Sensitivity Analysis and Visualization of Biofilms of Clinically Relevant Bacteria Exposed to Disinfectants

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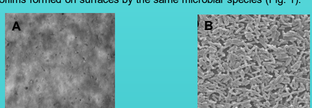
ABSTRACT

Objective: In clinical settings, surface disinfection represents one of the primary means by which the spread of infection is minimized. The main objectives of this study are to examine the effectiveness of disinfectants on clinically relevant bacterial biofilms and to directly visualize the effect of commercially available disinfectants on these biofilms to monitor death of the cells. **Method:** Biofilms of *P. aeruginosa* MPAO1, *B. subtilis* JH642 and clinical isolates of *E. coli* and *S. aureus* were grown at 37°C for 48hr. Minimum biofilm-eliminating concentration (MBEC) assays were performed using 96-well plates containing serially-diluted disinfectants. MBEC values were determined as the lowest concentration of disinfectant that inhibited growth. For fluorescence microscopy, biofilms were grown in 6-chamber flow cells and stained with BacLight Live/Dead stain. Disinfectants were injected through each chamber, using PBS as a control. Images of the biofilm were captured every 5 seconds for 2 minutes, then every 30 seconds for 10 minutes. **Result:** Each strain exhibited different susceptibility profiles to the disinfectants tested, with *B. subtilis* being the most resistant, and clinical isolates being the least. Fluorescence microscopy revealed that ethanol-based products were most effective, with cells appearing to be dead in as little as 5 seconds after exposure. Products containing quaternary ammonium compounds were least effective, with little to no change in cell survival after 12 minutes. Use of peroxide products resulted in some cell death by the end of the exposure period, but effects were much slower compared to alcohol-based products. **Conclusion:** Our study demonstrates that disinfectants exhibit varying effectiveness on biofilm cells. This is the first report on directly visualizing the changes of bacterial biofilms during exposure to disinfectants. Results from this study will provide further knowledge into how disinfectants act on biofilms, thereby 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, which are known to be more sensitive to antimicrobial agents than biofilms formed on surfaces by the same microbial species (Fig. 1).



Planktonic Methods	Biofilm Methods
USP <61> Microbial Limits	ASTM E2799 Disinfectant Efficacy on *P. aeruginosa* Biofilm using MBEC Assay
AOAC 961.02 Germicidal Spray	
AOAC 955.14, 955.15, 964.02 Use-Dilution Methods	
EN1040 Basic Bactericidal Activity of Chemical Disinfectants	

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])

The recent development of a device to study biofilms and determine the Minimum Biofilm- Eliminating Concentration (MBEC) of antimicrobial agents and disinfectants has allowed for a rapid, high-throughput assessment of antibiofilm activity of antibiotics, biocides and metals at varying concentrations[2]. This is the first study that has examined the effect of disinfectants on biofilms using the MBEC assay.

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. 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 objectives of this study are to examine the effectiveness of disinfectants on clinically relevant bacterial biofilms and to directly visualize the effect of commercially available disinfectants on these biofilms to monitor death of the cells.

METHODS

Disinfectants:

25% glutaraldehyde, 99% isopropanol, 20% chlorhexidine gluconate (CHG), 30% hydrogen peroxide, 5% sodium hypochlorite and 70% ethanol (ETOH). Four commercially available products were also tested: Product S (70.5% ETOH and 0.2% CHG), Product T (19.9% ETOH and 0.1% CHG), Product L (9.5% ETOH and 0.12% CHG), and Product V (0.5% hydrogen peroxide). Product C (15% Isopropanol, 7.5% ETOH, 0.76% quaternary ammonium chloride) was also tested in fluorescence microscopy studies.

Bacterial Strains and Growth Conditions:

Bacillus subtilis JH642, *Pseudomonas aeruginosa* MPAO1, clinical isolates of *Staphylococcus aureus* and *Escherichia coli*.
Strains were maintained on Luria Bertani (LB) agar. For MIC/MBEC assays, overnight cultures were prepared in brain heart infusion (BHI) broth and diluted 1:20 in 40ml of fresh media. 20µl of the diluted culture was used to inoculate 96-well plates for MIC assays and biofilm growth for MBEC assays. Biofilms were grown for 48hr at 37°C.
For fluorescence microscopy, overnight cultures were prepared in LB (for *E. coli*) or BHI (for *P. aeruginosa, S. aureus*) and diluted 1:10 in 1/8 LB or 1/8 BHI, respectively. The diluted culture was then used to inoculate flow cell chambers. Biofilms were grown for 48hr at 37°C.

Minimum Inhibitory Concentration (MIC) Assay:

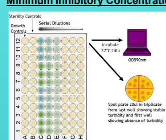


Fig. 2 MIC assay. Diluted overnight cultures of each strain were inoculated into 96-well plates containing serial 10-fold dilutions of the test disinfectants. OD_{590} was measured after 24hr relative cell densities were calculated. Spot plating was performed to confirm presence or absence of growth. The MICs were determined as the minimum disinfectant concentration needed to ensure that culture did not grow to over 10% of the relative cell density. (Figure modified from Innovotech's MBEC High-throughput (HTP) assay instructions)

Minimum Biofilm-Eliminating Concentration (MBEC) Assay:

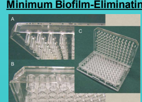


Fig. 3 MBEC device. Biofilms grow on pegs (A) that fit into the wells of a special 96-well plate (B and C). Biofilms for all test strains were grown for 48hr prior to exposure to disinfectants.

Serial dilutions were prepared similarly as described above for MIC assays, except the first dilution for all test disinfectants was 1:2, followed by serial 10-fold dilutions thereafter. MBEC lids containing biofilms were transferred to the exposure plate and incubated for 24hr at 37˚C.

Following the 24hr recovery period, plates were spot plated and OD_{590} was measured to calculate relative cell densities as described for MIC assays. The MBECs were determined as the lowest concentration of disinfectant needed to ensure eradication of biofilm, as evidenced by a relative cell density <10%.

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.

Fig. 4 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

Table 1 MIC and MBEC Values for *B. subtilis* JH642

	MIC	MBEC
Glutaraldehyde	2.5%	1.25%
Hydrogen peroxide	0.03%	15%
Chlorhexidine gluconate	0.0002%	0.01%
Ethanol	3.5%	>35%
Isopropanol	4.95%	>49.5%
Sodium hypochlorite	2.5%	2.5%
Product S	1/1000 dilution 0.0705% ETOH and 0.0002% CHG	1/20 dilution 3.525% ETOH and 0.01% CHG
Product T	1/100 dilution 0.199% ETOH and 0.001% CHG	1/20 dilution 0.995% ETOH and 0.005% CHG
Product L	1/100 dilution 0.095% ETOH and 0.0012% CHG	1/20 dilution 0.475% ETOH and 0.006% CHG
Product V	1/100 dilution 0.005% H_2O_2	½ dilution 0.25% H_2O_2

Table 2 MIC and MBEC Values for *P. aeruginosa* MPAO1

	MIC	MBEC
Glutaraldehyde	2.5%	--
Hydrogen peroxide	0.3%	15%
Chlorhexidine gluconate	0.02%	0.01%
Ethanol	0.35%	35%
Isopropanol	0.495%	4.95%
Sodium hypochlorite	2.5%	2.5%
Product S	1/100 dilution 0.705% ETOH and 0.002% CHG	1/20 dilution 3.525% ETOH and 0.01% CHG
Product T	1/10 dilution 1.99% ETOH and 0.01% CHG	--
Product L	1/10 dilution 0.95% ETOH and 0.012% CHG	½ dilution 4.75% ETOH and 0.06% CHG
Product V	1/10 dilution 0.05% H_2O_2	½ dilution 0.25% H_2O_2

-- indicates data was inconclusive

Table 3 MIC and MBEC Values for *E. coli* Clinical Isolate

	MIC	MBEC
Glutaraldehyde	2.5%	1.25%
Hydrogen peroxide	0.03%	0.15%
Chlorhexidine gluconate	0.002%	0.01%
Ethanol	3.5%	35%
Isopropanol	4.95%	49.5%
Sodium hypochlorite	0.25%	0.25%
Product S	1/100 dilution 0.705% ETOH and 0.002% CHG	1/20 dilution 3.525% ETOH and 0.01% CHG
Product T	1/100 dilution 0.199% ETOH and 0.001% CHG	1/200 dilution 0.0995% ETOH and 0.0005% CHG
Product L	1/100 dilution 0.095% ETOH and 0.0012% CHG	1/200 dilution 0.0475% ETOH and 0.0006% CHG
Product V	1/10 dilution 0.05% H_2O_2	1/20 dilution 0.025% H_2O_2

Table 4 MIC and MBEC Values for *S. aureus* Clinical Isolate

	MIC	MBEC
Glutaraldehyde	2.5%	1.25%
Hydrogen peroxide	0.3%	1.5%
Chlorhexidine gluconate	0.0002%	0.01%
Ethanol	35%	35%
Isopropanol	49.5%	49.5%
Sodium hypochlorite	0.25%	0.25%
Product S	1/100 dilution 0.705% ETOH and 0.002% CHG	1/20 dilution 3.525% ETOH and 0.01% CHG
Product T	1/1000 dilution 0.0199% ETOH and 0.0001% CHG	1/200 dilution 0.0995% ETOH and 0.0005% CHG
Product L	1/100 dilution 0.095% ETOH and 0.0012% CHG	1/20 dilution 0.475% ETOH and 0.006% CHG
Product V	1/100 dilution 0.005% H_2O_2	1/20 dilution 0.025% H_2O_2

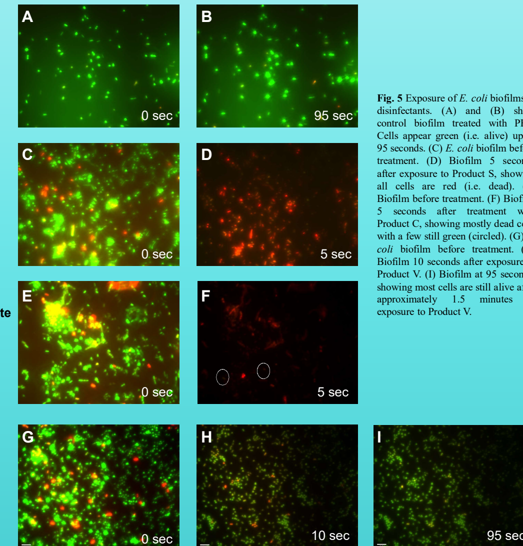


Fig. 5 Exposure of *E. coli* biofilms to disinfectants. (A) and (B) show control biofilm treated with PBS. Cells appear green (i.e. alive) up to 95 seconds. (C) *E. coli* biofilm before treatment. (D) Biofilm 5 seconds after exposure to Product S, showing all cells are red (i.e. dead). (E) Biofilm before 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.

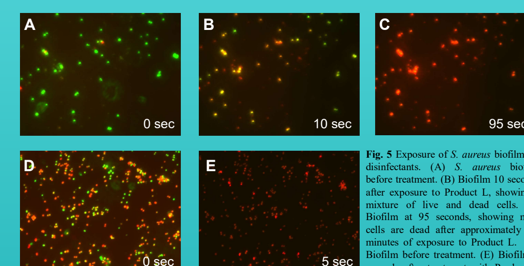


Fig. 5 Exposure of *S. aureus* biofilms to disinfectants. (A) *S. aureus* biofilm before treatment. (B) Biofilm 10 seconds after exposure to Product L, showing a mixture of live and dead cells. (C) Biofilm at 95 seconds, showing most cells are dead after approximately 1.5 minutes of exposure to Product L. (D) Biofilm before treatment. (E) Biofilm 5 seconds after treatment with Product S, showing all cells are dead. (PBS control showed live cells from 0-95 seconds).

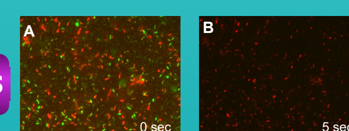


Fig. 5 Exposure of *P. aeruginosa* biofilm to Product S. (A) *P. aeruginosa* biofilm before treatment. (B) Biofilm 5 seconds after exposure to Product S, showing all cells are dead. (PBS control showed live cells from 0-95 seconds).

CONCLUSIONS

- Biofilms of the four test strains were more resistant than their planktonic counterparts to all disinfectants with the exception of glutaraldehyde and sodium hypochlorite
- Each strain exhibited different susceptibility profiles to the test disinfectants; however, *B. subtilis* JH642 and *P. aeruginosa* MPAO1 biofilms were generally most resistant to the test disinfectants and products, while biofilms of the clinical isolates of *E. coli* and *S. aureus* were most susceptible
- Overall, the MIC and MBEC of the commercial products demonstrated lower concentrations of active ingredients than MIC and MBEC for the active ingredients alone, indicating that formulation is key for product effectiveness.
- Fluorescence microscopy results show that products containing ethanol rapidly kill biofilm cells in as little as 5 seconds after exposure, while hydrogen peroxide-based products show live cells even after 3 minutes exposure.
- Products containing lower concentrations of alcohol (<70%) require longer exposure times to achieve complete killing of biofilm cells.

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

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