Treatment of plastic and extracellular matrix components with chlorhexidine or benzalkonium chloride: effect on *Candida albicans* adherence capacity *in vitro*

Christine Imbert*, Edith Lassy, Gyslaine Daniault, Jean-Louis Jacquemin and Marie-Hélène Rodier

Laboratoire de Parasitologie et Mycologie Médicales, Centre Hospitalier Universitaire La Milétrie, BP 577, 86021 Poitiers Cedex, France

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This study investigates the influence of treatment of plastic and extracellular matrix (ECM) proteins with chlorhexidine or benzalkonium chloride on subsequent adherence of *Candida albicans*. Three concentrations were tested for each antiseptic: (i) chlorhexidine, MIC (6.25–12.5 mg/L), 80 × MIC and 800 × MIC; and (ii) benzalkonium chloride, MIC (3.12 mg/L), 40 × MIC and 1600 × MIC. Chlorhexidine and benzalkonium chloride activities were correlated with the tested concentrations. Antiseptics used at MIC were unable to modify the adherence to plastic or ECM proteins. Chlorhexidine (80 × MIC) induced a decrease in plastic adherence of 31% of the 15 strains used and an increase in ECM protein adherence of 13% of strains. Benzalkonium chloride (40 × MIC) induced a decrease in adherence to ECM proteins or plastic of 13–27% of strains. Our results indicated that the treatment with 1600 × MIC benzalkonium chloride could induce the opposite effect on adherence, depending on the surface: 60% of the strains showed an increase in their adherence to ECM proteins, whereas 93% of the strains showed a decrease in their adherence to plastic. A similar phenomenon was observed after treatment with 800 × MIC chlorhexidine: 60% of the strains showed an increase in their adherence to ECM proteins, whereas 67% showed a decrease in adherence to plastic. Treatment of medical devices with at least 5000 mg/L of chlorhexidine or benzalkonium chloride could therefore reduce *C. albicans* adherence to plastic surfaces, but would be unable to prevent fungal adherence to ECM proteins.

Keywords: *Candida*, adherence, antiseptic agents

Introduction

*Candida* species are increasingly important nosocomial pathogens and *Candida albicans* remains the most frequently reported, even if other species of *Candida* are reported with an increased frequency.1 According to a widespread American study, the rate of invasive fungal infections among hospital patients approximately doubled between 1980 and 1990, and the incidence of nosocomial candidaemia alone increased five-fold.2 There are multiple reasons for the increase in incidence of invasive fungal infections in patients, including intensity of chemotherapy employed in cancer patients, the prolonged use of immunosuppressive agents in bone marrow transplant patients, the use of broad-spectrum antibiotics and the increasing use of intravenous catheters.3 Infections associated with the use of central venous catheters (CVCs) can result in serious medical complications and expensive care, and this is the most frequent factor limiting their prolonged use.4,5 Infectious complications remain frequent for all types of CVC.6 Antiseptics are used to clean and disinfect traumatic wounds, mucous membranes, hands, operation sites and sometimes to impregnate catheters.7–11 The increased number of *C. albicans* infections among denture wearers also calls for effective prevention, and numerous authors are interested in evaluating the efficacy of surface antiseptics or those impregnated into various denture materials.12–15 *In vitro* studies have suggested that catheters, denture materials and other non-
biological materials impregnated with some antiseptics, antibiotics or silver could resist infection more efficiently.\textsuperscript{12,14,16–20}

In the present study, chlorhexidine gluconate and benzalkonium chloride were chosen as the candidate drugs. The aim of this work was to evaluate and compare the in vitro effect of a pre-treatment of two different surfaces—untreated plastic or plastic overlaid with extracellular matrix (ECM) proteins—with chlorhexidine or benzalkonium chloride on subsequent infection by \textit{C. albicans} isolates. The concentrations used corresponded to: (i) MIC; (ii) concentrations usually used on mucosa; and (iii) concentrations usually used on plastic surfaces.

**Materials and methods**

**Organisms and growth conditions**

Fifteen isolates of \textit{C. albicans} were studied; they were all isolated in our laboratory from patients with septicemia. The identification of these clinical isolates was carried out by using conventional physiological and morphological studies such as the germ-tube test in serum, agglutination (Bichromatic Latex, Fumouze, Levallois Perret, France) and metabolic properties (API 20C, bioMérieux, Marcy-L’Étoile, France). Yeasts were first grown for 48 h at 28°C on Sabouraud agar slants (Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France), to obtain a fresh culture of synchronous stationary yeast-phase \textit{C. albicans}.\textsuperscript{21} A loopful of this culture was transferred to 25 mL of Yeast Nitrogen Base medium (YNB, Difco, Detroit, MI, USA), supplemented with 300 mM galactose (Sigma, St Louis, MO, USA; YNB-gal) and incubated for 36 h at 37°C, without shaking. Galactose promotes adherence of \textit{C. albicans} yeasts to cellular and plastic surfaces.\textsuperscript{22,23}

Before use in the adherence experiments, blastospores were harvested, washed twice in 0.1 M phosphate-buffered saline (PBS, pH 7.2; bioMérieux) and adjusted to 1.5 × 10^7 blastospores/mL.

**MICs of antiseptics**

Chlorhexidine digluconate 20% (Sigma) and benzalkonium chloride (Sigma) were used. The MICs of chlorhexidine and benzalkonium chloride were assessed by a broth dilution method using RPMI-1640 medium with L-glutamine but without bicarbonate, buffered with 0.165 M MOPS at pH 7. Chlorhexidine and benzalkonium chloride were prepared as stock solutions of 5000 mg/L in sterile water and serial dilutions of antiseptics (50–0.024 mg/L) were prepared in RPMI medium. Yeast inocula were prepared by suspension in RPMI medium, adjusted to a final concentration of 10^4 yeasts/mL and the MICs of each of the antiseptic agents were determined after incubation for 48 h at 37°C, without shaking. The MIC was defined as the lowest drug concentration showing no visible fungal growth. All tests were carried out on two occasions.

**Immobilized ECM proteins**

Extracellular matrix gel (ECM gel, Sigma) was coated on to wells of 96-well tissue culture plates (polystyrene, Evergreen Scientific, Los Angeles, CA, USA) according to the manufacturer’s instructions. This gel was composed primarily of laminin, collagen type IV, heparan sulphate proteoglycan and entactin.

**Treatment of plastic and biological surfaces with antiseptics**

The culture plates of ECM gel-coated and uncoated polystyrene were treated for 5 min with different concentrations of chlorhexidine or benzalkonium chloride. The test concentrations of chlorhexidine corresponded to the MIC, 80 × MIC and 800 × MIC for each isolate of \textit{C. albicans}, and the test concentrations of benzalkonium chloride corresponded to the MIC, 40 × MIC and 1600 × MIC for each isolate. These concentrations were selected to correspond with those usually used on mucosa (chlorhexidine 500–1000 mg/L and benzalkonium chloride 125 mg/L) or plastic surfaces (chlorhexidine and benzalkonium chloride 5000 mg/L). The MICs of antiseptics were also determined.

**Adherence of \textit{C. albicans} to polystyrene**

Adherence experiments were carried out in untreated 96-well tissue culture plates as described previously.\textsuperscript{21} Tetrazolium salt XTT was used to assess the adherence of \textit{C. albicans} blastospores to wells of tissue culture plates: the principle was based upon the reduction of XTT tetrazolium to tetrazolium formazan by mitochondrially active \textit{C. albicans} blastospores in the presence of an electron-coupling agent, menadione. Briefly, \textit{C. albicans} blastospores were added to 96-well tissue culture plates at an inoculum of 1.5 × 10^7 cells/mL in 150 µL of PBS and were allowed to adhere to the polystyrene for 2 h at 37°C; half of the wells were then washed twice with PBS to remove the non-adherent yeasts. Thereafter, 300 mg/L XTT (Sigma) and 0.13 mM menadione (Sigma) were added to all wells. Plates were incubated for 3 h at 37°C without shaking, then gently agitated and XTT formazan measured at \textit{A}_{492nm} (LP400 micro-plate reader, Sanofi Diagnostics Pasteur) in washed and unwashed wells. The % adherence capacity of each isolate was calculated as a mean of absorbance units in washed wells/absorbance units in unwashed wells.

Background formazan values were determined with plates that contained PBS only or PBS, XTT and menadione; these values did not exceed 0.005 absorbance units and therefore were not significant. All experiments were carried out twice with six replicates.
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Adherence of C. albicans to ECM proteins

Adherence of C. albicans to ECM proteins was measured following a 3 h incubation with XTT and menadione, as outlined above.

Statistical analyses

An analysis of variance (ANOVA) and a Scheffé's test were conducted to determine differences among the test groups (P < 0.05).

Results

MICs of antiseptics

Results showed that MICs of chlorhexidine for the 15 isolates of C. albicans, obtained in RPMI broth, ranged between 6.25 and 12.5 mg/L, and the MIC of benzalkonium chloride was 3.12 mg/L in all cases.

Plastic surfaces treated with chlorhexidine

Effect on the adherence capacity to plastic. The influence of the treatment of plastic with chlorhexidine on the adherence capacity of C. albicans was dependent on the antiseptic concentration. Chlorhexidine used at its MIC had no significant effect. Chlorhexidine at 80 × MIC induced a decrease (P ≤ 0.001) in the adherence capacity of five strains (31%) compared with the control or with MIC chlorhexidine treatment (Figure 1). Chlorhexidine at 800 × MIC was the most effective concentration to modify adherence: 10 strains (67%) were less adherent (P ≤ 0.001) than after no treatment, nine strains (60%) were less adherent (P ≤ 0.001) than after MIC treatment and four strains (27%) were less adherent (P ≤ 0.001) than after the use of 80 × MIC chlorhexidine.

Effect on the adherence capacity to ECM proteins. The influence of the treatment of ECM components with chlorhexidine was dependent on the antiseptic concentration, and the chlorhexidine used at its MIC had no significant effect (Figure 2). Chlorhexidine at 80 × MIC induced an increase (P ≤ 0.001) in the adherence capacity of two strains (13%) compared with the control and of four strains (27%) compared with MIC chlorhexidine treatment (Figure 2). Chlorhexidine at 800 × MIC had the greatest effect on adherence: nine strains (60%) were more adherent (P ≤ 0.001) than without treatment, 10 strains (67%) were more adherent (P ≤ 0.001) than after MIC treatment and seven strains (47%) were more adherent than after 80 × MIC treatment.

Plastic surfaces treated with benzalkonium chloride

Effect on the adherence capacity to plastic. The effect on adherence capacity of treatment of a plastic with benzalkonium chloride was dependent on the antiseptic concentration. The use of benzalkonium chloride at its MIC did not induce any modification of the adherence capacity but the use of benzalkonium chloride at a concentration of 40 × MIC (125 mg/L) inhibited the adherence to plastic (P ≤ 0.001) of C. albicans strains

Figure 1. Effect of the treatment of plastic with chlorhexidine on the adherence of C. albicans. These data represent the average and standard deviation for two experiments carried out with six replicates.
Yeasts were significantly less adherent to plastic ($P \leq 0.001$) when it was pre-treated with the highest tested concentration of benzalkonium chloride ($1600 \times$ MIC, 5000 mg/L); 14 strains (93%) showed a decrease in adherence capacity compared with control or with results obtained after treatment with benzalkonium chloride at its MIC (Figure 3).

Our results also showed that 10 strains (67%) were less adherent ($P \leq 0.001$) after treatment of plastic with $1600 \times$ MIC benzalkonium chloride than after treatment with $40 \times$ MIC benzalkonium chloride (Figure 3).
**Effect on the adherence capacity to ECM proteins.** The influence of treatment of ECM components with benzalkonium chloride was dependent on the antiseptic concentration. The chlorhexidine used at its MIC (3.12 mg/L) modified adherence ($P \leq 0.001$) of three strains (20%); the adherence of strain 182 was increased, whereas it was inhibited for strains 43 and 253. The use of benzalkonium chloride at a concentration of $40 \times \text{MIC}$ (125 mg/L) inhibited adherence to ECM components ($P \leq 0.001$) of two strains (13%) compared with control or MIC treatment (Figure 4). The use of benzalkonium chloride at a concentration of $1600 \times \text{MIC}$ (5000 mg/L) increased adherence of nine strains (60%) compared with control, of 11 strains (73%) compared with MIC treatment and of nine strains (60%) compared with $40 \times \text{MIC}$ (Figure 4).

**Discussion**

Binding antiseptic substances to a catheter surface or to any implanted medical prosthesis is one possible strategy to overcome the problem of infections associated with these implanted devices. We investigated the efficiency of two major antiseptics, chlorhexidine and benzalkonium chloride, on adherence of *C. albicans* blastospores after the pretreatment of plastic surfaces and ECM components.

Adherence is a prerequisite for *C. albicans* colonization and is considered as an initial step in the process leading to infection. When a non-biological material is implanted, there is an initial attachment and a biofilm layer rapidly forms on its surface. This biofilm is a monolayer or multilayer of cells embedded within an ECM material. Biofilm microorganisms are known to be resistant to host defence mechanisms and antibiotic therapy, and can be released from the layer into the surroundings, thus sustaining the infection. There is considerable interest in understanding the mechanisms involved in biofilm formation on catheters, which could lead to control and intervention strategies for the prevention of systemic candidiasis. Some authors have, for example, recently suggested that filamentation promoted the formation and persistence of cell populations on the biofilm that overlaid the catheter.

We have observed that the influences of chlorhexidine and benzalkonium chloride on *C. albicans* adherence capacity were correlated with the antiseptic concentration used to treat the surfaces. These antiseptics, used at their corresponding MIC, were unable to modify adherence to plastic or ECM proteins efficiently. This result corroborated the recommendations established for the use of antiseptics in the treatment of plastic surfaces and mucosa, i.e. concentrations corresponding to the MIC were not enough to prevent colonization.

The higher concentrations of chlorhexidine ($800 \times \text{MIC}$) and benzalkonium chloride ($1600 \times \text{MIC}$) have been chosen to correspond with those usually used to treat hard surfaces. Our results indicated that surface treatment with $1600 \times \text{MIC}$ of benzalkonium chloride could enhance fungal adherence, depending on the nature of the surface; 60% of the strains showed a significant increase in their adherence capacity to ECM proteins, whereas 93% of the strains showed a significant decrease in their adherence capacity to plastic after treatment. A similar phenomenon was observed after treatment of...
the tested surface with chlorhexidine at 800 × MIC; 60% of the strains showed an increase in adherence to ECM proteins, whereas 67% of the strains showed a decrease in adherence to plastic after treatment. This phenomenon appeared as soon as the chlorhexidine concentration reached 80 × MIC but was not observed with benzalkonium chloride at 40 × MIC. Benzalkonium chloride (40 × MIC) induced a decrease in adherence to biological or plastic surfaces of 13–27% of the strains, whereas an enhanced adherence was never observed. The benzalkonium chloride concentration of 40 × MIC and the chlorhexidine concentration of 80 × MIC were chosen to correspond to those usually used to treat mucous surfaces, and these results confirmed that they were unable to reduce fungal adherence. The highest concentrations of chlorhexidine and benzalkonium chloride that were tested were very efficient in preventing fungal adherence to biological or plastic surfaces. These results confirmed that they were unable to reduce fungal adherence. However, our results also suggested that antiseptic pretreatment could promote adherence to ECM components. C. albicans is able in vivo to adhere to a plastic device and then to form a biofilm. Our results were obtained in vitro, but raise questions about the benefit of treating medical devices with antiseptic in the prevention of candidiasis if we only consider the step of biofilm development. Cell surface hydrophobicity plays a major role in adherence of C. albicans to plastic, whereas adherence to ECM proteins involves specific adhesins present at the surface of the fungal cell. The processes implicated in adherence to a plastic or biological surface are very different and this could explain why we observed opposing influences of antiseptic treatments. Our results correlate with other studies which suggested that chlorhexidine is effective in preventing C. albicans plastic attachment and growth on acrylic resin. McCourtie et al. suggested that the pre-treatment of denture acrylic with chlorhexidine gluconate reduced the subsequent adherence of C. albicans. More recently, Ivanovski et al. have evaluated the efficiency of disinfecting solutions incorporated into dental stone casts against one strain of C. albicans and suggested that chlorhexidine was unable to achieve a satisfactory level of disinfection. Other authors have shown that chlorhexidine is an effective antifungal agent in vitro. It is nevertheless difficult to compare these results because of the varying experimental conditions. Each study has been carried out with only a few strains of C. albicans, and the test concentrations of the antiseptics were often different.

To summarize, our results indicate that chlorhexidine and benzalkonium chloride had opposing effects on the in vitro adherence capacity of C. albicans depending on the nature of the surface; adherence was inhibited on plastic surfaces and increased on ECM proteins. This study also suggests that chlorhexidine and benzalkonium chloride have a comparable effect on modifying fungal adherence to ECM components when these antiseptics were used between 5000 and 10000 mg/L. However, these concentrations of benzalkonium chloride were more efficient than chlorhexidine in preventing adherence of the yeast cells to plastic surfaces.

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References

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