

EVALUATION OF DISINFECTANT EFFICACY AGAINST BIOFILMS AND PLANKTONIC CELLS OF SLIME PRODUCING BACTERIA AND YEASTS

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Purpose: Biofilms are communities of special microorganisms colonizing a surface, and biofilm cells are more resistant to biocides. The aim of this study was to investigate the effects of polyvinylpyrrolidone iodine (PVP), sodium hypochlorite (NaOCl) and glutaraldehyde on biofilms and planktonic cells of slime producing bacteria and yeasts.

Materials and Methods: Biofilms of slime producing isolates of coagulase-negative *Staphylococcus* (CoNS), *Pseudomonas aeruginosa* and *Candida albicans* were grown on polystyrene coupons for 72 hours in Tryptic soy and Sabouraud broth with the addition of glucose. These biofilms and planktonic cell suspensions of tested microorganisms were treated with 10% PVP, 5% NaOCl and 2% glutaraldehyde. The effects of disinfectants were evaluated according to the plate counting method.

Results: Glutaraldehyde was the most effective agent on biofilms of the tested microorganisms. Furthermore, PVP had no effect on biofilms of any of the tested microorganisms. NaOCl was effective on biofilms of *C. albicans* but not effective on biofilms of CoNS or *P. aeruginosa*. PVP, NaOCl and glutaraldehyde were all effective against planktonic cell suspensions.

Conclusion: Glutaraldehyde is an effective agent on biofilms and could be used for disinfection of CoNS, *P. aeruginosa* and *C. albicans* biofilms. In addition, biofilms seem to be more resistant to disinfectants than planktonic cell suspensions.

Key Words: Biofilm, Planktonic cells, Disinfectants.

DEZENFEKTANLARIN, SLAYM MADDESİ ÜRETEBEN BAKTERİ VE MAYALARIN BİYOFİMLERİ VE PLANKTONİK HÜCRELERİNE KARŞI ETKİNLİĞİNİN DEĞERLENDİRİLMESİ

Amaç: Biyofilmler mikroorganizmaların yüzeylerde oluşturdukları özel yapılarıdır ve biyofilm içindeki hücreler biyosidlerin öldürücü etkilerine karşı direnç göstermektedirler. Bu çalışmada polivinilpirolidon iyot (PVP), sodyum hipoklorit (NaOCl) ve glutaraldehit'in, slaym maddesi üreten bazı bakteriler ve mayaların oluşturdukları biyofilmler ve planktonik hücre süspansiyonlarına karşı etkilerinin araştırılması amaçlanmıştır.

Gereç ve Yöntem: Çalışmada, slaym maddesi ürettiği saptanan, koagülaz-negatif stafillokok (KNS), *Pseudomonas aeruginosa* ve *Candida albicans* klinik izolatlarının, glikoz eklenmiş Triptik soy ve Sabouraud sıvı besiyerinde, polistren yüzeyler üzerinde, 72 saat içinde biyofilm oluşturmaları sağlanmıştır. Daha sonra %10'luk PVP, %5'lik NaOCl ve %2'lik glutaraldehit'in, test edilecek mikroorganizmalara ait biyofilmler ve planktonik hücre süspansiyonları üzerindeki etkinlikleri değerlendirilmiştir. Dezenfektanların etkinlikleri plak koloni sayma yöntemiyle hesaplanmıştır.

Bulgular: Glutaraldehit'in test edilen tüm mikroorganizmaların biyofilmlerine karşı etkili, PVP'nin ise etkisiz olduğu gözlenmiştir. NaOCl'in *C. albicans*'ın oluşturduğu biyofilmlere karşı etkili, *P. aeruginosa* ve KNS'un oluşturduğu biyofilmlere karşı etkisiz olduğu bulunmuştur. PVP, NaOCl ve glutaraldehit'in test edilen bütün mikroorganizmaların planktonik hücre süspansiyonlarına karşı etkili olduğu saptanmıştır.

Sonuç: Bu çalışmada elde edilen veriler glutaraldehit'in biyofilmler üzerinde etkili olabileceğini ve KNS, *P. aeruginosa* ve *C. albicans*'ın oluşturduğu biyofilmlerin dezenfeksiyonunda kullanılabilirliğini göstermektedir. Aynı zamanda bu veriler biyofilmi oluşturan hücrelerin, dezenfektanlara planktonik hücrelerden daha dirençli olduğunu ortaya koymuştur.

Anahtar Kelimeler: Biyofilm, Planktonik hücreler, Dezenfektanlar.

INTRODUCTION

A biofilm is a community of microbes embedded in an organic polymer matrix, adhering to a surface (1).

Virtually any surface—animal, mineral, or vegetable—(i.e. biotic or abiotic) is fair game for bacterial colonization and biofilm formation, including ship hulls, dairy and petroleum pipelines, rocks in streams, and medical implants, such as catheters, prosthetic heart valves and joint replacements. Biofilms can also form on the surface of containers used for disinfectants, cleaning buckets, wash-hand basins, contact lenses, thermometers, sutures and dental prosthetics (1, 2).

A diverse number of microorganisms are capable of generating biofilms. *Staphylococcus epidermidis*, *Pseudomonas aeruginosa* and *Candida albicans* are important pathogens causing nosocomial infections that produce slime substance and form biofilms in appropriate conditions.

Bacteria in biofilms are phenotypically different from planktonic or suspended cells, notably as they resist killing by antibiotics, biocides, and disinfectants (2, 3).

In some extreme cases, the concentrations of antibiotics required to achieve bactericidal activity against adherent organisms can be three to four orders of magnitude higher than for planktonic bacteria, depending on the species-drug combination (1).

In view of previous findings, the purpose of this study was to compare the activities of disinfectants on biofilms and planktonic cells.

MATERIALS AND METHODS

Microorganisms

Clinical isolates of *C. albicans* (n=1), coagulase-negative *Staphylococcus* (CoNS) (n=1) and *P. aeruginosa* (n=1) were used in the experiments. All three isolates were producing slime.

Slime production

Slime production was determined by using a modification of the tube adherence method (4). Sabouraud broth supplemented with glucose (final concentration, 8%) (5) for *C. albicans* and Tryptic soy broth supplemented with glucose (final concentration, 2%) (6, 7) for CoNS and *P. aeruginosa* were prepared to promote adhesion and slime production.

A loopful of organisms was inoculated into a tube containing 10 ml of broth and it was incubated aerobically at 37 °C for 48 h. Then the cultures were aspirated. The tubes were washed twice with sterile demineralized water and then stained with 0.25% safranin (8). The tubes were examined for the presence of a viscid

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slime layer. A continuous film on the surface of the glass tube was considered positive. Strong slime producing organisms were chosen for the experiments.

Biofilm formation

Broth medium for each organism, prepared for slime production as described above, was used for biofilm growth. A standardized inoculum (1×10^5 to 5×10^5 CFU/ml) of each microorganism prepared in broth medium and 10 ml of broth were aliquoted into sterile glass tubes. Small polystyrene coupons (surface area, 1 cm^2) were submerged in growth medium in the tubes and then incubated aerobically at 37°C for 72 h (9). The growth medium was discarded and fresh medium added every 12 h (6).

Disinfectants and neutralization medium

The disinfectants used in this study were 10% polyvinylpyrrolidone iodine (PVP) (Adeka), 5% sodium hypochlorite (NaOCl) (Sigma) and 2% glutaraldehyde (Antiseptica), and 0.5% sodium thiosulfate (Merck) and 2% glycine (Serva) were used as neutralization medium.

Treatment of biofilms with disinfectants

After biofilm formation, the medium was aspirated and polystyrene coupons were removed from the tubes. Non-adherent cells were removed by gently washing the coupons in sterile demineralized water. Then the coupons were submerged in 1 ml of disinfectant in glass tubes and incubated for 5 min at room temperature. After the incubation period, 9 ml of neutralization medium was added to each tube and they were incubated for another 5 min. Then the coupon, which was still in the liquid, was smeared with a cotton swab on both sides, and the tube containing the swab and the coupon was vortexed at full speed for 60 s to remove all biofilm cells from the swab and the surface (9). A 40-fold dilution of the neutralized suspension was made in sterile demineralized water, and 20 μl of the suspension was plated on Tryptic soy agar (TSA) immediately after dilution (9).

Sensitivity of planktonic cells to disinfectants

For evaluation of the activities of disinfectants against planktonic cells, 1 ml of bacterial and yeast suspension, which was adjusted to 0.5 McFarland, was added to 9 ml of disinfectant in a sterile glass tube. After 5 min contact, 1 ml of each

mixture was transferred to 9 ml of neutralization medium and allowed to remain in contact for 5 min. A 10-fold dilution was made in sterile demineralized water, and 20 μl of the suspension was plated on TSA immediately after dilution (10).

All experiments were performed in duplicate. Sterile demineralized water was used instead of disinfectant in the control tests. The culture plates were incubated aerobically at 37°C for 48 h. After incubation, the colonies of the control and assayed microorganisms were counted.

RESULTS

We investigated the activities of glutaraldehyde, NaOCl and PVP on planktonic cells and biofilms of slime producing *C. albicans*, *P. aeruginosa* and CoNS.

The mean numbers of viable cells, measured by colony formation on TSA plates after removing attached microorganisms from the coupons, were 1.25×10^5 CFU/cm² for *C. albicans*, 1.3×10^6 CFU/cm² for *P. aeruginosa*, and 3.2×10^7 CFU/cm² for CoNS. The initial mean densities of cells in the planktonic cell suspensions were 2.5×10^7 CFU/ml for *C. albicans*, 2.3×10^8 CFU/ml for *P. aeruginosa*, and 1.3×10^8 CFU/ml for CoNS.

The reduction rates after treatment with disinfectants for 5 min were evaluated as log reduction values. The log reduction was calculated by subtracting the assayed log density from the control log density (10). The log reduction values of tested disinfectants on planktonic cells and biofilms are given in Table 1.

Bacterial exopolysaccharides are the main component of the biofilm glycocalyx, which has also been named the slime layer (1). The glycocalyx provides a certain degree of protection for its inhabitants against certain environmental threats, including biocides, antibiotics, antibodies, surfactants, bacteriophages, and foraging predators such as free-living amoebae and white blood cells (1).

At least three mechanisms have been proposed to account for the increased resistance of biofilms to antimicrobial agents. The first is that the glycocalyx prevented the perfusion of biocides to cellular targets, while the second is that the nearly dormant growth pattern of bacterial populations in the bio-

Table 1: Log₁₀ reduction values of tested disinfectants on planktonic cells and biofilms.

Log ₁₀ reduction*	<i>C. albicans</i>		<i>P. aeruginosa</i>		<i>Staphylococcus</i>	
	Planktonic	Biofilm	Planktonic	Biofilm	Planktonic	Biofilm
Glutaraldehyde	>5	>5	>5	>5	>5	>5
NaOCl	>5	>5	>5	3	>5	3
PVP	>5	0	>5	1	>5	2

* log₁₀ value for the control–log₁₀ value for the assayed sample

films rendered organisms indifferent to antibiotic activity. The third is that the microenvironment of the biofilm adversely affected the activity of the antimicrobials (1).

Glutaraldehyde is an important dialdehyde that has found usage as a disinfectant and sterilant, in particular for low-temperature disinfection and sterilization of endoscopes and surgical equipment. The mechanism of action of glutaraldehyde involves a strong association with the outer layers of bacterial cells, specifically with unprotonated amines on the cell surface, possibly representing the reactive sites.

Chlorine- and iodine-based compounds are the most significant microbicidal halogens used in the clinic, and they have been traditionally used for both antiseptic and disinfectant purposes. Despite being widely studied, the mechanism of action of chlorine-releasing agents (CRAs) is not fully known. CRAs are highly active oxidizing agents and thereby destroy the cellular activity of proteins. Similar to chlorine, the antimicrobial action of iodine is rapid. Iodine rapidly penetrates into microorganisms and attacks key groups of proteins (in particular the free sulfur amino acids cysteine and methionine), nucleotides, and fatty acids, culminating in cell death (11).

In order to prove disinfectant efficiency, there has to be a 5-log reduction in initial cell concentrations. Nevertheless, although this is true for suspension tests, some modifications may be needed for other tests.

Luppens et al. reported that a disinfectant that resulted in more than a 4-log reduction in of biofilm cell concentration (4×10^7 to 1.3×10^8 CFU/cm²) should be considered an effective agent on biofilms (9). Wirtanen proposed that for a biofilm test only a 3-log reduction was necessary, but Luppens et al. pointed out that a 3-log reduction is too small for biofilms that can contain up cells to 1.3×10^8 CFU/cm² (9, 10, 12).

In the present study, all tested disinfectants had high bactericidal activity (>5 logs) on planktonic cell suspensions of the tested microorganisms.

Glutaraldehyde resulted in more than a 5-log reduction on biofilms of all the tested microorganisms. PVP had no effect on *C. albicans* biofilms and resulted in a 2-log reduction on *P. aeruginosa*, and a 1-log reduction on CoNS biofilms. However, NaOCl achieved a 3-log reduction on *C. albicans* (initial cell concentration, 1.25×10^5 CFU/cm²) and *P. aeruginosa* (initial cell concentration, 2×10^7 CFU/cm²) biofilms. These data indicate that a 3-log reduction was not enough for this NaOCl to prove its efficiency on biofilms of *C. albicans* and *P. aeruginosa* according to Luppens et al. (9).

Campanac et al. showed that *P. aeruginosa* biofilms were resistant, while planktonic cells were sensitive to six quaternary ammonium compounds (QACs) tested, and they noted that the factors involved in biofilm resistance to QACs vary according to the bacterial modifications induced by the formation of a biofilm. They underlined the involvement of the exopolysaccharide and particularly the three-dimensional structure of *P. aeruginosa* biofilm (10). In addition, biofilms of *P. aeruginosa* also showed resistance to different antibiotics. Coquet et al.

reported that biofilms of *P. aeruginosa* showed much more resistance to tobramycin and imipenem than planktonic cultures (13). In our study, although high levels of bactericidal activity (>5 logs) were observed against planktonic cells of *P. aeruginosa* with PVP, NaOCl and glutaraldehyde, bactericidal activity was only obtained with glutaraldehyde against biofilms of this bacterium.

Oie et al. showed that benzalkonium chloride (0.1%) and alkyldiaminoethyl glycine (0.1%) were ineffective for the eradication of biofilm cells of methicillin resistant *Staphylococcus aureus* even after 1 h, but were effective for the eradication of planktonic cells within 20 s. Sodium hypochlorite (0.01%) was also ineffective for the eradication of biofilm cells even after 30 min, but was lethal to planktonic cells within 20 s (14). In agreement with these findings, our results reveal that 5% NaOCl and 10% PVP were ineffective for the eradication of CoNS biofilm but showed bactericidal activity on planktonic cells after 5 min. However, Merritt et al. found that 10% NaOCl was effective on *S. epidermidis* cells adhered to polystyrene. They reported that 10% NaOCl reduced the number of microorganisms by at least 4-5 logs (15).

Lamfon et al. reported that *C. albicans* biofilms were ≥ 1000 -fold more resistant to fluconazole and miconazole and eight-fold more resistant to chlorhexidine than the planktonic cells of the same organism (16). Barnabe et al. showed that the combination of coconut soap and 5% NaOCl was effective in controlling *C. albicans* and *Streptococcus mutans* denture biofilms (17). Chandra et al. found that MIC values of fluconazole for biofilm-grown *C. albicans* were 128 times greater than those for planktonic cultures. They stated that it was unclear whether the increase in the drug resistance of *C. albicans* biofilms was due to the production of extracellular material or to genetic and biochemical alterations in fungal cells; they also proposed an alternative explanation for antifungal resistance in biofilms, namely metabolic quiescence of cells (18).

Standard culture collection strains are more suitable for repeating disinfection tests and obtaining reliable results. However, microorganisms that cause nosocomial infections show much more resistance to antibiotics and antiseptics than the standard strains. The aim of disinfection, especially in hospitals, is to eradicate mostly these resistant microorganisms. Therefore, we preferred to use clinically isolated strains in the disinfectant efficacy tests.

On the basis of these experiments, we conclude that glutaraldehyde is more effective than PVP and NaOCl on biofilms of *C. albicans*, *P. aeruginosa* and CoNS. It is plausible to use glutaraldehyde for disinfection of medical devices that have a risk of biofilm formation. NaOCl had little effect and this agent and PVP are not suitable for the disinfection of biofilms.

Different mechanisms can play a role in the resistance of biofilms. Further studies on the disinfection of biofilms and factors promoting biocide resistance will be useful.

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