Egyptian Journal of Medical Microbiology Volume 27 / No.3 / July 2018 127-133 Online ISSN: 2537-0979

ORIGINAL ARTICLE

Effectiveness of Different Disinfectants Used in ICUs on *Candida* Biofilms at Different Concentrations and Contact Times

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Key words:
Candida; biofilm; disinfectants, ethanol, sodium hypochlorite, peracetic acid

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ABSTRACT

Background: *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.* ***Objectives:*** *The aim of this study was to evaluate the efficiency of the commonly used hospital disinfectants [ethanol, chlorine (sodium hypochlorite; SH) and peracetic acid (PA)] on biofilms induced by clinical Candida isolates.* ***Methodology:*** *Isolation and identification of Candida spp. were conducted by the various conventional methods, in the Microbiology laboratory, Faculty of Medicine, Beni-Suef University. Biofilms were grown in 96 well flat-bottomed microtiter plates and they were evaluated by crystal violet (CV) assay method. Thereafter, the selected disinfectants concentrations were adjusted to manufacturer's recommendations for instrument disinfection: 70% ethanol, 5.25% SH (5000 ppm of chlorine) and 0.2% PA. They were also prepared at the 1/2 and 1/4 of their recommended concentrations to evaluate the activity of lower concentrations. The biofilms were then treated with the disinfectants at contact times of 1, 5 and 10 minutes.* ***Results:*** *Positive samples for Candida were distributed as follows; urine samples 23 (76.7%), sputum samples 5 (16.7%), a blood sample 1(3.3%) and a pus sample 1 (3.3%). C.albicans were detected in 23 (76.7%) of yeast yields isolated, while, 7 (23.3%) were C.non albicans. Strong biofilm formation was noticed in 9 (30%) isolates, moderate in 9 (30%), while 12 out of 30 (40%) showed weak biofilm formation. Degree of biofilm reduction using the three disinfecting agents was assessed with different concentrations and different contact times. The findings showed that increasing the concentration of the used disinfectants (1/2, 1/1) together with exposure for longer contact times (5,10 min) were leading to more increase in the percentage of reduction of biofilm formation that was evidently higher in C.albicans than that of C.non albicans.*

INTRODUCTION

Great progresses in the medical field, especially in critical care, attained during the last decades have contributed not only to longer survival of patients, but also to the increasing incidence of opportunistic infections caused by fungi. Complex medical and surgical problems, disruption of natural barriers, several invasive procedures and lengthy antibiotic treatment are some of the factors contributing to the alarming increase of fungal infections in the Intensive Care Unit (ICU) settings [1,2]. The leading fungal infection, as documented by several studies, is *Candida*. In 2007, the results of extended study of prevalence of infection in intensive care units (EPIC II); including 1,265 ICUs in 75 countries revealed that 19% of pathogens isolated in ICU patients were fungi [3]. *Candida* species (spp.) were predominantly isolated (17%) followed by *Aspergillus* species.

Candida species are mostly harmless and comprise part of the normal human flora. Only a small percentage of the identified species cause diseases in humans. *Candida* spp. is responsible for an extremely large spectrum of diseases [4,5] such as peritonitis, other abdominal infections, meningitis and infective endocarditis. The source of *Candida* infection can be endogenous (gastro-intestinal flora or mucocutaneous colonization) or exogenous (hands of health workers, contaminated IV fluids) that occasionally led to local outbreaks [6]. Amongst the most important virulence factors of *Candida* species is biofilm production (**7**) that leads to less susceptibility to disinfectants than the planktonic cells of the same organisms. Biofilms act as pools for pathogens and cannot be easily removed [8]. They are responsible for about 65% of nosocomial infections, consequently, developing effective practices to combat biofilms in the hospital environment is critically important [9].

Disinfectants are broad-spectrum biocidal compounds that inactivate microorganisms on inanimate surfaces [10,11]. Several disinfectants are used in hospital settings to combat such infections; they are used for environmental decontamination and disinfect many of the medical devices [12,13]. Among the mostly used disinfectants in ICUs are alcohols, chlorine and chlorine compounds, formaldehyde, glutaraldehyde, orthophthal-aldehyde, hydrogen peroxide, iodophors, peracetic acid, phenolics and quaternary ammonium compound.

There is very limited information on the effectiveness of disinfectants against fungal biofilms and their killing efficacy, especially against biofilms is questionable [8,13]. Therefore, the current study aimed to assess the effect of commonly used disinfectants in Intensive Care Units (ICUs); chlorine, ethanol, and peracetic acid in different concentrations and contact times against *Candida* species induced biofilms isolated from clinical samples.

METHODOLOGY

The study was conducted on 87 patients admitted to the Intensive Care Units (paediatric and adult) in Beni-Suef University Hospital during the period from February 2016 to the end of November 2016. Clinical data were collected including; sex, age, residence, site of sample and cause of admission.

Fungal isolates:

Out of the 87 patients examined, 30 cases were positive for *Candida* yields. They were isolated and identified in the Microbiology Laboratory, Faculty of Medicine, Beni-Suef University, from various clinical samples. *C. albicans* ATCC 90028, and *C. albicans* ATCC 10231 were used as quality control strains.

Isolation and identification of Candida isolates

The samples, except for blood samples, were subjected to direct film stained by Gram stain, then, cultured on Sabouraud Dextrose Agar medium (SDA) for isolation of fungi. The plates were incubated at 35^0 C aerobically for 24-48 hours, and were examined for fungal growth. Identification of the isolates was conducted by the conventional microbiological tests for *Candida*[14] as regards (Gram's stain, colony morphology, germ tube test and chlamydospore production test).

Biofilm formation and evaluation

The identified isolates were grown on SDA at 35°C for 24 h and saline washed. The turbidity of each suspension was adjusted to 0.5 McFarland.

Commercially available pre-sterilized, polystyrene, flat-bottomed, 96-well microtiter plates (Nunclon; Nalge Nunc International, Roskilde, Denmark) were used for biofilm formation. Each well was inoculated with aliquots of 20 µl of yeast cell suspension and 180 µl aliquoted of Yeast Nitrogen Base (YNB) broth media (Difco Laboratories) containing 0.9% D-glucose to form *Candida* biofilms. Plates were then incubated at 35 °C for 48 h without agitation [16,17].

The formed biofilms were washed three times with 200 µL of PBS. Washed biofilms were fixed by adding 200 µL of methanol to each well (15 min), after which the supernatants were removed, and the plates were air-dried for 45 min. Subsequently, 200 µL of a 0.1% (w/v) Crystal Violet (CV) solution was added to each well and incubated at room temperature for 20 min, after which, excess CV solution was removed by washing the plates gently under running tap water. Two hundred microliters of 33% (v/v) acetic acid (decolorizing solution) was then added to the wells to release the bound CV.

Finally, 100 µL of this decolorizing solution was transferred to a fresh 96-well microtiter plate and the absorbance levels were determined using a microtiter plate reader at wave length of 620 nm. Thereafter, quantification of the biofilms was calculated according to the values of the optical density (OD) of 620 nm using ELISA reader [18,19].

Biofilm calculation

The optical density (OD) of each strain was obtained by the arithmetic mean of the absorbance of three wells and this value was compared with the mean absorbance of negative controls (OD nc). The following classification was used for the determination of biofilm formation: no biofilm production (ODs = ODnc), weak biofilm production (ODnc< ODs> 2ODnc), moderate biofilm production (2ODnc < ODs> 4ODnc) and strong biofilm production (4ODnc<ODs)[20,21].

Disinfectants Tested

Selected disinfectants were obtained from their manufacturers, in concentrations of 5000 ppm of Chlorine, 70% Alcohol and 0.2% Peracetic Acid. The selected disinfectants were evaluated on the formed biofilms in concentrations recommended by the manufacturer, as well as ½ and ¼ of the recommended concentrations of each.

Biofilm treatment by selected disinfectants

The 9 strong biofilm formers of the *Candida* isolates together with 7 moderate and 7 weak biofilm formers were selected. Using the previously mentioned technique, each of the selected strains were left in incubator for 48 h for biofilm formation, thereafter, the medium were aspirated; to remove planktonic cells, and the wells were washed three times with sterile PBS. A 200 µl aliquot of disinfectants were then added to each prewashed wells leaving 2 wells for each strain as a control; one containing no organism and only media and disinfectant and the other containing no disinfectant with only the tested organism.

Each concentration of the selected disinfectant was tested in different well, and at contact times of 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[16,17].

Statistical methodology

Data were collected and analyzed statistically using Statistical Package for Social Sciences program (SPSS v21). The following tests were used in this study: mean, standard deviation, T tests for independent samples, ANOV A test (analysis of variance). Significance levels: $p>0.05$ insignificant, $p\leq0.05$ significant and $p<0.001$ highly significant.

RESULTS

The present study was conducted on 87 patients admitted to the Intensive Care Units (ICUs) of Beni-Suef University Hospital; 53 of them were females (61%), ages of the patients ranged between 0.08 and 83 years with mean ±SD 40.1 ±17.3 years and median of 35.5 years. Thirty (34.4%) out of the 87 cases examined revealed candidal infections.

Their ages ranged between 0.08 and 77 years with mean ±SD 31.4±27.6 years and median of 27.5 years. Fourteen cases (46.7%) were from Urban areas and 16 (53.3%) were from Rural areas. Sex of the patients was equally distributed; 15 males and 15 females (50% each).

The samples positive for *Candida* yields were distributed as follows: urine samples 23 (76.7%), sputum samples 5 (16.7%), blood samples 1(3.3%) and pus samples 1 (3.3%). *C. albicans* were detected in 23 (76.7%) of yeast yields isolated (positive germ tube and chlamydospore production tests), while, 7 (23.3%) were *C. non-albicans*.

Biofilm production was assessed using Crystal Violet (CV) staining assay. Strong biofilm formation was noticed in 9 (30%) isolates, moderate in 9 (30%), while 12 out of 30 (40%) showed weak biofilm formation.

The 9 strong biofilm formers of the *Candida* isolates together with 7 moderate and 7 weak biofilm formers were tested for the effect of the selected disinfectants. The results of this study showed that the effectiveness of disinfectants varies depending on the species, time and concentration of the disinfectant used. Increasing the concentration of the used disinfectants (1/2, 1/1) together with exposure for longer contact times (5,10 min) led to more reduction of biofilms produced that was evidently higher in *C. albicans* than that of *C. non albicans* (Figures1-6, Table 1).

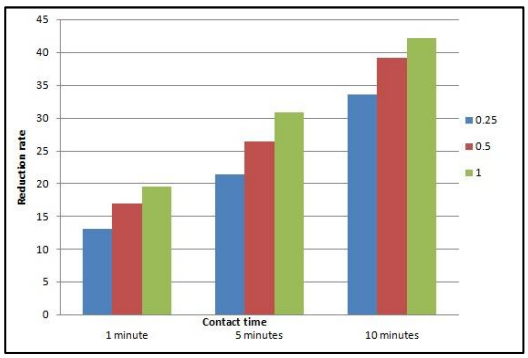

Fig. 1: Reduction rate of biofilms by ethanol on calbicans

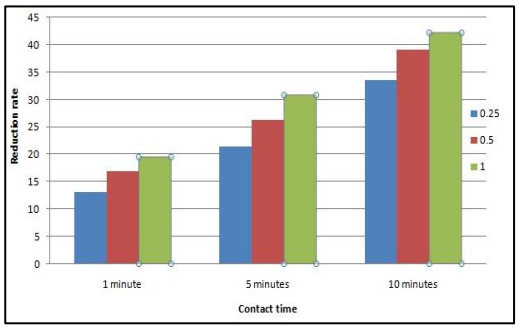

Fig. 2: Reduction rate C. non albicans of biofilms by ethanol on candida non alibcans

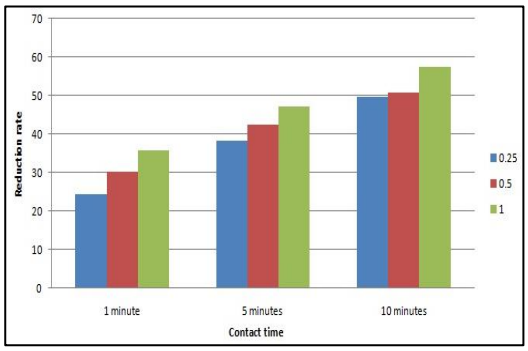

Fig. 3: Reduction rate of biofilm by chlorine at different contact times and concentrations on *C. albicans.*

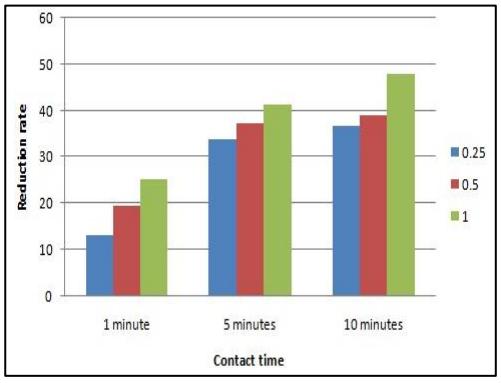

Fig. 4: Reduction rate of biofilm by chlorine at different contact times and concentrations on *C. non-albicans* respectively.

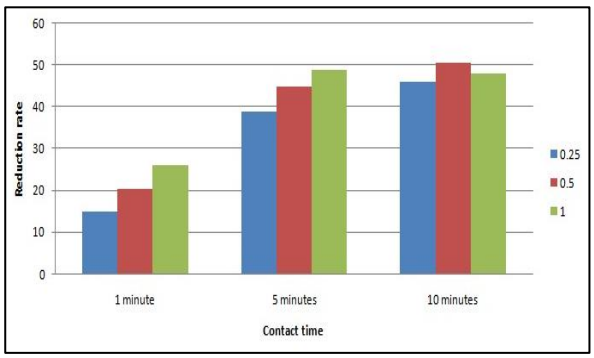

Fig. 5: Reduction rate of biofilm by peracetic acid at different contact times and concentrations on *C. albicans.*

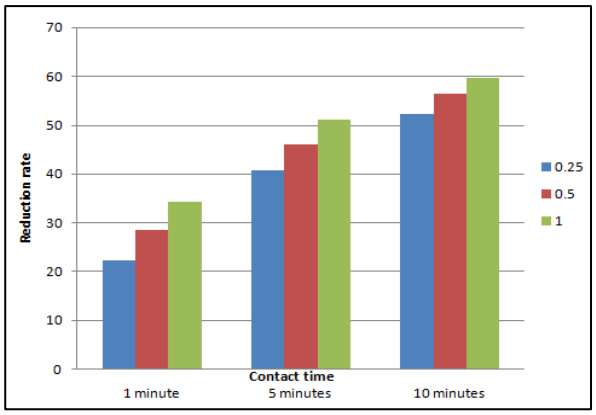

Fig. 6: Reduction rate of biofilm by peracetic acid at different contact times and concentrations on *C. non-albicans* respectively.

The effect of the disinfectants at different concentrations and contact times on the reduction rate of biofilm of *Candida in vitro* was demonstrated. The results revealed that there was a significant statistical difference between the three disinfectants at different contact times and concentrations (*p*-value ≤0.05)**.** Correlations between the different disinfectants' effectiveness revealed the following;

- Reduction rate was more evident with peracetic acid followed by chlorine then ethanol, but the difference was statistically significant between ethanol and peracetic acid (*P*-value=0.004*). However, chlorine was not statistically different from peracetic acid or ethanol (table1, table 2).
- Regarding the concentration of the three disinfectants there was a statistical significant difference between the three concentrations, increasing the concentration increased the reduction rate, the statistical difference between (0.25 - 0.5), (0.25- 1) and (0.5-1) showed *P*-value of <0.05*(table1, table 3).

Regarding contact time the best contact time was 10 minutes followed by 5 minutes then 1 minutes and the difference between the three contact times was statistically significant (*P*-value <0.05*) (table1, table 4). Surprisingly, the reduction rate of *C. albicans* biofilms when subjected to peracetic acid in 0.5 concentration exceeded that of the full concentration at contact time of 10 min (fig.5, table 1), nevertheless, in all other concentrations in different species the reduction rates were, as expected, increasing with higher concentration and more contact time (fig 5,6, table1).

Table 1: The percentage of biofilm reduction among *Candida albicans and C. non-albicans* isolates by the three disinfectants in different concentrations and at different contact times

Species	Disinfectant	Reduction rates %								
		1 minute			5 minutes			10 minutes		
		0.25	**0.5**	**1**	**0.25**	**0.5**	**1**	**0.25**	**0.5**	**1**
C. albicans	**Ethanol**	21.7	24.9	29.2	35.6	39.4	43.2	47.1	50.7	**55.5**
	Chlorine	24.4	30.1	35.7	38.2	42.4	47.1	49.6	50.7	**57.5**
	Peracetic acid	22.3	28.5	34.2	40.8	46.1	51.2	52.2	56.5	**59.7**
C. nonalbicans	**Ethanol**	13.1	16.9	19.5	21.3	26.3	30.8	33.6	39.1	**42.2**
	Chlorine	13.2	19.4	25.3	33.8	37.2	41.2	36.8	39.1	**47.8**
	Peracetic acid	15	20.3	26	38.9	44.8	48.8	46.1	**50.6**	48

Table 2: Comparison between the effectiveness of the three disinfectants on the reduction rate of the selected *Candida* biofilms

Disinfectant 1	Disinfectant 2	Mean Difference	*P*-value
Ethanol	Chlorine	-3.8007	0.096
	Peracetic acid	-5.8626*	0.004
Chlorine	Peracetic acid	-2.0619	0.499

**p- value is significant* ≤0.05

Table (3): The difference in biofilm reduction rates in the three concentrations used among the selected *Candida* isolates

Concentration 1	Concentration 2	Mean difference	*P*- value
0.25 concentration	0.5 concentration	-4.544*	0.040
	1 concentration	-8.976*	0.000
0.5 concentration	1 concentration	-4.432*	0.048

**p- value is significant* ≤0.05

Collectively, there was a statistically significant difference between the three contact times on affecting the reduction rate (*P*-value<0.001); the highest difference was between one minute and ten minutes followed by the difference between one minute and five minutes and lastly the difference between five and ten minutes (table 4).

Table 4: The difference in biofilm reduction rates at the different contact times among the selected *Candida* isolates

Contact time 1	Contact time 2	Mean Difference	*P*-value
One minute	5 min	**-15.2326***	0.000
	10 min	**-25.3735***	0.000
Five minute	10 min	**-10.1408***	0.000

**Significant p- value* ≤0.05

DISCUSSION

Candida species are major human fungal pathogens. Recent evidences suggest that the majority of infections produced by this pathogen are associated with biofilm growth. Biofilm production is associated with a high level of antimicrobial resistance[22].

Disinfectants are broad-spectrum biocidal compounds that inactivate microorganisms on living tissue and inanimate surfaces. Their mechanisms of action have been extensively studied, as has bacterial resistance to them [23]. However, there is very limited information on the effectiveness of disinfectants against fungal biofilms especially, *Candida* spp.

The current study was conducted on patients admitted to ICUs in Beni-Suef University Hospital. Thirty yields were isolated from different clinical specimens. *C. albicans* were detected in 23 (76.7%) of yeast yields isolated, while, 7 (23.3%) were *C. non-albicans*, isolated from urine samples 23 (76.7%), sputum samples 5 (16.7%), a blood sample 1(3.3%) and a pus sample 1(3.3%). This was in agreement with Ibrahim *et al.* [21], who found that the most isolated organisms were *C. albicans* (65.3%) followed by *C. tropicalis* and *C. glabrata*. However, in another study done in Turkey by Ece *et al.* [24], they reported that *C. albicans* (38.6%) and *C. tropicalis* (13.9%) were the most prevalent isolates, nevertheless, the most prevalent specimens were from UTI which agrees with the present findings as the isolated samples were 23 representing 76.7%. Moreover, Seddiki et al. [25] study showed that twelve strains (19.04%) of *Candida* spp. were isolated during the study period; nine of which were *C. glabrata* and only three strains of *C. albicans* were identified, *C. glabrata* was predominant compared with *C. albicans*; a ratio of 3:1 of *C.glabrata* versus *C.albicans* was observed which contradicts our results. These variations may be due to the difference in geographic distribution, the sample size, site of isolation and the associated risk factors.

The present study revealed that, the isolated *Candida* species varied in their *in vitro* biofilm forming ability. Results showed that 9 (30%) isolates were strong biofilm formers and 9 (30%) isolates were moderate while most of isolates showed weak biofilm formation12 (40%). These findings matched Alnuaimi et al. [26], who found that the majority of clinical strains showed low biofilm production.

The results of this study showed that the effectiveness of the tested disinfectants varied, depending on the isolated species, time and concentration. In general, at different concentrations of the disinfectants, longer contact times (5 and 10 min) were more effective than the short contact time (1 min) on biofilms, the effect of the disinfectants on biofilm was variable; according to species type, contact time and disinfectant type, however, none of the tested disinfectants completely eradicated the biofilm. Similarly many studies clearly proved the generally accepted fact of the decreased sensitivity of biofilm cells to disinfectants [27,28]. The present study revealed that disinfectants were more effective at the higher concentrations and at longer contact time for ethanol, chlorine and peracetic acid (at concentration1/1) 10 min for *C. albicans* isolates than those for *C. non-albicans* isolates (table 1). Unexpectedly, the reduction rate

among the selected *C. albicans* biofilms when subjected to peracetic acid in 0.5 recommended concentration exceeded that of the full concentration at contact time of 10 min. This finding needs more evaluation regarding; the effectiveness of different peracetic acid concentrations at longer contact times, age of biofilm, effect of yeast storage on disinfectant resistance and different mechanisms of resistance. However, in all other concentrations in different species the reduction rates were, increasing with higher concentration and more contact time. In the same context, Nett *et al.* [27] tested the impacts of three biocides (ethanol, hydrogen peroxide and sodium dodecyl sulfate) on *C. albicans*, *C. parapsilosis*, and *C. glabrata* biofilms. Their findings suggested that the higher concentrations of the biocides were required for efficacy against biofilms. The concentrations needed to decrease the burden of mature biofilm cells by 50% were from 2 to 10 fold higher for biofilm cell inhibition than for planktonic cell inhibition. Moreover**,** Oz *et al.*[29] proved that the use of disinfectants reduced the biofilm at all concentrations, however, none of them completely eradicated the biofilm. Interestingly, when they were used at lower concentrations, longer contact times were more effective. This also concurs with the current findings.

Pires et al. [30] tested the impacts of four biocides (Acetic acid, hydrogen peroxide, sodium hypochlorite, and a commercial biocide made of peracetic acid and hydrogen peroxide) used for the disinfection of hemodialysis systems against *Candida parapsilosis*, *Candida orthopsilosis* and *Candida albicans* biofilms, the results were similar for all the tested species. However, the standard biocide (sodium hypochlorite), 500 ppm or 0.5 g/liter) failed to destroy the *Candida* biofilms tested completely, nevertheless, the former and peracetic acid and hydrogen peroxide decreased biofilm formation, and hydrogen peroxide reduced the burden of *Candida orthopsilosis* and *C. albicans* biofilm formation at concentrations below 0.3%, which agrees with the present study.

CONCLUSION AND RECOMMENDATIONS

In conclusion, there was a positive benefit for the use of disinfectants in the reduction of biofilm formation. Our data suggested that, each of the isolated species need to be tested separately for each disinfectant application. Since biofilm formation is 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 eradication. So, further studies are necessary to clarify the concentrations of disinfectants and contact times on different *Candida* biofilms.

The proper disinfection of the reusable devices 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 disinfectants (such as the use of disinfectants with low concentration or short contact time) may cause the emergence of disinfectant-resistant microorganisms.

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