Effect of disinfectants on biofilm development by five species of *Candida*

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Accepted 7 February, 2012

*Candida* biofilms have become an increasingly significant clinical problem for hospital-acquired and device-associated infections due to their increased levels of resistance to the antifungals and disinfectants. This study examines the biofilm formation of five *Candida* isolates (*Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida krusei* and *Candida tropicalis*), using the XTT (2, 3-Bis[2-methoxy-4-nitro-5-(sulfonylamino) carbonyl-2H-tetrazolium-hydroxide]), and compares the susceptibility of biofilm cells to five disinfectants (glutaraldehyde, hydrogen peroxide, peracetic acid, ortho-phtalaldehyde and sodium hypochlorite) at the different times of biofilm formation (6th, 12th, 24th and 48th). Thus, the aim of our study was to investigate whether these disinfectants can interfere on *Candida* biofilm development phases. Our results indicated that the many variations in biofilm formation in clinical *Candida* species following disinfectant stress, however, none of the disinfectants completely removed the biofilm. Also, our transmission electron microscopic findings suggest that the disinfectants showed an important effect on the planktonic *Candida* cell. All these results also emphasize the importance of regular disinfection, before the starting of biofilm formation. On the other hand, XTT assay may not be the only adequate method to evaluate the efficacy of disinfectants. For these reasons, further studies are necessary to evaluate the disinfectant effectiveness on *Candida* biofilms.

Key words: Biofilm, *Candida*, tetrazolium salt (XTT), disinfectant, transmission electron microscope (TEM).

INTRODUCTION

*Candida* species are now recognized as causing disease with high frequency and are one of the most prevalent sources of nosocomial infections (Douglas, 2003; Neppelenbroek et al., 2006). Although *C. albicans* remains the most frequent cause of candidiasis, the incidence of the disease caused by other species of *Candida* has been increasing steadily (Mihrendi and Makimura, 2008). A major virulent attribute of *Candida* is its ability to adhere and form surface attached microbial communities known as biofilms. It has been shown that more than 65% of human infections have a biofilm-related origin (Chaieb et al., 2011). The ability of *Candida* species to form biofilms has great importance due to their increased resistance to antifungals and biocides and cells within biofilms to withstand host immune defences (Silva et al., 2008). In the hospital environment, *Candida* biofilms have also been observed in most medical devices, such as stents, implants, endotracheal tubes or various types of catheters (d'Enfert, 2006). For this purpose, it is important to evaluate the effects of biocides used in hospitals to eradicate cells growing in biofilms.

As known, *C. albicans* biofilm formation can be divided in three sequential steps: first, early phase (0 to 11 h), involving adhesion of fungal cells to the substrate; second, intermediate phase (~12 to 30 h), during which
the blastospores coaggregate and proliferate, forming communities while producing a carbohydrate-rich extracellular matrix (ECM); and third, maturation phase (~31 to 72 h), in which the fungal cells are completely encased in a thick ECM (Mukherjee et al., 2003). An understanding of biofilm response of Candida species to vary biocides in these biofilm development phases may be important to the challenge against candidal infections, because any intervention to this complex steps may inhibit the biofilm formation and development (Sangetha et al., 2009).

Disinfectants are chemical or physical agents that inactivate microorganisms. They are multitargeted agents and often used as environmental disinfectants or for medical devices. To reduce the spread of infections, it is very important that appropriate disinfecting agents and methods are used (Stanizewska et al., 2010). So far, although biofilm resistance to antifungals has been well described, Candida biofilm susceptibility to biocides has received less attention (Nett et al., 2008). Limited studies of C. albicans biofilm have examined the activities of variety biocides, but their findings have not been consistent (Suci and Tyler, 2002; Szymanska, 2006; Nett et al., 2008).

For many years, the XTT assay has been the mainstay for the estimation of biomass in yeast biofilms (Honraet et al., 2005). The tetrazolium salt (XTT) is intracellularly reduced to a watersoluble formazan, which is colorimetrically determined in the cell supernatant (Roehm et al., 1991). XTT reduction depends on cell activity, instead of cell mass. In a mature biofilm, while nutrient-rich upper layers exhibit a relatively higher metabolic activity, nutrient-deficient basal layers have been shown to have lower metabolic activity and biofilm bottom cell layers tend to be relatively quiescent at later stages of biofilm formation (Silva et al., 2008). For this reason, some authors have questioned its accuracy in biofilm quantification. Nevertheless, XTT assay has become the preferred tool due to the rapidity and the ability to detect live yeast and hyphal organisms in the biofilm (Nett et al., 2011).

In our study, we assessed the effect of the five disinfectants commonly used in clinical environments on the Candida and its biofilm formation phases. A colorimetric XTT assay was used to determine the percentage of viable cells in biofilms following disinfectant treatment. Additionally, transmission electron microscope (TEM) was also used to view the effect of biocides on this fungus. To our knowledge, we report here for the first time, the effects of disinfectants mentioned on Candida and its biofilm development phases.

MATERIALS AND METHODS

In this study, five clinical Candida isolates (C. albicans 1467431, C. glabrata 1472762, C. parapsilosis 427600, C. krusei 1506110 and C. tropicalis 1508018) were used obtained from our culture collection. These isolates were categorized as apparent biofilm producers previously by congo red agar, visual tube and spectrophotometric microtiter plate methods (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.

Disinfectants tested

Disinfectants were obtained from their manufacturers; hydrogen peroxide (HP), (Akkim, Istanbul, Turkey), sodium hypochlorite (SH), (Mooncid Pulverex, Doganyal Chemstry, Istanbul, Turkey), peracetic acid (PA) (Akkim, Istanbul, Turkey) and glutaraldehyde (GLU) (Cidex, Johnson and Johnson Medical GmbH Norderstedt, Germany) as active ingredient; ortho-phtalaldehyde (OPA) (Johnson & Johnson Medical GmbH Norderstedt, Germany) and as commercial ready-to-use form. The biocide 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.

Effect of disinfectants on Candida biofilms

The biofilms of Candida strains 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 total of twenty plates were designed for each isolate and each time period (6, 12, 24, and 48th h). For each disinfectant, a separate column was designed: 1, 3, 5, 7, and 9th columns. The first three wells were chosen to be control (without disinfectant) and the next three wells were used for a disinfectant in each column. However, the first three wells in 11th column were served as negative controls including sterile Sabouraud dextrose broth alone. Dilutions of disinfectant were freshly prepared on the same day testing was done.

Prior to study, organisms were grown on SDA at 35°C 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 3×10^5 CFU/ml with Sabouraud dextrose broth (SDB) supplemented with glucose (final concentration, 8%). Each well was inoculated with aliquots of 20 μl of yeast cell suspension and 180 μl of SDB supplemented with glucose. Each plate was incubated for the previously determined time points at 35°C. After biofilm formation, the medium was aspirated and planktonic cells were removed by thoroughly washing (BIO-TEK ELx50) at three times with sterile phosphate-buffered saline (PBS). A 200 μl aliquot of disinfectants was then added except the control wells. According to the manufacturer’s recommendations, the contact times of SH, HP, OPA and PA were selected as 10 min. Only for GLU, the contact time was 20 min. For this reason, firstly GLU was added on plates and 10 min later, other disinfectants was added. So, 20 min exposure time for GLU was ensured. All experiments were performed in triplicate.

Quantification of the biofilm by XTT assay

The XTT assay was used to analyze the presence of viable cells in each of the wells following disinfectants supplementation in comparison with disinfectant-free controls (Chaieb et al., 2011; Kiraz et al., 2011). It measures the reduction of a tetrazolium salt (2, 3-Bis [2-methoxy-4-nitro-5-(sulfenylamino) carbonyl-2H-tetrazolium-hydroxide] (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) was dissolved in acetone at a concentration of 10 mM and then diluted 1:10 with saline. Final solutions including 0.5 mg/ml XTT with 125 μM menadione were prepared in saline. After the disinfectant exposure, plates were washed with PBS for three times to remove the disinfectant. Two hundred μl SDB and 50 μl XTT-menadione solution were added to each wells and the plates were incubated at 37°C in the dark chamber for 2 h. Oxidative activity was then measured at 405 nm using a microtiter plate reader (BIO-TEK, ELx800, USA).

Transmission electron microscopy

For TEM observations, 1 milliliter of the cell suspension at the concentration of 1×10⁶ cells/ml were inoculated on a Sabouraud dextrose agar plates and incubated at 37°C for 12 h. Each disinfectant was prepared at the recommended concentration and dropped onto the plates, separately. After the disinfectants were treated with test isolate at contact times 10 min, a small block of yeast containing agar was withdrawn from plate. The blocks were fixed with 2.5% glutaraldehyde, rinsed in buffer, postfixed with 1% osmium tetroxide in 0.1 M potassium phosphate for 2 h at room temperature, dehydrated in ethanol and embedded in Epon-Araldite resin. Ultrathin sections (60 nm) were stained with uranyl acetate and lead citrate and observed under a TEM (JEOL JEM 1220) (Sangetha et al., 2009).

Statistical analysis of data

The data from experiments were analysed using the SPSS 19.0 statistics package. The differences in the degree of biofilm formation of disinfectant supplementation were examined by the Mann Whitney U test, Kruskal Wallis H test, Wilcoxon test and Friedman test. P<0.05 were considered significant.

RESULTS

Important variations in metabolic activities of Candida species following the disinfectant exposure was observed by XTT method. According to the Mann Whitney U test results, the absorbance values of the control group were significantly higher than those from the disinfectant treatment groups (p<0.05). Thus, the disinfectants apparently reduced the biofilm compared to the control group. In this study, the disinfectant effectiveness showed the significantly differences according to the biofilm development phase. On the other hand, this effect varied time- and species-dependently.

Friedmant test data demonstrated a time-dependent effectiveness of disinfectants on Candida biofilms (p<0.05) Wilcoxon test results revealed that the disinfectants showed a minimum effect against biofilms at 24th h whereas it was maximum at 48th h (p<0.05). (Figure 1).

Kruskal Wallis H test also showed the differences in the effectiveness among the disinfectants. While SH, OPA, PA and GLU were not observed important differences at 6th h, the effectiveness of HP was found significantly
TEM of C. albicans

According to the TEM findings, the disinfectants we tested were able to affect Candida cell viability and the structural integrity of the cells. Figure 3 shows the TEM photomicrographs of the disinfectant untreated cells and treated planctonic cells of C. albicans. Control C. albicans cell as examined by TEM showed typical morphology of Candida with a uniform control density, typically structured nucleus and vacuoles. The cytoplasm was enveloped by a typical structure of cell membrane and cell wall (Figure 3a.) After 10 min of exposure to the disinfectants, the tested products showed essential effects on the viability and the structural integrity of the cells. The damage of the blastoconidia exposed to the SH was found mainly inside the cells and characterized as cytoplasm congealing (protein denaturation) (Figure 3d). The PA and OPA treated cells exhibited notable alterations in the cell membrane and the cell wall (Figures 3c and 3f). The cytoplasmic volume decreased and the cell membrane invaginated with notable structural disorganization within the cell cytoplasm. HP (Figure 3b) showed especially structural changes in the blastoconidial cytoplasm, that is, the cytoplasm appeared rougher when compared with the untreated blastoconidia. Cytoplasm congealings and vacuoles were shown in the cytoplasm. However, cell membrane and cell wall were regular and intact, generally. GLU treated cells showed the membrane damage and denaturated rough cytoplasm (Figure 3e).

DISCUSSION

Biofilms are microbial communities, embedded in a polymeric matrix, growing attached to a surface. They have characteristic architecture and distinct phenotypic properties (Nett et al., 2007). The cells in biofilm exhibit antimicrobial resistance that is often 50-1000 times higher than that in their planktonic counterparts (Lee et al., 2007). The explanation of this resistance may be due to slow growth rate and nutrient limitation, differential expression of drug resistance genes or extracellular matrix (Theraud et al., 2004). Recently, Candida infections are commonly associated with biofilm formation and they are difficult to treat. This paper describes the effect of the five disinfectants commonly used in clinical environments on the Candida and its biofilm formation phases.

Our results showed that the many variations in biofilm formation in clinical Candida species following disinfectant stress. For example, at 0th -12th h which consist of early phase biofilm, C. parapsilosis 427600 (at 12th h) and C. krusei 1506110 (at 6th h) isolates have resisted to disinfectants. However, at 24th - 48th h the disinfectant activities have increased compared with the early phase. In fact, it is expected the thickening of biofilm layer and the decrease of disinfectant activity with time. In the present study, only for C. tropicalis 1508018 isolate, the killing effect of the disinfectants decreased by the time as expected. This may probably be also since the difficulties of disinfectant to reach to inner cells as the biofilm layer gets thicker. But in other isolates, either disinfectants have been more effective with time or they have shown similar effects at all time points. We can not explain the cause of this condition exactly but this may be probably since the XTT assay have become more sensitive as the biofilm matures. Perhaps, it might be also easier to remove by mechanical effect when the biofilm mass was abundant. In the present study, none of the tested disinfectants completely removed the Candida biofilm. This is not surprising because the results of many studies clearly proved the generally accepted fact of the decreased sensitivity of biofilm cells to disinfectants in the comparison to planctonic cells (Nett et al., 2008; Smith and Hunter, 2008).

We used XTT method in our study as it is considered ideal for quantification of Candida biofilms and the testing of their susceptibility to antimicrobials (Ramage et al., 2001), (Figure 2). XTT depends on cell activity, but some authors have questioned its accuracy in biofilm quantification (Silva et al., 2008).

Thein et al. showed that the XTT assay results can vary in accordance with the sensitivity of different species of Candida to tetrazolium salts (Thein et al., 2007). Kuhn et al. also (2003) reported that the XTT method is useful for comparisons involving one strain; its use may be more problematic in attempts to compare different fungal strains and species. Similarly, Nett et al. also indicated that the processing of XTT may differ among Candida strains (Nett et al., 2008). In our study also, XTT method showed the many variations and this results are in agreement with those reported earlier.

As known, biocides must traverse the outer cell layer to reach their target sites, usually present within microbial cells. However the information as to how this uptake is achieved is somewhat limited (Russell, 2003). Although the variations in cell structure or complexity, it is clear that some common target sites might be present in vegetative cells of different species. In generally, our electron microscopic findings showed that interaction between the tested disinfectants and the blastoconidia mainly occurred on the surface of the cells. Apparently, the cytoplasm appeared rougher when compared with the control. Apparently, OPA has also shown its main effect on cell wall membrane, whereas HP did not exhibit an important change in cell wall and cell membrane.

The results are in general agreement with those reported previously (Kurzatkowski et al., 2010; Anil et al.,

high. At 48th h, HP was found to be more effective than PA. The differences were not seen among the other all disinfectants in terms of biofilm effectiveness.
As a result, our studies showed that the activity of different disinfectants on Candida biofilms varied greatly even between different species of the same genus. However, although the biofilms have been repeatedly shown to have a decreased susceptibility to antimicrobials, this may not always be the case. So, each isolate must be considered on its own as to its susceptibility of biocides. In this study, although the disinfectants have caused serious damage on the fungi itself, they have remained inadequate against its biofilms. The mechanisms of this resistance also may be multifactorial. So we think that the detailed studies should be investigated about the biocide response on early-, intermediate-, and mature-phase biofilms. On the other hand, XTT assay may not be the only adequate method to evaluate the efficacy of disinfectants. Because, the

Figure 2. Percentage reduction rates in Candida biofilms following treatment with different disinfectants method at 6, 12, 24 and 48th h by XTT method.
cells that were submitted to a stress by the use of disinfectants may not respond well to XTT evaluation. So, usage of XTT assay should be combined with the other analyses for comparisons across different strains and species.

ACKNOWLEDGEMENT

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

REFERENCES


