Visualization of Clinically Relevant Biofilms During Exposure to Disinfectants

D. Del Re[1], K. Dhyani[1], R. Mair[1], M. Legner[2], D. G. Cvitkovitch[2], and D. Swift[1]
[1]Biolennia Laboratories, Toronto, ON; [2]University of Toronto, Toronto, ON

ABSTRACT

Background: Microbial biofilms play a major role in the progression of infection and have been shown to be more difficult to eradicate than planktonic cells. In a clinical setting, surface disinfection represents one of the primary means by which the spread of infection is minimized. The main objective of this study was to directly visualize the effect of disinfectants on clinically relevant biofilms to determine their efficacy. **Methods:** Biofilms of *Pseudomonas aeruginosa* MPAO1, and clinical isolates of *Escherichia coli* and *Staphylococcus aureus* were grown at 37˚C for 48hr in 6-chamber flow cells. Biofilms were either first stained with BacLight Live/Dead probe for 15min, followed by disinfectant treatment, or treated first and then stained with BacLight. Disinfectants were injected through each chamber, using PBS as a control. Images of the biofilm cells were captured every 5 seconds for 2 minutes, followed by every 30 seconds for an additional 10 minutes (only for biofilms treated after staining with BacLight). All experiments were performed in duplicate. **Results:** Each disinfectant showed different efficacies against the test strains. Ethanol-based products appeared most effective, with a shift from green to red fluorescence (bacteria apparently killed) in as little as 5 seconds. Products containing quaternary ammonium compounds and peroxide exhibited some cell death by the end of the exposure period, but effects were much slower. **Conclusions:** This study demonstrates that different disinfectants exhibit varying degrees of effectiveness in killing biofilm cells. This is the first study that has directly visualized bacterial biofilms during the course of exposure to disinfectants. This will provide further knowledge into how disinfectants act on biofilms, leading to more effective infection control strategies.

INTRODUCTION

The use of disinfectants is the primary means employed at the community, institutional and household levels to kill microorganisms that reside on inanimate surfaces in order to control the spread of infectious agents[1]. Before commercial products can be approved for use by the public, they must be tested and certified; however, most standardized test methods rely on the response of planktonic cells. Research has shown that biofilm cells are more resistant to antimicrobial agents compared to planktonic cultures of the same species; however, few studies have compared effectiveness of disinfectants on biofilms and planktonic cultures.

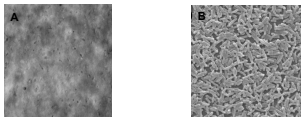

Fig. 1 Planktonic and biofilm cultures in standardized test methods. Images of planktonic (A) and biofilm (B) cultures of *E. coli* showing examples of standardized testing methods for assessing efficacy of disinfectants. (Figure modified from Innovotech's MBEC High-throughput (HTP) assay instructions[2])

Little is known about the immediate effects of disinfectants on bacteria and it can be difficult to determine just how quickly commercial products actually begin to kill their bacterial targets. For this study, we wished to directly visualize bacterial biofilms as they are exposed to disinfectants in order to determine their efficacy and monitor their effects on cells over time. In order to achieve this, biofilms were stained with fluorescent probes and then exposed to various disinfectants. Time-lapse images of pre-stained biofilms were taken during 5-10 min after the onset of exposure in order to record the effects of the disinfectants over time.

This is the first study that has undertaken the task of direct visualization of bacterial cells as they are exposed to disinfection agents. Results from this study will provide further knowledge into how disinfectants act on biofilms, thereby leading to more effective infection control strategies.

OBJECTIVES

The main objective of this study was to directly visualize the effect of commercially available disinfectants on clinically relevant biofilms to monitor death of the cells.

METHODS

Disinfectants:
Five commercially available products were tested: Product S [70.5% ETOH and 0.2% chlorhexidine glugonate (CHG)], Product T (19.9% ETOH and 0.1% CHG), Product L (9.5% ETOH and 0.12% CHG), Product V (0.5% hydrogen peroxide), and Product C (15% Isopropanol, 7.5% ETOH, 0.76% didecyldimethyl ammonium chloride).

Bacterial Strains and Growth Conditions:
Pseudomonas aeruginosa MPAO1, clinical isolates of *Staphylococcus aureus* and *Escherichia coli*.
Strains were maintained on Luria Bertani (LB) agar. Overnight cultures were prepared in brain heart infusion (BHI) broth (for *P. aeruginosa* and *S. aureus*) or LB broth (for *E. coli*) and diluted 1:10 in 1/8 BHI or 1/8 LB, respectively. The diluted culture was then used to inoculate flow cell chambers. Biofilms were grown for 48hr at 37˚C.

Fluorescence Microscopy

Biofilms were grown in 6-channel flow cells for 48hr at 37˚C. Following incubation, the biofilm cells were stained with BacLight Live/Dead probe for 15min. The fluorescence microscope software was used to set up a time-lapse program to capture images of the biofilms before and during treatment. Disinfectants were injected into each channel at specified time points and images were captured in 5-second intervals for approximately 1.5 minutes and then at 30-second intervals for 10 minutes.

Products C and V were incompatible with the Live/Dead probe for direct visualization. As a result, biofilms were first treated with the disinfectants for 20 second, 1 minute, 2 minute, 3 minute and 5 minute exposure times, rinsed with PBS and then stained with BacLight Live/Dead probe for visualization. Images were taken every 5 seconds for 45 seconds.

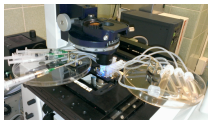

Fig. 2 Apparatus for visualization of bacterial biofilms during exposure to disinfectants. Six-channel flow cell slides were placed on the microscope stage. Syringes containing the test disinfectants were attached to the inflow tubing, while sterile collection vials were attached to the outflow.

RESULTS

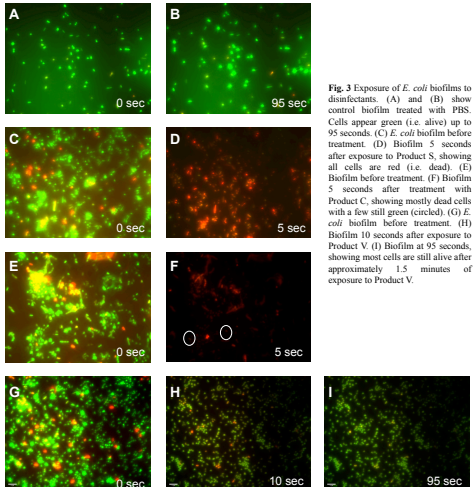

Fig. 3 Exposure of *E. coli* biofilms to disinfectants. (A) and (B) show control biofilm treated with PBS. Cells appear green (i.e. alive) up to 95 seconds. (C) *E. coli* biofilm before treatment. (D) Biofilm 5 seconds after exposure to Product S, showing all cells are red (i.e. dead). (E) Biofilm before treatment. (F) Biofilm 5 seconds after treatment with Product C, showing mostly dead cells with a few still green (circled). (G) *E. coli* biofilm before treatment. (H) Biofilm 10 seconds after exposure to Product V. (I) Biofilm at 95 seconds, showing most cells are still alive after approximately 1.5 minutes of exposure to Product V.

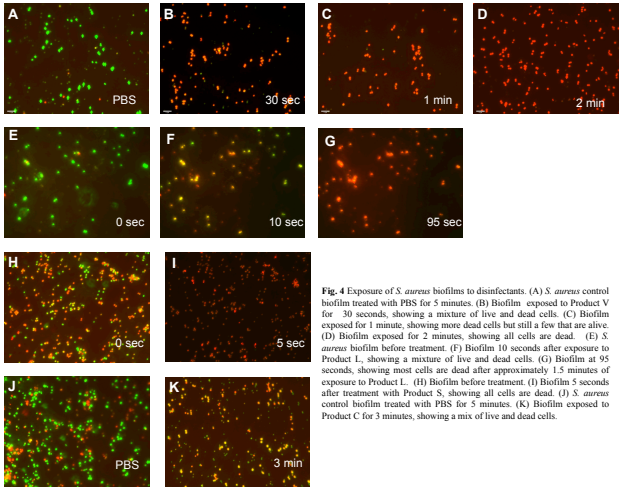

Fig. 4 Exposure of *S. aureus* biofilms to disinfectants. (A) *S. aureus* control biofilm treated with PBS for 5 minutes. (B) Biofilm exposed to Product V for 30 seconds, showing a mixture of live and dead cells. (C) Biofilm exposed for 1 minute, showing more dead cells but still a few that are alive. (D) Biofilm exposed for 2 minutes, showing all cells are dead. (E) *S. aureus* biofilm before treatment. (F) Biofilm 10 seconds after exposure to Product L, showing a mixture of live and dead cells. (G) Biofilm at 95 seconds, showing most cells are dead after approximately 1.5 minutes of exposure to Product L. (H) Biofilm before treatment. (I) Biofilm 5 seconds after treatment with Product S, showing all cells are dead. (J) *S. aureus* control biofilm treated with PBS for 5 minutes. (K) Biofilm exposed to Product C for 3 minutes, showing a mix of live and dead cells.

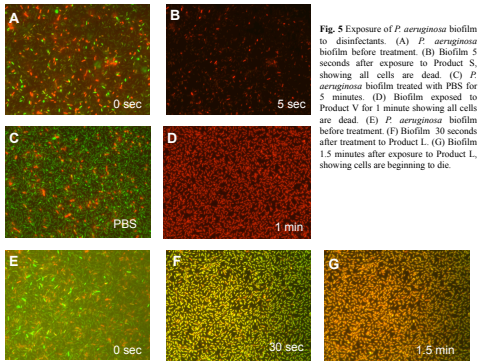

Fig. 5 Exposure of *P. aeruginosa* biofilm to disinfectants. (A) *P. aeruginosa* biofilm before treatment. (B) Biofilm 5 seconds after exposure to Product S, showing all cells are dead. (C) *P. aeruginosa* biofilm treated with PBS for 5 minutes. (D) Biofilm exposed to Product V for 1 minute showing all cells are dead. (E) *P. aeruginosa* biofilm before treatment. (F) Biofilm 30 seconds after treatment to Product L. (G) Biofilm 1.5 minutes after exposure to Product L, showing cells are beginning to die.

CONCLUSIONS

- Disinfectant effectiveness depends on product formulation, as well as test strain.
- Products containing ethanol rapidly kill biofilm cells for all test strains in as little as 5 seconds after exposure.
- Products containing lower concentrations of alcohol (<70%) require longer exposure times to achieve complete killing of biofilm cells.
- The hydrogen peroxide-based product required a longer exposure time than ethanol-based products to kill *E. coli and S. aureus* cells, showing live cells even after 3 minutes exposure, whereas *P. aeruginosa* cells were killed after 1 minute exposure.
- This study presents a novel method for microscopic analysis of bacteria that allows for immediate visualization and monitoring of cells over the course of exposure to disinfectants and a variety of antimicrobial agents.

REFERENCES

1. Myer, B. and B. Cookson. 2010. Journal of Hospital Infections 76: 200-205.
2. Harrison, J. 2011. The MBEC High-throughput (HTP) Assay for Antimicrobial Susceptibility Testing of Biofilms. Innovotech Inc.

ACKNOWLEDGEMENTS

This project was partially funded by NRC-IRAP Grant 790040