

Efficacy of antiseptics and disinfectants on clinical and environmental yeast isolates in planktonic and biofilm conditions

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The aim of this study was to evaluate the efficacy of five antiseptics, three surface disinfectants and UV radiation against a wide range of clinical and environmental yeast isolates. Their efficacy against pure cultures, yeast mixtures and biofilms (prepared by culturing yeasts in Sabouraud broth containing a final concentration of 8% glucose) was tested. Three clinical isolates of *Candida albicans*, *Cryptococcus neoformans* and *Rhodotorula rubra*, and two environmental isolates of *Candida albicans* and *Cryptococcus uniguttulatus* were selected. For seven out of eight biocides tested (Betadine, Dermacide, Chlorhexidine, Dosisepsine, hydrogen peroxide, sodium hypochlorite, 70% alcohol, 0.5% Ecodiol) and for UV radiation, susceptibility did not differ according to genus, species or origin. Hydrogen peroxide, 0.25% Ecodiol and UV radiation were ineffective against the five isolates tested. On pure planktonic cultures, and, to a lesser extent, on free-living yeast mixtures, the other products were active and were then tested against biofilms: eight out of nine biocides were ineffective. Chlorhexidine at 0.5% was the only fungicidal agent on pure cultures, yeast mixtures and biofilms. The importance of the test method, including agent concentration, is discussed.

INTRODUCTION

Although the environment is a well-known source of human *Aspergillus* infection (Anaissie & Costa, 2001; Carter & Barr, 1997; Leenders *et al.*, 1999), there are few data on environmental sources of human pathogenic yeasts. *Cryptococcus* spp., *Histoplasma capsulatum* and *Rhodotorula* spp., reported as serious opportunistic yeasts in humans, are widespread in the environment. Like *Candida* spp., they have been isolated from animals (particularly birds and mammals) and also from sea water and trees (Blaschke-Hellmessen, 1999, 2000; Camin *et al.*, 1998; De Vroey, 1979; Odds, 1984; Younglove *et al.*, 1968). Yeast survival outside the host is poorly understood (Koike *et al.*, 1992; Odds, 1991; Valdes-Collazo *et al.*, 1987). We have found that *Cryptococcus* spp. and also *Candida albicans* survive well in the environment, i.e. more than 24 weeks in an experimental mixture of soil and water, at 20 °C and 30 °C (Théraud *et al.*, 2003). Therefore, fighting against yeast reservoirs in the hospital environment would contribute to diminishing the risk of nosocomial infections.

In France, all antiseptics for living tissue and surface disinfectants are tested against reference strains of two pathogenic fungi (*Candida albicans* and *Aspergillus niger*) before marketing (French AFNOR standards NF T 72-202 and XP T 72-300/1, available from <http://www.boutique.afnor.fr>). Limited data are available on the impact of fungal biodiversity and testing practical conditions on the antifungal activity of biocides. Here we tested the *in vitro* efficacy

of several antiseptics and disinfectants against a variety of yeasts. Tests were done with both pure and mixed suspensions of various genera and species of planktonic yeasts (*Cryptococcus* spp., *Candida albicans* and *Rhodotorula rubra*) isolated from humans or the environment. As yeast cells in biofilm conditions display markedly increased resistance to antifungal agents used in human therapy (Chandra *et al.*, 2001b; Hawser & Douglas, 1995; Ramage *et al.*, 2001), we also tested the activity of biocides against yeast cells in biofilms, in comparison to planktonic conditions.

METHODS

Yeast strains. We tested three clinical isolates (*Candida albicans*, *Cryptococcus neoformans* and *R. rubra*) and two environmental isolates (*Cryptococcus uniguttulatus* isolated from a patient's room in Rennes teaching hospital and *Candida albicans* isolated from a starling dormitory). All cryopreserved isolates were obtained from the culture collection of the Laboratoire de Parasitologie-Mycologie, Faculté de Médecine de Rennes, France.

Planktonic cell treatment and evaluation of efficacy. In the first set of experiments, 5 ml pure suspensions of each isolate (final density 6×10^8 c.f.u. ml⁻¹) were incubated at room temperature for 5 min following AFNOR methodologies, with an equal volume of each of five antiseptics (Table 1) and three disinfectants (Table 2) at different concentrations, or were exposed to a physical disinfecting method using UV radiation for 30 min (Table 2). Consecutive treatment with 0.5% Ecodiol followed by 1.2° sodium hypochlorite was also tested.

Table 1. Antiseptics for living tissues tested

Trade name	Active compound and concentration
Betadine, Asta medica, Mérignac, France	10 % Iodine polyvinylpyrrolidone
Dermacide, C.S. Laboratoires, Paris, France	2 % Sodium laurylsulfate
Chlorhexidine, Gilbert, Hérouville, France	0.5 % Chlorhexidine digluconate
Dosisepsine, Gifrer and Barbezat, Decines, France	0.05 % Chlorhexidine digluconate
Hydrogen peroxide, Gifrer-Barbezat, Decines, France	3 % Hydrogen peroxide

Table 2. Surface disinfectants used

Name/trade name	Active compound
Sodium hypochlorite (Javel ideale), Pieri Chimie, Portes-Les-Valence, France	1.2 ° Sodium hypochlorite (= 0.38 %, w/v = 3800 p.p.m.) 12 ° sodium hypochlorite (= 3.8 %, w/v = 38 000 p.p.m.)
70% Alcohol	70 % Ethanol
Ecodiol, Prodene Klint, Alby/Cheran, France	Surface active agents, alkylamine
Ultraviolet radiation	365 nm, 50 cm

In a second set of experiments, the most effective agents at optimal concentration (Betadine, Chlorhexidine, 1.2 ° sodium hypochlorite, 70 % alcohol, 0.5 % Ecodiol and 0.5 % Ecodiol) followed by 1.2 ° sodium hypochlorite) were then tested (5 min, v/v) on mixed suspensions prepared with equal quantities of each isolate (final density 6×10^8 c.f.u. of each isolate ml^{-1}), as follows: (i) an 'environmental' mix of *R. rubra* plus the environmental *Candida albicans* and *Cryptococcus* isolates and (ii) a 'clinical' mix of *R. rubra*, *Candida albicans* and *Cryptococcus neoformans* isolates.

For these two sets of experiments, yeast cells were harvested after treatment and washed three times in 0.9 % NaCl. Survival rates of treated and untreated control suspensions were determined by retro-culture in 96-well tissue culture microplates (Tissue Culture Testplate; TPP, Switzerland) and the serial dilution assay method (tenfold dilutions from 10^8 to 1 c.f.u. per well), with Sabouraud broth (20 g glucose l^{-1} and 10 g pancreatic peptone l^{-1} ; Prolabo) after 48 h incubation at 30 °C. Each experimental condition was done twice, tested ten times in duplicate, and results are expressed as means \pm SD \log_{10} viable yeast cells ml^{-1} , compared to the appropriate untreated control.

Biofilm treatments and evaluation of efficacy. Antiseptic and disinfectant efficacy was tested on biofilms formed by pure and mixed suspensions of the five isolates. Biofilms were produced in 96-well microtitre plates, by using a modification of the methods described by others (Ramage *et al* 2001; Shin *et al.*, 2002). Briefly, biofilms of each suspension were prepared using a serial dilution method (tenfold dilutions from 10^8 to 1 c.f.u. per well), and growing for 24 hours at 37 °C in Sabouraud broth supplemented with glucose (final concentration 8 %). After biofilm formation as assessed by light microscopy, the medium was aspirated, and nonadherent cells were removed by thoroughly washing the biofilms three times in sterile 0.9 % NaCl. Biofilms were then treated for 5 min with 200 μl of the most effective agents on planktonic suspensions. After three washes in 0.9 % NaCl, 250 μl Sabouraud broth was added. After 48 h at 37 °C, biofilms were disrupted and 20 μl were plated in Sabouraud agar (48 h at 37 °C, Sabouraud Chloramphenicol agar; Bio-Rad). Each experimental condition was tested ten times in duplicate, and results are expressed as means \pm SD \log_{10} viable yeast cells ml^{-1} , compared to the appropriate untreated control. As a positive control we also tested 12 ° hypochlorite.

Statistical analysis. Analysis of variance (ANOVA test) was run on StatView software (Abacus Concepts, Berkeley, CA, USA). *P* values < 0.05 were considered significant.

RESULTS

Pure planktonic cultures

Antiseptic and disinfectant efficacy on pure suspensions of *R. rubra*, *Candida albicans* and *Cryptococcus* species is shown in Fig. 1. UV radiation, 0.25 % Ecodiol and hydrogen peroxide resulted in a decrease of the fungal load by less than 3 \log_{10} yeast cells ml^{-1} compared to controls for the five isolates tested. These agents thus cannot be considered fungicidal according to French AFNOR standards, which require $\geq 10^4$ -fold reduction, i.e. 4 \log_{10} (AFNOR, 1995). Dosisepsine (0.05 % chlorhexidine) was effective on *R. rubra* and on both environmental and clinical *Candida albicans* isolates ($P < 0.05$), but had little effect on *Cryptococcus* species (decrease of the fungal load by less than 1 \log_{10} yeast cells ml^{-1}). In contrast, Betadine, Dermacide, 0.5 % chlorhexidine, 1.2 ° sodium hypochlorite, 70 % alcohol, 0.5 % Ecodiol and the sequence of 0.5 % Ecodiol followed by 1.2 ° sodium hypochlorite were fungicidal, reducing the fungal load by more than 7 \log_{10} yeasts ml^{-1} for the five isolates, compared to controls ($P < 0.05$).

Mixed planktonic cultures

Betadine, 0.5 % chlorhexidine, 1.2 ° sodium hypochlorite, 70 % alcohol, 0.5 % Ecodiol and the combination of 0.5 % Ecodiol + 1.2 ° sodium hypochlorite were then tested on mixed yeast suspensions (Fig. 2). With the exception of 0.5 % Ecodiol, these agents were still fungicidal on mixed yeast suspensions, but were less effective than on pure suspensions ($P < 0.05$). While 0.5 % Ecodiol used alone reduced fungal

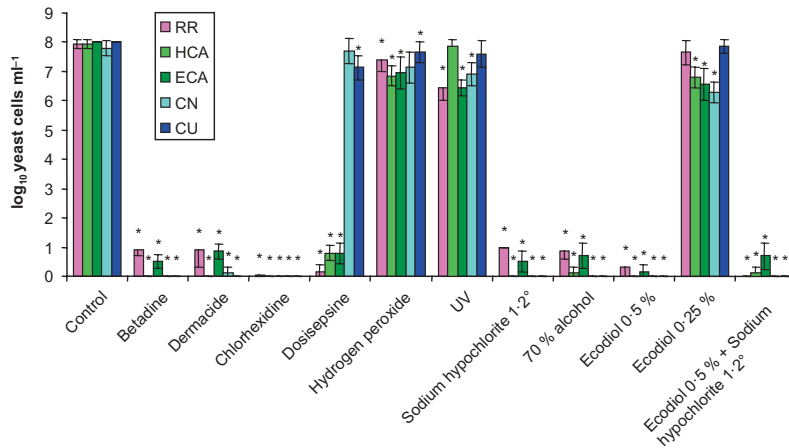


Fig. 1. Reduction in fungal load after antiseptic and disinfectant action on pure yeast suspensions. RR, *R. rubra* isolate; HCA, human *Candida albicans* isolate; ECA, environmental *Candida albicans* isolate; CN, *Cryptococcus neoformans* isolate; CU, *Cryptococcus uniguttulatus* isolate. *Significant difference vs control ($P < 0.05$)

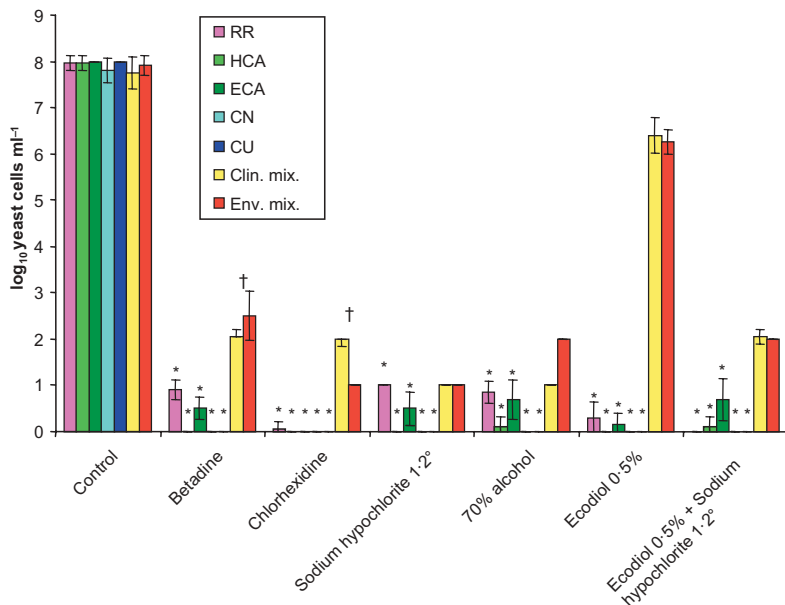


Fig. 2. Reduction in fungal load after antiseptic and disinfectant action on mixed suspensions. RR, *R. rubra* isolate; HCA, human *Candida albicans* isolate; ECA, environmental *Candida albicans* isolate; CN, *Cryptococcus neoformans* isolate; CU, *Cryptococcus uniguttulatus* isolate; Clin. mix., mixed clinical suspension (RR+HCA+CN); Env. mix., mixed environmental suspension (RR+ECA+CU). *Significant difference between planktonic and mixed suspensions ($P < 0.05$). †Significant difference between mixed environmental and clinical suspensions ($P < 0.05$).

load by only 1 to 2 \log_{10} yeast cells ml^{-1} , the combination of 0.5% Ecodiol + 1.2° sodium hypochlorite maintained its fungicidal activity.

Small but significant differences in the efficacy of some agents against mixed clinical and environmental yeast suspensions were observed. Betadine and 70% alcohol were less effective on the clinical mix than the environmental one, conversely, 0.5% chlorhexidine was less effective on the environmental mix than on the clinical one ($P < 0.05$).

Biofilms

Antiseptic and disinfectant efficacy on pure and mixed suspensions of *R. rubra*, *Candida albicans* and *Cryptococcus* species grown in biofilms is presented in Fig. 3. Only 0.5% chlorhexidine reduced fungal load in biofilm conditions by more than 4 \log_{10} yeast cells ml^{-1} and was thus fungicidal for

the five isolates and the two mixed suspensions ($P < 0.05$). None of the other agents were active on either pure or mixed yeast populations in these conditions.

As a positive control we also tested 12° sodium hypochlorite. All biofilms were sterilized (data not shown).

DISCUSSION

Antiseptics and disinfectants are broad-spectrum biocidal compounds that inactivate micro-organisms on living tissue and inanimate surfaces. Their mechanisms of action have been extensively studied, as has bacterial resistance to them (McDonnell & Russell, 1999). However, limited data are available on the susceptibility and mechanisms of resistance of reference strains of yeasts (mainly *Candida albicans* and *Saccharomyces cerevisiae*) to biocides. The aim of this study was to evaluate the efficacy of several antiseptic and disinfectant agents against various species and genera of yeasts, of

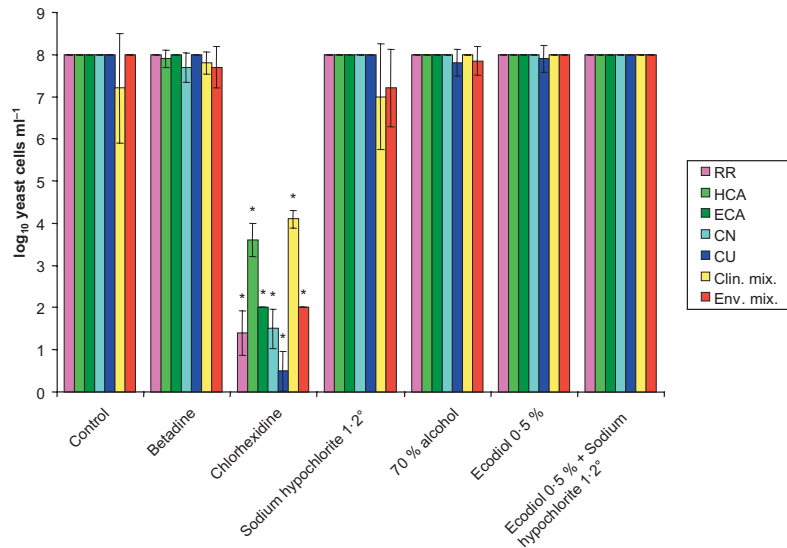


Fig. 3. Reduction in yeast load after antiseptic and disinfectant action on biofilms. RR, *R. rubra* isolate; HCA, human *Candida albicans* isolate; ECA, environmental *Candida albicans* isolate; CN, *Cryptococcus neoformans* isolate; CU, *Cryptococcus uniguttulatus* isolate; Clin. mix., mixed clinical suspension (RR+HCA+CN); Env. mix., mixed environmental suspension (RR+ECA+CU). *Significant difference vs control ($P < 0.05$).

clinical and environmental origin, in pure and mixed planktonic culture and in biofilm conditions. The 5 min contact time used here corresponds to that generally considered effective for living tissue and inanimate surface yeast decontamination.

In the first set of experiments, no difference in biocide efficacy was observed between *Candida albicans*, *Cryptococcus* species and *R. rubra* (except with Dosisepsine), or according to the clinical or environmental origin of the yeasts. The most effective agents against planktonic yeasts were Betadine, Dermacide, Chlorhexidine, 70% alcohol, 0.5% Ecodiol, 1.2° sodium hypochlorite and the combination of 0.5% Ecodiol + 1.2° sodium hypochlorite. Efficacy was closely related to the concentration of the active compound. The manufacturers of Ecodiol recommend a concentration of 0.25%. We found that Ecodiol failed to kill efficiently at this concentration, whereas 0.5% Ecodiol was fungicidal and thus should be used in practice. Similar results were observed with chlorhexidine digluconate, the 0.5% solution was more effective than 0.05%. Regarding hydrogen peroxide, which alters yeast outer membranes (Dusseau *et al.*, 1998), we found it to be ineffective on *Cryptococcus neoformans* and *Candida albicans* in our experimental conditions. These results are in accordance with those of Huahua *et al.* (1991), who found that *Cryptococcus neoformans* grew significantly better in culture bottles in the presence of hydrogen peroxide, and those of Jamieson *et al.* (1996), who showed that trace hydrogen peroxide created an acid environment suitable for *Candida albicans* growth, and induced an adaptive response.

However, results obtained with pure planktonic suspensions of yeasts may have little relevance to environmental contamination of material surfaces, medical devices or skin. We therefore also tested the agents in conditions of planktonic and biofilm polycontamination. The activity of the products was mostly 10–100-fold reduced on mixed suspensions compared to pure cultures. One hypothesis could be that

co-culture of yeasts may influence the protein load and mimic dirty conditions, affecting the efficacy of the biocides. Bessems (1998) previously showed that the killing activity of hypochlorite and quaternary ammonium compounds on *Candida albicans* was reduced in the presence of a high protein load. Regarding 0.5% Ecodiol, it is noteworthy that combination of this latter product with sodium hypochlorite was effective, albeit less so than sodium hypochlorite single-agent treatment, as previously reported. In general, combinations are only slowly fungicidal compared to hypochlorite (Scott *et al.*, 1986). Paired combinations of iodine potassium iodide, chlorhexidine acetate and sodium hypochlorite were no more effective than hypochlorite used alone (Waltimo *et al.*, 1999).

Candida albicans forms biofilms and exhibits *in vitro* levels of phenotypic resistance to antifungal agents (Hawser & Douglas, 1995; Chandra *et al.*, 2001b; Kumamoto, 2002), contributing to increased risk of infection in the medical field (Kuhn *et al.*, 2002; Shin *et al.*, 2002; Douglas, 2003). Much less is known about the efficacy of biocides in inactivating yeasts such as *Candida albicans*, *Cryptococcus* spp. and *Rhodotorula* spp. that contribute to biofilm formation in the environment. Chlorhexidine is the most studied biocide regarding its action against *Candida albicans* biofilms. Chandra *et al.* (2001a) showed that the susceptibility of *Candida albicans* biofilms to chlorhexidine was significantly reduced compared to the susceptibility of planktonic cells. Suci & Tyler (2002, 2003) demonstrated that early stage and mature *Candida albicans* biofilms exhibit levels of phenotypic resistance to chlorhexidine. In our study, the efficacy of chlorhexidine against the different biofilms tested was dramatically decreased compared to planktonic yeast suspensions, but it is the only biocide evaluated that maintained a fungicidal activity. The 5 min contact time used here does not allow, however, evaluation of the risk of emerging phenotypic resistance.

All other biocides were ineffective against the seven yeast

biofilms tested. Biofilm organization has been reported to confer resistance to antifungal agents (Hawser & Douglas, 1995; Douglas, 2003), through mechanisms such as slow growth rate and nutrient limitation (Baillie & Douglas, 1998) and differential expression of drug resistance genes (Kolaczowska *et al.*, 2002). A protective effect of the extracellular matrix was suggested (Hawser *et al.*, 1998) but recently forsaken (Baillie & Douglas, 2000) for the benefit of 'persister' cells (Lewis, 2001). Using mixed species biofilms of *Candida albicans* and *Staphylococcus epidermidis*, Adam *et al.* (2002) suggested that polycontamination can affect the action of antibiotics and antifungal agents. To increase the efficacy of antiseptic and disinfectant agents on bacterial biofilms, Takeo *et al.* (1994) recommended increasing the concentration and/or contact time. All these difficulties emphasize the importance of regular disinfection, before biofilm formation starts (Meyer, 2003).

In conclusion, our results show the importance of experimental test conditions when studying the efficacy of antiseptics and disinfectants on yeasts, pure planktonic suspensions being more sensitive than mixed suspensions. Only one of the five antiseptics tested (hydrogen peroxide) and two of the four disinfectants (0.25 % Ecodiol and UV) were ineffective. In our study, only high concentration of chlorhexidine (0.5 %) and hypochlorite (12 °) had fungicidal activity on yeast biofilms. However, this latter concentration, corresponding to 3.8 % active chlorine, is toxic for users (Rutala & Weber, 1997).

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