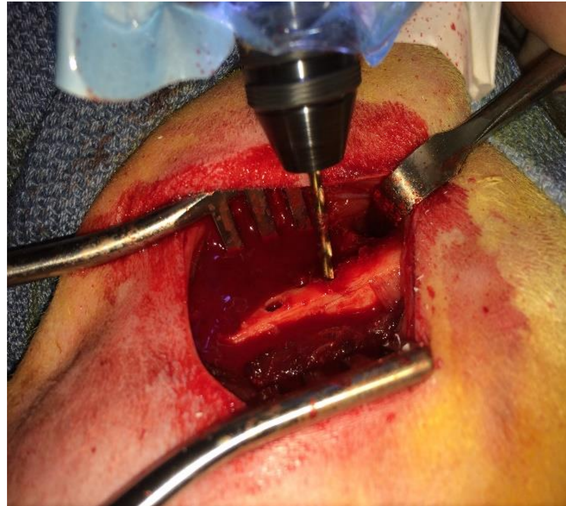


Fig. 2.2 Surgical Inoculation Procedure.

Drilling into the femur prior to irrigation and inoculation with bacterial inoculum soaked (biofilm coated) suture, metal k-wire and bacterial inoculum.

2.2.3 Necropsy and Harvest of Infected Tissues

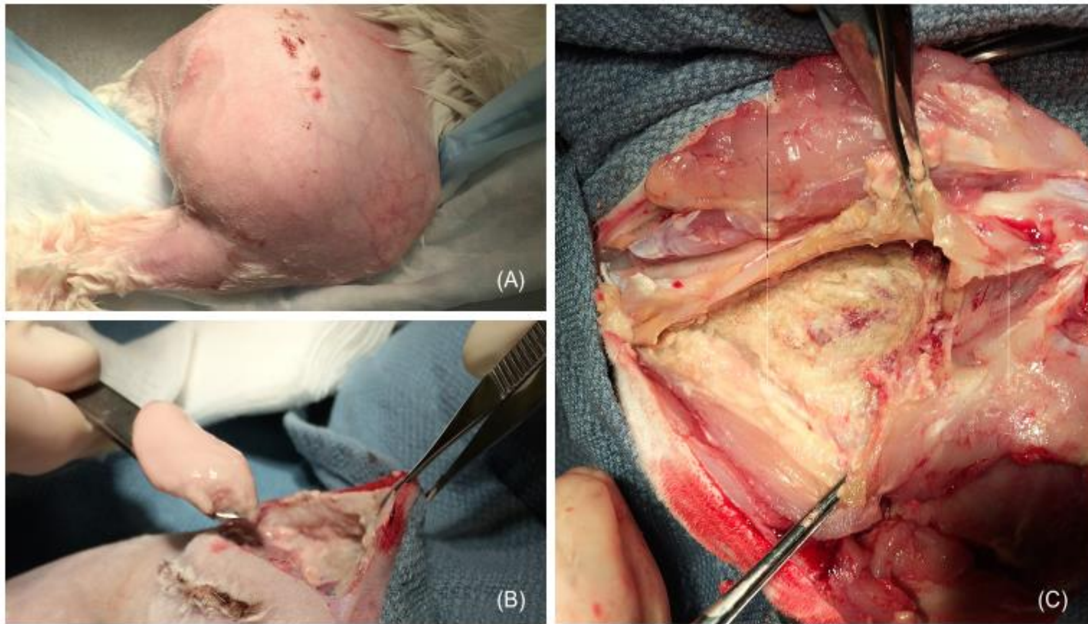


Fig. 2.3 Recovery of infected tissues from euthanized rabbit (thigh)

(A) Swelling at the surgery site (B) Recovery of Pus from infection site (C) Necrotic Muscle

Upon observation of apparent infection [Fig. 2.3 (A)], rabbits were sedated with ketamine/xylazine and euthanized by pentobarbital injection. A necropsy procedure was then performed to collect all foreign bodies and necrotic/infected tissues from the infected site. Using sterile instruments and standard aseptic surgical technique including skin preparation with 2% chlorhexidine scrub, the infected metal wire, suture, and samples of infected bone, muscle, and pus were recovered and stored in labeled, sterilized screw-top tubes for each animal.

Bone was assumed to be infected based on the presence of pus in the intramedullary canal and harvested muscles were recovered based on the presence of visible micro-abscesses or plaque/biofilm formation on the tissues. Each vial was sealed tightly with a screw cap and transported immediately to ASU for antibiotic susceptibility tests to determine the extent of infection quantitatively (MBEC). The antimicrobial exposure was started within four hours after the recovery from euthanized animal.

2.2.4 Results

Among the 26 rabbits, as shown in the tables (2.2 and 2.3) below, 8 were tested for *S. aureus* (ATCC 49230) with a successful infection in all the cases. 4 rabbits were inoculated with *S. aureus* (ATCC BAA 1556) with a successful infection in 3 cases and the 4th rabbit was prematurely euthanized on displaying elevated symptoms of discomfort (loss of appetite, weight and increased fatigue). 3 rabbits were inoculated with *S. aureus* (ATCC BAA 1680) with a successful infection in 2 rabbits and the 3rd rabbit was prematurely euthanized due to symptoms mentioned previously. 3 rabbits were inoculated with *P. aeruginosa* (ATCC 27853) with a successful infection all 3 cases. Microorganisms that failed to establish an infection were *S. epidermidis* [(ATCC 35984), 5 rabbits], *S. epidermidis* [(ATCC 12228), 1 rabbit], *S. agalactiae* [(ATCC 13813), 2 rabbits] and *E. faecalis* [(ATCC 29212), 1 rabbit].

Table 2.2 List of Rabbit Procedures

Rabbit #	Micro Organism	ATCC	Notes
1	*S. aureus*	49230	Infected 2nd inoculation
2	*S. aureus*	49230	Infected 2nd inoculation
3	*S. aureus*	49230	Infected 1st inoculation
4	*S. aureus*	49230	Infected 1st inoculation
5	*S. aureus*	49230	Infected 1st inoculation
6	*S. aureus*	49230	Infected 1st inoculation
7	*S. aureus*	49230	Infected 1st inoculation
8	*S. aureus*	49230	Infected 1st inoculation
9	*S. epidermidis*	35984	No infection after 1st inoculation
10	*S. epidermidis*	35984	No infection after 2nd inoculation
11	*S. epidermidis*	35984	No infection after 2nd inoculation
12	*S. epidermidis*	35984	No infection after 2nd inoculation
13	*P. aeruginosa*	27853	Infected after 1st inoculation
14	*P. aeruginosa*	27853	Infected after 1st inoculation
15	*P. aeruginosa*	27853	Infected after 1st inoculation
16	*S. epidermidis*	35984	No infection after 1st inoculation
17	*S. aureus* (MRSA)	BAA 1556	Infected after 1st inoculation
18	*S. aureus* (MRSA)	BAA 1556	Euthanized (no tissues collected)
19	*S. aureus* (MRSA)	BAA 1556	Infected after 1st inoculation
20	*S. aureus* (MRSA)	BAA 1556	Infected after 1st inoculation
21	*E.faecalis*	29212	No infection after 1st inoculation
22	*S. agalactiae*	13813	No infection after 1st inoculation
23	*S. aureus* (MRSA)	BAA 1680	Infected after 1st inoculation
24	*S. epidermidis*	12228	No infection after 1st inoculation
26	*S. aureus* (MRSA)	BAA 1680	Euthanized (no tissues collected)
27	*S. aureus* (MRSA)	BAA 1680	Infected after 1st inoculation

Table 2.3 Rabbit surgeries summary (Femur Model)

Microorganism	Result	Infection Development
S. aureus (BAA 1556)	3 of 4 infected + 1 Euthanized	2.5 ~ 3 weeks
S. aureus (BAA 1680)	2 of 3 infected + 1 Euthanized	1 ~ 1.5 weeks
S. aureus (49230)	8 of 8 infected	3 ~ 3.5 weeks
P. aeruginosa (27853)	3 of 3 infected	4 ~ 6 weeks
S. epidermidis (35984)	0 of 5 infected	No infections
S. epidermidis (12228)	0 of 1 infected	No infections
E. faecalis (29212)	0 of 1 infected	No infections
S. agalactiae (13813)	0 of 1 infected	No infections

2.2.5 Discussion

Among the procedures, all 3 strains of *Staphylococcus Aureus* (ATCC BAA 1556, BAA 1680 and 49230) resulted in a chronic local infection which supports this experimental procedure to be promising for *Staphylococcus aureus* and *Pseudomonas aeruginosa* infections. However, half of the strains that were inoculated did not result in an infection, including *Staphylococcus epidermidis, Streptococcus agalactiae and Enterococcus faecalis*.

For cases in which the femoral model failed to result in infection, a second surgery was attempted, inoculating a devascularized segment of radius using a model developed by Nelson *et al.*[45-47]

2.3 Forelimb Radius Model

2.3.1 Introduction

A rabbit model of radial osteomyelitis[45-47] has been reported in literature to produce a chronic bone infection because it relies on a devascularized segment of bone inside which bacteria are isolated from the host response of the rabbit. This is not totally possible in the femur site because cutting fully through the femur would lead to instability whereas removal of a large portion of the radius does not require fixation and allows the rabbit to ambulate and bear some weight on the limb as the ulna remains intact.

2.3.2 Surgery and Inoculation

The radius of the left forelimb was accessed via a skin incision, and a 1.5-2 cm diaphyseal segment was removed using a sterile fine-toothed saw. The bone segment was inoculated with bacterial suspension approximating the turbidity of a 1.5 McFarland standard and placed back in the original site. A biofilm-contaminated suture was also placed in the site along with a small stainless steel wire as one of the goals of the study was to cause an implant-related infection.

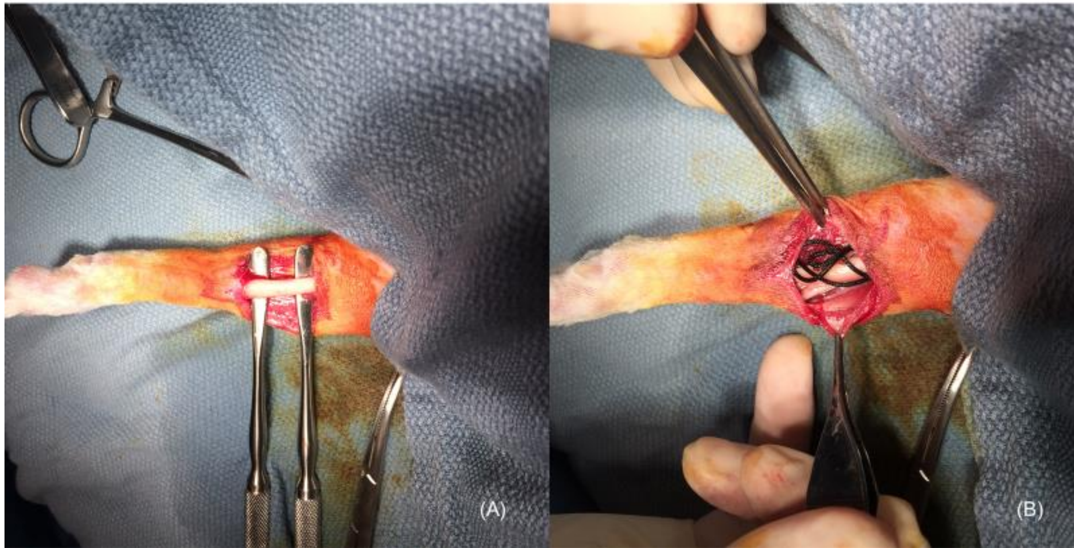


Fig. 2.4 Radial infection model (A) Accessing the femur and removing a small section of the rabbit ulna to devascularize. (B)Inserting the devascularized bone segment back into the ulna and additional inoculum, biofilm coated suture and metal wire.

2.3.3 Results

The table below represents the results of the rabbit radial model inoculations after the administration of highest volume possible (150µL, limited due to the space in the bone cavity) turbid inoculum of the intended microorganism.

Table 2.4 Rabbit Surgeries Summary (Radial Model)

Rabbit #	Micro Organism	ATCC #	Notes
16	*S. epidermidis*	35984	No infection
25	*S. agalactiae*	13813	No infection

2.3.4 Discussion

The forelimb radius infection model was adopted after the femur infection model failed to establish *S.epidermidis* (35984), *S.agalactiae* (13813), *E.faecalis* (29212), and *S.epidermidis* (12228) infections.

In this model, two rabbits were inoculated with S*.epidermidis* (35984) and *S.agalactiae* (13813) separately with a turbid inoculum containing approximately 225 µL (6.75x10^7 CFU) in addition to the biofilm coated suture. Infection failed to establish in both the cases even after a development time of 4-5 weeks after the surgical procedure, and the rabbits were euthanized. The macroscopic appearance of tissues at the inoculated site showed no evidence of infection upon dissection. Tissues from the non-operated limbs were recovered aseptically for separate studies.

This showed that the radial osteomyelitis model does not apply broadly to all bacteria, including some other staphylococci even after the administration of high. In part, based on the lack of consistency in rabbit infection outcomes for *ex vivo* MBEC testing, an alternative experiment was planned to grow biofilm on biological tissue specimens *in vitro* where the growth is uninhibited due to the absence of the host response of an animal. Although this would result in a biofilm that may not perfectly mimic *in vivo* biofilm given the uninhibited growth, we hypothesized that the MBEC values obtained would be no greater than the antimicrobial concentrations necessary to eradicate biofilms established *in vivo* on comparable surfaces.

CHAPTER 3: EX VIVO TISSUE MINIMUM BIOFILM ERADICATION CONCENTRATION (MBEC) AND MINIMUM INHIBITORY CONCENTRATION (MIC)

3.1 *Ex vivo* Tissue Minimum Biofilm Eradication Concentration (MBEC)

3.1.1 Materials and Methods

Infected, freshly recovered samples of bone, muscle, pus, implant and suture obtained from necropsy (2.1.5) were aseptically divided into smaller specimens (40 mg each) and individually placed into the wells of a 96 well plate. MBEC was measured for all possible permutations that were limited by the recovered infected tissues. Four microorganisms (ATCC # BAA 1556, BAA 1680, 49230, 27853) resulted in established infections. Five specimen types and 3 drug combinations over 3 exposure durations were measured for *S. aureus* (BAA 1556), *S. aureus* (49230) and *P. aeruginosa* (27853). For the microorganism *S. aureus* (BAA 1680), the recovered tissue specimens were limited to one rabbit. Tissues from a single rabbit yielded enough specimens to evaluate MBEC for a single antimicrobial combination for all 5 materials at all 3 exposure times. Therefore, only one drug combination (TOB) was tested for 5 specimen types over 3 exposure durations for BAA-1680. Plated specimens were exposed to 200 µL antimicrobial media [antimicrobial agent(s) in tryptic soy broth (TSB)] ranging from 4000 µg/mL to 31 µg/mL (4000, 2000, 1000, 750, 500, 375, 250, 125, 62, 31 in µg/ml) of three drug combinations (TOB, VANC, TOB+VANC) over three exposure durations (6 h, 24 h and 72 h). TOB+VANC denotes equal amounts of tobramycin and vancomycin, where 1000 µg/ml TOB+VANC consists of 500 µg/ml of each agent. The inclusion of 750 and 375 µg/mL was done to accommodate more concentration levels to be tested in our range of interest which is consistent with the general local antimicrobial delivery levels (100 – 1000 µg/mL) achieved by an investigational formulation being developed in our lab and reported in literature for other high-dose formulations.[45] After the exposure duration, the specimens were rinsed 4 times with 200 µL of sterile medium to remove residual antimicrobial. Each rinsed sample was subcultured in separate screw top culture tubes with 3 mL TSB at 37°C to observe the microbial growth over a

period of 3 weeks. The lowest concentration at which there was no visible growth of the microorganism in the subculture tube was determined to be the minimum biofilm eradication concentration.

Subculture tubes were classified as positive (turbid) or negative (transparent) based on visible inspection at up to 21 days following subculture. MBEC was generally interpreted as the lowest concentration at which a negative subculture was obtained. Single negative subcultures between multiple positive subcultures were interpreted as false negative and the negative subculture at the next highest concentration was selected as the MBEC value. Also, a positive subculture was interpreted as a false negative if it was seen with multiple negative values at lower concentrations. Additionally, if there were multiple gaps (inconsistent positive and negative subcultures), the MBEC values was marked as *undetermined*.

The figure below represents a typical antimicrobial exposure plate where the tissue specimens were divided into small pieces to fit into the 96 well plate. They were exposed to antimicrobials of different drug combinations (TOB, VANC and TOB+VANC) over three distinct exposure durations (6 hour, 24 hour, 72 hour).

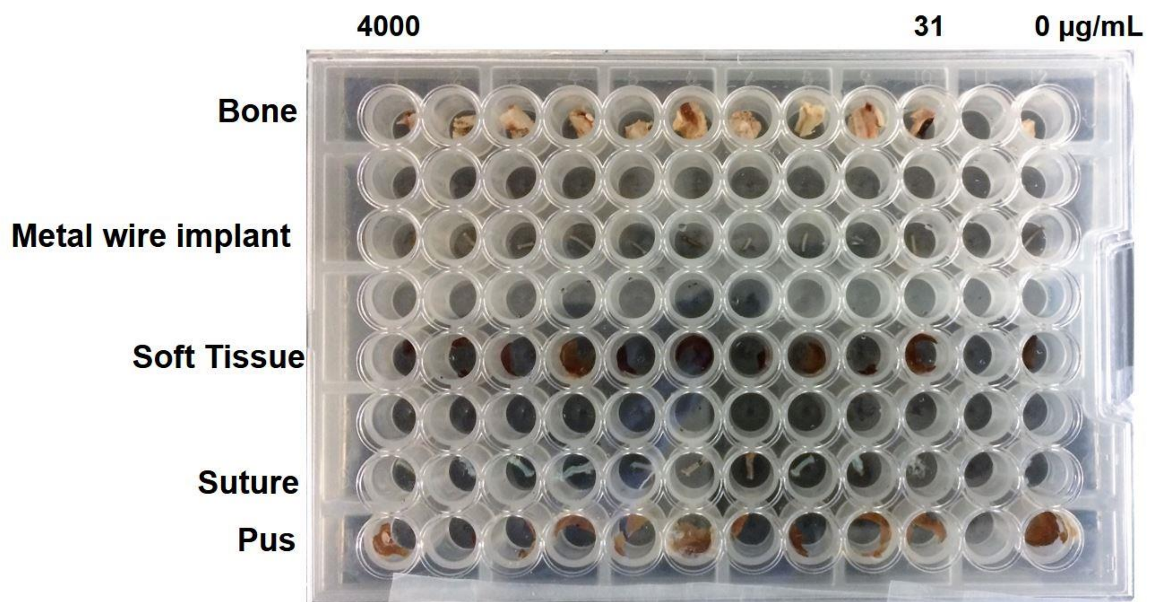


Fig. 3.1 MBEC plate setup for *ex vivo* tissue antimicrobial exposure

The figure below represents the subculture analysis where the specimens exposed to antimicrobials were subcultured. The numbers in white represent the antimicrobial concentration to which the specimens were exposed. In this particular case, the MBEC was determined to be 500 µg/mL after interpreting it to not show any microbial growth evident by the non turbid growth medium.

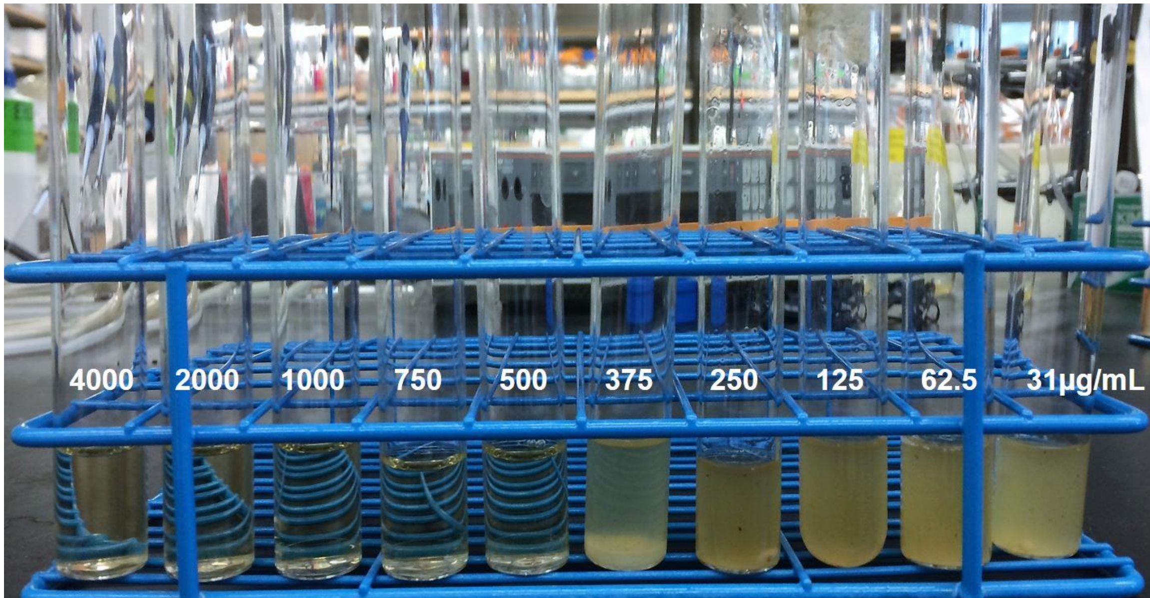


Fig. 3.2 Results after subculture, all concentrations in µg/mL; values shown in white correspond to the antimicrobial concentration (µg/mL) to which the specimens were exposed prior to subculture. The MBEC for this series of samples was determined to be 500 µg/mL.

3.1.2 Results

Table 3.1 Raw data table of *ex vivo* MBEC representing the microorganism *S. aureus* (BAA 1556) vs VANC

6 h exposure
Antibiotic Concentration in µg/mL

	4000	2000	1000	750	500	375	250	125	62	31	control	MBEC
Bone	-	-	-	-	-	-	+	+	+	+	+	375
Muscle	-	+	-	-	-	-	-	+	+	+	+	250
Pus	-	-	-	+	+	+	+	+	+	+	+	1000
Implant	+	+	+	+	+	+	+	+	+	+	+	>4000
Suture	-	-	-	+	+	+	+	+	+	+	+	1000

24 h exposure
Antibiotic Concentration in µg/mL

	4000	2000	1000	750	500	375	250	125	62	31	control	MBEC
Bone	-	-	+	-	+	-	+	+	+	+	+	*undetermined*
Muscle	-	-	-	-	-	-	-	+	+	+	+	250
Pus	-	-	-	-	+	+	+	+	+	+	+	750
Implant	-	-	+	-	-	+	+	+	+	+	+	500
Suture	-	-	-	-	+	-	+	+	+	+	+	750

72 h exposure
Antibiotic Concentration in µg/mL

	4000	2000	1000	750	500	375	250	125	62	31	control	MBEC
Bone	-	-	-	-	+	-	-	+	-	+	+	*undetermined*
Muscle	-	-	-	+	-	-	-	+	+	-	+	*undetermined*
Pus	+	-	-	-	-	+	+	+	+	+	+	500
Implant	-	-	-	-	-	-	+	-	-	-	+	31
Suture	-	-	-	+	-	-	+	-	+	+	+	*undetermined*

There were undetermined values (24 hour exposure of antimicrobial to implant specimen, muscle sample at 72 hour exposure and suture sample at 72 hour exposure) where false negatives and positives were observed and hence were not able to determine the actual MBEC.

Table 3.2 *Ex vivo* MBEC of *S. aureus* (BAA 1556)

	TOB			**VANC**			**TOB+VANC**		
Material	6 h	24 h	72 h	6 h	24 h	72 h	6 h	24 h	72 h
Bone	2000	125	750	375	*n/a*	*n/a*	2000	500	125
Muscle	2000	250	375	250	250	*n/a*	250	375	125
Pus	>4000	>4000	>4000	1000	750	500	375	125	31
Implant	31	31	31	>4000	500	31	31	31	31
Suture	1000	750	1000	1000	750	375	125	31	31

*n/a – values *undetermined* due to multiple gaps

Table 3.3 *Ex vivo* MBEC of *S. aureus* (BAA 1680)

		TOB	
Material	6 Hr	1 Day	3 Day
Bone	250	1000	125
Muscle	4000	375	750
Pus	>4000	2000	1000
Implant	250	31	31
Suture	31	31	31

Table 3.4 *Ex vivo* MBEC of *S. aureus* (49230)

	TOB			**VANC**			**TOB+VANC**		
	6 h	24 h	72 h	6 h	24 h	72 h	6 h	24 h	72 h
Bone	31	31	125	>4000	4000	>4000	>4000	1000	31
Muscle	2000	375	375	2000	1000	n/a	4000	750	125
Pus	2000	2000	125	ns	4000	ns	1000	>4000	2000
Implant	31	31	31	>4000	n/a	31	31	31	31
Suture	375	31	125	4000	2000	n/a	>4000	250	250

n/a – undetermined due to inconsistency
ns – no sample obtained from the euthanized, infected rabbit.

Table 3.5 *Ex vivo* MBEC of P. aeruginosa (27853)

	TOB			**VANC**			**TOB+VANC**		
	6 h	24 h	72 h	6 h	24 h	72 h	6 h	24 h	72 h
Bone	4000	125	125	4000	>4000	>4000	>4000	4000	1000
Muscle	>4000	750	375	>4000	>4000	>4000	4000	4000	4000
Pus	ns	4000	ns	ns	>4000	ns	>4000	>4000	>4000
Implant	31	31	31	500	125	125	31	31	31
Suture	>4000	>4000	2000	>4000	>4000	750	>4000	4000	125

ns – no sample was obtained from the euthanized, infected rabbit. Due to pus being runny and the consistent specimen of pus being a smaller sample, it was tested only for 24 hour exposure times.

Table 3.6 *Ex vivo* Tissue MBEC in bone and muscle				
		Bone		
ATCC#	**Exposure Time**	**MBEC**		
		TOB	**VANC**	**TOB+VANC**
BAA 1556	6 h	2000	375	2000
	24 h	125	2000	500
	72 h	750	*undetermined*	125
BAA 1680	6 h	250	*no data*	*no data*
	24 h	1000	*no data*	*no data*
	72 h	125	*no data*	*no data*
49230	6 h	*undetermined*	>4000	>4000
	24 h	31	4000	1000
	72 h	125	>4000	31
27853	6 h	4000	>4000	>4000
	24 h	*undetermined*	>4000	4000
	72 h	125	>4000	1000

		Muscle		
ATCC#	**Exposure Time**	**MBEC**		
		TOB	**VANC**	**TOB+VANC**
BAA 1556	6 h	2000	250	250
	24 h	250	250	375
	72 h	375	250	125
BAA 1680	6 h	4000	*no data*	*no data*
	24 h	375	*no data*	*no data*
	72 h	750	*no data*	*no data*
49230	6 h	2000	2000	375
	24 h	375	1000	750
	72 h	375	2000	*undetermined*
27853	6 h	>4000	>4000	4000
	24 h	4000	>4000	4000
	72 h	375	>4000	4000

3.1.3 Discussion

Although the experimental approach pursued in this study generally allowed for determination of MBEC on tissue and biomaterial surfaces infected *in vivo*, the method has a number of weaknesses. First, 50% of the microorganisms that were inoculated failed to cause an infection. Second, the raw data were difficult to interpret due to inconsistency in subculture results leading to many undetermined MBEC values. Third, the ones that infected successfully, produced inconsistent data due to a majority of MBEC values obtained did not decrease with time which in

contrast to the research showing the increase in susceptibility of biofilm to antimicrobials with increase in exposure time.[48]

3.2 Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration (MIC) can be defined as the lowest antimicrobial agent concentration preventing the visible growth of microorganisms after an overnight incubation. A low MIC value indicates high sensitivity of the microorganism towards corresponding antimicrobial agent.

3.2.1 Materials and Methods

Minimum inhibitory concentration was measured by the CLSI standard broth microdilution method[49] and median MIC (antimicrobial concentration required to inhibit the growth of at least 50% of the tested strains) and MIC_{max} (antimicrobial concentration required to kill all the tested strains) was used in comparing the spectrum of activity of the antimicrobial combinations.

For a given study, the microorganism of interest was grown overnight in cation-adjusted Mueller-Hinton Broth (Sigma-Aldrich 90922), and the concentration was adjusted to approximately 1.5x10^6 CFU using the same media. Bacteria were exposed to three antimicrobial combinations (100% Tobramycin, 100% Vancomycin and 1:1 combination of each) at each of ten total antimicrobial concentrations (128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25 µg/mL) and one negative control in sterile round bottomed 96 well tissue culture plate (Biofil TCP 002096) before incubation for 18-20 hours at 37°C. The lowest antimicrobial concentration at which there was no visible growth of the microorganism was considered the MIC.

Table 3.7 MIC of S. aureus (49230)

	Concentration of Antimicrobial Combination (µg/ml)											
	128	64	32	16	8	4	2	1	0.5	0.25	Control	MIC
TOB	-	-	-	-	-	-	-	-	+	+	+	1
VANC	-	-	-	-	-	+	+	+	+	+	+	8
TOB+VANC	-	-	-	-	-	-	-	+	+	+	+	2

3.2.2 Results

The data interpretation shows the single agents TOB and VANC had MIC ≤ 8 μg/mL against 6 out of 8 tested microorganisms and the antimicrobial combination of TOB+VANC had MIC ≤ 8 μg/mL against all the tested 8 microorganisms. On a closer observation with median MIC as a standard, TOB measured as 2 μg/mL and VANC measured 4 μg/mL as well whereas the antimicrobial combination measured as 4 μg/mL

Table 3.8 Minimum Inhibitory Concentration Results

ATCC #	MIC TOB (μg/mL)	MIC VANC (μg/mL)	MIC TOB+VANC (μg/mL)
S.aureus (BAA 1556)	2	4	1
S.aureus (BAA 1680)	2	2	2
S.aureus (49230)	1	8	2
S.epidermidis (35984)	128	2	4
P.aeruginosa (27853)	8	>128	8
E.faecalis (29212)	64	2	4
E.coli (25922)	2	>128	8
Median MIC	**2**	**4**	**4**
MIC_{max}	**128**	**>128**	**8**

3.2.3 Discussion

For the tested microorganisms, the use of combination of antimicrobial agent combination (TOB+VANC) showed a wider coverage over microorganisms, which can be shown by its MIC_{max} value that was 8 μg/mL. In comparison, for treatments with TOB it was 128 μg/mL and VANC it was greater than 128. Also, the median MIC value (inhibiting the growth of at least half of the tested strains) for the antimicrobial treatment TOB+VANC was comparable to both the single agents (TOB or VANC).

CHAPTER 4: IN VITRO TISSUE MINIMUM BIOFILM ERADICATION CONCENTATION

4.1 Materials and Methods

From the previous chapter, studies resulted in incomplete and inconsistent data. Therefore, a different approach was adopted to grow the biofilms on animal tissues *in vitro* and estimate the MBEC values. It was expected that the data generated would be more consistent as the variability of infection found in different animals or within animals would be reduced and that MBEC values could be determined for all biofilm-forming bacteria.

4.1.1 Materials

Tissues (bone and muscle) were obtained from healthy uninoculated limbs aseptically harvested from euthanized female New Zealand White rabbits used in the rabbit implant infection model (Section 2.1.5). Specimens of bone were obtained from femora and the muscle samples were obtained from the adjacent muscles.

4.1.2 Methods

Eight microorganisms were chosen for this study which included *S. aureus* (BAA 1556), *S. aureus* (BAA 1680), *S. aureus* (49230), *S. epidermidis* (35984), *S. epidermidis* (12228), *E. Faecalis* (29212), *P. aeruginosa* (27853) and *E. coli* (25922). *P. aeruginosa* and *E. coli* are gram-negative and the other bacteria used are gram-positive.

Tissues were divided into specimens of approximately 40 mg each and plated in sterile 96 well tissue culture plate (Celltreat 229197). To the specimens, 200 μL bacterial inoculum (grown overnight at 37°C and diluted 1:30 in TSB with 1% glucose) was added and incubated for 72 hours at 37°C to allow for biofilm growth on the tissues. After incubation, the biofilm-contaminated tissue specimens were transferred into a new sterile 96 well plate. Specimens were exposed to 200 μL antimicrobial combinations (antimicrobial in TSB) ranging from 4000 μg/mL to 31 μg/mL (4000, 2000, 1000, 750, 500, 375, 250, 125, 62, 31 in μg/ml) to three drug combinations (tobramycin, vancomycin, 1:1 weight combination of both tobramycin and vancomycin), three

distinct exposure durations (6 hr, 24 hr and 72 hr) and subsequently rinsed to remove residual antimicrobials. Each rinsed sample was subcultured in separate screw-top culture tubes with 3 mL TSB at 37°C to observe microbial growth over a period of 3 weeks. The subculture tube representing the specimen exposed to the lowest concentration at which there was no visible growth of the microorganism was determined as the *in vitro* MBEC.

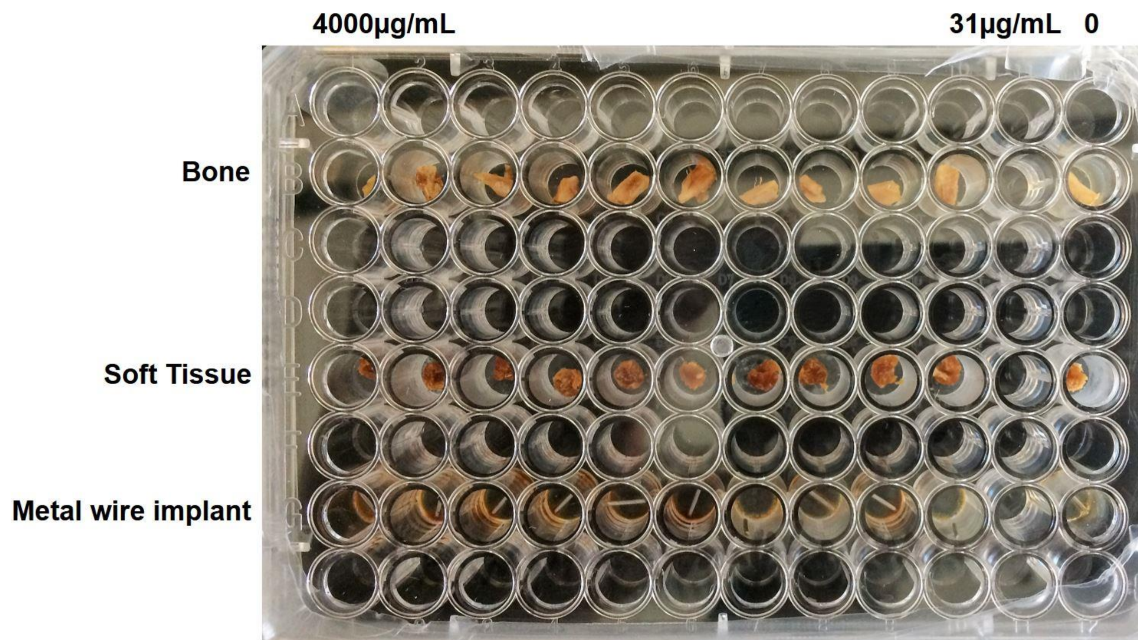


Fig. 4.1 MBEC plate setup for *in vitro* tissue antimicrobial exposure

Subculture tubes were classified as positive (turbid) or negative (transparent) based on visible inspection at up to 21 days following subculture. MBEC was generally interpreted as the lowest concentration at which a negative subculture was obtained. Single negative subcultures between multiple positive subcultures were interpreted as false negative and the negative subculture at the next highest concentration was selected as the MBEC value. Also, a positive subculture was interpreted as a false negative if it was seen with multiple negative values at lower concentrations. Additionally, if there were multiple gaps (inconsistent positive and negative subcultures), the MBEC values was marked as *undetermined*.

Table 4.1 Example of a Raw data table of *in vitro* tissue MBEC representing the microorganism *S. aureus* (49230) vs TOB+VANC

6 h exposure

	Antibiotic Concentration in µg/ml											
	4000	2000	1000	750	500	375	250	125	62	31	control	MBEC
Bone	-	-	-	-	+	+	-	+	+	+	+	750
Muscle	-	-	-	-	-	-	+	+	+	+	+	375
Implant	+	-	-	+	-	+	+	-	+	+	+	*undetermined*

24 h exposure

	Antibiotic Concentration in µg/ml											
	4000	2000	1000	750	500	375	250	125	62	31	control	MBEC
Bone	-	-	-	+	+	+	+	+	+	+	+	1000
Muscle	-	-	-	-	-	+	+	+	+	+	+	500
Implant	-	-	-	-	-	-	-	-	+	-	+	125

72 h exposure

	Antibiotic Concentration in µg/ml											
	4000	2000	1000	750	500	375	250	125	62	31	control	MBEC
Bone	-	-	-	-	-	+	+	+	+	+	+	500
Muscle	-	-	-	-	-	+	+	+	+	+	+	500
Implant	+	-	-	-	-	-	-	-	-	-	+	31

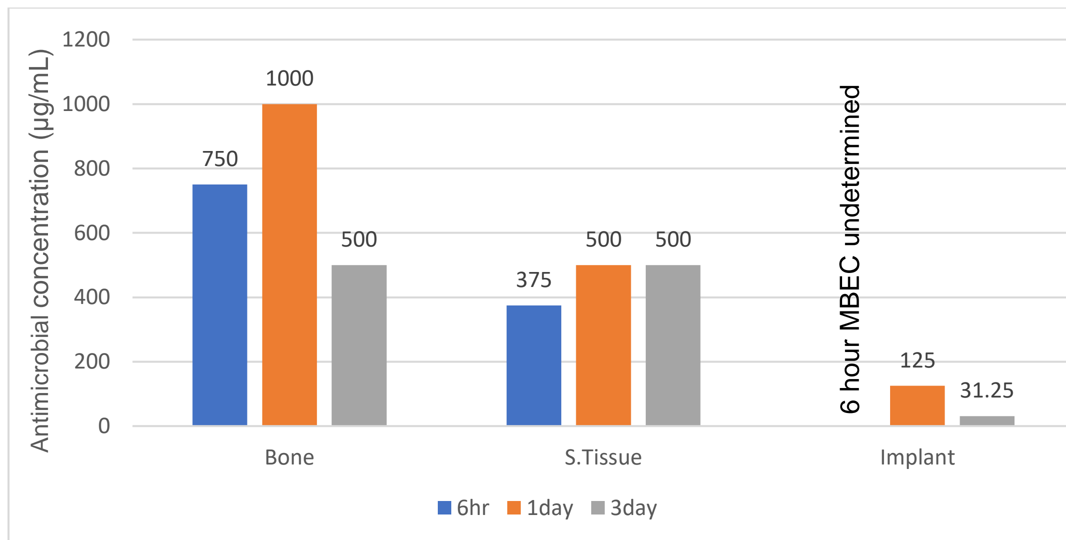


Fig. 4.2 *In vitro* tissue MBEC representing S. aureus (49230) vs TOB+VANC

4.2 Results

Samples were sent to Antech Diagnostics and culture analysis and were confirmed that biofilms grown on tissues were of the intended organism.

Table 4.2 *In vitro* tissue MBEC of *S. aureus* (BAA 1556)

	TOB (µg/mL)			VANC (µg/mL)			TOB+VANC (µg/mL)		
	Bone	S.Tissue	Implant	Bone	S.Tissue	Implant	Bone	S.Tissue	Implant
6 h	500	2000	4000	>4000	4000	>4000	>4000	4000	-
24 h	2000	375	>4000	>4000	2000	>4000	4000	125	250
72 h	250	250	>4000	>4000	1000	>4000	750	125*	62

* - Possible synergy

Table 4.3 *In vitro tissue* MBEC of *S. aureus* (BAA 1680)

	TOB (µg/mL)			VANC (µg/mL)			TOB+VANC (µg/mL)		
	Bone	S.Tissue	Implant	Bone	S.Tissue	Implant	Bone	S.Tissue	Implant
6 h	>4000	>4000	4000	>4000	4000	>4000	4000	750*	1000
24 h	>4000	>4000	31	>4000	2000	>4000	>4000	250*	-
72 h	>4000	4000	-	>4000	2000	>4000	4000	250*	-

* - Possible synergy
** - value could not be determined due to inconsistencies in sub culture data

Table 4.4 *In vitro tissue* MBEC of *S. aureus* (49230)

	TOB (µg/mL)			VANC (µg/mL)			TOB+VANC (µg/mL)		
	Bone	S.Tissue	Implant	Bone	S.Tissue	Implant	Bone	S.Tissue	Implant
6 h	4000	500	>4000	>4000	2000	>4000	750	375	-**
24 h	500	375	-	>4000	>4000	>4000	1000	500	125
72 h	125	250	750	4000	1000	>4000	500	500	31

** – value could not be determined due to inconsistencies in sub culture data

Table 4.5 *In vitro tissue* MBEC of *S. epidermidis* (35984)

	TOB (µg/mL)			VANC (µg/mL)			TOB+VANC (µg/mL)		
	Bone	S.Tissue	Implant	Bone	S.Tissue	Implant	Bone	S.Tissue	Implant
6 h	>4000	>4000	>4000	>4000	4000	>4000	>4000	750	31
24 h	>4000	>4000	>4000	4000	750	>4000	>4000	2000	-**
72 h	>4000	>4000	>4000	2000	375	>4000	4000	750	31

** - value could not be determined due to inconsistencies in sub culture data

Table 4.6 *In vitro tissue* MBEC of *E. faecalis* (29212)

	TOB (μg/mL)			VANC (μg/mL)			TOB+VANC (μg/mL)		
	Bone	S.Tissue	Implant	Bone	S.Tissue	Implant	Bone	S.Tissue	Implant
6 h	>4000	>4000	31	2000	250	>4000	>4000	2000	4000
24 h	>4000	>4000	-**	1000	250	-**	>4000	500	>4000
72 h	>4000	>4000	-**	1000	750	>4000	4000	250	1000

Table 4.7 *In vitro tissue* MBEC of *P. aeruginosa* (27853)

	TOB (μg/mL)			VANC (μg/mL)			TOB+VANC (μg/mL)		
	Bone	S.Tissue	Implant	Bone	S.Tissue	Implant	Bone	S.Tissue	Implant
6 h	>4000	2000	31	>4000	>4000	375	750	750	500
24 h	>4000	375	31	>4000	>4000	250	125	750	250
72 h	4000	62	31	>4000	>4000	125	125	375	375

Table 4.8 *In vitro tissue* MBEC of *E. coli* (25922)

	TOB (μg/mL)			VANC (μg/mL)			TOB+VANC (μg/mL)		
	Bone	S.Tissue	Implant	Bone	S.Tissue	Implant	Bone	S.Tissue	Implant
6 h	>4000	125	31	>4000	>4000	250	>4000	4000	31
24 h	4000	375	125	>4000	>4000	375	>4000	4000	31
72 h	4000	125	31	>4000	4000	250	-**	2000	31

** - value could not be determined due to inconsistencies in sub culture data

The table below represents the summary of *in vitro* bone MBEC. The values highlighted in red indicate high MBEC values and the values in blue text indicate MBEC values obtained using cancellous bone.

Table 4.9 Summary of Bone *in vitro* MBEC

Micro organism	Exposure time	MBEC		
		TOB	VANC	TOB+VANC
S.aureus BAA 1556	6 h	>4000	>4000	>4000
	24 h	2000	>4000	4000
	72 h	250	>4000	750
S.aureus BAA 1680	6 h	>4000	>4000	4000
	24 h	>4000	>4000	>4000
	72 h	4000	>4000	4000
S.aureus (49230)	6 h	4000	>4000	750
	24 h	500	>4000	1000
	72 h	125	4000	500
S.epidermidis (35984)	6 h	>4000	>4000	>4000
	24 h	>4000	4000	>4000
	72 h	>4000	2000	4000
E.faecalis (29212)	6 h	>4000	2000	>4000
	24 h	>4000	1000	>4000
	72 h	>4000	1000	4000
P.aeruginosa (27853)	6 h	>4000	>4000	750
	24 h	>4000	>4000	125
	72 h	4000	>4000	125
E.coli (25922)	6 h	>4000	>4000	>4000
	24 h	4000	>4000	>4000
	72 h	4000	>4000	*undetermined*

The table below represents the summary of *in vitro* muscle MBEC. The values highlighted in red indicate high MBEC values.

Table 4.10 Summary of Muscle *in vitro* MBEC

Micro organism	Exposure time	MBEC		
		TOB	VANC	TOB+VANC
S.aureus BAA 1556	6 h	2000	4000	500*
	24 h	375	2000	125*
	72 h	250	1000	125*
S.aureus BAA 1680	6 h	>4000	4000	750*
	24 h	>4000	2000	250*
	72 h	4000	2000	250*
S.aureus (49230)	6 h	500	>4000	375
	24 h	375	>4000	500
	72 h	250	2000	500
S.epidermidis (35984)	6 h	>4000	4000	750*
	24 h	>4000	750	2000
	72 h	>4000	375	750
E.faecalis (29212)	6 h	>4000	250	2000
	24 h	>4000	250	500
	72 h	>4000	750	250*
P.aeruginosa (27853)	6 h	2000	>4000	750
	24 h	375	>4000	750
	72 h	62	>4000	375
E.coli (25922)	6 h	125	>4000	4000^
	24 h	375	>4000	4000^
	72 h	125	4000	2000^

* - Possible Synergy

^ - Possible Antagonism

The below table represents the *in vitro* MBEC values of the implant samples. The majority of values were either undetermined or did not fall within the measured range (31 - 4000µg/mL). Data were not analyzed further.

Table 4.11 Summary of Implant *in vitro* MBEC

Micro organism	exposure time	MBEC		
		TOB	VANC	TOB+VANC
S.aureus BAA 1556	6 h	4000	>4000	*undetermined*
	24 h	>4000	>4000	250
	72 h	>4000	>4000	62
S.aureus BAA 1680	6 h	4000	>4000	1000
	24 h	31	>4000	*undetermined*
	72 h	*undetermined*	>4000	*undetermined*
S.aureus (49230)	6 h	>4000	>4000	*undetermined*
	24 h	*undetermined*	>4000	125
	72 h	750	>4000	31
S.epidermidis (35984)	6 h	>4000	>4000	31
	24 h	>4000	>4000	*undetermined*
	72 h	>4000	>4000	31
E.faecalis (29212)	6 h	31	>4000	4000
	24 h	*undetermined*	*undetermined*	>4000
	72 h	*undetermined*	>4000	1000
P.aeruginosa (27853)	6 h	31	375	500
	24 h	31	250	250
	72 h	31	125	375
E.coli (25922)	6 h	31	250	31
	24 h	125	375	31
	72 h	31	250	31

4.2.1 Comparison of *in vitro* tissue median MBECs of Bone and Muscles

Table 4.10 Median of *in vitro* Bone MBEC for tested 7 microorganisms

Table 4.12 Median of *in vitro* Bone MBEC for tested 7 microorganisms

	6 h	**24 h**	**72 h**
TOB	>4000	>4000	4000
VANC	>4000	>4000	>4000
TOB+VANC	>4000	>4000	4000

Overall, the vast majority of the bone MBEC values were high and did not fall into the range of concentrations evaluated in this study (31 – 4000 µg/mL) as seen in the figure below. Given the low ratio of MBEC values determined in the bone, an analysis would not account for an unbiased one and therefore, it was not included further.

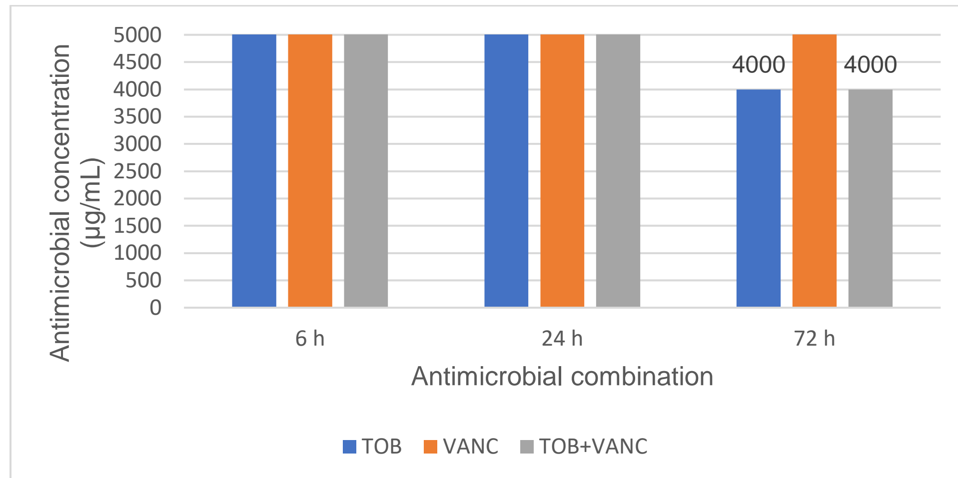


Fig. 4.3 Median for *in vitro* Bone MBEC

Table 4.13 Median of *in vitro* Muscle MBEC for tested 7 microorganisms

	6 h	**24 h**	**72 h**
TOB	2000	375	250
VANC	4000	2000	2000
TOB+VANC	750	500	375

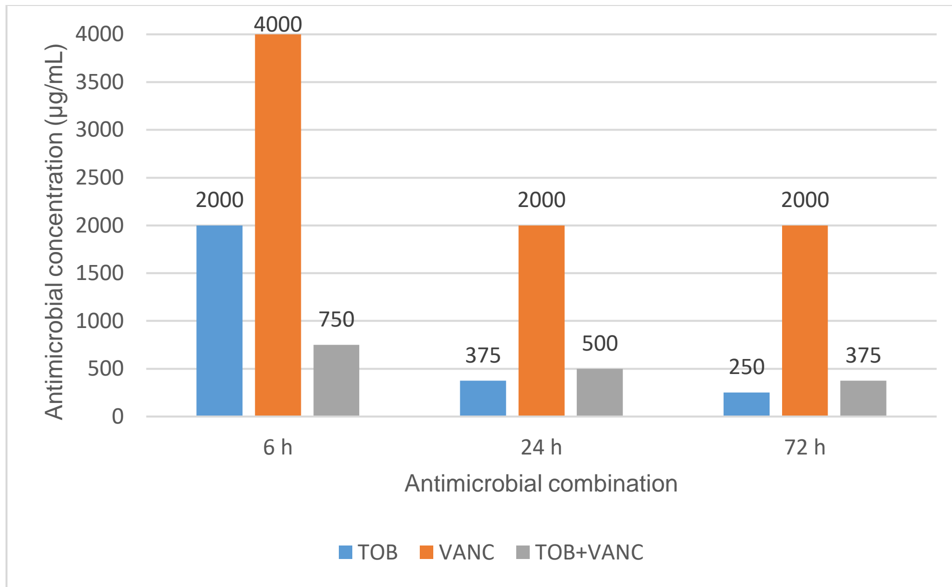


Fig. 4.4 Median for *in vitro* Muscle MBEC

Median values indicate the concentration of antimicrobial concentrations required to kill at least fifty percent of the tested microorganism strains (4 out of 7). The table indicates an increase of susceptibility with increase in exposure duration to the antimicrobials.

Table 4.14 *In Vitro* Muscle MBEC of Microorganisms at 6 hour

ATCC #	MBEC TOB (µg/mL)	MBEC VANC (µg/mL)	MBEC TOB+VANC (µg/mL)
S.aureus (BAA 1556)	2000	4000	500*
S.aureus (BAA 1680)	>4000	4000	750*
S.aureus (49230)	500	>4000	375
S.epidermidis (35984)	>4000	4000	750
P.aeruginosa (27853)	2000	>4000	750
E.faecalis (29212)	>4000	250	2000
E.coli (25922)	125	>4000	4000^
Median	**2000**	**4000**	**750**
$MBEC_{max}$	**>4000**	**>4000**	**4000**

* - Possible Synergy
^ - Possible Antagonism

Table 4.15 *In Vitro* Muscle MBEC of Microorganisms at 24 hour

ATCC #	MBEC TOB (µg/mL)	MBEC VANC (µg/mL)	MBEC TOB+VANC (µg/mL)
S.aureus (BAA 1556)	375	2000	125*
S.aureus (BAA 1680)	>4000	2000	250*
S.aureus (49230)	375	>4000	500
S.epidermidis (35984)	>4000	750	2000
P.aeruginosa (27853)	375	>4000	750
E.faecalis (29212)	>4000	250	500
E.coli (25922)	375	>4000	4000^
Median	**375**	**2000**	**500**
$MBEC_{max}$	**>4000**	**>4000**	**4000**

* - Possible Synergy
^ - Possible Antagonism

Table 4.16 *In Vitro* Muscle MBEC of Microorganisms at 72 hour

ATCC #	MBEC TOB (µg/mL)	MBEC VANC (µg/mL)	MBEC TOB+VANC (µg/mL)
S.aureus (BAA 1556)	250	1000	125*
S.aureus (BAA 1680)	4000	2000	250*
S.aureus (49230)	250	2000	500
S.epidermidis (35984)	>4000	375	750
P.aeruginosa (27853)	62	>4000	375
E.faecalis (29212)	>4000	750	250
E.coli (25922)	125	4000	2000**
Median	**250**	**2000**	**375**
$MBEC_{max}$	**>4000**	**>4000**	**2000**

* - Possible Synergy
** - Possible Antagonism

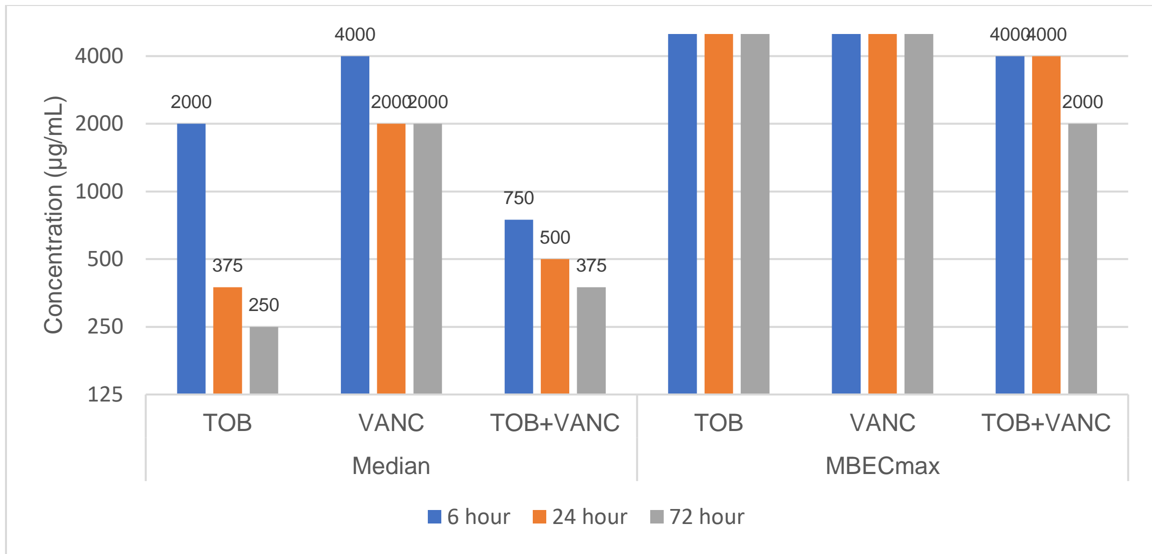


Fig. 4.5 *In vitro* muscle median and maximum MBEC

Analyzing the median *in vitro* muscle MBEC values, the antimicrobial combination TOB+VANC is either comparable to or lower than that of the single agents alone. The $MBEC_{max}$ shows all 7 microorganisms' biofilms are susceptible to TOB+VANC in the tested range which is not the case for the single agents alone.

4.3 Discussion

Biofilms were successfully established on animal tissues *in vitro* as expected for seven out of the eight organisms. The microorganism *S. agalactiae* (13813) did not form biofilms which was inferred from negative subculture results of the positive control.

The data from this study was more internally consistent as compared to the ex vivo tissue MBEC. The subculture data allowed for straightforward interpretation of the MBEC with fewer "gaps" due to false positive or false negative results, indicating less variability between individual samples. This method also requires reduced use of animals and affords more flexibility in experimental timing. Overall, most MBEC values measured using cortical bone exceeded 4000 µg/mL, which was the maximum concentration evaluated in this study. However, most of the MBEC values for muscle samples were in the tested range and showed increased susceptibility with antimicrobial exposure duration in most cases. A possible explanation for the extremely high MBECs observed in bone is that the bacteria were able to grow throughout the bone and the transport of the antimicrobials was limited, either due to the physical dense structure of the bone or its chemical interactions with the antimicrobials. This suggests that local delivery of antimicrobials may not be effective at any level against infection in intact cortical bone, placing high priority on thorough surgical debridement.

Because the antimicrobial combination TOB+VANC covers a broader spectrum (all the tested microorganisms are sensitive to TOB+VANC) of microorganisms and its action of susceptibility being comparable to that of TOB alone, a dual agent strategy appears superior in formulations for local delivery.

For the duration of antibiotic exposure, the MBEC values at 24 hour exposure are lower than 6 hour exposure and comparable to 72 hour exposure.

4.4 Comparison of MIC against MBEC of muscle at 24 hour antimicrobial exposure

Table 4.17 MIC of Microorganisms

ATCC #	MIC TOB (µg/mL)	MIC VANC (µg/mL)	MIC TOB+VANC (µg/mL)
S.aureus (BAA 1556)	2	4	1
S.aureus (BAA 1680)	2	2	2
S.aureus (49230)	1	8	2
S.epidermidis (35984)	128	2	4
P.aeruginosa (27853)	8	>128	8
E.faecalis (29212)	64	2	4
E.coli (25922)	2	>128	8
Median	**2**	**4**	**4**
MIC_{max}	**128**	**>128**	**8**

Table 4.18 *In vitro* MBEC of Muscle

		TOB (µg/mL)	VANC (µg/mL)	TOB+VANC (µg/mL)
6 hour	**Median**	2000	4000	750
	MBECmax	>4000	>4000	4000
24 hour	**Median**	375	2000	500
	MBECmax	>4000	>4000	4000
72 hour	**Median**	250	2000	375
	MBECmax	>4000	>4000	2000

Compared against MIC (Table 4.12), the MBEC values (Table 4.11) generally lie between 100 – 1000x higher as seen in the tables above. This allows for an interpretation that delivering an antimicrobial agent locally at the infected site, even if sufficient to kill planktonic bacteria, it is not sufficient to eradicate bacteria in biofilm.

4.5 Correlation between MIC and *in vitro* tissue MBEC

The below figure represents a plot correlating the logarithmic (base 2) values of MIC and corresponding *in vitro* muscle MBEC at 24 hour antimicrobial exposure for the same microorganism and same antimicrobial treatment.

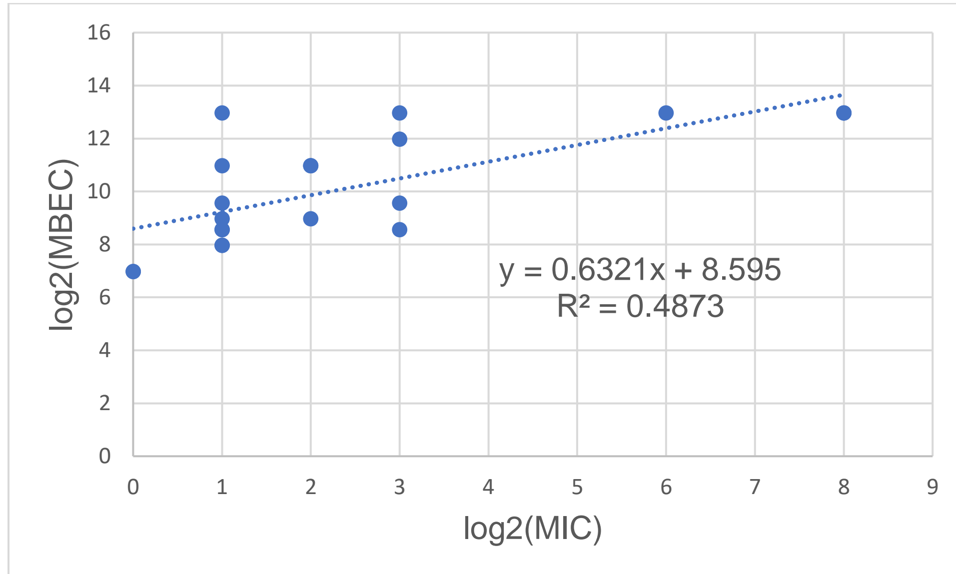


Fig. 4.6 Correlation plot of log2(MIC) vs log2(*in vitro* MBEC)

The R-squared value of 0.48 shows a weak but a possible correlation between MIC and MBEC data when pooled across all strains tested. With more careful analysis limited within specific species of organisms, the correlation between MIC and MBEC could improve and possibly support the use of MIC to estimate relative MBEC values of different antimicrobial treatments or exposure times.

CHAPTER 5: CONCLUSIONS

5.1 Rabbit implant infection model

This research document reports on the experimental approach in developing an *in vivo* rabbit biofilm related bone infection model on biological tissues and to measure the *ex vivo* minimum biofilm eradication concentration on those tissues. A chronic infection was established using four out of eight microorganisms tested. The microorganisms that successfully caused the infection were three *S.aureus* species (ATCC strains BAA 1556, BAA 1680 and 49230) and *P. aeruginosa* (ATCC 27853) and the tissues obtained from these animals were tested for MBEC.

Infection did not establish using one biofilm forming *S. epidermidis* (ATCC 35984) and one non-biofilm forming *S. epidermidis* strain (ATCC 12228), *S. agalactiae* (ATCC 13813) and *E. Faecalis* (ATCC 29212). A second surgery was performed and the rabbits were reinoculated for *S.epidermidis* (35984) and *S. agalactiae* (13813). A different approach with the rabbit radius (Nelson et al.) was adopted after the failure of infection establishment which also failed to establish infection for *S. epidermidis* (35984) and *S. agalactiae* (13813).

5.2 *Ex vivo* tissue minimum biofilm eradication concentration (MBEC) and Minimum inhibitory concentration (MIC)

The project measured the *ex vivo* tissue MBEC values for the tissues obtained from infected rabbits. Analysis showed anomalies and inconsistencies that did not result in the determination of the actual MBEC values. Since the data was inadequate to establish a definite conclusion, a newer approach had to be adopted to measure the MBEC by growing biofilms on biological tissues in laboratory conditions. This allowed us to grow the biofilms on biological tissues without the presence of host immunity response thereby expecting a more consistent biofilm infected tissues and a larger data generated in a shorter duration.

MIC tests were performed to assess the susceptibility of the microorganisms of interest against three antimicrobial combinations (TOB, VANC, TOB+VANC). MIC_{75} values for TOB+VANC were

lower than the single agents (TOB or VANC) and TOB+VANC had a wider coverage given findings show only 6 out of 8 microorganisms were sensitive to either TOB or VANC, but all the 8 microorganisms were sensitive to the combination TOB+VANC.

5.3 *In vitro* tissue minimum biofilm eradication concentration (MBEC)

As anticipated, biofilms were successfully grown *in vitro* on the biological surfaces. Broth dilution antimicrobial susceptibility tests were performed on tissues after growing biofilms. MBEC obtained were significant, consistent and antimicrobial susceptibility increased with time. The bone MBEC values were too high and similar for statistical analysis.

Statistical studies of muscle MBEC values showed TOB+VANC to be the superior option among the three antimicrobial combinations for treatment with its median MBEC being comparable to the best treatment (TOB) and $MBEC_{max}$ eradicating all eight microorganisms in contrast to only 6 out of 8 for the antimicrobial combinations as single agents (TOB or VANC) alone.

Analyzing the antimicrobial exposure durations, 24 hour exposure showed optimum results with the 24 hour exposure time resulted in lower MBEC compared to 6 hour exposure and similar MBEC compared to 72 hour exposure.

A comparison was made between the MIC of microorganisms and corresponding MBEC values of muscle at 24 hour exposure for all 3 antimicrobial combinations. On an average the MBEC values were 100 – 1000x higher than the MIC values proving MIC level antimicrobial delivery at infection sites would not eliminate the biofilm bacteria. An additional observation showed a possible correlation between MIC and MBEC showing MIC to be a possible metric to determine the relative susceptibility of biofilm bacteria to various antimicrobial treatments.

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