Susceptibility of Candida albicans biofilms grown in a constant depth film fermentor to chlorhexidine, fluconazole and miconazole: a longitudinal study

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Objectives: The aim of this study was to assess the resistance of Candida albicans biofilms to both antifungal and antimicrobial agents in vitro.

Methods: Biofilms of C. albicans were grown on denture acrylic discs in a constant depth film fermentor and maintained with artificial saliva. The MIC of fluconazole, miconazole and chlorhexidine for C. albicans was first determined. Using these data, 72 h biofilms were exposed to these agents at different MIC levels. In order to assess growth, biofilms were removed from the fermentor, incubated in the test agent for various periods, the biofilms disrupted and the viable yeast cells present determined. The MIC for these cells was then also determined. In a separate experiment, biofilms of various ages (2–72 h) were exposed to sub-biofilm MIC concentrations for two different periods.

Results: C. albicans biofilms were found to be highly resistant to fluconazole and miconazole compared with the same cells grown in suspension (≥1024 × MIC). In contrast, chlorhexidine inhibited the growth of C. albicans biofilms at a concentration up to 8 × MIC. When the susceptibility of biofilms over time was investigated, higher reductions were observed for chlorhexidine and miconazole than fluconazole for biofilms of 2 and 6 h.

Conclusions: We have shown in this study that the susceptibility of C. albicans to antifungal and antimicrobial agents changes throughout biofilm development.

Keywords: in vitro, antifungal, antimicrobial, MIC

Introduction

The long-term survival of microorganisms is dependent on their ability to attach to surfaces and form adherent biofilms.1 When a community of microorganisms become irreversibly attached to a surface the organisms exhibit distinctive phenotypic properties.2 Contact with a solid surface triggers the expression of a panel of bacterial enzymes, which catalyse the formation of sticky polysaccharides that promote colonization and protection. Bacteria express new, and sometimes more virulent, phenotypes when growing within a biofilm. The microorganisms tend to be far more resistant to antimicrobial agents3 and it becomes particularly difficult for the host immune system to render an appropriate response.3

Biofilm formation is critical in the development of denture-associated erythematous candidiasis, which is a common condition occurring in patients with an oral prosthesis. Despite the use of antifungal agents to treat candidiasis, colonization is often re-established soon after treatment. Indeed, these clinical observations emphasize the importance of biofilm formation and the inability of current antifungal therapy to treat such conditions. The mechanisms by which Candida albicans biofilms resist the action of antifungal agents are not known; however, it has been suggested that drug resistance may arise as a result of surface-induced gene expression4 and may also depend on the phase of biofilm growth.5

Several biofilm systems have been developed to study C. albicans biofilm formation on various materials, such as catheter or denture material, by incubating them in a growth medium. However, biofilm structure is highly dependent upon the conditions under which it is formed;6 hence, we have used a model which is particularly suited to studies of biofilms of oral microorganisms.

The aim of this study was to assess the in vitro susceptibility of C. albicans biofilms of different ages to chlorhexidine, fluconazole and miconazole.

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and miconazole using a model that simulated biofilm formation in the oral cavity.

Materials and methods

Inoculum and media

A clinical isolate (no. 64) of C. albicans genotype A was used in all of the experiments. The nutrient source was mung-containing artificial saliva.8

Production of biofilms

Polymethylmethacrylate cold-cure acrylic denture discs were prepared using self-cure acrylic (Dentsply Limited, Weybridge, UK) in a 5 mm × 1 mm polytetrafluoroethylene (PTFE) mould. All disc surfaces were prepared using silicon carbide grit (16–20 µm). Biofilms were grown on the acrylic discs in a constant depth film fermentor (CDFF; University of Wales, Cardiff, UK) run aerobically at 37°C, as described previously.3 The CDFF consists of a glass vessel with stainless steel end-plates that contain ports for the entry of medium, gas and for sampling. Artificial saliva was pumped over the biofilms at a rate of 0.5 mL/min. The vessel houses a stainless steel disc containing 15 PTFE sampling pans and a PTFE sampling pan that rotates under a PTFE scraper blade, smearing the incoming medium over the biofilms and maintaining them at a pre-determined depth. Each sampling pan has five cylindrical holes containing PTFE plugs into which discs were recessed (300 µm) to create a space in which the biofilms could form.

Susceptibility

The MICs of fluconazole (Pfizer Ltd, Tadworth, UK), miconazole (Johnson and Johnson, Beerse, Belgium) and chlorhexidine digluconate (Sigma, Poole, UK) for C. albicans derived from Sabouraud dextrose agar (Oxoid, Basingstoke, UK) plates were determined using the broth dilution susceptibility method described in NCCLS guidelines.9 Discs, containing the biofilms, were removed from the CDFF after 72 h growth and treated with fluconazole or miconazole (0.25, 2, 16, 64, 256 mg/L) for 24 or 48 h, or chlorhexidine (0.019%, 0.15%, 0.3%, 1.25%, 2.5%) for 5 or 15 min. In separate experiments, the susceptibility against different aged biofilms (2, 6, 24, 48 and 72 h) was investigated. The biofilms were treated at 37°C for 24 and 48 h in fluconazole and miconazole (256 mg/L) and in chlorhexidine (0.019%) for 5 and 15 min under the same conditions. The biofilms were then vortexed vigorously for 1 min to remove any cells from the surface, and plated onto Sabouraud dextrose agar (Oxoid). Subsequently, the post-biofilm MIC of any remaining colonies on the agar was determined. Duplicate CDFF experiments were carried out in order to produce biofilms over a 72 h period. The MIC testing (in triplicate) and statistical analysis performed using the Student’s t-test (two-sample) to determine P values.

Results and discussion

The susceptibility of C. albicans biofilms to fluconazole, miconazole and chlorhexidine is shown in Table 1. The organisms grown in the biofilms were ≥1000-fold more resistant to fluconazole and miconazole and eight-fold more resistant to chlorhexidine than the same organisms grown planktonically.

At 2 h, an average of 2.28 × 10⁸ cfu/biofilm were found to be attached to the denture acrylic. This number increased steadily over time (6 h—7.2 × 10⁹, 24 h—8.3 × 10⁹, 48 h—9.1 × 10⁹ cfu/biofilm) until there was an average of 1.67 × 10⁹ cfu/biofilm comprising the mature biofilm at 72 h. Figure 1 shows the percentage reduction in the number of C. albicans exposed to fluconazole 256 mg/L, miconazole 256 mg/L and 0.019% chlorhexidine compared with PBS controls.

Biofilms removed from the CDFF at 2 h and exposed to fluconazole for 24 h (Figure 1a) were significantly reduced in numbers, by 83.4% and 94.5%, respectively (P < 0.05). After 24 h, as the biofilm matured, the biofilms became more resistant and the reductions observed were not significantly different from the control (P > 0.05). The 2 h biofilms were also susceptible after 48 h exposure, although there was no significant difference at any other time of biofilm development (P > 0.05). Previous studies have shown a modest effect of fluconazole against pre-formed biofilms with an ~30% decrease in biofilm activity after 48 h.10

Exposure of 2 and 6 h biofilms to miconazole (Figure 1b) for 24 h resulted in 99.2% and 99.9% reductions in viability, respectively, a significant reduction from the control biofilms (P < 0.05). However, after this time there was no significant reduction in viability from 24 to 72 h (P > 0.05). In contrast, 2, 6 and 24 h biofilms exposed to chlorhexidine (Figure 1c) were all highly susceptible to sub-MIC levels. However, no significant reductions (P > 0.05) were observed for 48 or 72 h biofilms.

Table 1. MICs of fluconazole, miconazole and chlorhexidine for C. albicans

<table>
<thead>
<tr>
<th>Agent</th>
<th>Planktonic</th>
<th>72 h biofilms</th>
<th>Fold increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluconazole (mg/L)</td>
<td>0.25</td>
<td>≥256</td>
<td>≥1024</td>
</tr>
<tr>
<td>Miconazole (mg/L)</td>
<td>0.25</td>
<td>≥256</td>
<td>≥1024</td>
</tr>
<tr>
<td>Chlorhexidine (digluconate (%))</td>
<td>0.04</td>
<td>0.3</td>
<td>8</td>
</tr>
</tbody>
</table>

No significant differences were observed between 24 and 48 h exposure times (P > 0.05). When the resuspended cells were grown on solid media and then tested, they were no longer resistant to the three agents, demonstrating that the phenotype was reversible, a phenomenon demonstrated previously.10

Earlier studies, which have evaluated the structure of C. albicans biofilms, have indicated three phases of growth, early, intermediate and maturation extending over 72 h.6 Hence, our studies were carried out over the same period to encompass these phases. Chandra et al.6 also showed that the proportions of yeast and hyphal cells present in the biofilm were dependent upon the nutrient source. For example, biofilms grown in a yeast–nitrogen-based medium contained mainly the yeast form, whereas filaments predominated in RPMI-grown biofilms. In the present study, where artificial saliva was used, the yeast form predominated at an average ratio of 25:1.

In order to study biofilm development and perturbation, data must be comparable and reproducible from experiment to experiment. One approach to reproducibility is to develop constant depth reactors where surface growth is periodically removed to maintain a constant geometry. One such device, which employs a mechanical scraper bar, is the CDFF, which was designed to investigate the growth of dental plaque organisms. Although previous studies have indicated differences in susceptibilities between antifungal compounds over time, none has demonstrated this using an in vitro model designed to study oral biofilms. Indeed, although this model has been used extensively for studying the susceptibility of bacterial biofilms, this study describes its first application in fungal biofilms.
Susceptibility of Candida albicans biofilms

The MIC data have shown that, as we might expect, these biofilms are highly resistant to both antifungal and antimicrobial agents. Interestingly, however, there were large differences in the degree of susceptibility of C. albicans biofilms among fluconazole, miconazole and chlorhexidine, with the latter being the most effective. Such information may help guide therapeutic decisions affecting the outcome for patients.

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References