South West Sydney
Limb Preservation and Wound Research

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DebX Medical: The Effectiveness of Debrichem on In Vitro Biofilms Report

Methods

All testing was performed in triplicate and testing was completed twice, separated by 2 weeks apart. This was to ensure reproducibility (Parker et al., 2018). Testing was undertaking in accordance with ASTM methods.

Bacteria

The strains used for testing all three models were the ATCC strains *P. aeruginosa* 15442 and *S. aureus* 6538. The strains were cultured using Typtone Soy Agar (TSA) and Tryptone Soy Broth for the MBEC and CDC and Luria-Bertani (LB) Broth and Agar for the Semi solid model. All saline used was sterile 0.9% saline.

Agent tested

Debrichem (DebX Medical) an acid used for chemical debridement in wounds. Control samples were treated with 0.9% sterile saline.

Biofilm Models

- MBEC® Assay, CDC Biofilm Reactor and Semisolid Biofilm *in vitro* models. MBEC® Assay is a standardised *in vitro* model for screening agents for antimicrobial inhibition and efficacy.
- The CDC Biofilm Reactor is a standardised *in vitro* model commonly used in biofilm research. It produces reproducible biofilms on individual coupons under flow.
- Semi-solid model has been previously described by Crone et al. (2015). It simulates a wound environment through the growth of bacteria encapsulated in agar.

Imaging Preparation – Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) was used to visualise and confirm growth of microbial biofilms in both MBEC® and CDC models. Microbial biofilms were sampled at 5-200 µm. The pegs and discs were fixed in glutaraldehyde overnight, dehydrated through serial dilutions of ethanol and hexamethyldisilazane (HDMS) (Polysciences, Inc., Warrington Pa.) before being air dried for at least 48 hours. Specimens were then mounted, gold coated and examined.

MBEC model

MBEC® Testing was completed according to the ASTM International Standard Test Method for Testing Disinfectant Efficacy against *Pseudomonas aeruginosa* Biofilm using the MBEC® Assay (Designation E2799-17). Briefly, an overnight suspension of each test strain was diluted to $2x10^7$ and $1x10^7$ for ATCC 15442 and ATCC 6538, respectively. 150uL of each inoculum was then added to a 96-well MBEC Assay Plate (Innovotech, Edmonton, Canada), prior to the 96-peg lid (Innovotech being added and the plate and incubated for 24 hours at 35°C ± 2°C with continuous shaking (110 rpm).

Biofilm pegs were washed in sterile saline for 10 seconds, then transferred to a new 96-well plate containing 200 µL of Debrichem solution or sterile saline (control) for a contact time of 30 seconds. Following treatment, the pegs were rinsed twice in saline for 10 seconds each. To facilitate the disaggregation of the biofilm, the recovery plate was sonicated in an ultrasonic water bath (SoniClean™ 160TD, Australia) high for 20 minutes. The recovery solution was then serially diluted, and spot plated in triplicate onto TSA and incubated at 35°C ± 2°C for 18-24 hours and utilised for plate counts and quantitative analysis.

In total, nine pegs were treated with Debrichem and utilized for quantification analysis (6 pegs) and SEM imaging (3 pegs), with an identical number of control pegs treated with sterile saline.

Quantification of Log10 results of MBEC® Assay was calculated using the following equation:
Log_{10} (CFU/mm^2) = Log_{10} [(X/B) (V/A)(D)]
Where:
X = CFU counted in the spot
B = volume plated (0.01 mL)
V = well volume (0.20 mL)
A = peg surface area (46.63 mm^2)
D = dilution

Optical density (OD) of MBEC® plate
To determine OD of the MBEC®, 100µL of sterile TSB was added to each recovery plate well and covered with a sterile non-pegged lid and placed in the incubator at 35 ± 2°C for 24 hours. The plate was then read using a spectrophotometer (SPECTRAmax M2e) at 650nm.

Results of MBEC® testing
Debrichem demonstrated 100% eradication of biofilm from both ATCC 15442 and ATCC 6538 after 30 seconds using the MBEC Assay®. This was a mean log reduction of 2.7 x log10^4 CFU/mm^2 for ATCC 6538 and 7.6 x log10^5 CFU/mm^2 for ATCC 15442 (triplicate testing).

Strain	Average Log10 Control	Average Log10 Debrichem	Log10 reduction
ATCC 15442	4.7x10^5 (±4.2x10^5)	0	4.7x10^5
ATCC 6538	4.1x10^7 (± 5.7x10^7)	0	4.1x10^7

Qualitative Determination of MBEC - OD
The OD of both 15442 and *S. aureus* biofilm treated with Debrichem was OD_{650} <0.1.

SEM of the *Pseudomonas Aeruginosa* MBEC™ peg
SEM of select pegs to illustrate the presence of bacteria and biofilm. A) Demonstrates ATCC 15442 *Pseudomonas aeruginosa* biofilm growth on the control peg post-treatment, B) Picture B demonstrates lysed cells of ATCC 15442 *Pseudomonas aeruginosa* on the peg treated with Debrichem.

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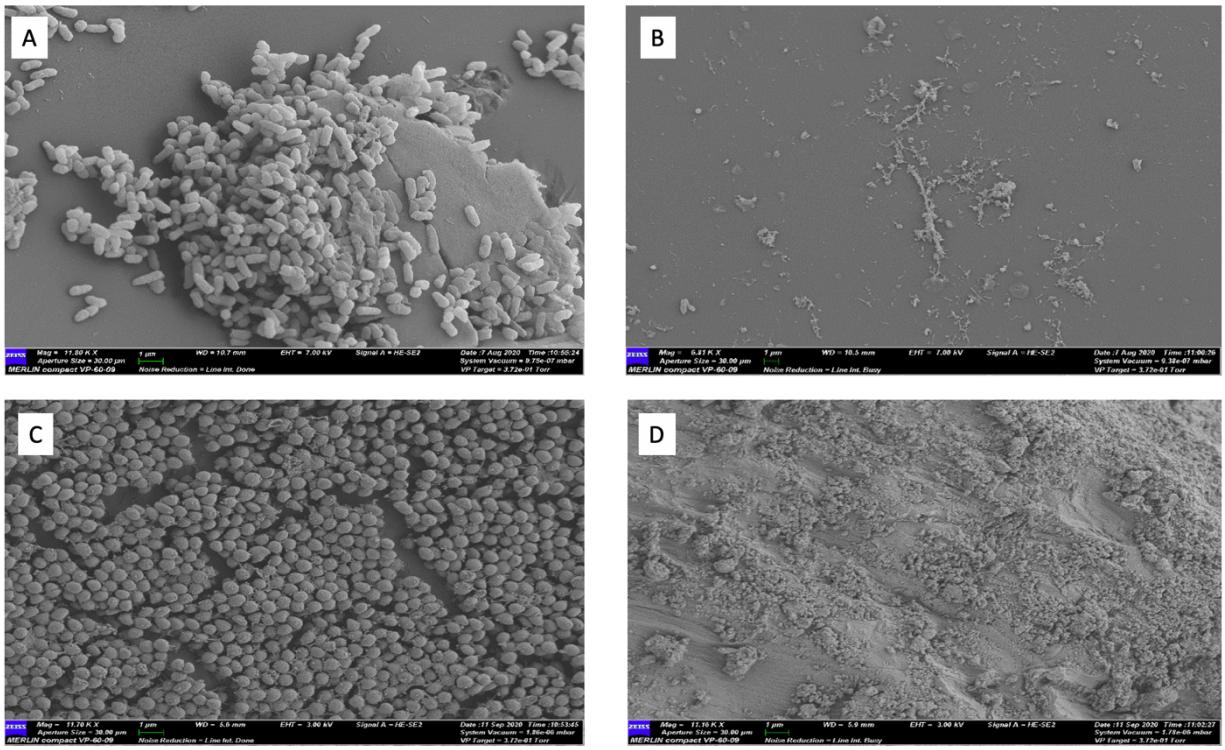
SEM of the *Staphylococcus aureus* MBEC™ peg
C) ATCC 6538 *Staphylococcus aureus* biofilm pre-treatment, D) ATCC 6538 *Staphylococcus aureus* post treatment with Debrichem for 30 seconds.



CDC Biofilm Reactor model
The CDC Biofilm reactor (BioSurface Technologies Corporation) was prepared using the ASTM Method E3161-18 'Standard practice for preparing a *Pseudomonas aeruginosa* or *Staphylococcus aureus* Biofilm using the CDC Biofilm Reactor', and the effect of Debrichem was determined using the ASTM Method E2871-19 'Standard Test Method for Determining Disinfectant Efficacy Against Biofilm Grown in the CDC Biofilm Reactor Using the Single Tube Method'.

Briefly, an overnight culture of ATCC 15442 was diluted to 2 x 10^7 CFU/mL, then 1 mL was added to the CDC reactor containing 500mL sterile TSB solution (Add concentration here). The reactor was placed on a magnetic stir plate (Talboys™ 4x4 Ceramic Stirrer 230V, USA) at 125 ± 5 r/min at room temperature for a 24-hour batch phase growth. A growth medium concentration of 100 mg/L TSB continuously flowed for the continuously stirred tank reactor (CSTR) phase with a residence time of 30 ±2 minutes using a Masterflex Digital Pump (John Morris, Masterflex Digital Drive and Pump Head, USA) for a further 24 hours.

For ATCC 6538 an overnight culture was diluted to 1.2 x 10^7 CFU/mL before being added to the reactor containing 500mL of sterile TSB solution (Add concentration here). The reactor

was placed on the magnetic stir plate at 60 ± 5 r/min and incubated at 36 ±2 °C for 24 hours of batch phase growth. The CSTR phase was then run for an additional 24 h at 36 ±2 °C using 1 g/L TSB, with a residence time of 30 ±2 minutes.

Following the CSTR phase, the treated coupons were rinsed in a Falcon tube (50mL conical centrifuge tube, ThermoFisher) containing 30mL 0.9% saline. Four individual coupons were each dropped into individual Falcon tubes containing 4mL of Debrichem for 30 seconds ensuring the coupons were covered. Coupons were then rinsed in a tube containing 10mL of 0.9% saline, then dropped into a new tube containing 36mL of 0.9% saline. Control coupons underwent the same process with sterile saline.

Each tube was then vortexed on the highest setting for 30 ±5 seconds at room temperature and then placed in an ultrasonic water bath (SoniClean™) at 45 ±5 kHz for 30 ±5 seconds at room temperature. This process was repeated for a total of 3x rounds of vortexing and 2x rounds of sonication.

For the treated coupons, the entire recovery solution was processed through a Nalgene Reusable Filter Unit with a 0.2µm filter, followed by placing the filter membrane directly onto a TSA plate. The control coupon tubes were briefly vortexed and diluted from 10^0 to 10^6 and plated in duplicate using the spread plate method on TSA plates. The plates were then incubated at 35°C for 48 h and 72 h for the control and treated plates, respectively.
Quantification of the $\log_{10}$ density (LD) for each control coupon was calculated using the following:

$$\mathrm{Log}_{10}\left\{\left[\frac{\sum_{i=1}^{n} X_i}{\sum_{i=1}^{n}(B_i \times D)}\right] \times V\right\}$$

Where:
n = number of dilutions
X = average CFU of the replicate plates
B = volume plated
V = total volume of buffered dilution water plus neutralizer
$D = 10^{-k}$
k = dilution

The treated coupons were calculated using the above calculation, using the volume of Debrichem instead of buffered dilution for V.

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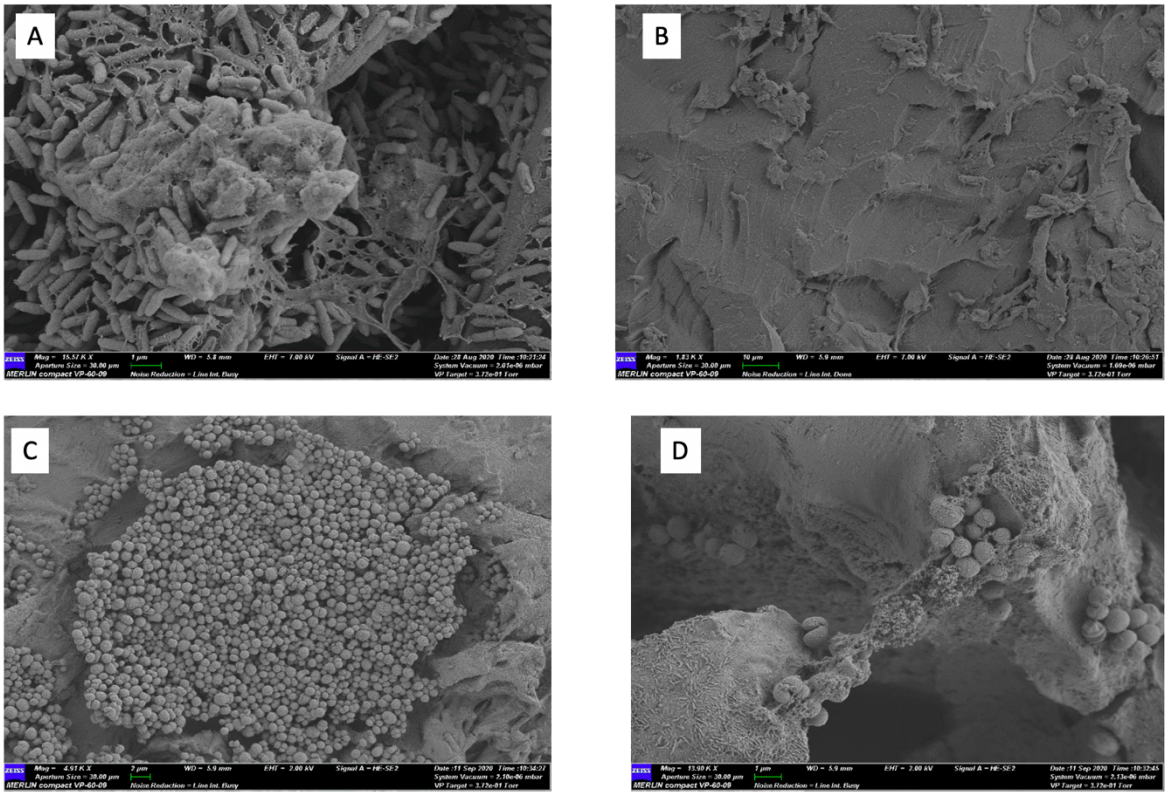
Results of the CDC Biofilm Reactor

The use of Debrichem of 30 seconds of application resulted in complete eradication of any bacterial growth with both ATCC biofilm strains.

Strain	Average Log Density	Average Log10 Debrichem	Log10 reduction
ATCC 15442	8.685 log10 (±0.09)	0	8.685 log10
ATCC 6538	6.7 log10 (±0.1)	0	6.7 log10

SEM of CDC polycarbonate discs

SEM image of ATCC 15442 biofilm on a polycarbonate coupon pre (A) and post (B) treatment with Debrichem for 30 seconds. SEM image of ATCC 6538 biofilm on a polycarbonate coupon pre (C) and post (D) treatment with Debrichem for 30 seconds.



Semi solid biofilm model

Wound Simulation Medium (WSM) was prepared using Bolton Broth, agar, Bovine Foetal Serum (Sigma, Australia) and sterile Defibrinated Horse Blood (Australian Ethical Biologicals, VIC, Australia), for a final concentration of; 5mL Bolton Broth with 1% agar, mixed with 2.5mL of serum and blood respectively. Overnight cultures of each strain were grown in LBB and diluted to a final dilution of 10^{-4}. The inoculum for the model was prepared by adding 100µL of the 10^{-4} dilution to 900µL of WSM. The semi solid model was assembled by adding 180 uL of the WSM to the centre of a gene frame (Thermofisher) which was added to a glass microscope slide (26mmx76mm). The medium was allowed to set before 2uL of the ATCC 15442 and 4uL of the ATCC 6538 inoculum were added to the centre of the medium and allowed to set. Finally, another 150uL of WSM was added over the top of the inoculum and allowed to set. The slides were then placed in a sterile container with tissue paper wet with sterile water, before being sealed with Parafilm and incubated at 37°C for 24 h.
Following incubation, 150 µL of Debrichem was pipetted over the middle of the slide and incubated at RT for 30 sec, before being rinsed off with approximately 3 mL of sterile saline. Control slides were rinsed with sterile saline.

Each gel was then aseptically transferred to a 2mL Eppendorf tube containing 1.5mL of sterile saline and a 5mm stainless steel bead. The tubes then underwent bead beating in a TissueLyser II at 20Hz for 15 sec to facilitate the breaking up of the agar. Following bead beating, the contents of each tube were transferred to 45mL of 0.9% saline and centrifuged at maximum speed (6000g) for 10 minutes. Following centrifuging, the supernatant was carefully decanted, and a further 45 mL of saline was added and centrifuged as described above. All but two mL of the supernatant was removed before the tubes were vortexed at low speed to resuspend the pellet. The tubes were then placed in the ultrasonic water bath (SoniClean™) and degassed for 5 minutes followed by sonication on high for an additional 5 min. Each solution was then serially diluted to 10^6 in saline, and spot plated in triplicate on LBA plates. The plates were then incubated at 37±2°C for 18-24 hours and colony counted.
Quantification of log counts was performed using the following equation:

$$\frac{\text{Number of colonies X dilution (eg. } 10^5)}{\text{Volume plated (mL)}}$$

<u>Results of Semi-Solid Biofilm Model</u>

Complete eradication of any bacterial growth with both strains.

Strain	Average Log10 Control	Average Log10 Debrichem	Log10 reduction
ATCC 15442	1.5×10^6 ($\pm 5.7 \times 10^5$)	0	1.5×10^6
ATCC 6538	1.1×10^7 ($\pm 8.3 \times 10^6$)	0	1.1×10^7

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References

Parker AE, Hamilton MA, Goeres DM. Reproducibility of antimicrobial test methods. Sci Rep. 2018;8(1):12531. Published 2018 Aug 22. doi:10.1038/s41598-018-30282-3.

Crone S, Garde C, Bjarnsholt T, Alhede M. A novel in vitro wound biofilm model used to evaluate low frequency ultrasonic-assisted wound debridement. J Wound Care 2015. 24:2, 64-72.

I can confirm that all test results are a true and accurate reflection of our testing methods completed in compliance with the standard operating procedures of the laboratory.

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