Complex biofilms show variability of bacteria survival over long durations: implications for *in vitro* screening of antimicrobial actives.

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ABSTRACT

Development of new antimicrobials has not kept pace with the rise in drug resistant bacterial infections seen today, requiring clinicians to use multiple antibiotics in larger doses, for longer periods of time. Modified Robbins Devices (MRDs), designed with small uniform sampling coupons have greatly increased the efficiency in testing novel agents against biofilms, known to play a critical role in drug resistance and chronic infections. Due to the slow metabolism of bacteria in biofilms, long exposure times are often required to demonstrate efficacy of antimicrobial agents. However, in order to establish the relevancy of long exposure times, it is important to know first determine how long control (untreated) biofilms can survive on without the benefit of nutrient media which can interfere with the function of some antimicrobial agents. This is further complicated when the biofilm is composed of a mixed culture biofilm with more than one microorganism. In this study, we grew a complex biofilm matrix composed of *S. aureus, P. aeruginosa*, and *E. coli.* After the biofilm reached maturity, it was removed from nutrient media and submerged in a non-nutritive buffered saline solution (PBS) for up to 36 hours. Total and species-specific colony forming unit (CFU) counts were determined using both selective and non-selective agar plates. The results of this study have important implications for the testing of antimicrobial and anti-biofilm agents.

METHODS

Preparation of Inoculum:

Two days prior to the experiment, streak a small loopful of test organisms from frozen culture collection and culture on a non-selective agar plate (e.g. TSA). Incubate overnight at 37°C ± 1°C and examine the culture for purity prior to use.

Test organisms:
- *Pseudomonas aeruginosa* (ATCC 27317)
- *Staphylococcus aureus* (ATCC 25923)
- *Escherichria coli* (ATCC 47022)

Incubate at 37°C ± 1°C for approximately eighteen hours.

Preparation of each inoculum:

Staphylococcus aureus (ATCC 25923)
Pseudomonas aeruginosa (ATCC 27317)
Escherichria coli (ATCC # 47022)

The day prior to the experiment a small loopful of each microorganism from the above-mentioned strains were placed into full strength Trypic Soy Broth and vortexed.
Plate s were then incubated at 37°C ± 1°C for approximately eighteen hours.

Formation of biofilm using of the MRD apparatus (Figure 1)

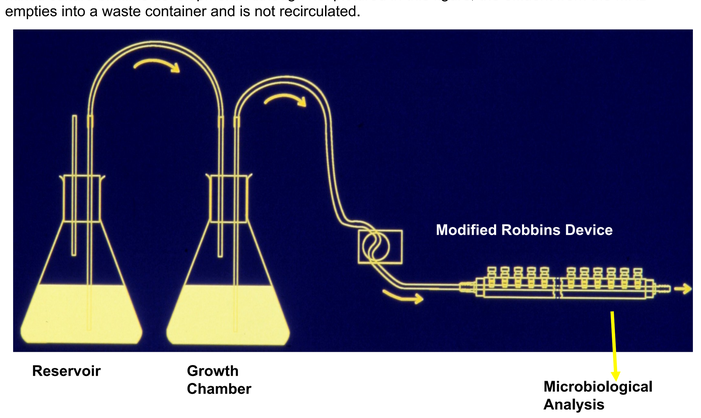
The entire flow system including MRD was sterilized using ethylene oxide sterilization system.
The MRD was connected by siliconized tubing (Tygon, Pittsburgh, PA) to a 2 L reservoir flask filled with half strength of Trypic Soy Broth media, and held at 37C. The reservoir was seeded with 2% inoculum of *Staph aureus* (ATCC 25923) so that the reservoir delivers logarithmic-phase microbes to the MRD. To ensure adequate aeration of the cultures, each reservoir was connected to an Elite 800 fish tank pump (Rolf C. Hagen Corp., Mansfield, MA). Air is vigorously pumped through an air filter and bubbled through the media. A fresh half strength of TSB media reservoir was also connected to the growth vessel. The MRD sampling ports were loaded with PVC sampling coupons and inserted into the MRD prior to starting the flow of media. The sampling coupons must be flush with the upper surface of the MRD fluid chamber to ensure the proper laminar flow properties.
Media containing logarithmic growth phase bacteria was continuously circulated through the MRD by means of a peristaltic pump (Masterflex, Cole Parmer). The pump ran for approximately 18 hours and delivered approximately 60 mL per hour of media with logarithmic growth-phase bacteria through the MRD. During the first four hours of incubation, only the media and *Staph aureus* were circulated through the MRD. After four hours of incubation, the initial biofilm was established and the pump connected to the fresh half strength of TSB media reservoir was turned on. The fresh media was delivered at approximately 30-40 mL per hour to the growth vessel. The overflow was discarded through an overflow spout into a waste beaker. After twenty four hours of circulation, the growth vessel containing of *Staph aureus* was disconnected and replaced with fresh half strength of TSB media . The fresh TSB media circulated through the MRD for one hour to rinse off the planktonic *Staph aureus* then the growth vessel was seeded with a 2% inoculum of *Pseudomonas aeruginosa*, so that the reservoir delivered logarithmic-phase microbes to the MRD and was circulated through the MRD for three hours. After three hours of circulation, the growth vessel containing of *Pseudomonas aeruginosa* was disconnected and replaced with fresh half strength of TSB media. The fresh TSB media circulated through the MRD for one hour to rinse away the planktonic bacteria in the MRD then the growth vessel wasseeded with 2% of the third bacteria *E. coli* (ATCC 47022) so that the reservoir delivered logarithmic-phase microbes to the MRD and was circulated through the MRD for three hours. After three hours of circulation, the growth vessel containing the *E. coli* was disconnected and replaced with fresh half strength of TSB. The fresh half strength of TSB was continuously circulated through the MRD by means of a peristaltic pump for another 18 hours. The pump delivered approximately 60 mL per hour of media through the MRD. After another overnight incubation (approx 18 hours), the sampling coupons were removed to and placed in PBS for 0, 2 ,4, 6, 24 and 36 hours and then sampled for the surviving sessile population as described below.

To sample the sessile population:
A sample ports were aseptically removed and rinsed under a stream of PBS (10 mL) to remove any loosely adherent bacteria. The sample coupons were then scraped with a sterile scalpel blade and the scrapings, along with the sample were added to a tube of sterile PBS. The tube and contents were ultrasonicated using a low output (50-60Hz) bath ultrasonicator (Branson, Shelton, CT) for five minutes to remove any cells still attached to the surface and to disperse any aggregates of bacteria. A 100 µL aliquot of the culture fluid was removed and serially diluted in sterile PBS. Four samples of the dilutions were plated, in duplicate, on TSA plates to determine the total number of bacteria. Four samples of the dilutions are plated, in duplicate, on Pseudomonas Isolation Agar (PIA), on Manitol Salt Agar (MSA) and, on MacConkey Agar (MAC) to determine the number of Pseudomonas, Staph aureus and E. coli, respectively.

Calculations:
Use dilutions giving 30 to 300 colonies per plate. Multiply colonies counted by inverse of dilution and inverse of fractional volume plated.
CFU/mL = (dilution factor)-1 X (correction factor)-1 X average number of colonies
EXAMPLE: If the 10^{-3} dilution gives an average plate count of 42 colonies per plate, and 100µL volumes were plated, then:
CFU/mL = (10^{-3})-1 X $(0.1)^{-1}$ X 42
= 1000 X 10 X 42
= 4.2 X 105

Figure 1: Schematic diagram of the Modified Robbins Device (MRD)

The schematic below demonstrates the setup of the MRD for the studies described in this paper. Note that the growth chamber is inoculated with a new species of bacteria successively over the course of the mixed culture biofilms development. Although not pictured in this figure, the effluent from the MRD empties into a waste container and is not recirculated.



RESULTS

Over the 36-hour time-frame of the experiment, the total log CFUS on non-selective TSA indicated a modest overall decline of approximately 1 log (Figure 2). When the bacterai were plated on selective media, no significant variance in the number of gram negative bacteria (*P. aeruginosa* and *E. coli*) was observed. (Figures 3 & 5, respectively) The average bacteria concentration for *S. aureas,* however, steadily declined with increasing incubation times, with a significant (P-value<0.001) drop after a 24hr incubation period. After 36hrs of incubation, viable *S. aureas* could not be recovered on MSA plates (Figure 5).

Figure 2. Log CFUs of surviving total bacteria held in PBS over 36 hours and plated on non-selective trypic soy agar (TSA)

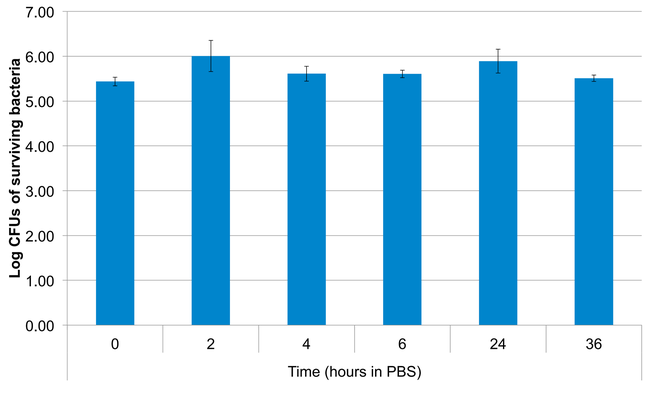


Figure 2:

Over a 36 hour incubation period, the total number of viable biofilm bacteria remained relatively constant with a modest 1 log reduction by 6 hours that persisted through the remainder of the study.

Figure 3. Log CFUs of surviving Ps aeruginosa held in PBS over 36 hours and plated on selective Pseudomonas Isolation Agar (PIA)

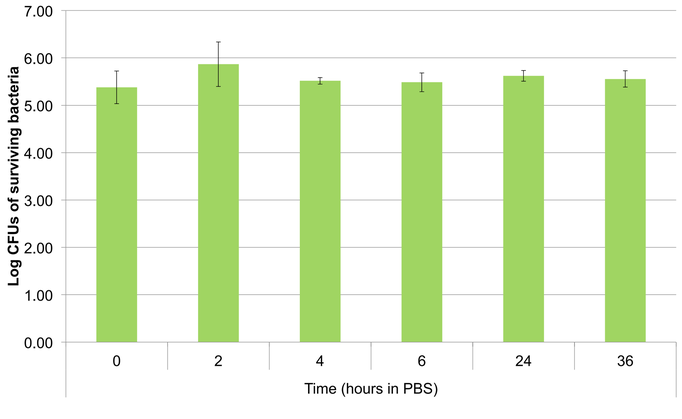


Figure 3:

Over a 36 hour incubation period, the total number of viable *Pseudomonas aeruginosa* biofilm bacteria remained constant..

Figure 4. Log CFUs of surviving Staph aureus held in PBS over 36 hours and plated on selective Mannitol Salts Agar (MSA)

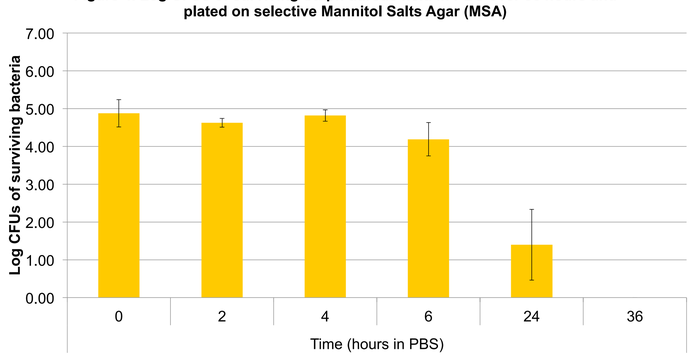


Figure 4:

Over a 36 hour incubation period, the total number of viable *Staphylococcus aureus* biofilm bacteria declined dramatically culminating in a reduction to zero viable CFUs at the terminal time point.

Figure 5. Log CFUs of surviving E. coli held in PBS over 36 hours and plated on selective MacConkey Agar (MAC)

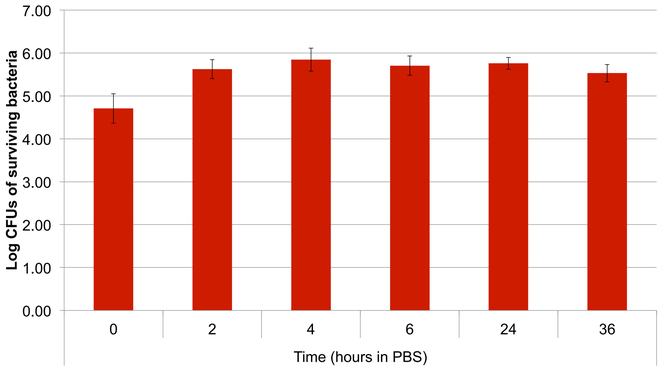


Figure 5:

Over a 36 hour incubation period, the total number of viable *E. coli* biofilm bacteria remained constant..

CONCLUSIONS

The results reflect the sustainability of gram negative bacteria from a mixed culture biofilm for up to 36 hrs. However, the data supports viability only up to six hours for *S. aureas* within the complex matrix, possibly due to secreted cytotoxic factors from the gram negative microorganisms.
These findings demonstrate the importance of establishing a baseline of long-term survival in the absence of extraneous factors and for the need to examine the survival of each organism independently rather than simply counting the total number of viable organisms in a non-selective manner.

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1) Antibiotic resistance of Pseudomonas aeruginosa colonizing a urinary catheter in vitro. Nickel JC et al. Eur J Clin Microbiol. 1985 Apr;4(2):213-8.
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