Commercial mouthwashes are more effective than azole antifungals against Candida albicans biofilms in vitro

Gordon Ramage, PhD, a Anto Jose, BDS, MSc, a Brent Coco, PhD, a Ranjith Rajendran, MSc, a Riina Rautemaa, DDS, PhD, b Colin Murray, BDS, PhD, a David F. Lappin, PhD, a and Jeremy Bagg, BDS, PhD, a Glasgow and Manchester, United Kingdom; and Helsinki, Finland

GLASGOW DENTAL SCHOOL, UNIVERSITY OF MANCHESTER, AND UNIVERSITY OF HELSINKI

Objective. The aim of this study was to evaluate and compare the activity of prescription and over-the-counter antimicrobial compounds against planktonic and biofilm forms of Candida albicans isolated from cases of oral candidiasis in vitro.

Study design. The efficacy of azoles, polyenes, an echinocandin, and 4 over-the-counter mouthwashes were tested against C. albicans–derived planktonic and biofilm cells.

Results. Planktonic cells were shown to be highly sensitive to all of the antifungal agents tested. Sessile cells were highly resistant to azoles (≥128 mg/L) but equally sensitive to caspofungin and short treatments with Corsodyl, Listerine, and Oraldene.

Conclusions. Although C. albicans is sensitive to azole antifungal agents in planktonic form, it is highly resistant within the biofilm. The good efficacy of the over-the-counter mouthwashes against candidal biofilms in vitro suggests that clinical trials should now be designed to establish their role in the clinical management of oral candidal infections. (Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2011;111:456-460)

Oropharyngeal candidiasis (OPC) is a frequent problem within immunocompromised and elderly populations. It may manifest in different clinical presentations, including pseudomembranous and erythematous forms, causing symptoms such as pain, burning sensation, and altered taste. These can subsequently lead to nutritional compromise. OPC is typified by a complex mixture of yeasts and hyphae surrounded by extrapolymeric matrix material, which encases the cells within to form an impenetrable barrier to host defenses and antimicrobial therapy. These are predominantly formed by Candida albicans adherent to the surfaces of oral mucosa and teeth as well as prosthetic appliances. These biofilm consortia have a community structure, dominant metabolic processes, and interorganism interactions.

Antifungal resistance is a key characteristic of candidal biofilms, which is associated with exopolymeric matrix, increased cell density, metabolic inactivity of cells and up-regulation of efflux pumps. In addition, Candida spp. other than C. albicans, such as C. glabrata and C. krusei, that are commonly resistant to azole antifungals, are frequently detected within the oral cavity. Nevertheless, azole antifungal drugs are frequently prescribed for the treatment of these infections. Fluconazole and itraconazole are often used, particularly for patients with underlying systemic illness, but recurrence of infection is a common clinical problem. Defined resistance mechanisms induced through azole use have been reported, such as up-regulation of mRNA from genes involved in the ergosterol biosynthetic pathway or from multidrug efflux pumps.

Given that OPC are common infections and induced by adherent yeasts within a biofilm consortium, it is important to investigate the effect of antifungal agents by using a system that more accurately reflects the lifestyle of C. albicans within the oral cavity. The present in vitro study therefore aimed to examine antifungal susceptibility profiles of planktonic and sessile C. albicans isolated from patients with oral candidiasis to a range of prescription antifungal agents and over-the-counter mouthwashes.
MATERIAL AND METHODS

Culture conditions and standardization

Candida albicans strain ATCC 90028 and 34 clinical strains described in a previous investigation by our group were used for biofilm sensitivity testing.1 All working stocks of C. albicans were maintained at 4°C on Sabouraud (Oxoid, Cambridge, UK) agar, and stored indefinitely in Microbank vials (Pro-Lab Diagnostics, Cheshire, UK) at −80°C. Isolates were propagated in yeast peptone dextrose medium (1% w/v yeast extract, 2% w/v peptone, 2% w/v dextrose [Oxoid]), washed by centrifugation, resuspended in RPMI-1640 buffered with morpholinepropanesulfonic acid (MOPS [Sigma, Poole, UK]) to a cellular density equivalent to 1.0 × 10⁶ cells/mL.

Antifungal agents and mouthwashes

The following antifungal agents were used in the course of this study: fluconazole (FLZ; Diflucan; Pfizer Pharmaceuticals, Sandwich, UK); voriconazole (VRZ; Vfend; Pfizer Pharmaceuticals); itraconazole (ITZ; Sporanox; Janssen-Cilag, Buckinghamshire, UK); caspofungin (CSP; Cancidas; Merck Sharp and Dohme, Hertfordshire, UK); amphoterin B (AMB; AmBisome; Gilead Sciences, Cambridge, UK); and nystatin (NYS; Bristol-Meyers Squibb; Middlesex, UK).

The following over-the-counter mouthwashes were used in the course of this study and are listed with their active ingredient(s) and the recommended application time: Listerine (Pfizer Consumer Health Care, UK): eucalyptol (0.092% v/v), thymol (0.064% v/v), methyl salicylate (0.060% v/v), menthol (0.042% v/v), ethanol (26.9% v/v), 30-second rinse; Oralene (Warner-Lambert Consumer Health Care, UK): hexetidine (0.1% v/v), 60-second rinse; Colgate Peroxyl (Colgate Palmolive, UK): hydrogen peroxide (1.5% v/v), 60-second rinse; and Corsodyl (GlaxoSmithKline Consumer Health Care, UK): chlorhexidine gluconate (0.2% w/v), ethanol (7% v/v), 60-second rinse.

Antifungal susceptibility testing

Antifungal testing to determine minimum inhibitory concentrations (MICs) of planktonic cells was performed using the CLSI M-27A broth microdilution method.12 The spectrophotometric method of inoculum preparation was used to obtain a density corresponding to 0.5-2.5 × 10⁶ cells/mL in the RPMI test medium for each of the isolates. These were added 1:1 within round-bottomed microtiter plates to serially double-diluted concentrations (0.0625 to 16 mg/L) of each antifungal prepared directly in MOPS-buffered RPMI medium. Antifungal-free controls were also included. Microtiter trays were then incubated at 37°C and end points read visually at 48 hours. Isolates were tested in triplicate.

Sessile susceptibility testing was performed as previously described.13 Briefly, biofilms from 34 C. albicans isolates were formed on commercially available presterilized polystyrene flat-bottomed 96-well microtiter plates (Corning, Corning, NY, USA) by pipetting standardized cell suspensions into selected wells of the microtiter plate and incubated for 48 hours at 37°C. After biofilm formation, the medium was aspirated and nonadherent cells removed by thoroughly washing the biofilms 3 times in sterile phosphate-buffered saline solution (PBS). Each antifungal agent was then added to the biofilms in serially double diluted concentrations from stock solutions of each antifungal prepared in RPMI medium directly, and incubated for a further 48 hours at 37°C. For these experiments, antifungal agents were tested at a concentration range of 0.25 to 128 mg/L except for caspofungin, which was tested at a concentration range between 0.0625 and 16 mg/L. A series of antifungal-free wells and biofilm-free wells were also included to serve as positive and negative controls, respectively. Sessile minimum inhibitory concentrations (SMICs) were determined at 80% inhibition using an XTT [2,3-bis(2-methoxy-4-nitro-5-sulfo-phe- nyl)-2H-tetrazolium-5-carboxanilide; Sigma] reduction assay, adapted from previous studies to quantify anti–C. albicans biofilm activity,13-15 as described below. Testing of isolates was performed in triplicate.

Susceptibility testing of commercial mouthwashes

Planktonic and sessile susceptibility testing was performed as described above with each of 4 commercially available mouthwashes. Planktonic testing was performed using the CLSI M27-A assay. For sessile testing, mouthwashes were added directly to 10 replicate biofilms for each isolate, including appropriate control samples. Biofilms were treated at room temperature according to the recommended rinse time for each manufacturer (30-60 s). After each defined treatment, the mouthwashes were replaced with European neutralizing solution (1% v/v phosphate buffer, 0.1% w/v L-histidine, 0.5% w/v sodium thiosulfate, and 0.3% w/v lecithin [soya refined] and 10% v/v Tween-80) for 5 minutes. Biofilms were then washed with sterile PBS before quantification of the biofilm metabolic activity. Testing of these isolates was performed in triplicate.

Quantification of biofilm metabolic activity

A semiquantitative measure of each biofilm was calculated using a formazan salt–based XTT reduction assay. Briefly, XTT was prepared as a saturated solution of 0.5 g/L in PBS. The solution was then filtered
through a 0.22-μm filter, aliquoted, and stored at −80°C. Before use, XTT was thawed and menadione (Sigma, 10 mmol/L prepared in acetone) added to a final concentration of 1 μmol/L. A 100-μL aliquot of the XTT-menadione solution was subsequently added to each prewashed biofilm and to control wells (for measurement of background XTT-reduction levels). Plates were then incubated in the dark for up to 2 hours at 37°C. A colorimetric change in the XTT-reduction assay, representing a direct correlation of metabolic activity of the biofilm, was then measured at 492 nm in a microtiter plate reader (FluoStar Omega; BMG Labtech, UK).

Statistical analyses
Statistical analyses of biofilm formation were performed using SPSS Software ver. 16.0 (SPSS, Chicago, IL, USA). For multigroup comparisons, the Kruskal-Wallis test and χ² statistic were used to determine if any groups exhibited a statistically significant different percentage of biofilm metabolism. If the Kruskal-Wallis test demonstrated ≥1 of the groups to be statistically different, a post hoc analysis using the Mann-Whitney U test and Bonferroni correction was used to adjust the significance value (P) for the number of comparisons.

RESULTS
Antifungal susceptibility profiles of C. albicans isolates
The C. albicans oral isolates were susceptible to all antifungal agents tested in the planktonic state (Table I). CSP and VRZ were the most effective antifungal agents, with MIC₅₀/₉₀ values of <0.0625 mg/L for both antifungal agents. The MIC range for CSP was slightly lower (<0.0625-0.25 mg/L) than for VRZ (<0.0625-1 mg/L). AMB and ITZ shared identical MIC₅₀ and MIC₉₀ values of 0.625 and 0.125 mg/L, respectively. FLZ displayed higher MIC₅₀ and MIC₉₀ values of 0.5 and 2 mg/L, respectively, with a range from 0.0625-4 mg/L. NYS activity was relatively poor, with MIC₅₀/₉₀ of 2 mg/L and a narrow range of 1-4 mg/L.

<table>
<thead>
<tr>
<th>FLZ</th>
<th>VRZ</th>
<th>ITZ</th>
<th>CSP</th>
<th>AMB</th>
<th>NYS</th>
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<tr>
<td>PMIC</td>
<td>SMIC</td>
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<td>SMIC</td>
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<tr>
<td>0.5</td>
<td>&gt;128</td>
<td>&lt;0.0625</td>
<td>&gt;128</td>
<td>0.0625</td>
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<td>2</td>
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<td>&lt;0.0625</td>
<td>&gt;128</td>
<td>0.125</td>
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<tr>
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<tr>
<td>High</td>
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<td>&gt;128</td>
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AMB, Amphotericin B; CSP, caspofungin; FLZ, fluconazole; ITZ, itraconazole; NYS, nystatin; VRZ, voriconazole; MIC, minimum inhibitory concentration; PMIC, planktonic minimum inhibitory concentration; SMIC, sessile minimum inhibitory concentration.

The most potent antifungal against the sessile C. albicans isolates was shown to be CSP, with MIC₅₀ <0.0625 and MIC₉₀ 0.0625 mg/L. AMB was the second-most effective antifungal with an MIC₅₀ of 2 and an MIC₉₀ of 4 mg/L (range 1-8 mg/L). All sessile-grown C. albicans isolates were resistant to NYS, with MIC values ranging between 16 and 32 mg/L. Azole drugs, including FLZ, ITZ and VRZ, were highly ineffective, with MIC values >128 mg/L.

Effect of mouthwashes on C. albicans
Planktic MIC testing demonstrated that Corsodyl was the most effective (0.15 to .3%), followed by Colgate Peroxyl (0.6 to 3.27%), Oralene (0.6 to 6.75%), and Listerine (6.75 to 25%; Table II). The antibiofilm activity of the 4 mouthwashes was assessed using the exposure times recommended by the manufacturers. Corsodyl, Listerine, and Oralene were the most effective agents, exhibiting ~75%–80% reduction in metabolism (Fig. 1). No significant differences between these 3 treatments were observed. These 3 mouthwashes were all significantly superior to Colgate Peroxyl (P < .001), which resulted in only a 40% metabolic reduction.

<table>
<thead>
<tr>
<th>Mouthwash</th>
<th>Range</th>
<th>MIC₅₀</th>
<th>MIC₉₀</th>
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<tr>
<td>Listerine</td>
<td>6.75-25</td>
<td>25</td>
<td>25</td>
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<tr>
<td>Colgate Peroxyl</td>
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<td>1.2</td>
<td>3.27</td>
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<tr>
<td>Corsodyl</td>
<td>0.15-0.3</td>
<td>0.3</td>
<td>0.3</td>
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<tr>
<td>Oralene</td>
<td>0.6-6.75</td>
<td>1.2</td>
<td>3.27</td>
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Each concentration described represents a proportion of the commercially available concentration.

DISCUSSION
The ability of C. albicans to exist within complex biofilm consortia provides protection from mechanical forces, and the biofilm polymeric matrix provides a barrier of diffusion to antifungal agents.3 Therefore,
infections caused by *Candida* biofilms are frequently recalcitrant to a range of treatment regimens.\(^{16,17}\) In the present paper, we report that conventional antifungal agents, azoles and polyenes, though effective against planktonic cells, have reduced activity against *C. albicans* biofilms in vitro. However, 3 of the over-the-counter mouthwashes, as well as caspofungin, exhibited significant antibiofilm activity and may therefore serve as a more appropriate oral chemotherapeutic strategy. As well as being potentially more effective, this approach would also avoid repeated subfungicidal exposure of biofilm-embedded yeasts and hyphae to azoles, which is an important driving force behind development of resistance.\(^{10,11}\)

*Candida albicans* continues to be the predominant organism associated with yeast biofilm infections.\(^{1,17}\) This is believed to result primarily from the selective advantage of morphogenesis that *C. albicans* utilizes as part of its pathogenic repertoire.\(^{18}\) Although the ability to form hyphae has been shown to be central to *C. albicans* biofilm development, not all clinical isolates are capable of forming biofilms to equivalent levels.\(^{19}\) Tortorano et al. found a significant correlation between *C. albicans* biofilm formation and mortality due to candidemia, whereas mortality was not associated with antifungal sensitivity of planktonic cells.\(^{19}\) These data, together with those reported in the present paper, indicate that *C. albicans* does not necessarily require the acquisition of defined molecular mechanisms to resist treatment.\(^{11,20}\) Instead, the ability to form biofilms upon various substrates provides adequate protection from antifungal agents.\(^{3,4}\)

The cell wall–active antifungal caspofungin proved to be highly active against all OPC strains tested in both planktonic and biofilm states in vitro. This is in accordance with previous publications.\(^{21}\) However, caspofungin can only be administered intravenously and is prohibitively expensive for the treatment of OPC. Nevertheless, if a topical oral formulation of caspofungin could be produced, then not only could it be used for the treatment of biofilms, but also for azole-insensitive yeasts, such as *C. glabrata* and *C. krusei*. There are, however, caveats to this approach, because recent data reported echinocandin resistance through the FKS1 and FKS2 gene loci.\(^{20,22}\) Although this is still relatively rare, caution should be exercised regarding its overuse, because resistance has been described in several *Candida* spp.\(^{23}\)

Our results demonstrate that the commercially available mouthwashes Corsodyl, Oraldene, and Listerine have the capacity to reduce the biofilm viability in vitro by \(\sim 80\%\), which equates to the breakpoint for sessile MIC testing. These agents both killed and removed biofilm biomass (data not shown) when used according to the manufacturer’s instructions. Earlier studies have reported similar data, where chlorhexidine (0.12%) and Listerine mouthwashes were shown to be effective against fungal biofilms, but unfortunately they did not compare these compounds with antifungal agents.\(^{24}\) However, in a comparison of the efficacy of fluconazole and miconazole to chlorhexidine against *C. albicans* biofilms grown in a constant-depth film fermenter, it was shown that chlorhexidine was significantly more effective than the azoles.\(^{25}\) These mouthwashes are also highly active against planktonic cells that are likely to be present in saliva and contribute to colonization and subsequent biofilm formation, which is in agreement with earlier studies.\(^{26,27}\) The active components in commercially available mouthwashes have broad-spectrum antimicrobial activity through membrane disruption, which, unlike the azoles and polyenes, is not related to cell membrane ergosterol, resistance, and toxicity. Moreover, compounds such as chlorhexidine gluconate demonstrate substantivity by adsorbing to protein and extracellular material. Tomas et al. recently reported that substantivity could be improved by duration of exposure, so it is likely that an increased duration of exposure to \(>60\) seconds would improve overall *C. albicans* biofilm killing.\(^{28}\)

Nevertheless, there are also some drawbacks to the use of over-the-counter mouthwashes, for example, superficial staining of enamel, burning sensation, and alterations of taste. Many of them also have a high alcohol content, which has been implicated in oral cancer.\(^{29,30}\) As a result, alternative compounds have attracted some attention. For example, it has been demonstrated that both azole-sensitive and azole-resistant yeasts could be inhibited effectively using the membrane-disrupting agent tea tree oil.\(^{31}\) The use of compounds such as these may be a more desirable alternative to prescription antifungals. However, because the

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**Fig. 1.** Metabolic reduction of *Candida albicans* biofilms with 4 over-the-counter oral mouthwashes. Reduction in the biofilm metabolism of 16 *C. albicans* denture stomatitis strains and the *C. albicans* strain ATCC 90028. Each strain was treated for the time indicated per manufacturers’ instructions (\(n = 8\)). The box-and-whiskers plot illustrates the median and distribution of all strains tested. Post hoc statistical analysis using the Mann-Whitney *U* test demonstrated a significant difference in mouthwash treatment (\(*P < .001\)).
data in this paper were generated entirely in vitro, appropriate clinical trials that would establish both effectiveness and patient acceptability of these over-the-counter preparations in vivo need to be carried out.

REFERENCES