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The comparison of various disinfectants' efficacy on *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilm layers

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Background/aim: Because biofilms are resistant to antibiotics and biocides, they usually cause chronic persistent infections, which are arduous to cure and have high mortality and morbidity. Our study aimed to investigate the efficiency of orthophthalaldehyde, peracetic acid, hydrogen peroxide, and sodium hypochlorite on *Pseudomonas aeruginosa* and *Staphylococcus aureus* biofilm layers and live microbial cells.

Materials and methods: Biofilm layers were determined by crystal violet assay and live microbial cells were determined using a resazurin assay.

Results: For *Pseudomonas aeruginosa*, sodium hypochlorite showed the most influential disinfection because it diminished 83.6% of the biofilm layer and decreased 99.7% of live microbial cells. For *Staphylococcus aureus*, hydrogen peroxide was determined the most active disinfectant with 80.3% reduction of the biofilm layer. Sodium hypochlorite was also determined to be the most efficient disinfectant with 99.8% reduction of live microbial cells. Sodium hypochlorite was the most influential disinfectant on biofilm layers and live microbial cells of both microorganisms.

Conclusion: We concluded that if we use sodium hypochlorite at a high level as a disinfectant for both surfaces and medical equipment, it is beneficial to prevent infections related to biofilms. More studies about prevention of biofilm occurrence and standardization of the methods for investigating disinfectants' effects are necessary.

Key words: Biofilm, orthophthalaldehyde, peracetic acid, hydrogen peroxide, sodium hypochlorite

1. Introduction

A biofilm can be defined as a well-organized microbial community in the extracellular polymeric substance (EPS) that adheres to living or inanimate surfaces (1). Owing to their resistance to antibiotics and antiphagocytic effects, biofilms can frequently cause persistent chronic infections that are difficult to treat. Biofilms are significant causes of morbidity and mortality (2,3). At least 65% of all bacterial infections are associated with biofilm (2,3). Infections such as natural valve endocarditis, otitis media, chronic bacterial prostatitis, cystic fibrosis, and periodontitis develop as a result of biofilms that form on living surfaces. The medical devices on which biofilms can develop include prosthetic heart valves, central venous catheters, urinary catheters, contact lenses, and intrauterine devices (4). Due to the high rates of mortality and morbidity associated with biofilms, several studies have been conducted on antimicrobials and particularly on the effectiveness of antibiotics against biofilms. It has been demonstrated that antibiotics show limited efficacy against biofilms as the biofilm layer persists. For this reason, it should be borne in mind that disinfectants serve as a significant alternative against human mucosa and biofilms on the surfaces of medical device (5). This study aims at investigating the effectiveness of orthophthalaldehyde, peracetic acid, hydrogen peroxide, and sodium hypochlorite against *Pseudomonas aeruginosa* and *Staphylococcus aureus* biofilm layers and the live microbial cells on the biofilm layers.

2. Material and methods

2.1. Bacterial strains

S. aureus (ATCC 6538) and *P. aeruginosa* (PA01) bacterial strains that are known to produce biofilms were selected (5,6). Bacterial cells stocked at –80 °C were passaged to a tryptic soy agar (TSA) plate (Merck). After being incubated for 24 h at 35–37 °C, a bacterial solution was

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prepared, adjusted to 0.5 McFarland turbidity (10^8 cfu/mL), and diluted with a concentration of 10^6 cfu/mL.

2.2. Disinfectants

Orthophthalaldehyde (OPA; Savanol 8-9), peracetic acid (PAA; Merck), hydrogen peroxide (H_2O_2; Sigma-Aldrich), and sodium hypochlorite (NaClO; Waterlife) were selected as disinfectants. We selected biocides at their maximum allowed concentrations. The concentrations used were as follows: OPA: 0.55%, PAA: 0.3%, H_2O_2: 5%, NaClO: 10,000 ppm (5,7).

2.3. Neutralization test

2.3.1. For neutralization of disinfectants

Sodium bisulfate (0.5%, Merck) for OPA, 3 g/L sodium thiosulfate (Amresco) for PAA, 50 ml/L catalase (Sigma-Aldrich) for H_2O_2, and 5 g/L sodium thiosulfate (Amresco) for NaClO were used (5,7,8).

The neutralizer solutions were tested in order to determine the effectiveness and toxicity for both bacterial strains.

2.3.2. For determination of activity of neutralizer solutions

Neutralizer solution (100 µL) and disinfectant (800 µL) were mixed in an Eppendorf tube for 5 min and 100 µL of diluted bacterial suspension (10^6 cfu/mL) was added. Following 30 min of incubation at room temperature, 10 µL was taken from the mixture and inoculated onto the TSA plate. After 24 h of incubation, the number of colonies was counted (5).

2.3.3. For determination of the toxicity of neutralizer solutions

Distilled water (100 µL) and neutralizer solution (800 µL) were mixed in an Eppendorf tube. Following 5 min of incubation at room temperature, 100 µL of diluted bacterial suspension (10^6 cfu/mL) was added. Thirty minutes later, 10 µL was taken from the mixture and inoculated onto TSA medium. After 24 h of incubation, the number of colonies was counted.

The disinfectants, distilled water, neutralizer solutions, and bacterial suspensions were adjusted at room temperature before the tests. All test solutions were renewed aseptically before each application.

2.4. Antibacterial susceptibility tests

First the efficacy of all disinfectants at 30 min was tested. After holding 100 µL of disinfectant and 800 µL of sterile distilled water in an Eppendorf tube for 5 min, 100 µL of bacterial suspension (10^6 cfu/mL) was added, and following 30 min of incubation, 10 µL was taken and inoculated onto a TSA plate. After 24 h of incubation, the number of colonies was counted and cfu/mL was calculated. Afterwards 800 µL of disinfectant + 100 µL of microbial suspension (10^8 cfu/mL) and 100 µL of distilled water were mixed together. At each designated minute (at 1, 5, 15, 30, and 60 min), 100 µL of test mixture was taken and added into 800 µL of neutralizer and 100 µL of distilled water. After 5 min of incubation it was inoculated onto the medium. Following 24 h of incubation at 37 °C, the number of colonies was counted and cfu/mL was calculated (5).

2.5. Determination of disinfectant activity on biofilm layer

Bacterial suspensions (200 µL) of *S. aureus* and *P. aeruginosa* strains at concentrations of 10^6 cfu/mL suspended in TSB medium were added to 96-well plates. As a negative control, 200 µL of noninoculated TSB was added. Plates were incubated at 37 °C for 24 h. After this time, the TSB was removed and washed with 300 µL of phosphate-buffered saline (PBS; BD). Disinfectant (200 µL) was added, and after each contact period the disinfectants were gently removed by means of a pipette without causing any damage to the biofilm. It was washed twice more with PBS. Neutralizer solution (200 µL) was brought into contact with the biofilm for 5 min. The biofilm was then washed with PBS two more times after gently removing the neutralizer solution with a pipette. In negative and positive controls, the washing was performed by adding 200 µL of sterile distilled water instead of disinfectant (5).

2.6. Identifying the biofilm layer

In order to fix the biofilm layer, 150 µL of 99% methanol (Sigma-Aldrich) was added to each well for a period of 15 min. Following 15 min of incubation, the methanol was removed and the plate was dried in air. A 2% crystal violet solution (150 µL) was added into wells for the *S. aureus* biofilm layer, and 150 µL of 0.7% crystal violet solution was added into wells for the *P. aeruginosa* biofilm layer. Following incubation for 5 min, the crystal violet solution was removed with a pipette and washed with running water. The plate was dried in the air. A 33% glacial acetic acid solution (150 µL) was added in order to remove the crystal violet adhering to the biofilm. After 15 min of incubation, optic density was measured by means of a plate reader (BioTek plate reader) at a wavelength of 570 nm.

2.7. Identifying live microbial cells

TSB (190 µL) and 10 µL (0.5 µg) of resazurin were added to the cells after forming the biofilm layer and performing the disinfectant tests. Following 30 min of incubation for *S. aureus* and 60 min of incubation for *P. aeruginosa* in the dark at 37 °C, fluorescence was measured by means of the plate reader at 530 nm excitation wavelength and 590 nm emission wavelength.

2.8. Calculating the number of tests and statistical analyses

With the aim of determining the effectiveness of disinfectants against the biofilm layers and live microbial cells, identification of the biofilm layers and identification

of live microbial cells were done three different times in order to calculate the required test number. With the data obtained, it was determined that five tests should be done through the Number Cruncher Statistical System (NCSS) Power Analysis Sample Size (PASS) program with 95% power to determine the disinfectants' effectiveness on biofilm layers, and that three tests were necessary for determining the live microbial cells. These preliminary tests were not included in the assessment of the data. For both bacteria, these tests were studied for a total of eight times on two different occasions.

The absorbance rates obtained with the plate reader were transferred to SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA). The significance was set at $\alpha = 0.05$ for hypothesis tests in the study. The Friedman test was conducted in order to demonstrate the effectiveness of disinfectants against the biofilm layers at the designated time intervals. For paired groups, the Wilcoxon test was used in order to determine the significance according to minutes.

3. Results

3.1. Neutralization test

Sodium thiosulfate at 5 g/L successfully neutralized NaClO while 3 g/L sodium thiosulfate successfully neutralized PAA and 50 ml/L catalase neutralized H_2O_2. As a result of neutralizing OPA at a 0.5% concentration with sodium bisulfate, 5000 cfu/mL reproduction of bacteria was observed in TSA.

It was found that the neutralizer solutions used in this study were not toxic against either of the bacterial strains.

3.2. Antibacterial sensitivity test

No growth was observed for *S. aureus* or *P. aeruginosa* after 30 min of contact with OPA, PAA, H_2O_2, and NaClO.

For planktonic *P. aeruginosa*, OPA and PAA diminished it 100%, H_2O_2 diminished it 99.98%, and NaClO diminished it 99.99% at the 1st minute. H_2O_2 and NaClO diminished it 100% at the 5th minute.

For planktonic *S. aureus*, OPA, PAA, and NaClO diminished it 100% while H_2O_2 diminished it 99.96% at the 1st minute. H_2O_2 diminished it 100% at the 5th minute.

3.3. Determining the effectiveness of disinfectants against the biofilm layers

3.3.1. *Pseudomonas aeruginosa*

The mean absorbance ± standard deviation values, the percentage (%) decrease for *P. aeruginosa* biofilm layers obtained after the application of the disinfectants at the designated time intervals, and the P-values obtained from the Friedman test are shown in Table 1. It was determined that H_2O_2 did not have a significant effect on *P. aeruginosa* biofilm layers, while OPA, PAA, and NaClO each had the highest effect at the 30th minute and NaClO was the most effective disinfectant against *P. aeruginosa.*

3.3.2. *Staphylococcus aureus*

The mean absorbance ± standard deviation values, the percentage (%) decrease for *S. aureus* biofilm layers obtained after the application of the disinfectants at the designated time intervals, and the P-values obtained from the Friedman test are shown in Table 2. It was determined that H_2O_2 and NaClO were the most effective disinfectants.

3.4. Determining the effect of disinfectants on the live microbial cells on the biofilm layers

3.4.1. *Pseudomonas aeruginosa*

Table 3 demonstrates the mean fluorescence ± standard deviation values, the percentage decrease obtained after the application of disinfectants at the designated times for live microbial cells on the biofilm layers, and the P-values obtained from the Friedman test. It was observed that NaClO was the most effective disinfectant, causing a 99.789% decrease at the 60th minute.

3.4.2. *Staphylococcus aureus*

Table 4 demonstrates the mean fluorescence ± standard deviation values, the percentage decrease obtained after

Table 1. The mean absorbance ± standard deviation, P-values and percentage (%) decrease for *P. aeruginosa* biofilm layers.

	Control	1st minute	5th minute	15th minute	30th minute	60th minute	
OPA	0.643 ± 0.149	0.443 ± 0.228 (31.2%)	0.343 ± 0.145 (46.6%)	0.259 ± 0.108 (59.8%)	0.225 ± 0.101 (65.1%)	0.244 ± 0.093 (62.1%)	$\chi^2 = 21.5$ P = 0.001
PAA	0.289 ± 0.115	0.171 ± 0.065 (41%)	0.154 ± 0.072 (46.6%)	0.109 ± 0.051 (62.2%)	0.107 ± 0.059 (63%)	0.174 ± 0.085 (39.7%)	$\chi^2 = 23.5$ P = 0.000
H_2O_2	0.366 ± 0.158	0.180 ± 0.082 (50.7%)	0.244 ± 0.257 (33.4%)	0.265 ± 0.275 (27.5%)	0.239 ± 0.175 (34.6%)	0.253 ± 0.108 (30.7%)	$\chi^2 = 9.5$ P = 0.089
NaClO	0.621 ± 0.226	0.566 ± 0.238 (9%)	0.387 ± 0.136 (37.7%)	0.242 ± 0.177 (61%)	0.102 ± 0.050 (83.6%)	0.105 ± 0.131 (83.1%)	$\chi^2 = 30.8$ P = 0.000

Table 2. The mean absorbance ± standard deviation, P-values, and percentage (%) decrease of *S. aureus* biofilm layers.

	Control	1st minute	5th minute	15th minute	30th minute	60th minute	
OPA	2.675 ± 0.253	2.154 ± 0.685 (19.5%)	2.068 ± 0.634 (22.7%)	1.726 ± 0.463 (35.5%)	1.683 ± 0.349 (37.1%)	1.047 ± 0.642 (60.9%)	χ^2 = 27.8 P = 0.000
PAA	0.844 ± 0.067	0.727 ± 0.100 (14%)	0.574 ± 0.061 (32.1%)	0.574 ± 0.061 (28.6%)	0.574 ± 0.061 (18.1%)	0.574 ± 0.061 (17.6%)	χ^2 = 21.0 P = 0.001
H_2O_2	2.486 ± 0.625	0.747 ± 0.148 (70%)	0.579 ± 0.164 (77%)	0.531 ± 0.131 (78.7%)	0.520 ± 0.168 (79.1%)	0.491 ± 0.104 (80.3%)	χ^2 = 20.6 P = 0.001
NaClO	1.886 ± 0.245	2.387 ± 0.257 (126%)	1.624 ± 0.364 (13.9%)	0.926 ± 0.334 (51%)	0.452 ± 0.122 (76.1%)	0.950 ± 0.272 (49.7%)	χ^2 = 29.0 P = 0.000

Table 3. The mean fluorescence ± standard deviation, the percentage (%) decrease, and the P-values for the live microbial cells on the biofilm layers.

	Control	1st minute	5th minute	15th minute	30th minute	60th minute	
OPA	6550.7 ± 313.0	15.71 ± 24.34 (99.7%)	25.23 ± 20.35 (99.6%)	90.23 ± 79.41 (98.6%)	30.76 ± 47.68 (99.5%)	72.48 ± 57.97 (98.8%)	χ^2 = 21.7 P = 0.001
PAA	6608.6 ± 354.4	54.00 ± 59.31 (99.1%)	36.40 ± 56.39 (99.4%)	57.56 ± 70.82 (99.1%)	11.75 ± 14.82 (99.8%)	53.50 ± 65.58 (99.1%)	χ^2 = 17.5 P = 0.004
H_2O_2	6997.4 ± 32.7	1129.36 ± 771.98 (83.9%)	131.96 ± 188.25 (98.1%)	64.13 ± 73.92 (99.0%)	235.51 ± 419.23 (96.6%)	500.05 ± 665.68 (92.8%)	χ^2 = 21.3 P = 0.001
NaClO	6363.6 ± 1145.5	29.00 ± 24.55 (99.5%)	22.06 ± 23.94 (99.6%)	14.73 ± 75.49 (99.7%)	17.51 ± 13.33 (99.7%)	13.43 ± 90.27 (99.7%)	χ^2 = 18.0 P = 0.003

Table 4. The mean fluorescence ± standard deviation, the percentage (%) decrease, and the P-values for the live microbial cells on the biofilm layers.

	Control	1st minute	5th minute	15th minute	30th minute	60th minute	
OPA	10391.7 ± 962.8	60.00 ± 18.70 (99.4%)	44.00 ± 43.77 (99.5%)	26.75 ± 49.22 (99.7%)	66.62 ± 59.69 (99.3%)	100.75 ± 39.26 (99.0%)	χ^2 = 25.0 P = 0.000
PAA	2689.0 ± 2089.7	19.12 ± 11.84 (99.2%)	18.75 ± 19.45 (99.3%)	8.00 ± 11.84 (99.7%)	7.00 ± 12.46 (99.7%)	43.62 ± 28.29 (98.3%)	χ^2 = 26.1 P = 0.000
H_2O_2	5108.0 ± 2637.7	1079.75 ± 992.75 (78.8%)	233.87 ± 120.83 (95.4%)	169.87 ± 46.05 (96.6%)	87.37 ± 17.19 (98.2%)	53.12 ± 32.10 (98.9%)	χ^2 = 26.8 P = 0.000
NaClO	5210.5 ± 315.3	7.62 ± 9.47 (99.8%)	248.37 ± 435.12 (95.2%)	42.25 ± 37.88 (99.1%)	50.62 ± 28.54 (99.0%)	24.50 ± 18.89 (99.5%)	χ^2 = 23.1 P = 0.000

the application of disinfectants at the designated times for live microbial cells on the biofilm layers, and the P-values obtained from the Friedman test. It was found that NaClO was the most effective disinfectant against *S. aureus* with a 99.854% decrease at the 1st minute.

4. Discussion

Biofilm can be defined as a microbial community well organized in the EPS that adheres to living or inanimate surfaces (1). Several gram-positive bacteria, gram-negative bacteria, and fungi of clinical significance can form

biofilms. Biofilms have a profound effect on health care and they are associated with 65% of all infections (9,10).

In order to prove the effectiveness of a disinfectant against the bacterium to be tested, the disinfectant should cause a 5 log (99.999%) decrease after its contact with the bacterium (11). According to FDA recommendations, as a high-level disinfectant in reusable medical devices, OPA should be used at 20 °C at a concentration of 0.55% and 0.6% for 12 min, at a concentration of 0.575% for 10 min at 50 °C, and at a concentration of 5.75% for 5 min. H_2O_2 can be used at 20 °C at a 2% concentration for 8 min and a 7.5% concentration for 30 min. It is also recommended that hypochlorite containing 400–450 ppm active chlorine can be used for 10 min at 30 °C, while that containing 650–675 ppm active chlorine can be used for 10 min at 25 °C. It is suggested that PAA should be used at 3300–3800 ppm at 25 °C for a period of 5 min and 3100–3400 ppm at 20 °C for 7 min (12).

The material compatibility of disinfectants is significant. Hypochlorite that includes >500 ppm active chlorine is corrosive to metal instruments. Hypochlorite solution is used to disinfect tonometer heads, noncritical surfaces, and equipment. Dilutions in the range of 1:10 to 1:100 of 5.25%–6.15% sodium hypochlorite are recommended for decontaminating blood spills. Other health care uses of hypochlorite include as an irrigating agent in endodontic treatment, disinfecting laundry, dental appliances, hydrotherapy tanks, applanation tonometers, and water distribution systems in hemodialysis centers and hemodialysis machines. H_2O_2 is incompatible with brass, zinc, copper, and silver/nickel plating. It is used in concentrations from 3% to 6% for the disinfection of soft contact lenses, tonometer biprisms, ventilators, fabrics, and endoscopes. It is effective in spot-disinfecting fabrics in patients' rooms. OPA has perfect material compatibility but it makes protein gray like skin, mucous membranes, clothing, and environmental surfaces. Peracetic acid is compatible with many materials and instruments but is corrosive to copper, brass, bronze, plain steel, and galvanized iron (13).

Among the disinfectants used in this study, it was observed that OPA, PAA, and NaClO were effective at the 1st minute against both *S. aureus* and *P. aeruginosa*. It was also found that H_2O_2 was effective at the 5th minute.

In order to ensure the effective treatment of biofilm-related infections, not only should the microorganisms be killed, but the matrix should also be removed, since the slime layer can rapidly recolonize. For this reason, in an ideal biofilm model, both the matrix and the microorganisms should be identified (14). We used crystal violet (CV) and resazurin methods simultaneously. The biofilm layer (matrix and dead/live microorganism) can be identified by CV, and live microbial cells can be identified by the resazurin method.

The most important limitation of biofilm formation is that there is no standard method to assess disinfectant susceptibility. Bacterial strains, disinfectants and concentrations, contact times, and neutralizer solutions must be standardized to compare them with other studies. The CV method is a static test system for biofilm formation by a plate. It is less used as there is no free flow of nutrients and waste products. It takes a long time to work, is incompatible with high-throughput, and measures mass instead of viability (14).

In the CV method, a significant absorbance increase for *S. aureus* was obtained according to the control of NaClO during the 1st minute. It was thought that this problem might have resulted from the fact that CV was tied more to the biofilm layer during the short contact of the disinfectants with the bacteria. For this reason, the biofilm layers of both bacterial strains were studied again for each designated minute by adding a negative control (200 µL of noninoculated TSB). The disinfectant was put into contact with the negative controls. In the wells used as negative controls, to which no bacteria were added, and which were thus lacking a biofilm layer, it was seen that the CV was kept, and this was more noticeable at the 1st minute. In the previous studies that we could access, we did not find any data reporting an absorbance increase following the addition of a disinfectant or antimicrobial by means of the CV method.

We think that the resazurin method can be used in order to identify the live microbial cells in determining the effectiveness of antibiotics and disinfectants within the scope of the plate model in biofilm studies. In identifying the biofilm layer, on the other hand, the literature indicates that it is more appropriate to use direct physical methods such as confocal laser scanning microscopy to detect the biofilms instead of CV, which is an indirect method, and that the quantity of biofilms should be measured by means of software like COMSTAT or PHYLIP (15). The most significant disadvantage of this study was that there was not a standard method available for identifying biofilms.

The findings of this study indicated that H_2O_2 was not effective against *P. aeruginosa* biofilm layers while PAA and OPA were effective at similar levels, causing 63% and 83% decreases in the biofilm (matrix + live/dead microbial cells), respectively. It was observed that the disinfectants did not preserve their effects after the 30th minute. It was found that all the disinfectants used in this study were effective against *S. aureus* biofilm layers. The most effective disinfectants were found to be H_2O_2, achieving 80.3% reduction in biofilm at the 60th minute, and NaClO, which achieved 76.1% reduction at the same minute. OPA achieved 60.9% reduction after 60 min. The least effective disinfectant was PAA, which achieved 32.1% reduction.

The findings indicated that OPA, PAA, and NaClO were 99% effective at the 1st minute against the live microbial cells on the *Pseudomonas aeruginosa* biofilm layer while H_2O_2 reached nearly the same effectiveness only at the 15th minute. It was determined that NaClO was the most effective disinfectant, achieving 99.7% reduction at the 60th minute. On the other hand, OPA, PAA, and NaClO were effective against the live microbial cells of *Staphylococcus aureus* at the 1st minute with about 99% decrease, whereas H_2O_2 could not reach this level of effectiveness, achieving 98.9% reduction only at the 60th minute. The findings also demonstrated that NaClO was the most effective disinfectant, causing a 99.8% decrease of the live microbial cells at the 1st minute.

Given the fact that we used a 10^6 cfu/mL bacterial suspension in the plate model of biofilm formation, it can be calculated that 1460 cfu/mL bacteria might have survived even with NaClO, the most effective disinfectant against live microbial cells on *S. aureus* biofilm. With a 10^8 cfu/mL bacterial suspension, with which we examined the efficacy of the disinfectants against planktonic microorganisms, 146,000 cfu/mL bacteria would have survived. However, it was observed that NaClO was 100% effective at the 1st minute against the bacteria in suspension. It is known that bacterial biofilms are 1000 times as resistant to antimicrobials and disinfectants as planktonic bacteria, and that bacteria develop resistance against the host defense mechanism as a result of biofilm formation (16). Our findings also support these existing data. The resistance of the bacteria on biofilms against the biocides can be intrinsic, genetically acquired, or phenotypical (tolerance). As is known, the resistance mechanisms involve restriction in the diffusion and reaction of the disinfectants in biofilms, phenotypic adaptation at sublethal concentrations, gene transfer, and mutations. Since the disinfectants are chemically active molecules, they lose their efficacy in the presence of organic substances such as proteins, nucleic acids, and carbohydrates (17). Identifying the mechanisms by which the disinfectants cannot be precisely effective against the biofilms is crucial in the fight against biofilms and in developing new treatment alternatives.

It is known that the diffusion of chlorine composites into biofilms is restricted due to the EPS and that alkaline-based disinfectants have less penetration ability due to their reaction with biofilm matrix. De Beer et al., through a microelectrode method that they developed, determined that the penetration of chlorine composites into *P. aeruginosa* and *Klebsiella pneumonia* biofilms was 20% lower (18).

It is known that PAA becomes inactive owing to biofilm surface layers. The other reason for the low efficacy of PAA against biofilms is that oxidation strengthens the covalent bonds and fixes the biofilm layer (19).

The reasons for the low efficacy of H_2O_2 against biofilm layers are that it is inactivated by catalase, alginate, and free oxygen radicals and that it cannot penetrate into the EPS (20–22). According to the hypothesis proposed by Cochran et al., biofilm-forming bacteria express the genes that reduce the sensitivity against the oxidizing disinfectants such as hydrogen peroxide and monochloramine (23).

It is known that the effectiveness of OPA is reduced as a result of its reaction with the proteins in the biofilm layer and as a result of its fixing the biofilm (24).

Tote et al., in their study that compared the efficacy of a variety of disinfectants against *S. aureus* and *P. aeruginosa* biofilm matrices and the live microbial cells in the biofilm layers, reported that H_2O_2 and NaClO were effective against both of the biofilms and PAA was the least effective disinfectant against live microbial cells (5). Since the authors stated that using a neutralizer solution for the biofilm layer and the disinfectant test would not change the results of their tests, they did not use a neutralizer solution. In parallel with this, we also assume that our not precisely neutralizing OPA did not affect the results of the current study.

Perumar et al., in their study investigating the efficacy of H_2O_2, H_2O_2 and ethanol, PAA, and 2-furoic acid combinations against *P. aeruginosa*, *K. pneumonia*, and *Acinetobacter* spp. biofilm layers, reported that H_2O_2 alone had low efficacy against the biofilms but more successful results could be achieved by its combination with a strong acid (peracetic acid) or ethanol. They stated that the effectiveness of H_2O_2 emerged during the first 30 min and no additional benefit was achieved with longer contact, but that in the case of its combination with ethanol, ethanol could yield more benefits for longer contact periods by dehydrating the biofilm matrix. Studies on combination treatments are currently being done since it has been shown that H_2O_2 alone has limited effects against biofilm layers (20). Tachikawa et al. reported that contact of *Pseudomonas fluorescence* with H_2O_2 following ozone (O_3) resulted in a synergistic effect. They explained that the reason for this was that hydroxide radicals and the extracellular polymeric matrix were damaged (25).

Jahid et al., in their study that compared the effectiveness of ethanol, NaClO, H_2O_2, PAA, and benzalkonium chloride against *Aeromonas hydrophila* biofilm layers, found that ethanol was the most effective disinfectant at 70% concentration, and PAA was more effective than NaClO (21).

Presterl et al. compared the efficacy of povidone iodine, alcohol, and H_2O_2 against *Staphylococcus epidermidis* biofilm layers and alcohol-H_2O_2 had a rapid effect on the biofilm layer whereas povidone iodine was comparatively less effective (26).

In another study performed with methicillin-resistant *Staphylococcus aureus* it was determined that 10% povidone iodine reduced the biofilm by up to 90%, the combination of 7% H_2O_2 with 0.2% PAA had an equal effect with 1% NaClO, and 70% alcohol was not effective against the biofilm bacteria. They attributed the inefficacy of alcohol to the fact that it fixes the biofilm bacteria (27).

Cabeça et al. reported that NaClO was the most effective disinfectant against *S. aureus* biofilm and biguanide was the least effective, while NaClO and PAA were the most effective disinfectants against *Listeria monocytogenes* biofilm while biguanide, iodine, and quaternary ammonium composites were the least effective (28).

The findings of this study demonstrated that H_2O_2 did not have the desired effect against *P. aeruginosa* biofilm and it had lower efficacy against live microbial cells on the biofilm. For this reason, it is thought that we must be more cautious when using 5% concentrations of H_2O_2 on biofilm-forming surfaces and reusable medical devices. It can be argued that NaClO is a better choice in treating bacterial biofilms as it is the most effective disinfectant against both the biofilm matrix and live bacteria. However, it can be suggested that more successful results can be obtained with periods of contact longer than 10 min as recommended by the FDA.

As biofilm matrices resist the disinfectants to be tested, the researchers studying biofilms have turned their attention to finding other molecules and treatment strategies. Recently, prospective treatment alternatives have been tested for biofilm-related infections, particularly in the food sector. Bacteriophages, bacteriocin, titanium dioxide photocatalysts, ionization or ultraviolet radiation, surfactant treatment, ultrasonic treatment, ozone, microemulsion, and nanoemulsion can be given as examples of such new methods of treatment (29).

In conclusion, previous research on the comparison of a variety of disinfectants indicates that there is no disinfectant that is totally effective against biofilm matrixes. It is thought that the reason why different results were obtained in the relevant studies in which different disinfectants were compared in terms of effectiveness against biofilms was because different methods and different concentrations were used in order to form biofilms and conduct measurements after the application of disinfectants. For this reason, methodic standardization is needed for biofilm studies in order to generalize the results achieved in the studies conducted and put them into practice. Since it is not possible to completely eradicate biofilms, taking preventive measures against biofilm formation is more important than trying to eradicate it.

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