In Vitro Analyses of Ethanol Activity against Candida albicans Biofilms

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*Candida albicans* is a common cause of catheter-related bloodstream infections (CR-BSI). Ethanol (EtOH) lock therapy has been attempted despite limited data on optimal dose and duration. Concentrations of 35% EtOH or higher for a minimum of 4 h demonstrated a >99% reduction in mature *C. albicans* biofilm metabolic activity and prevented regrowth. Concentrations of 10% EtOH or higher reduced *C. albicans* biofilm formation by >99%. Further investigation of EtOH lock therapy for treatment and prevention of *C. albicans* CR-BSI is warranted.

Biofilms are a major component of catheter-related bloodstream infections (CR-BSI) due to *Candida* species. Clinical trials have demonstrated worse outcomes in candidemic patients when indwelling venous catheters are retained, particularly in nonneutropenic populations; therefore, Infectious Diseases Society of America guidelines for treatment of CR-BSI due to *Candida* species recommend line removal in nonneutropenic patients and, if possible, in neutropenic patients (7). However, catheter removal may be high risk in a significant proportion of patients due to thrombocytopenia, coagulopathies, lack of alternative venous access, or critical illness. Antifungal lock therapy (A/fLT) is a potential alternative strategy for catheter salvage. A limited number of *in vitro* studies, animal models, and clinical reports have used ethanol (EtOH) as an antiseptic agent as part of an A/fLT strategy (1–3, 6, 9, 12, 13). These strategies have used differing concentrations of EtOH (12.5 to 70%) with various dwell times (2 to 24 h). However, there are few data that systematically examine the effects of EtOH concentration and dwell time in an A/fLT model.

To determine the optimal concentration and time of EtOH needed to (i) inhibit mature *C. albicans* biofilms and (ii) prevent biofilm formation, we used static microplate, silicone disk, and colony regrowth models for systematic *in vitro* analyses. Wild-type *C. albicans* SC5314 was used as a reference strain for detailed studies. *C. albicans* reference strains ATCC 10231, ATCC 14053, and ATCC 24433 and strains 42379 and 53264, two clinically derived *C. albicans fks1* mutant isolates characterized by echinocandin resistance, were also studied (5, 11). Biofilm formation in 96-well microtiter plates was performed as described previously (10). A total of 100 μl of 200-proof EtOH diluted in buffered RPMI 1640 to concentrations of 5 to 50% (vol/vol) was added to biofilm-containing wells. The plates were incubated at 37°C for selected time points and analyzed using the XTT reduction assay (10). Each experiment was performed in triplicate or quadruplicate. Antifungal activity was expressed as a percentage relative to the metabolic activity of untreated biofilms.

Sessile MICs (sMIC) were defined as the concentrations of EtOH needed to reduce metabolic activity of the biofilms by 50% (sMIC50) and 80% (sMIC80). Metabolic activities of the treatment groups were compared to those of controls by using one- or two-way analyses of variance (ANOVs) and Dunnett’s multiple-comparison posttest. Differences were considered significant at a *P* value of <0.05. GraphPad Prism 5.0 (GraphPad Software, San Diego, CA) was used for statistical analyses and graphing.

The antifungal effect of 5 to 50% EtOH against mature *C. albicans* SC5314 biofilms was tested after 0.5, 1, 2, 4, and 24-hour treatments (Fig. 1). EtOH concentrations of 20% or higher had statistically significant antifungal activity at all time points. The sMIC50 ranged from 10 to 25% EtOH depending on the treatment duration. The sMIC80 ranged from 15 to 30%. EtOH concentrations of 30% or higher inhibited biofilms by >99% for all time points tested. Concentrations of 35% EtOH inhibited biofilms by 99.85%, 99.86%, and 99.97% at 1, 2, and 4 h, respectively.

To test *C. albicans* SC5314 biofilm formation in the presence of EtOH, cells in RPMI were coincubated with 5 to 50% EtOH at 37°C for 24 h. Biofilm formation was reduced by >99% at concentrations of 10% or greater. Metabolic activity of biofilms incubated with 5% EtOH decreased 66.2%.

The effect of 5 to 50% EtOH on mature biofilms of three *C. albicans* reference isolates (ATCC 10231, ATCC 14053, and ATCC 24433) and two echinocandin-resistant strains (42379 and 53264) was tested at 2 and 24 h. The effect of EtOH on the biofilms varied significantly among strains for both incubation times (*P* < 0.0001). For the 2-h EtOH incubation, the sMIC50 ranged from 15 to 20% and the sMIC80 ranged from 20 to 25%. Concentrations of 25% and higher inhibited biofilms formed by all strains by >99%. For the 24-hour incubation, the sMIC50 was 10% and the sMIC80 ranged from 15 to 20%. Concentrations of 20% and higher fully inhibited the biofilms of all strains.

We next used a silicone disk model in 96-well plates to study the effect of EtOH on inhibition and formation of *C. albicans* SC5314 biofilms formed on silicone surfaces (4). Mature biofilms on silicone disks were treated with EtOH for 2 h. The sMIC50 of EtOH was 10%, and the sMIC80 was 20%. EtOH concentrations of 25% and higher inhibited the biofilms by >99%. EtOH signifi-
cantly decreased biofilm formation at all concentrations and reduced biofilm formation by >99% at concentrations of 10% or greater.

Regrowth after EtOH treatment was tested as described by Raad et al., with slight modifications (8). *C. albicans* SC5314 biofilms formed on silicone disks were treated with 5 to 50% EtOH for 2 or 4 h (Table 1). Then, silicone disks were removed aseptically, scrape sonicated for 3 min, and incubated for 24 and 48 h at 30°C in Sabouraud’s dextrose broth. Optical densities were read, and 100-μl dilutions were plated onto Sabouraud’s dextrose agar and incubated for 48 h at 30°C for colony counting. For both 2- and 4-h treatments, cell regrowth was observed after treatment with 5 to 25% EtOH. EtOH concentrations of 35% or higher were sufficient to prevent regrowth after a 4-h treatment; a concentration of 50% was required to prevent regrowth after a 2-h treatment at both 24- and 48-h incubation periods.

Limited data currently preclude recommendations on the use of AfrLT in the management of CR-BSI due to *Candida* species. Nonetheless, EtOH lock therapy offers a promising means of catheter salvage when removal is not viable. Blackwood et al. (2) described the successful use of 70% EtOH lock solutions instilled for at least 2 h daily for 14 days to treat three pediatric patients with candidemia. Venkatesh et al. (13) reported that 12.5% EtOH reduced *C. albicans* biofilm mass by 50% after 24 h of incubation in a 96-well plate model; however, biofilms were not eradicated. Raad et al. (9) investigated the effects of a 25% EtOH solution on *Candida parapsilosis* biofilms; although biofilm growth was initially suppressed after 15 min (modified Robbins device) or 1 h (silicone disk model) of EtOH exposure, biofilm regrowth occurred after 24 h of incubation.

This study suggests that at 35% EtOH, incubation times should be at least 4 h to ensure adequate sterilization. Higher EtOH concentrations are effective at eradicating biofilms in vitro, but potential issues include catheter occlusion from precipitation of 100% EtOH (6) and alterations in mechanical integrity/elasticity of polyurethane catheters (3). Catheter type must therefore be taken into account when considering EtOH lock therapy. Limitations of this study include a focus on *C. albicans* and not non-*albicans Candida* species. Furthermore, this study utilized a series of in vitro biofilm models, which are highly simplified representations of central venous catheter (CVC)-related infection. Additional studies in a biofilm flow and/or animal model would assist development of a clinically useful EtOH lock protocol. Further studies of EtOH for prevention of CR-BSI due to *Candida* species are also warranted.

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**TABLE 1** Regrowth of *Candida* biofilm cells after 2- and 4-h EtOH treatments and 24 h of incubation of silicone disks

<table>
<thead>
<tr>
<th>EtOH concn (%)</th>
<th>OD&lt;sub&gt;600&lt;/sub&gt;</th>
<th>Mean CFU/ml</th>
<th>OD&lt;sub&gt;600&lt;/sub&gt;</th>
<th>Mean CFU/ml</th>
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<td>0</td>
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<td>3.0 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.492</td>
<td>2.6 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
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<tr>
<td>5</td>
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<td>3.0 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.480</td>
<td>2.6 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
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<td>3.0 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.120</td>
<td>5.2 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
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</tr>
<tr>
<td>50</td>
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<td>0</td>
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</tr>
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<td>Negative control</td>
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<sup>a</sup> OD<sub>600</sub>, optical density at 600 nm.


