British Microbiology Research Journal
2(2): 40-52, 2012

SCIENCEDOMAIN *international*
www.sciencedomain.org

Efficacy of Disinfectants on *Candida* Biofilms at Different Concentrations and Contact Times

Yasemin Oz[1], Ilknur Dag[2*] and Nuri Kiraz[3]

[1]Department of Microbiology, Faculty of Medicine, Division of Mycology, Eskisehir Osmangazi University, Eskisehir, Turkey.
[2]Vocational Health Services High School, Eskisehir Osmangazi University, Eskisehir, Turkey.
[3]Department of Microbiology and Clinical Microbiology, Cerrahpasa Medical Faculty, Istanbul University, Istanbul, Turkey.

Research Article

Received 7th March 2012
Accepted 25th April 2012
Online Ready 2nd May 2012

ABSTRACT

Aims: *Candida* species cause a wide spectrum of diseases, including hospital-acquired and device-associated infections. The biofilm formation is a major virulence factor in *Candida* pathogenesis and the cells in biofilm show enhanced resistance to disinfectants. Our aim was to evaluate the efficiency of the commonly used hospital disinfectants (glutaraldehyde (GLU), hydrogen peroxide (HP), peracetic acid (PA), ortho-phtalaldehyde (OPA) and sodium hypochlorite (SH) on biofilms of clinical *Candida* (*C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. krusei* and *C. tropicalis*) isolates.
Study Design: An experimental study.
Place and Duration of Study: Department of Microbiology, Faculty of Medicine and Electron Microscope Laboratory, Eskisehir Osmangazi University, between January 2011 and May 2011.
Methodology: These disinfectants were selected due to their common application in hospital environment. Their concentrations were adjusted to manufacturer's recommendations for instrument disinfection: 5% HP, 0.2% PA, 5.25% SH (5000 ppm of chlorine), 2% GLU and 0.55% OPA. They were also prepared at the 1:2 and 1:4 times of recommended concentration to evaluate the activity of lower concentrations. The biofilms were grown in microplates and treated with disinfectants at contact times 1, 5 and 10 minutes (20 min for GLU), then stained with the biomass indicator (2, 3-Bis [2-methoxy-4-nitro-5-(sulfenylamino) carbonyl-2H-tetrazolium-hydroxide]).
Results: The disinfectants reduced the biofilm for all concentrations studied, however none of them completely removed the biofilm. When they were used at low concentration, longer contact times were more effective. However, when the disinfectants were used in

**Corresponding author: Email: idag280@gmail.com;*

recommended concentration, results showed many variations depending on the disinfectant type, contact times and species.
Conclusion: Our results also emphasize the importance of regular disinfection, before the starting of biofilm formation.

Keywords: Biofilm; Candida spp.; XTT; disinfectant; scanning electron microscope.

1. INTRODUCTION

Candida species are opportunistic fungal pathogens, which cause superficial and systemic disease in immuncompromised individuals and are now recognized as major agents of hospital acquired infection (Douglas, 2003). Biofilm production is an important virulence factor in infections caused by *Candida* species*,* bearing in mind that infections caused by non-albicans species have increased (Uyanık et al., 2011).

When microorganisms attach to a surface and grow as biofilm, they become less susceptible to biocides and disinfectants than the planktonic cells of the same organisms. Biofilm cells are organized into structured communities embedded within a matrix of extracellular material (Donlan and Costerton, 2002). In this matrix, the microorganisms are highly protected from the action of several toxic substances (Perez et al., 2010). Biofilms can also act as reservoirs of pathogens in the hospital and cannot be easily removed (Rutala and Weber, 2008). They are responsible for approximately 65% of nosocomial infections, thus, developing effective procedures to combat biofilms in the hospital environment to control hospital-acquired infections is of critical importance (Song et al., 2012).

Disinfectants are both broad-spectrum and multi targeted biocidal compounds that inactivate microorganisms on living tissue and inanimate surfaces (Theraud et al., 2004). They are often used as environmental disinfectants or for medical devices (Russell, 2003*)*. These include alcohols, chlorine and chlorine compounds, formaldehyde, glutaraldehyde, orthophthalaldehyde, hydrogen peroxide, iodophors, peracetic acid, phenolics and quaternary ammonium compound, but their killing efficacy, especially against biofilms, is questionable (Rutala and Weber, 2008). Although they are used for the reduction of number of microorganisms, their simple use does not necessarily reduce the biofilm. It is essential to use the correct amount of the correct biocide with the correct frequency.

So far, the effectiveness of the variety biocides on bacterial biofilms has been demonstrated in many studies (Smith and Hunter, 2008; Purkrtova et al., 2010; Bardouniotis et al., 2003; Hassett et al., 1999; Sreenivasan and Chorny, 2005). However, there is very limited information on the effectiveness of disinfectant against fungal biofilms. Especially, *Candida* biofilms are important in clinical settings due to their role in device related infections. The objective of our study was to test the efficiency of the commonly used hospital disinfectants (GLU, HP, PA, OPA and SH) against *Candida* biofilms. For this purpose, the XTT (2, 3-Bis [2-methoxy-4-nitro-5-(sulfenylamino) carbonyl-2H-tetrazolium-hydroxide]) colorimetric assay was used to determine the decrease percentage of viable cells in biofilms following each treatment. On the other hand, morphological alterations caused by disinfectants on fungal cells in biofilm were also investigated using scanning electron microscopy.

2. MATERIALS AND METHODS

2.1 Isolates

Five clinical *Candida* isolates (*C. albicans* 1467431, *C. glabrata* 1472762, *C. parapsilosis* 427600, *C. krusei* 1506110 and *C. tropicalis* 1508018) were obtained from our culture collection. They were previously isolated and identified from blood and urine samples and categorized as apparent biofilm producers (Dag et al., 2010). Prior to being tested, all strains were subcultured at least twice on Sabouraud dextrose agar (SDA) to ensure viability and purity.

2.2 Disinfectants Tested

Disinfectants were obtained from their manufacturers; HP (50%), (Akkim, Istanbul, Turkey), SH (pure, dry, chlorine tablets), (Mooncid Pulverex, Doganay Chemstry, Istanbul, Turkey), PA (19%) (Akkim, Istanbul, Turkey) and GLU (25%) (Cidex, Johnson and Johnson Medical GmbH Norderstedt, Germany) as active ingredient; OPA (0,55%) (Johnson & Johnson Medical GmbH Norderstedt, Germany) as commercial ready-to-use form. They were diluted according to the manufacturer instructions. Dilution was made with tap water; 5% HP, 0.2% PA, 5.25% SH (5000 ppm of chlorine), 2% GLU and 0.55% OPA. In addition, we also evaluated the 1:2 and 1:4 times dilutions of recommended concentrations of these disinfectants to evaluate the activity of lower concentrations.

2.3 Biofilm Formation and Disinfectant Supplementation

The above five mentioned *Candida* species were used for biofilm studies. The biofilms were formed on commercially available preseterilized, polystyrene, flat bottomed, 96-well microtiter plates (Nunclon; Nalge Nunc International, Roskilde, Denmark) as described previously (Shin et al., 2002). A different plate was prepared for each isolate and; a different column was designed for each disinfectant. The first two wells of the each column were used for the negative control which contained sterile Sabouraud Dextrose Broth (SDB) with no disinfectant. The remaining six wells were used in duplicates for the concentrations (1:4, 1:2 and the recommended undiluted concentration). Dilutions of all disinfectants were freshly prepared on the same day testing was done.

Prior to study, organisms were grown on SDA at 35ºC for 24 h, and saline washed suspensions of each strain of *Candida* species were prepared. The turbidity of each suspension was adjusted to the equivalent of 3×10^7 CFU/ml with Sabouraud dextrose broth (SDB) supplemented with glucose (final concentration, 8%) (Shin et al., 2002). Each well in the odd number columns (1-9) of the microplates were inoculated with aliquots of 20 µl of yeast cell suspension and 180 µl of SDB. Plates were then incubated at 35ºC for 24 h without agitation.

After biofilm formation, the medium was aspirated and planktonic cells were removed by thoroughly washing the biofilms (BIO-TEK ELx50) at three times with sterile PBS. A 200 µl aliquot of disinfectants was then added to each prewashed wells. Two wells were used for each disinfectant concentration and they were treated at contact times 1, 5 and 10 minutes. The contact times of disinfectant were selected according to the manufacturer's recommendations. However, to evaluate the effect of short-term contact to the disinfectant, the contact times of 1 and 5 minutes have also been added to our study.

On the plate, in which cells contact time with the disinfectant was 10 min, firstly GLU was added and 10 min later, other disinfectants were added, therefore, 20 minutes contact time for GLU was ensured. All experiments were performed in duplicate.

2.4 Quantification of Viable Cells in the Biofilm

The tetrazolium salt (2,3-Bis [2-methoxy- 4-nitro-5-(sulfenylamino) carbonyl-2H-tetrazolium-hydroxide] (XTT) assay was used to quantify the number of viable cells in each of the wells following disinfectants supplementation in comparison with disinfectant-free controls (Chaieb et al., 2011; Hawser and Douglas, 1994). It measures the reduction of a XTT by metabolically active cells to a coloured water soluble formazan derivative that can be easily quantified colorimetrically. Briefly, XTT (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in saline at a concentration of 1 mg/ml and after it was completely dissolved, the solution was filtered through a 0.22 µm pore size filter. Menadione (Sigma-Aldrich, St. Louis, MO, USA), the electron mediator for the reaction, was dissolved in acetone at a concentration of 10 mM and then diluted 1:10 in saline. Final solutions including 0.5 mg/ml XTT with 125 µM menadione were prepared in saline (Imamura et al., 2008; Tumbarello et al., 2007, Ramage et al., 2002). Following the disinfectant contact, the plates were rinsed three times with PBS to remove the disinfectants. Two hundred microliter of SDB and 50 µl of the XTT-menadione solution were added to each prewashed and control wells, the plates were incubated at 37ºC for 2 h in a dark chamber. Oxidative activity was measured at 405 nm using a microtiter plate reader (BIO-TEK, ELx800, USA).

2.5 Ultrastructural Investigation of Disinfectant Activity on *Candida albicans* Biofilm

Candida biofilm was generated on a latex catheter and *C. albicans* was chosen as a test isolate. For this purpose, discs of 0,5 cm^2 surface area were cut from catheter, sterilized with ethylene oxide and placed in wells of 24-well tissue culture plates (Corning Incorporated Costar 3524). SDB (1.8 ml) and standardized cell suspension (200 µl) were applied to the surface of each disc, and the discs were incubated at 37ºC for 24 h. After incubation, the medium was aspirated and washing procedures and disinfectants exposure, as mentioned above, were performed. After the final washing, they were fixed with 2.5% (vol/vol) glutaraldeyde in 0,15 M PBS for 1 h at room temperature. They were then treated with 1% (wt/vol) osmium tetroxide for 1 h. Samples were subsequently washed in distilled water, dehydrated in a series of ethanol washes (70% for 10 min, 95% for 10 min. Specimens were mounted on aluminum stubs, with silver paste, coated with gold by using Polaron SC7620 Sputter Coater. The segments were then examined in a SEM (JEOL JSM-5600LV) (Hawser and Douglas, 1994).

2.6 Statistical Analysis

Data were statistically compared using 2-way analysis of variance (ANOVA-SPSS) (F test).

3. RESULTS AND DISCUSSION

The results of this study show that the effectiveness of disinfectant varies depending on the species, time and concentration. In general, at low concentrations of disinfectants, longer contact times (5 and 10 min) were more effective than the short contact (1 min) on biofilm.

Nevertheless, when the disinfectants were used in recommended concentration, the effect of the disinfectants on biofilm has changed according to species, contact time and disinfectant type. However, none of the disinfectants tested completely remove the biofilm (Table 1).

Table 1. The percentage reduction in biofilms of *Candida* species of five disinfectant agents with concentrations and contact time in XTT assay

Species	Disinfectants	% reduction rates								
		1 min			5 min			10 min (20 min for GLU[a])		
		1/4	1/2	1/1	1/4	1/2	1/1	1/4	1/2	1/1
Candida albicans	SH[b]	49.2	61	61.4	51.8	60.6	62.6	52.4	61.5	62.6
	OPA[c]	46.2	61.1	56.7	62.1	61	56.9	55	60.2	57.1
	PA[d]	37	48.3	52	58.7	61.8	62.3	60.2	61.3	62.5
	GLU	39.1	42.5	48.6	39.9	49	59.8	40.2	53.5	61
	HP[e]	38.3	50.5	60.1	37.3	56.2	61.4	39.3	56.6	64
Candida glabrata	SH	56.7	63.6	44	62.4	67.1	63.8	67.7	66.6	66.5
	OPA	58.3	67	68.1	62	67.1	68.2	66.3	68.2	67.2
	PA	53.3	66.1	68	67	67.3	69	66.6	64	68.2
	GLU	39	58	64.1	60.5	68.3	69.8	64	67.5	67
	HP	23.4	41	53.4	46.1	62	70.5	56	58.5	68.4
Candida krusei	SH	53.3	52.2	54.4	54.4	53.2	47.8	55.4	55.7	53.1
	OPA	52.7	53.3	53	45.9	52.5	52.5	47.6	51.4	53.6
	PA	49.3	52.8	53.8	48.5	51.7	53.5	51.4	51.9	53.6
	GLU	51.1	52.4	54.4	47.3	50.1	51.9	46	52	56
	HP	50.8	53.1	52.8	46.2	47.3	45.2	54	38.6	52.5
Candida tropicalis	SH	69.1	69.9	70.7	66.6	68.3	69.2	68	68	70.7
	OPA	69	70.1	68.3	63.8	61	61.8	65.9	66.3	67
	PA	66.8	68.3	70.4	66.4	69.1	70.8	69	69	70
	GLU	50	57	61.9	54.5	62.5	68.3	58	65.4	69.8
	HP	45.2	52.1	65.6	45.1	55.7	72	64.6	64.7	60.3
Candida parapsilosis	SH	25	39.1	32.7	38.2	38	41.3	32.4	37.4	40.6
	OPA	6.9	7.8	12	36.7	38	40	43	39	41.5
	PA	8.1	25.6	37.3	34.1	39.1	41	35.3	38.2	40.7
	GLU	23	6	29	34	36.7	35.5	28	37	40
	HP	18	28.2	34.3	23.7	42.5	34.2	29.5	35	39.6

[a]*GLU, glutaraldehyde,* [b]*SH, sodium hidroxyde,* [c]*OPA, ortho-phtalaldehyde,* [d]*PA, peracetic acid,* [e]*HP, hydrogen peroxide,* after 5 and 20 min of contact time

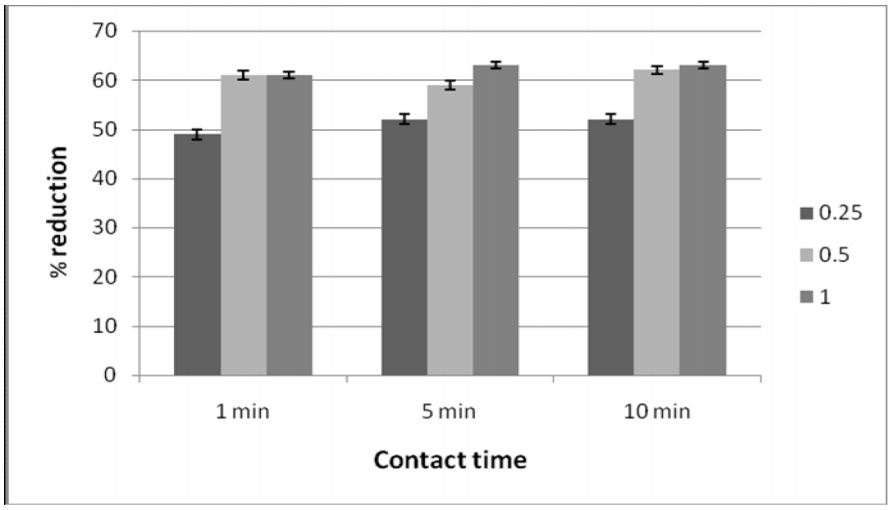
For *C. parapsilosis* isolate, disinfectants were more effective at the higher concentrations and at longer contact time: for SH, OPA and PA (at concentration of 1:1) 5 and 10 min; for GLU and HP (at concentration of 1:1) 10 min ($P<0.05$). *C. parapsilosis* was also affected at least from disinfectants within the species tested.

After contact times of 1 and 10 min, disinfectants exhibited the concentration-dependent effect on *C. albicans* biofilm ($P<0.05$) (Figure 1). The effects of PA and GLU after contact times of 5 and 10 minutes showed better results than 1 minute of contact time.

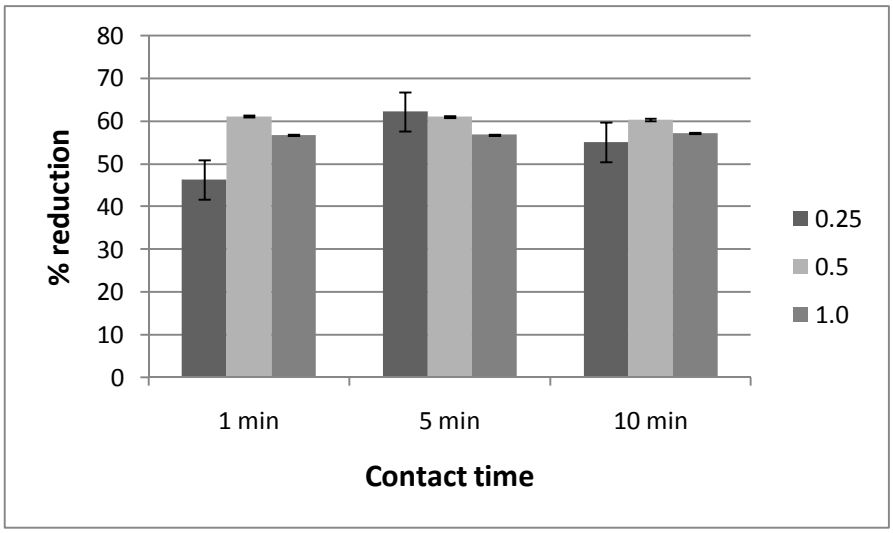
For *C. krusei* isolate, all disinfectants have shown similar effect on the biofilm. Increasing of their contact times had no significant effect on biofilm. However, disinfectants showed the concentration-dependent effect after 1 min of contact time ($P<0.05$). Also, the effect of disinfectants at 1:1 concentration was more high than those obtained by the lower concentration.

For *C. tropicalis*, as the time increases, SH and PA did not show reducing effect on biofilm, whereas GLU and HP after 5 and 20 min of contact time exhibited better results.

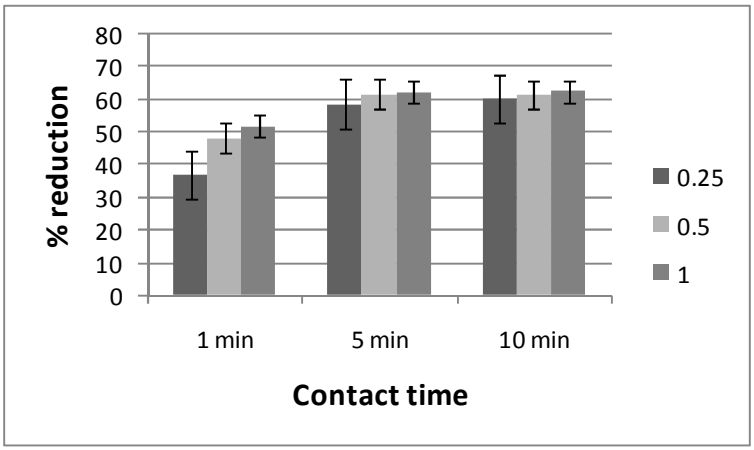
Disinfectants showed concentration-dependent effect at 5 min contact times on *C. glabrata* biofilm ($P<0.05$). The effects of GLU, HP and SH increased by the contact time increases.



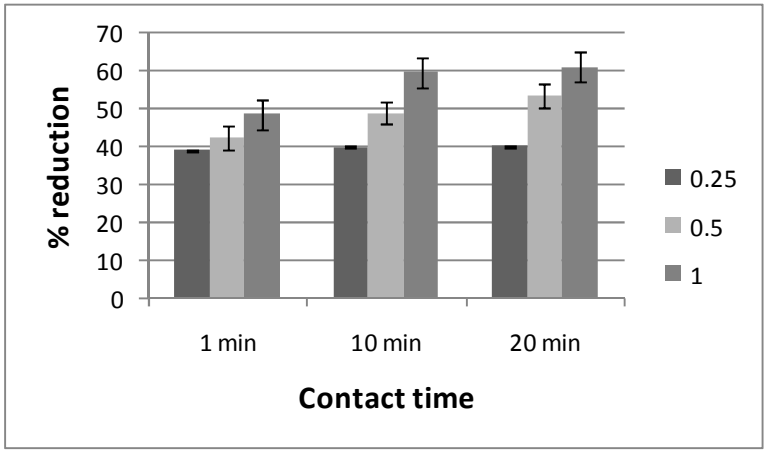
(a)SH



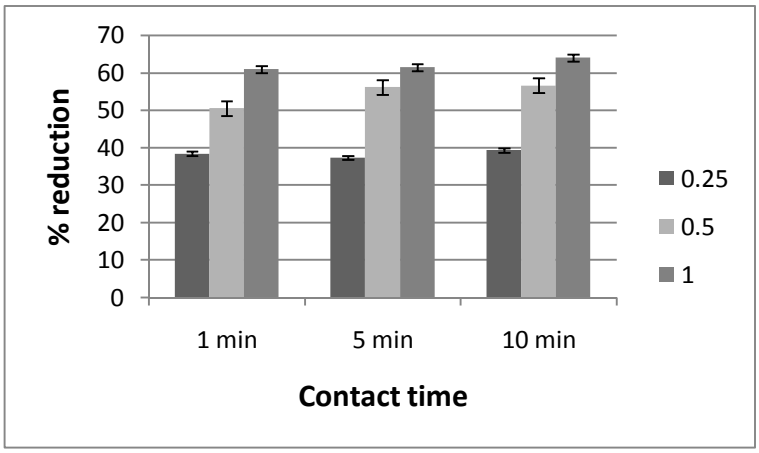
(b)OPA



(c)PA



(d)GLU

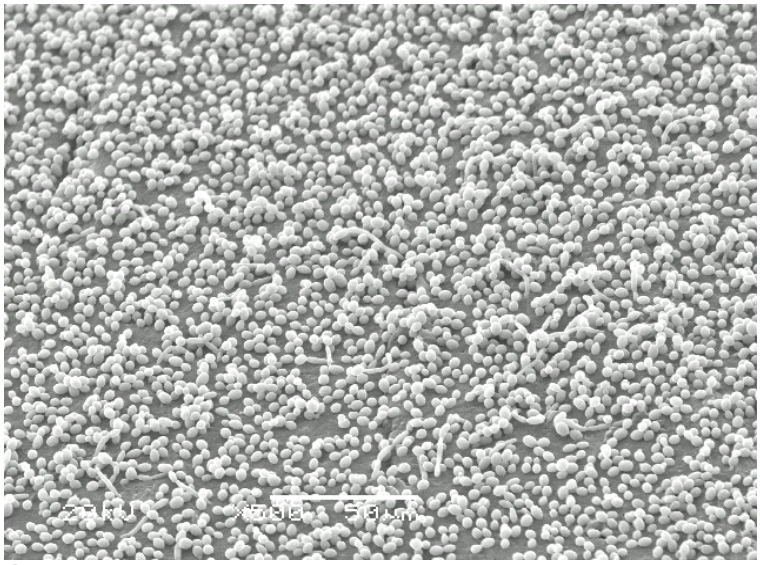


(e)HP

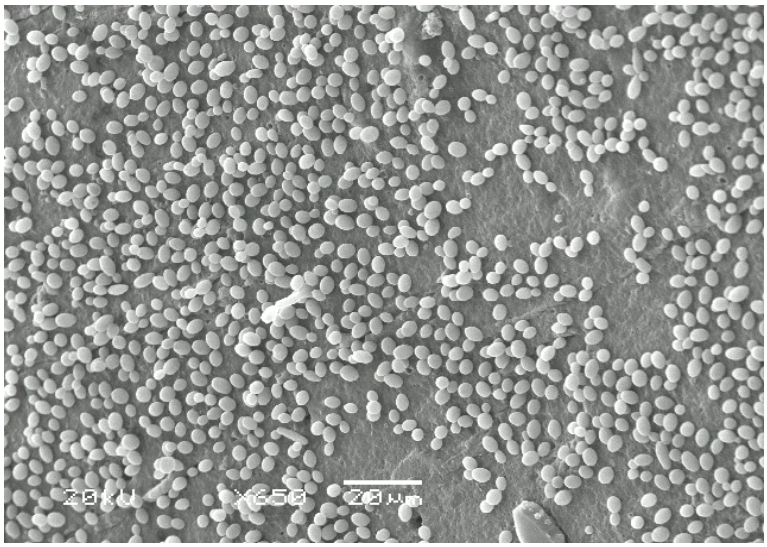
Fig. 1a-e. The activity of five disinfectants at three different exposure times and concentrations on *Candida albicans* biofilm (a) SH, (b) OPA, (c) PA, (d) GLU, (e) HP.

At a concentration of 1:4, all disinfectants showed the time dependent activity against *C. albicans*, *C. parapsilosis* and *C. glabrata* biofilms, so, as the time increases, disinfectant effectiveness increases too. Also, at a concentrations of 1:2 on *C. parapsilosis* biofilm and at a concentrations of 1:1 on *C. krusei* and *C. parapsilosis* biofilms were determined the time-dependent effect ($P<0.05$).

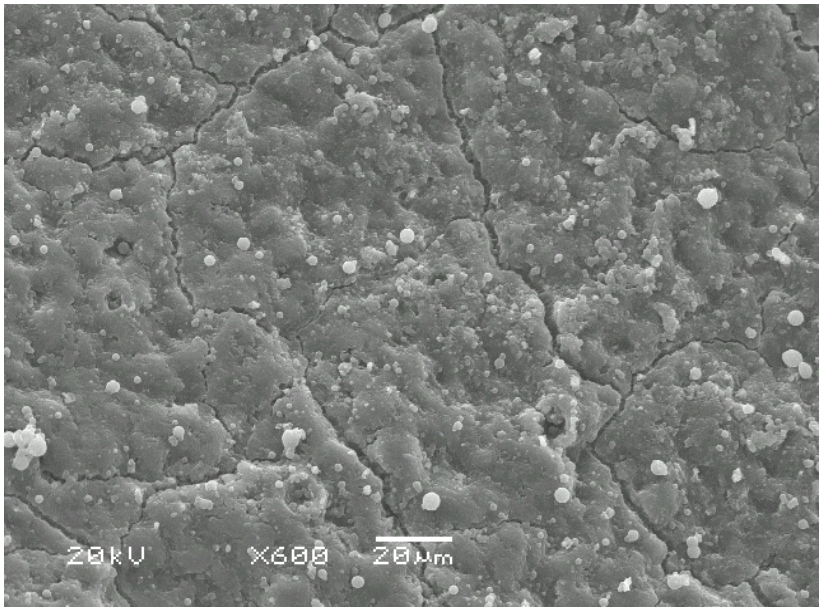
SEM micrographs in Fig. 2 show the time dependent effect on *C. albicans* biofilm of GLU. In also other disinfectants, when the contact time is increased, their activity increased too. However, in our study, we could not determined precisely any effect depending on concentration on biofilms by SEM.



(a)



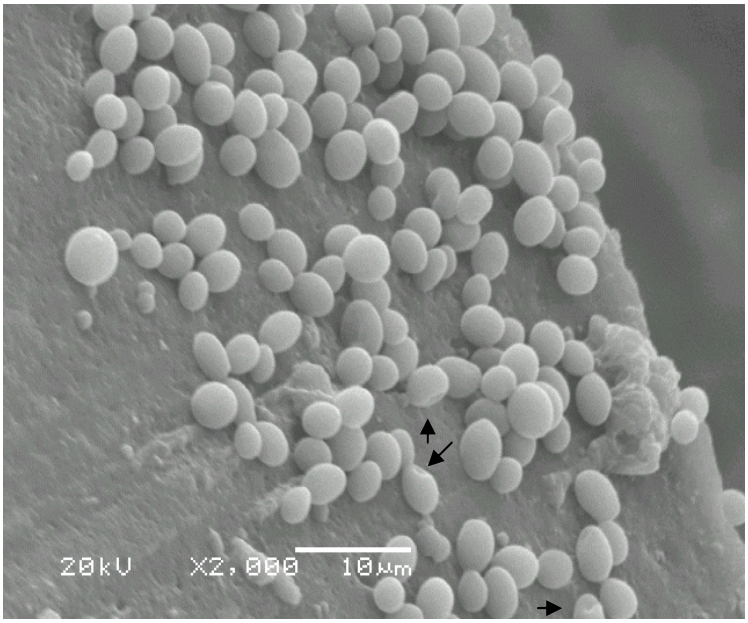
(b)



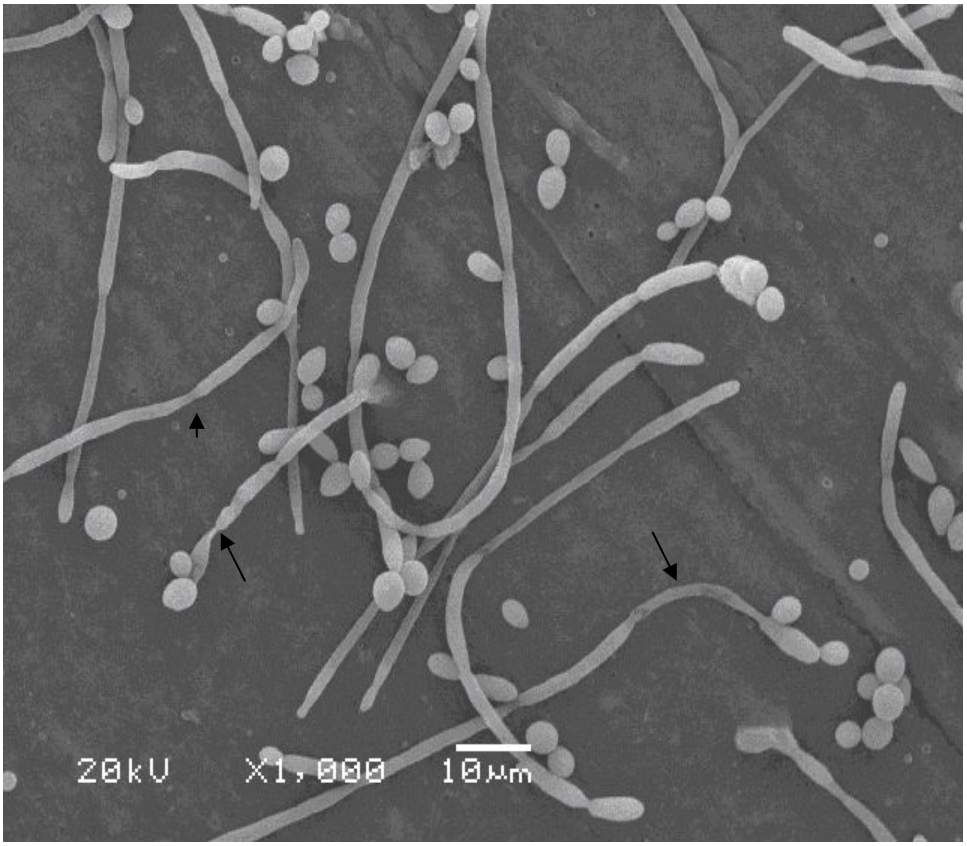
(c)

Fig. 2. The SEM micrographs of the GLU effect (a, b, c) at 1, 5 and 20 minutes on *Candida albicans* biofilm.

Apparently, after treatment with SH (1:1), *C. albicans* cells showed morphological alterations similar to invaginations into the cells (arrows in Figure 3-a). OPA exhibited morphologial changes such as contractions and deteriorations in *C. albicans* pseudohyphae (arrows in Fig. 3-b).



(3-a)



(3-b)

Fig. 3. The SEM micrographs of the effect of a. SH on *Candida albicans* biofilm; b. OPA on *Candida albicans* biofilm

The proper disinfection of the reusable device and surfaces is important in preventing medical device associated infections. Since there is also risk of biofilm development on these devices, using effective disinfection procedures is very necessary. On the other hand, improper use of disinfectant (such as the use of disinfectants with low concentration or short contact time) may cause the emergence of disinfectant-resistant microorganisms (Sidhu et al., 2002). The present study was aimed to determine the biofilm inhibiting effect of different disinfectants onto five *Candida* isolates.

The reduced susceptibility of microorganisms within a biofilm has been the subject of considerable experimentation (Russell, 2003). So far, there have been very limited studies about disinfectant activity against *Candida* biofilms, therefore, findings are mostly contradictive. A previous study tested the impacts of three biocides (ethanol, hydrogen peroxyde and sodium dodecyl sulfate) on *C. albicans, C. parapsilosis*, and *C. glabrata* biofilms. Their findings suggest that the higher concentrations of the biocides were required for efficacy against biofilms (Nett et al., 2008). This is in agreement with our results, when using the same disinfectants at concentrations and times recommended by the manufacturers, biofilm couldn't be completely removed.

In another study, antiseptic and disinfectant efficacy tested on biofilms formed by pure and mixed suspensions of the five yeast isolates showed that hydrogen peroxide (3%) was ineffective against these isolates. Also, the same study reported that only high concentration of hypochlorite (3.8%, w/v) had fungicidal activity on yeast biofilms (Theraud et al., 2004). In our study, although higher concentration (5.25%) of sodium hypochlorite has been used, its effect on biofilm varied depending on species and contact time. In agreement with our findings, Silva et al. also determined that *Candida* isolates exposed to 1% sodium hypochlorite showed invaginations into the cells, by scanning electron microscope (Silva et al., 2011).

In the present study, disinfectants we tested are found to be inadequate on *Candida* biofilms even in the suggested concentrations and contact times. Especially, short contact times (1 minute) and using low concentration doses (1:4) were quite insufficient for effective biofilm inhibition. In many healthcare center, the low concentrations of the disinfectants might be used due to cost of them. Similarly, lower contact times of disinfectants might be used in due to fast circulation in hospitals. Therefore, we also aimed to show the deficiency of lower concentration and contact time of disinfectants on biofilm.

To increase the efficacy of disinfectants on bacterial biofilms, Takeo et al. recommended for increasing the concentration and contact time (Takeo et al., 1994). Thus, it is also important that the resistance of microorganisms to different disinfectants be taken into account when planning the cleaning process. In addition, since there is a risk of biofilm development for devices that are used repeatedly in hospitals, using mechanical methods such as brushing in pre-cleaning may be important.

4. CONCLUSION

Our data suggest that each isolate needs to be tested separately for each disinfectant application. Since biofilm formation is very strain dependent, it is much more informative to study multiple isolates of each species. In addition, short contact times (1 minute) and using low concentration doses (1:4) were quite insufficient for effective biofilm inhibition. So, further studies are necessary to clarify the concentrations of disinfectants and contact times on different *Candida* biofilms.

ACKNOWLEDGEMENTS

This work was supported by a grant from Eskisehir Osmangazi University (Project no. 201041025).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

Bardouniotis, E., Ceri, H., Olson, M.E. (2003). Biofilm formation and biocide susceptibility testing of *Mycobacterium fortuitum* and *Mycobacterium marinum*. Curr Microbiol, 46, 28–32.

Chaieb, K., Zmantar, T., Souiden, Y., et al. (2011). XTT assay for evaluating the effect of alcohols, hydrogen peroxide and benzalkonium chloride on biofilm formation of *Staphylococcus epidermidis.* Microb Pathog, 50, 1-5.

Dag, I., Kiraz, N., Oz, Y. (2010). Evaluation of different detection methods of biofilm formation in clinical *Candida* isolates. Afr J Microbiol Res, 24, 2763-2768.

Donlan, R.M., Costerton, J.W. (2002). Biofilms: survival mechanisms of clinically relevant microrganisms. Clin Microbiol Rev., 15, 167-193.

Douglas, L.J. (2003). *Candida* biofilms and their role in infection. Trends Microbiol, 11, 30-36.

Hawser, S.P., Douglas, L.J. (1994). Biofilm formation by *Candida* species on the surface of catheter materials in vitro. Infect Immun, 62, 915-921.

Hassett, D.J., Elkins, G., Ma, J.F., McDermott, T.R. (1999). *Pseudomonas aeruginosa* biofilm sensitivity to biocides: use of hydrogen peroxide as model antimicrobial agent for examining resistance mechanisms. Methods Enzymol, 310, 599–608.

Imamura, Y., Chandra, J., Mukherjee, P.K., Lattif, A.A., Szczotka-Flynn, L.B., Pearlman, E., Lass, J.H., O'Donnell, K., Ghannoum, M.A. (2008). *Fusarium* and *Candida albicans* biofilms on soft contact lenses: model development, influence of lens type, and susceptibility to lens care solutions Antimicrob Agents Chemother, 52, 171–182.

Nett, J.E., Guite, K.M., Ringeisen, A., et al. (2008). Reduced biocide susceptibility in *Candida albicans* biofilms. Antimicrob Agents Chemother, 52, 3411–3413.

Perez, L.M., Alvarez, B.L., Codony, F., et al. (2010). A new microtitre plate screening method for evaluating the viability of aerobic respiring bacteria in high surface biofilms. Let. App Microbiol, 51, 331– 337.

Purkrtová, S., Turoňová, H., Pilchová, T., Demnerová, K., Pazlarová, J. (2010). Resistence of *listeria monocytogenes* biofilms to disinfectant. Czech Journal of Food Sciences, 28, 326–332.

Ramage G., Saville, S.P., Wickes, B.L., Lopez-Ribot, J.L. (2002). Inhibition of *Candida albicans* biofilm formation by farnesol, a quorum sensing molecule. Appl Environ Microbiol, 68, 5450–5463.

Russell, A.D. (2003). Similarities and differences in the responses of microorganisms to biocides. J Antimicrob Chemother, 52, 750–63.

Rutala, W.A., Weber, D.J., The Healthcare Infection Control Practices Advisory Committee (hIcPac) (2008). Guideline for disinfection and sterilization in healthcare facilities. Atlanta, GA:Centers for disease control and prevention.

Shin, J.H., Kee, S.J., Shin, M.G., et al. (2002). Biofilm production by isolates of *Candida* species recovered from nonneutropenic patients: comparison of bloodstream isolates with isolates from other sources. J Clin Microbiol, 40, 1244-1248.

Sidhu, M.S., Swrum, H., Holck, A. (2002). Resistance to quaternary ammonium compounds in food-related bacteria. Microb Drug Resist, 8, 393–399.

Silva, P.M.B., Acosta, E.J.T.R., Pinto, L.R., et al. (2011). Microscopical analysis of *Candida albicans* biofilms on heat-polymerised acrylic resin after chlorhexidine gluconate and sodium hypochlorite treatments. Mycoses, 54, 712-717.

Smith, K., Hunter, I.S. (2008). Efficacy of common hospital biocides with biofilms of multi-drug resistant clinical isolates. J Med Microbiol, 2008, 966-73.

Sreenivasan, P.K., Chorny, R.C. (2005). The effects of disinfectant foam on microbial biofilms. Biofoul., 21, 141-149.

Song, L., Jianfeng, W., Chuanwu, X. (2012). Biofilms on environmental surfaces: Evaluation of the disinfection efficacy of a novel steam vapor system. Am J Inf Control http://dx.doi.org/10.1016/j.ajic.2011.11.013.

Takeo, Y., Oie, S., Kamiya, A., et al. (1994). Efficacy of disinfectants against biofilm cells of *Pseudomonas aeruginosa*. Microbios, 79, 19–26.

Theraud, M.Y., Bedouin, Y., Guiguen, C., et al. (2004). Efficacy of antiseptics and disinfectants on clinical and environmental yeast isolates in planktonic and biofilm conditions. J Med Microbiol, 53, 1013–1018.

Tumbarello, M., Posteraro, B., Trecarichi, E.M., Fiori, B., Rossi, M., Porta, R., Gaetano Donati, K., La Sorda, M., Spanu, T., Fadda, G., Cauda, R., Sanguinetti, M. (2007). Biofilm production by Candida species and inadequate antifungal therapy as predictors of mortality for patients with candidemia. J Clin Microbiol, 45, 1843–1850.

Uyanık, M.H., Ayyıldız, A., Aktaş, A.E., et al. (2011). Investigation of biofilm production by *Candida* species isolated from various clinical samples. Br J Biomed Sci, 68, 43-45.