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Effectiveness of Disinfectants Used in Hemodialysis against both *Candida orthopsilosis* and *C. parapsilosis* Sensu Stricto Biofilms

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Biofilms have been observed in the fluid pathways of hemodialysis machines. The impacts of four biocides used for the disinfection of hemodialysis systems were tested against *Candida parapsilosis* sensu stricto and *Candida orthopsilosis* biofilms generated by isolates obtained from a hydraulic circuit that were collected in a hemodialysis unit. Acetic acid was shown to be the most effective agent against *Candida* biofilms. Strategies for effective disinfection procedures used for hemodialysis systems should also seek to kill and inhibit biofilms.

The water treatment system is a matter of major concern in hemodialysis, and reports have described its contamination by biofilms (1, 2). For disinfection, active chemical agents (biocides) have been introduced into routine practice (3). Hemodialyzers are disinfected with peracetic acid, and hemodialysis systems (which include hemodialysis machines, the water supply, water treatment systems, and distribution systems) are typically disinfected using chlorine-based disinfectants at an aqueous concentration of 500 ppm (3–5). Low-pH cleaning agents have also been used as disinfectants (4), and 3% hydrogen peroxide (vol/vol) may be used to treat biofilms on implants, on the implant-surrounding tissue, on the skin surface, or on infected wounds without devices (6).

In South American hospitals, the incidence of *Candida parapsilosis* is greater than that of *Candida albicans* (7, 8), and previous results from our laboratory (9–11) have shown that *C. parapsilosis* is predominantly implicated in the contamination of water samples collected at a hemodialysis center. Recently, *C. parapsilosis* isolates were classified into three distinct species: *C. parapsilosis* sensu stricto, *Candida orthopsilosis*, and *Candida metapsilosis* (12).

Although the nephrology community has been provided with information about the effects of disinfectants on microbial suspensions (3–5, 13, 14), few studies have evaluated the efficacy of disinfection in the presence of biofilms, particularly fungal biofilms. Therefore, the biocidal efficacy of the commercially available concentrations of biocides used for the disinfection of hemodialysis systems was evaluated against both *C. parapsilosis* sensu stricto and *C. orthopsilosis* biofilms. Additionally, the effects of concentration on the time required for effective treatment were compared between the more efficacious biocides and the standard established by legislation.

One hundred *C. parapsilosis* isolates were obtained from the sampling of water held in the hemodialysis unit studied (disinfection with a 30-min exposure to sodium hypochlorite at 500 ppm on a daily basis) located in the state of São Paulo, Brazil, between March 2006 and March 2007. The identities of the yeast isolates were determined by using a PCR-based method (12, 15) and specific primers directed against the secondary alcohol dehydrogenase (SADH) gene; 53 were assigned to the species *C. parapsilosis* sensu stricto, and 47 to *C. orthopsilosis*. The strains were stored in sterile, distilled water (16) in our laboratory. Fifteen strains each of two *Candida* species, namely, *C. parapsilosis* sensu stricto (WCP3,

WCP4, WCP6, WCP8, WCP10, WCP14, WCP16, WCP17, WCP24, WCP82, WCP83, WCP87, WCP88, WCP104, WCP108) and *C. orthopsilosis* (WCO33, WCO45, WCO52, WCO53, WCO125, WCO139, WCO147, WCO154, HMC0B, HMC0C, HMC0M, HMC0Q, HMC0Q1, HMC0R, HMC0U), were selected for this study. All *Candida* strains were subcultured on Sabouraud's dextrose agar (SDA; Gibco Ltd., Paisley, United Kingdom) and maintained at 4°C during the experimental period.

Acetic acid (C₂H₄O₂; 0.5% [vol/vol]; Merck, Darmstadt, Germany), hydrogen peroxide (H₂O₂; 3% [vol/vol]; Synth, SP, Brazil), sodium hypochlorite (NaOCl; 2% [wt/vol]; Merck), and a commercial biocide made of peracetic acid and hydrogen peroxide (CH₃CO₃H and H₂O₂; 1.34% [vol/vol] and 4.2% [wt/vol]; Proxitane; Fresenius Medical Care, Bad Homburg, Germany) were tested against *Candida* biofilms. All of the biocide stock solutions were diluted in filter-sterilized phosphate-buffered saline (PBS; 10 mM phosphate buffer, 2.7 mM potassium chloride, 137 mM sodium chloride, pH 7.4; Sigma Chemical Co., St. Louis, MO), followed by further dilution in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with L-glutamine and buffered with 0.165 M morpholinepropanesulfonic acid (MOPS; Sigma) at concentrations ranging from 0.002 to 2.7 g/liter for acetic acid, 0.01 to 15 g/liter for hydrogen peroxide, 0.02 to 20 g/liter for sodium hypochlorite, 0.006 to 6.7 g/liter for peracetic acid, and 0.02 to 21 g/liter for hydrogen peroxide.

All of the studied strains were screened for their ability to form biofilms by following the methodology previously described by Ramage et al. (17), and semiquantitative measurement of the growth biofilms was performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma) reduction assay (18–21) at 540 nm. *C. albicans* SC5314 was used as the biofilm control strain (22, 23).

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TABLE 1 The *in vitro* efficacy of biocides against *Candida orthopsilosis* and *C. parapsilosis* sensu stricto biofilms

<i>Candida</i> species	Compound ^a	Range(s) of concn (g/liter) tested	Concn required to kill biofilm cells in treatment assays (g/l) ^{b,c}	Concn required to inhibit biofilm formation (g/l) ^{b,d}
<i>C. orthopsilosis</i>	NaOCl	0.02–20	2.5 ± 1.36	1.25 ± 0.68
	H ₂ O ₂	0.01–15	3.75 ± 1.02	0.93 ± 0.09
	C ₂ H ₄ O ₂	0.002–2.7	0.33 ± 0.07	0.04 ± 0.01
	CH ₃ COOOH and H ₂ O ₂	0.006–6.7 and 0.02–21	0.83 ± 0.18 and 2.6 ± 0.58	0.21 ± 0.09 and 0.6 ± 0.22
<i>C. parapsilosis</i> sensu stricto	NaOCl	0.02–20	2.5 ± 0.66	2.5 ± 0.55
	H ₂ O ₂	0.01–15	1.87 ± 0.51	0.47 ± 0.03
	C ₂ H ₄ O ₂	0.002–2.7	0.33	0.04
	CH ₃ COOOH and H ₂ O ₂	0.006–6.7 and 0.02–21	0.83 ± 0.29 and 2.6 ± 0.93	0.41 ± 0.18 and 0.3 ± 0.13
<i>C. albicans</i> (SC 5314)	NaOCl	0.02–20	2.5 ± 0.55	2.5 ± 1.11
	H ₂ O ₂	0.01–15	1.87 ± 0.42	0.47 ± 0.06
	C ₂ H ₄ O ₂	0.002–2.7	0.33 ± 0.09	0.02 ± 0.01
	CH ₃ COOOH and H ₂ O ₂	0.006–6.7 and 0.02–21	0.83 ± 0.23 and 2.6 ± 0.71	0.21 ± 0.04 and 0.6 ± 0.16

^a NaOCl, sodium hypochlorite; H₂O₂, hydrogen peroxide; C₂H₄O₂, acetic acid; H₃COOOH and H₂O₂, peracetic acid and hydrogen peroxide (Proxitane).

^b The inoculum used for the inhibition assays was identical to the cell density used for the treatment assays (10⁶ cells/ml). The concentrations obtained from the optical density at 540 nm (MTT assay) correlated with the total viable count (CFU/ml). The values are means ± 1 standard deviation (SD) from four experiments for each disinfectant. The experiments were repeated three times with at least four replicates at each time point.

^c Strains: *C. orthopsilosis*, WCO33, WCO45, WCO52, WCO53, WCO125; *C. parapsilosis* sensu stricto, WCP3, WCP4, WCP6, WCP8, WCP10.

^d Strains: *C. orthopsilosis*, HMCOM, HMCOQ, HMCOQ1, HMCOR, HMCOU; *C. parapsilosis* sensu stricto, WCP83, WCP87, WCP88, WCP104, WCP108.

The experiments were divided into three set of experiments: (i) inhibition of biofilm formation, (ii) treatment of preformed biofilms with all biocides for 48 h of incubation, (iii) time-kill curves for more active biocides on established *Candida* biofilms (24 h old). For each group of the assays, five strains of *C. orthopsilosis*, five strains of *Candida parapsilosis* sensu stricto, and the strain *C. albicans* SC 5314 were used. For the inhibition assays, the biocides were added at the time of inoculation. For the treatment assays, the biocides were diluted in RPMI and added to established biofilms (24 h). Microtiter wells containing heat-killed *Candida* (10⁶ CFU/ml) were included as negative controls, and biofilms incubated in the absence of biocides were included as positive controls (24). After the desired contact times (0.5 min, 1 min, 5 min, 15 min, 30 min, 60 min, and 48 h), the test product was discarded, and the wells were washed twice with PBS. The use of a neutralizer was unnecessary; preliminary studies showed no differences between wells with and without neutralizer (data not shown). After each exposure to a biocide, the percentage of the metabolically active total cell population was determined by CFU enumeration on Sabouraud dextrose agar (Difco Laboratories, Detroit, MI) incubated at 30°C for 48 h. A standard curve was constructed to correlate the results obtained from the MTT assay with the calculated CFU/ml (limit of detection, 10 CFU/ml), and the data were expressed as the decimal logarithm. No change in optical density over the negative control was equal to ≤10 CFU/ml and was defined as the lowest concentration of biocide which resulted in total inhibition or in total killing of sessile cells. The experiments were repeated three times with at least four replicates for each time point.

The groups were compared using a *t* test and a one-way analysis of variance (ANOVA), followed by the *post hoc* Tukey test when all of the disinfectants were compared with the standard following 30 min of incubation. Logistic regression analyses were used to investigate relationships between MTT assay absorbance readings and the total viable count using the SPSS 15.0 software

package (SPSS Inc., Chicago, IL). All tests were performed using a significance level of *P* values of ≤0.05.

Overall, biofilm production by *Candida* was observed for 34 of *C. parapsilosis* sensu stricto strains and 38 of *C. orthopsilosis* strains. MTT activity was linearly associated with CFU counts, and the correlation coefficients (*R*²) were 0.8831, 0.9045, and 0.9178 for *C. parapsilosis* sensu stricto, *C. orthopsilosis*, and the reference strain *C. albicans* SC 5314, respectively. Strains for which the optical density was of 2.84 ± 0.059 and 2.3 ± 0.094 for *C. orthopsilosis* and *C. parapsilosis* sensu stricto, respectively, were scored as better biofilm formers and were selected for antibiofilm assays.

Candida biofilms were exposed to the biocides. After the biofilms were treated with NaOCl, C₂H₄O₂, or CH₃COOOH and H₂O₂, the results were similar for all the tested species (Table 1). However, when H₂O₂ was evaluated, the efficiency for *C. orthopsilosis* was the lowest compared to that of other *Candida* species. Still, the biocides concentration needed to inhibit the biofilm formation was dependent of the *Candida* species (Table 1). Additionally, a standard biocide (NaOCl, 0.5 g/liter) was compared to the experimental biocides with the best results (Fig. 1). A solution of NaOCl had little effect on the biofilm viability of all of the preformed (24-h) *Candida* biofilms (Fig. 1A to C). *C. orthopsilosis* and *C. parapsilosis* sensu stricto biofilm-embedded cells were killed after 1 min of C₂H₄O₂ (0.33 g/liter) contact, whereas for reference strain *C. albicans* SC 5314 biofilm cells (Fig. 2), the same effect was obtained after 2 min of exposure.

The presence of biofilm in dialysis systems is a point of concern, first because biofilms continuously release microbial components, such as toxin fragments, peptides, and polysaccharides, able to traverse dialysis membranes (14). To our knowledge, this is the first published report of the effects of biocides used for hemodialysis disinfection on *C. parapsilosis* sensu stricto and *C. orthopsilosis* biofilms.

In our study using preformed (24-h) *Candida* biofilms, 48 h of treatment with NaOCl (2.5 g/liter) killed biofilm cells of all *Can-*

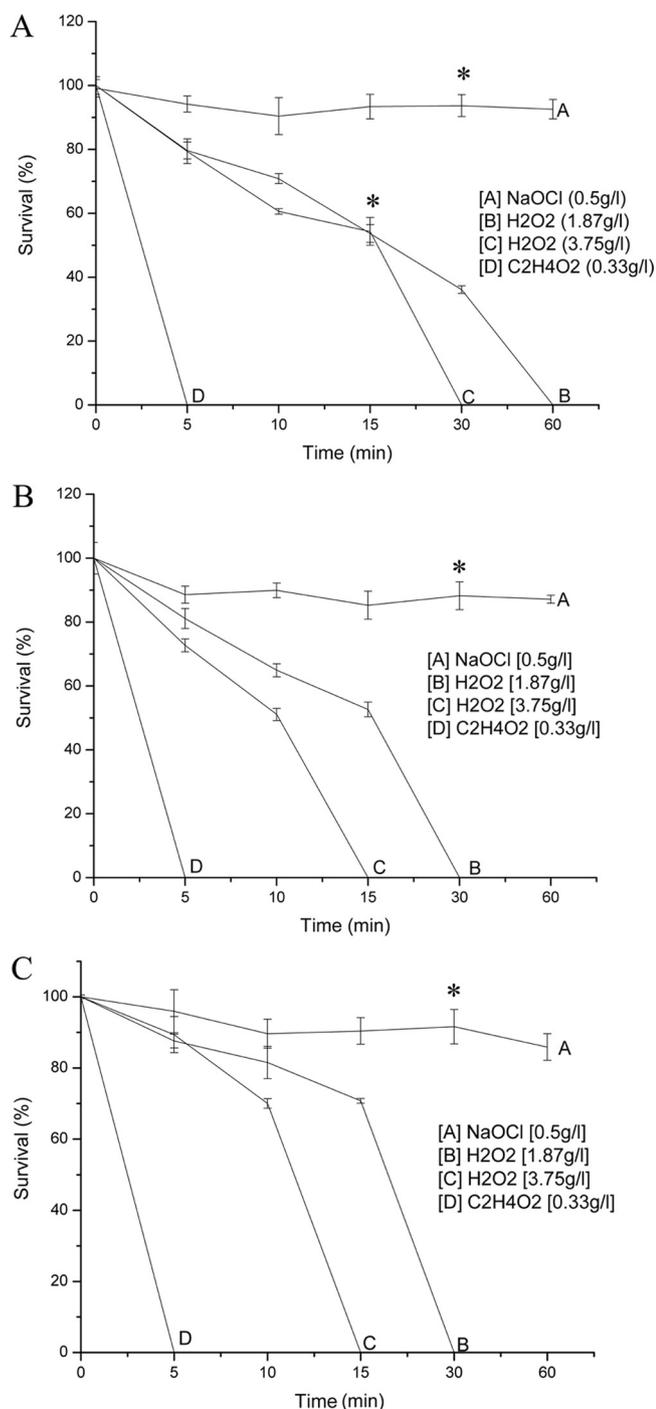


FIG 1 A comparison of the changes in the percent survival of *C. orthopsilosis* (WCO139, WCO147, WCO154, HMC0B, HMC0C) (A), *C. parapsilosis* (WCP14, WCP16, WCP17, WCP24, WCP82) (B), and *C. albicans* SC 5314 (C) biofilms after treatment with disinfectants (H_2O_2 [hydrogen peroxide], 1.87 g/liter or 3.75 g/liter; $\text{C}_2\text{H}_4\text{O}_2$ [acetic acid], 0.33 g/liter); or the standard biocide (NaOCl [sodium hypochlorite], 0.5 g/liter) obtained by the MTT reduction assay. Biofilms were cultured in RPMI medium for 24 h and tested against biocides for 5, 10, 15, 30, and 60 min of contact. Each point represents the average of four measurements, and the error bars represent the standard deviations. The experiments were repeated three times with at least four replicates at each time point, *, $P \leq 0.00001$, comparing all disinfectants with the standard in 30 min.

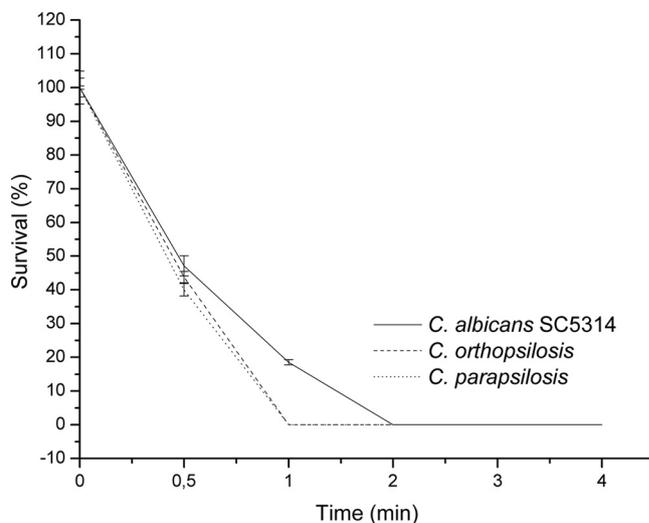


FIG 2 The effects of acetic acid on preformed *Candida* biofilms. The metabolic activities of *C. orthopsilosis* (HMCOM, HMCOQ, HMCOQ1, HMCOR, HMCOU), *C. parapsilosis* (WCP83, WCP87, WCP88, WCP104, WCP108), and *C. albicans* SC 5314 were measured by the MTT reduction assay. Sessile yeast cells were exposed to acetic acid at a concentration of 0.33 g/liter for periods of 30 s to 5 min, and the biofilm reduction was compared with the untreated control. The bars represent the averages of three MTT measurements, and the brackets denote the standard deviations. The experiments were repeated three times with at least four replicates at each time point.

*did*a biofilms and inhibited biofilm formation by *C. parapsilosis* sensu stricto and *C. albicans* SC 5314. For *C. orthopsilosis*, the concentration of 1.25 g/liter of NaOCl inhibited biofilm formation (Table 1). The tested concentrations are extremely corrosive, irritate mucosal surfaces, and are higher than those recommended by previous legislation (4, 5) for the disinfection of hemodialysis systems (500 ppm; 0.5 g/liter). However, these guidelines are not adequate for sessile microorganisms.

A previous work (25) focusing on microbial contamination in water treated for use in dialysis reported that the impact of treatment with hypochlorite on the water quality was negative due to detachment of the biofilm and resuspension of heavy loads of microorganisms in water immediately after disinfection. Roeder et al. (26), who investigated the effect of disinfectants against biofilms in drinking water, described that intervention eventually selects persistent microorganisms that can live on the waste of dead cells present in the biofilm. Limited disinfection efficacy against biofilm attached to a granular activated carbon filter has been demonstrated by LeChevallier et al. (27). Additionally, Norman et al. (28) have provided evidence that a 165- μm -thick biofilm chlorinated at 2.0 or 4.1 g/day rapidly returned to its original thickness as soon as the treatment was discontinued.

Peracetic acid and hydrogen peroxide also suppressed biofilm formation, and the former inhibited *Candida orthopsilosis* and *C. albicans* biofilm formation at concentrations below 0.3%. This combination of disinfectants has been successfully employed in Brazil (29) to reprocess dialyzers from the same patients. The effectiveness of the mixture of these biocides against bacterial biofilms in water treatment systems has also been described in the literature (26, 30, 31). However, in addition to being very expensive, the combination of hydrogen peroxide and peracetic acid can damage the hemodialysis hydraulic system.

Therefore, in an attempt to identify more economical and less harmful products, two compounds, hydrogen peroxide and acetic acid, were tested against *Candida* biofilms. In this work, H₂O₂ at concentrations below 3% (Table 1) effectively inhibited biofilm growth and killed the biofilm populations, with the exception of *C. orthopsilosis* (3.75 g/liter). Nett et al. (32) reported that a higher concentration of H₂O₂ was needed to reduce the *C. parapsilosis* biofilm burden. Additionally, H₂O₂ efficacy in water system disinfection has been described by Wong et al. (33).

Acid sanitizers are generally utilized at a concentration of 100 ppm or 0.1 g/liter (4). In this study, acetic acid proved to be a better antibiofilm agent against all of the assayed strains, a result similar to that obtained for biofilms consisting of *Listeria monocytogenes*, *Staphylococcus aureus*, and *Salmonella* spp. (34–36).

Since no method presently exists that detects biofilm *in vivo* with sufficient sensitivity to confirm its eradication, efforts in optimizing the cleaning and disinfection procedures used for hemodialysis systems should also seek to achieve biofilm, especially fungal biofilms. As demonstrated by this study, the standard current biocide (sodium hypochlorite, 500 ppm or 0.5 g/liter) failed to destroy the *Candida* biofilms tested. Our results showed that hydrogen peroxide or acetic acid may be alternatives, because they are more efficient than sodium hypochlorite. Further studies are needed to determine whether these agents can also be effective against additional *Candida* species. Ensuring water quality results in reduced patient morbidity and number of hospitalizations and improved quality of life.

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