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In vitro Evaluation of Antibiotic Lock Technique for the Treatment of *Candida albicans, C. glabrata,* and *C. tropicalis* Biofilms

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Candidaemia associated with intravascular catheter-associated infections is of great concern due to the resulting high morbidity and mortality. The antibiotic lock technique (ALT) was previously introduced to treat catheter-associated bacterial infections without removal of catheter. So far, the efficacy of ALT against *Candida* infections has not been rigorously evaluated. We investigated in vitro activity of ALT against *Candida* biofilms formed by *C. albicans, C. glabrata,* and *C. tropicalis* using five antifungal agents (caspofungin, amphotericin B, itraconazole, fluconazole, and voriconazole). The effectiveness of antifungal treatment was assayed by monitoring viable cell counts after exposure to 1 mg/mL solutions of each antibiotic. Fluconazole, itraconazole, and voriconazole eliminated detectable viability in the biofilms of all *Candida* species within 7, 10, and 14 days, respectively, while caspofungin and amphotericin B did not completely kill fungi in *C. albicans* and *C. glabrata* biofilms within 14 days. For *C. tropicalis* biofilm, caspofungin lock achieved eradication more rapidly than amphotericin B and three azoles. Our study suggests that azoles may be useful ALT agents in the treatment of catheter-related candidemia.

Key Words: *Candida*; Biofilms; Antibiotic Lock Technique

INTRODUCTION

Candida is one of the most common causes of nosocomial infections (1). In particular, the ability of *Candida* spp. to form the surface adherent growths termed biofilms is an important attribute in catheter-related infections, which increase mortality, morbidity, and medical costs. The current recommendation for catheter- or device-related infections caused by *Candida* spp. is the removal and replacement of the infected device (2). However, the process is accompanied by increased costs. In addition, in many patients with catheter-related infection, the removal of the infected device is sometimes difficult or has procedure-related risks (3). As an alternative, although the removal of the catheter is most appropriate for catheter-related candidemia, the antibiotic lock technique (ALT) has been recommended for the prevention and treatment of catheter-related infections in specific situations by the Infectious Diseases Society of America (IDSA) and the Centers for Disease Control and Prevention (CDC) (2, 4). In the ALT, a concentrated antibiotic solution is instilled into the central venous catheter (CVC) lumen and allowed to dwell ("lock") for several hours or days. So far, a few antifungal agents including caspofungin, micafungin, amphotericin B, and

fluconazole have been evaluated for their efficacies in ALT for the treatment of infections mainly caused by *C. albicans* (5-7). However, other clinically important *Candida* species such as *C. glabrata* and *C. tropicalis* have been rarely tested.

In previous studies, we demonstrated the in vitro effectiveness of ALT for the treatment of catheter-related infections by staphylococci, *Pseudomonas aeruginosa,* and *Klebsiella pneumoniae* (8, 9). The results of our studies as well as those by other investigators have suggested that selection of antimicrobial agents, their concentration, and their treatment duration depending on the organisms are important (10). In this study, we examined the in vitro activity of ALT to determine the adequate antifungal agents and duration of locking therapy for treatment of fungal biofilms formed by *C. albicans, C. glabrata,* and *C. tropicalis.*

MATERIALS AND METHODS

Strain

C. albicans SMC154, *C. glabrata* SMC128, and *C. tropicalis* SMC297 were isolated from catheterized blood of patients with underlying diseases such as diabetes mellitus-complicated foot infec-

tion (*C. albicans* SMC154), prostate cancer (*C. glabrata* SMC128), and hypoxic brain damage after cardiac arrest (*C. tropicalis* SMC297). All strains were confirmed to be biofilm-forming by using 96-well plate.

Planktonic minimal inhibitory concentrations (MICs)

Five antifungal agents commonly used to treat candidiasis were selected for this study: caspofungin (Merck, Basal, Switzerland), amphotericin B (Sigma-Aldrich, St. Louis, MO, USA), fluconazole, voriconazole (both from Pfizer, New York, NY, USA), and itraconazole (Janssen-Ortho, Toronto, ON, Canada). Caspofungin, fluconazole, and voriconazole were solubilized in sterile water, and amphotericin B and itraconazole were solubilized in dimethyl sulfoxide. MICs of the free-floating (planktonic) fungi were determined by the broth microdilution method according to the Clinical and Laboratory Standards Institute (11). MICs were determined using Roswell Park Memorial Institute (RPMI1640) medium and Yeast nirtogen base medium supplemented with 50 mM glucose (YNB-Glc) to maintain the experimental conditions related to the biofilm assay. *C. parapsilosis* ATCC 22019 was used as a quality control strain.

Sessile MICs

The MICs of the adherent (sessile) biofilm fungi (SMICs) were determined using the 2,3-bis(2–methoxy–4–nitro–5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) reduction assay (12, 13). All drugs were prepared in a series of two-fold dilutions, ranging in concentration from 4-256 mg/L. The inhibitory effects against biofilms were measured as the absorbance at 492 nm by using a VERSAmax Tunable microplate reader (Molecular Devices, Sunnyvale, CA, USA). The $SMIC_{50}$ and $SMIC_{80}$ for *Candida* biofilms were defined as the lowest drug concentration with a 50% and 80% reduction, respectively, in absorbance compared with the drug-free control.

Biofilm formation

A polyurethane (PU) sheet prepared as previously described was cut into 1×1 cm pieces (thickness, 0.3 mm) and was sterilized with ethylene oxide gas (14). *Candida* strains were grown on Sabouraud dextrose agar (SDA) plates at 37°C for 48 hr. A cell suspension was prepared and diluted with YNB-Glc, to yield a final inoculum of about $1×10^7$ colony forming units (CFU)/mL. Four sheets of the PU squares were placed in 10 mL of a cell suspension in YNB-Glc, prior to incubation with shaking at 75 rpm and 37°C for 5 days to allow biofilm formation.

In vitro ALT model

In vitro ALT was performed according to a previously described method with minor modification (8). Briefly, after 5 days of incubation, the PU pieces were washed twice with phosphate-buffered saline (PBS) to remove nonadherent cells. The colonized squares of PU were transferred to YNB-Glc containing 1 mg/mL of the individual antifungal agents. Drug-free YNB-Glc was used as a control for each experiment. The biofilm killing activities of each antifungal agent were assayed after lock periods of 1, 3, 5, 7, 10, or 14 days. Antifungal lock solutions were replaced every 2 days. Biofilm eradication was evaluated by the determination of viable cell counts. The PU pieces were moved into new 50 mL tubes and washed twice with 10 mL of sterile PBS to remove planktonic fungi and drug residue. Then, the sessile bacteria were recovered to complete release of adherent yeast by high-speed vortexing in 3 mL of sterile PBS, followed by sonication using a VCX-400 sonicator (Sonics & Materials, Danbury, CT, USA) using conditions of 120 s, 30% cycle, 3.5 s pulses. The number of viable cells was counted in 10-fold serial dilutions following plating on SDA and incubation. Each experiment was repeated three times.

RESULTS

MICs and SMICs

The MICs and SMICs of the five antifungal agents tested against the biofilms formed by three *Candida* spp. strains are shown in Table 1. With regard to MIC, all strains were susceptible to all the tested antifungal agents. However, *C. albicans* SMC154 showed a paradoxical growth effect for caspofungin, as growth in the presence of caspofungin concentrations above the MIC was observed, in agreement with previous observations (15). In the XTT reduction assay, the biofilms of the three *Candida* spp. displayed resistance to all tested antifungal agents. All antifungal agents except amphotericin B showed high SMICs for all strains.

Table 1. MICs (mg/L) and SMICs (mg/L) of antifungal agents for *C. albicans*, *C. glabrata*, and *C. tropicalis*

Antifungal agents	*C. albicans* SMC154		*C. glabrata* SMC128		*C. tropicalis* SMC297	
	MIC	SMIC	MIC	SMIC	MIC	SMIC
Caspofungin	0.06*	>256/>256	0.06	>256/>256	0.25	>256/>256
Amphotericin B	0.5	16/32	0.5	32/32	0.5	4/16
Itraconazole	≤0.03	256/>256	≤0.03	128/>256	0.06	>256/>256
Fluconazole	0.25	>256/>256	0.25	128/>256	1	>256/>256
Voriconazole	≤0.03	>256/>256	≤0.03	>256/>256	0.06	>256/>256

*Parodoxical growth effect.
MIC, minimal inhibitory concentration; SMIC, sessile minimal inhibitory concentration ($SMIC_{50}/SMIC_{80}$).

Effect of ALT

Fig. 1 displays the relative ALT effectiveness of the five antifungal agents against biofilms formed by *C. albicans*, *C. glabrata*, and *C. tropicalis* strains. Initial biofilm viable concentrations of each strain after 5 day incubation was $7.75\pm1.75\times10^5$ CFU/cm^2 for *C. albicans* SMC154, $1.32\pm0.43\times10^6$ CFU/cm^2 for *C. glabrata* SMC128, and $1.14\pm0.98\times10^5$ CFU/cm^2 for *C. tropicalis* SMC297. For *C. albicans* SMC154, the PU films were sterilized within 10, 7, and 14 days of exposure to itraconazole, fluconazole, and voriconazole, respectively (Fig. 1A). However, 1 mg/mL of caspofungin and amphotericin B did not eliminate *C. albicans* SMC154 within 14 days. *C. glabrata* SMC128 was also completely eradicated by azoles (Fig. 1B). Itraconazole was the most effective against *C. glabrata* as biofilms were eradicated within 3 days. Fluconazole and voriconazole eliminated viable fungi in *C. glabrata* biofilms within 5 and 7 days, respectively. However, *C. glabrata* SMC128 was not completely killed by 1 mg/mL of caspofungin and amphotericin B. Although about 90% of *C. glabrata* SMC128 sessile populations had been killed at 10 days, re-growth was detected at 14 days (Fig. 1B). Caspofungin showed the most efficiency in treating *C. tropicalis* biofilms as it eliminated detectable viability removed within 5 days (Fig. 1C). The three azole agents (itraconazole, fluconazole, and voriconazole) also removed *C. tropicalis* SMC297 within 7 or 10 days.

DISCUSSION

Use of indwelling medical devices such as CVCs may increase the risk of infection due to formation of biofilms. Such infections associated with the biofilms results in significant mortality and morbidity, prolong hospitalization, and increase medical costs. Currently, removal of the catheter is the most effective treatment for catheter-related infections, especially in patients with severe sepsis or septic shock. However, the catheter might be retained in patients with stable condition to avoid catheter replacement. As a conservative strategy, ALT is recommended as an alternative option for catheter-related infections (2, 10). Although catheter-related infections are the most frequently associated with coagulase-negative staphylococci and *Staphylococcus aureus*, other pathogens such as Gram-negative bacilli and *Candida* spp. can also be involved (10, 16). Although some in vitro and in vivo studies have evaluated the efficacy of ALT for *Candida* spp. reliable experimental data on the selection of effective antifungal agents and duration time for ALT have not been sufficient (3, 5, 7, 13, 17).

Presently, the azole compounds itraconazole, fluconazole, and voriconazole were found to be generally superior to caspofungin and amphotericin B in eradication of *Candida* biofilms. While 1 mg/mL of each of the three azole compounds eliminated viable cells in biofilms formed by all three *Candida* species
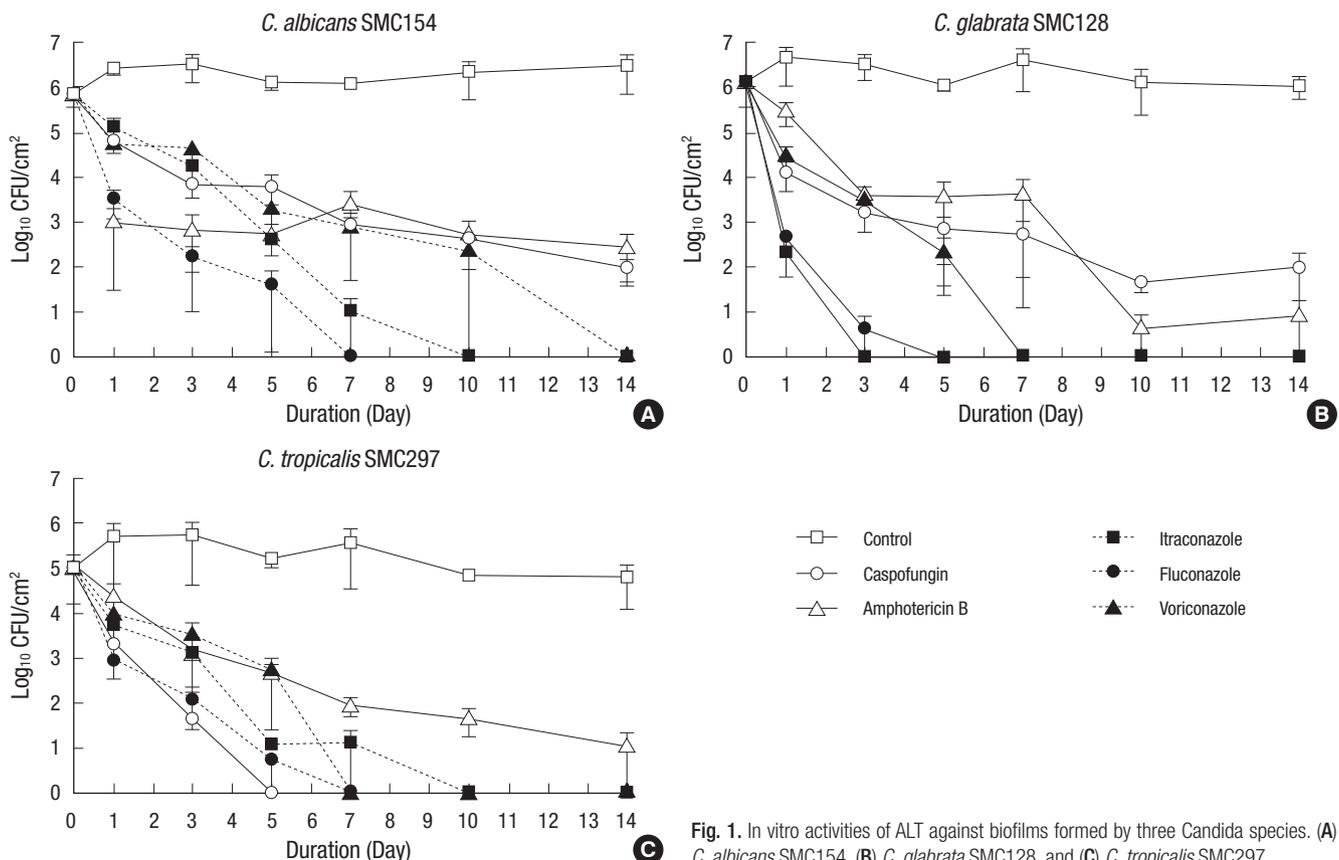

Fig. 1. In vitro activities of ALT against biofilms formed by three Candida species. (**A**) *C. albicans* SMC154, (**B**) *C. glabrata* SMC128, and (**C**) *C. tropicalis* SMC297.

within 14 days, caspofungin and amphotericin B did not. While caspofungin was most effective to treat *C. tropicalis* biofilms, viable cells were detected in *C. albicans* and *C. glabrata* biofilms treated were with 1 mg/mL of caspofungin within 14 days. One mg/mL of amphotericin B did not completely eliminate viability in biofilms formed by any of the three *Candida* species within 14 days.

Although the data is limited, previous studies have reported different results from our present data, suggesting that echinocandins like caspofungin and polyenes like amphotericin B might be effective against *Candida* biofilms (10). Some studies reported that ALT with amphotericin B may be effective in catheter-related candidemia (2, 3, 18). Cateau et al. (5) and Shuford et al. (7) showed that caspofungin may be useful in the treatment of *C. albicans* biofilms. Synergistic killing of *C. albicans* biofilms by caspofungin and fluconazole has been reported (19), although fluconazole alone is not necessarily effective in eradicating *C. albicans* biofilms (6, 17). Most studies have focused on *C. albicans* biofilms, to the exclusion of other important *Candida* species such as *C. glabrata* and *C. tropicalis*.

The different results from the previous studies may be due to the method used in determination of biofilm eradication. We evaluated the efficacy of ALT antifungal agents based on the reduction of viable cell counts as in our previous studies on bacterial infections (8, 9). This method of viable cell determination has been used in vivo (7, 17). In contrast, several in vitro studies monitored the reduction of metabolic activity using XTT (5, 6). Although the level of metabolic activity may be positively related with viable cell counts (6, 20), 50% reduction of metabolic activity was compared in the XTT reduction assay, rather than the complete eradication of biofilms as in the viable cell counting method presently used. Caspofungin and amphotericin B displayed more than 3-log reduction of viable cell counts (99.9% reduction), although they failed to eradicate biofilms completely in our study. Complete eradication of biofilm organisms may be more important in ALT as an alternative option of catheter removal to treat catheter-related infections to prevent the relapse of infection. In addition, cell density in PU films may be an important factor contributing to the different ALT results (21). Cell density can be influenced by several factors such as growth conditions, strains used in the study, and exposure time in biofilm formation. Because the initial cell density of biofilms could not be evaluated in the XTT assay, direct comparison of several ALT studies might not be possible with regard to cell density.

The different results might also reflect limitations in our study design. We used only one strain of each *Candida* species. However, the efficacy of ALT may strain-dependent as well as species-dependent. In addition, we evaluated only one concentration (1 mg/mL) of antifungal agents. Differing pharmacokinetics and pharmacodynamics of different antifungal agents may be reflected in differing optimal concentrations. Unlike previous studies, the reason that amphotericin B and caspofungin showed an inferior effect against *C. albicans* may be the higher MICs and SMICs (Table 1). SMIC$_{50}$ of caspofungin for *C. albicans* was >256 mg/L in this study, while it was 0.5 mg/L in another study (7). In addition, it could not be excluded that *C. albicans* biofilms might not be removed by 1 mg/mL of caspofungin because of paradoxical effect shown by *C. albicans* SMC154 for caspofungin. Further in vitro and in vivo studies incorporating diverse concentrations of antifungal agents against more strains are required. In vivo investigations should also be performed based on the results of in vitro studies to recommend the appropriate guidelines for treatment of catheter-related candidemia.

Our study suggests that azoles such as itraconazole, fluconazole, and voricanazole may be useful ALT agents in the treatment of catheter-related candidemia. Our in vitro results based on the treatment with 1 mg/mL of the selected antifungal agents demonstrates that fluconazole, itraconazole, and caspofungin are most effective in reducing the exposure time of antifungal lock solutions for *C. albicans*, *C. glabrata*, and *C. tropicalis* biofilms, respectively.

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