
BIOFILM FORMING CAPABILITY AND ANTIFUNGAL SUSCEPTIBILITY PROFILE OF *Candida* spp. FROM BLOOD

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ABSTRACT

Although *Candida albicans* remains the most frequent *Candida* species; however other species have emerged as important causes of candidiasis. In this work, we evaluated the *in vitro* susceptibility profile of *C. albicans*, *C. parapsilosis*, and *C. tropicalis* biofilms isolated from patients with candidemia to fluconazole, voriconazole, amphotericin B, and caspofungin. Differences between the biofilm ultrastructure of the three species were also determined. The isolates were phenotypically determined by growth on a Chromagar™ medium and assimilation profile on ID32C. The Scanning Electron Microscopy method (SEM) on biofilm was performed using polyurethane strips. For the *in vitro* susceptibility profile a microdilution in broth was used. Sessile cells were resistant to fluconazole, voriconazole and caspofungin. The resistance to amphotericin B was less pronounced and more variable between the tested isolates. In the SEM, slight differences in ultrastructural morphology for each species in biofilms were observed. Our results verified biofilm formation. Low susceptibility to the drugs in the three researched species confirmed the higher virulence of them.

KEY WORDS: Biofilm; *Candida* spp.; *in vitro* susceptibility; scanning electron microscopy.

INTRODUCTION

Candida species are important human pathogens because they cause high rates of morbidity and mortality in immunocompromised patients (Xie et al., 2008; Oberoi et al., 2012). The frequency of *Candida* infection varies in relation to causative species. Although *Candida albicans* remains the most common, *Candida parapsilosis*, *Candida tropicalis*, *Candida krusei*, and *Candida glabrata* have emerged as important cause of infection (Turner & Butler, 2014). Acquired resistance to antifungal agents has been observed amongst *Candida* species. Antifungal treatment can select microorganisms favoring infections caused by non-*C. albicans* species. Thus, continuous surveillance of species distribution trends and antifungal drug susceptibility profiles are necessary (Pfaller & Diekema, 2012).

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Virulence determinants that are selectively expressed can be involved in *Candida* species pathogenicity due to a host's dysfunctional defense system. Biofilm formation by *Candida* spp. has been implicated as a potential virulence factor in the development of candidiasis, mainly in patients using intravascular devices (Mane et al., 2012).

Biofilms are biological communities with a high degree of organization where microorganisms form structured, coordinated and functional communities. These biological communities are embedded in extracellular polymeric substances produced by the communities themselves (Donlan, 2002).

A biofilm has important clinical repercussions, as it is significantly less susceptible or resistant to antifungal agents (Mahmoudabadi et al., 2014). There are strong evidences that a *Candida* spp. biofilm is highly resistant to azoles, but there are discrepancies in the results of the effects of amphotericin B and caspofungin (Cocuaud et al., 2005; Mahmoudabadi et al., 2014).

Morphological examination by optical and electron microscopy allows evaluation of *in situ* activity and of the thickness of a biofilm (Mahmoudabadi et al., 2014). It is well known that the adsorption or neutralization of a drug could depend on the thickness of a biofilm, which presents differences among *Candida* species (Lattif et al., 2010; Prazynska & Gospodarek, 2014). Studies have demonstrated the resistance of *C. albicans* in biofilms to antifungal drugs (D'Enfert, 2006; Prazynska & Gospodarek, 2014), but there is relatively little knowledge about other *Candida* species.

Thus, the aim of this study was to investigate the *in vitro* susceptibility profile of *C. albicans*, *C. parapsilosis*, and *C. tropicalis* biofilms isolated from patients with candidemia to fluconazole, voriconazole, amphotericin B, and caspofungin. Differences between the biofilm ultrastructure of the three species were also assessed.

MATERIAL AND METHODS

Twenty-six isolates (10 *C. albicans*, 10 *C. parapsilosis*, and 6 *C. tropicalis*) recovered from blood from inpatients in the Intensive Care Unit (ICU) with candidemia from the “Hospital das Clínicas” of the Federal University of Goiás were examined. These previously isolated samples were stored at -70 °C in yeast extract peptone dextrose (YEPD) broth (Difco) using frozen milk.

Identification

The identification of the isolates, performed in the Mycology laboratory of Federal University of Goiás, were phenotypically determined by growth on a Chromagar™ (Chromagar company Paris, France) medium and assimilation profile on ID32C (BioMérieux France).

Antimicrobial Susceptibility Test for Plancktonic cells

Amphotericin B (Bristol-Myers Squibb, Woerden, The Netherlands), voriconazole (Pfizer Central Research, Sandwich, United Kingdom), and caspofungin (Merck & Co, Whitehouse Station, New Jersey), used in the *in vitro* susceptibility testing, were dissolved in dimethyl sulfoxide (DMSO) while fluconazole (Pfizer Central Research, Sandwich, United Kingdom) was dissolved in water and subsequently diluted in RPMI 1640 medium.

In vitro susceptibility tests of the cells in their free form were performed using a broth microdilution method, according to the Clinical and Laboratory Standards Institute (CLSI) document M27-S4 (CLSI, 2012). The final concentration ranged from 0.03 to 16 µg/mL for voriconazole and amphotericin B; from 0.015 to 8µg/mL for caspofungin, and from 0.125 to 64 µg/mL for fluconazole.

The minimal inhibitory concentrations (MIC) were determined visually after 24 h of incubation as the lowest drug concentration that significantly diminished growth ($\geq 50\%$) compared to the controls for voriconazole, fluconazole, and caspofungin. For amphotericin B, complete growth inhibition was considered.

The *in vitro* susceptibility interpretative criteria for fluconazole, voriconazole, and caspofungin were used according to the M27-S4 (CLSI, 2012) document. The isolates were considered resistant to fluconazole with MICs ≥ 8 µg/L and to voriconazole MIC ≥ 1 µg/L. For caspofungin, MIC ≥ 1 µg/L were considered resistant for *C. albicans* and *C. tropicalis*, while for *C. parapsilosis* MIC ≥ 8 µg/L were considered resistant. For amphotericin B, isolates with MIC > 1 µg/L were considered resistant.

Antimicrobial Susceptibility Test for Sessile Cells

Candida isolates were cultivated on Sabouraud dextrose agar for 24 h and used to induce biofilm formation in microtiter plates at an optical density (OD) of 550 nm (Prazynska & Gospodarek, 2014). The plates were incubated at 35°C for 48 h, washed with a 0.15 M phosphate buffer saline (PBS) to remove the planktonic cells and supplemented with fluconazole, amphotericin B, voriconazole, or caspofungin in concentrations used for sessile cells. The plates were sealed and incubated for 24 h at 37° C. After this period, the biofilm was quantified using the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) reduction assay. All antifungal agents were evaluated in concentrations 16 times higher, with the final concentrations ranging from 0.5 to 256 µg/mL for voriconazole and amphotericin B, from 0.25 to 128 µg/mL for caspofungin, and from 2 to 1,024 µg/mL for fluconazole. Microscopic examinations of biofilms formed in microtiter plates were performed by light microscopy using an inverted microscope. The sMIC (sessile Minimum

Inhibition Concentration) was defined as the lowest concentration capable of reducing the absorbance by 50% (sMIC 50) and 80% (sMIC 80) compared to the control. For each isolate, *in vitro* biofilm susceptibility was tested in triplicate.

Scanning Electron Microscopy

The method of scanning electron microscopy (SEM) on biofilm was performed according to Bizerra et al. (2008) with some modifications. Briefly, polyurethane (PU) strips were aseptically cut and placed into 24-well cell culture plates. An inoculum (10^6 cells/mL) of *Candida* isolates was added to the wells of the plates containing PU strips and incubated at 35 °C for 48 h. After the incubation period, non-adherent cells were gently washed twice with PBS (0.15 M, pH 7.0). The biofilm formed was fixed with 1.25% glutaraldehyde in PBS (0.15 M, pH 7.0) for 24 h at 4°C. After fixation, the cells were dehydrated with a series of ethanol dilutions (30, 50, 70, 90, 95, and 100 %) at room temperature, subjected to the critical point of the CO₂ chamber (Autosamdri®, 815, Series A), coated with gold (Denton Vacuum Desk V.), and examined in a scanning electron microscope (JEOL, JSM - 6610, equipped with EDS, Thermo scientific NSS Spectral Imaging).

RESULTS

The antifungals tested in this study showed decreased activity against sessile cells of all strains tested. The activity of amphotericin B and caspofungin was less pronounced than azole derivatives and more variable between the isolates tested (Table 1).

A range of 0.5 to 4 µg/mL for amphotericin B against *C. albicans* and of 0.5 to 2 µg/mL for caspofungin against *C. tropicalis* was found. sMIC 50 and sMIC 80 were lower for these drugs than for azole antifungals.

The data obtained in the *in vitro* susceptibility tests demonstrated increased values of sMIC 50 and sMIC 80 for all drugs evaluated compared to the values observed for planktonic cells as showed in Table 1.

All species were able to form biofilms. SEM analysis of representative isolates of these species showed biofilms with clusters of ovoid yeast cells with buddings and clear constriction rings. Differences were observed between the *Candida* species. The organization of *C. albicans* biofilm was composed of a large number of hyphae and blastoconidia in a thick biofilm mass (Figure 1A). In the ultrastructure of *C. parapsilosis*, enlarged blastospores or clumps of cells were verified, but no filamentation was observed, and the biofilms were thinner (Figure 1B). The morphology of *C. tropicalis* resembled that of *C. albicans*, but with lower filamentation (Figure 1C).

Table 1. Variation of the minimum inhibitory concentration ($\mu\text{g/mL}$) of amphotericin B, caspofungin, fluconazole and voriconazole in *Candida sessile* (sMIC50 and sMIC80) and planktonic (MIC) cells.

Antifungal	Planktonic cells			SMIC50			SMIC80					
	range	mean	MIC ₅₀	MIC ₉₀	range	mean	MIC ₅₀	MIC ₉₀	range	mean	MIC ₅₀	MIC ₉₀
AmphotericinB												
<i>C. albicans</i>	0.5-4	0.4	0.5	0.5	0.5-4	1.0	1	4	0.5-4	9.5	2	4
<i>C. parapsilosis</i>	1-2	0.3	0.5	0.5	1-8	56.3	1	2	1-8	158.4	1	8
<i>C. tropicalis</i>	0.5-1	-	0.5	0.5	-	-	1	1	1-4	-	4	4
Caspofungin												
<i>C. albicans</i>	1-2	0.09	0.06	0.25	2-4	88	1	2	2-4	192	2	4
<i>C. parapsilosis</i>	0.5-2	0.76	0.5	1	1-4	84.8	1	2	1-4	169.6	1	4
<i>C. tropicalis</i>	0.25-1	-	0.25	0.25	0.5-2	-	1	1	0.5-2	-	1	2
Fluconazole												
<i>C. albicans</i>	512->1024	26.16	1	>64	1024	972.8	>1024	>1024	1024	1024	>1024	>1024
<i>C. parapsilosis</i>	>1024	1.03	1	2	>1024	1024	>1024	>1024	>1024	1024	>1024	>1024
<i>C. tropicalis</i>	1024	-	0.5	0.5	>1024	-	>1024	>1024	>1024	-	>1024	>1024
Voriconazole												
<i>C. albicans</i>	128-256	6.45	0.125	>16	256->256	217.6	256	256	256->256	256	>256	>256
<i>C. parapsilosis</i>	32->256	0.05	<0.03	0.06	128->256	169.6	128	256	128->256	243.2	>256	>256
<i>C. tropicalis</i>	256->256	-	<0.03	0.5	>256	-	256	>256	>256	-	>256	>256

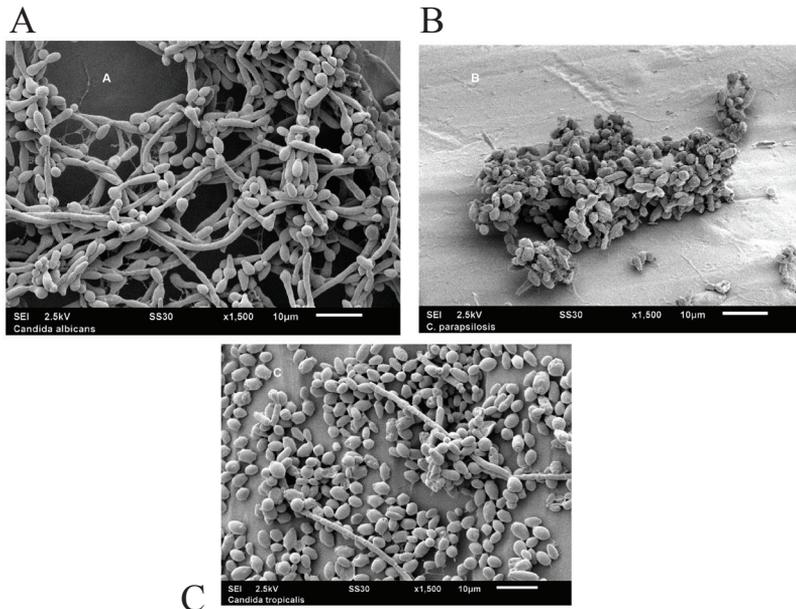


Figure 1. Ultrastructure of *Candida* species biofilms. A) *C. albicans*; B) *C. parapsilosis*; C) *C. tropicalis*. Scanning electron microscopy (SEM), 1500 X, the bar represents 10 µm.

DISCUSSION

Several studies have shown that MICs of planktonic cells can be potential predictors of the clinical response to antifungals (Aller et al, 2000). The most important feature of biofilm growth is the high resistance to antimicrobial agents that can be up to 1,000 fold greater than that of planktonic cells (Mah & O’Toole, 2001).

Although the resistance of *C. albicans* in biofilm to antimycotic drugs has been well described by D’Enfert (2006) and Prazaynska & Gospodarek (2014) there is relatively little about other *Candida* species. Therefore, research on the susceptibility of *Candida* spp. biofilm remains important. In our work, biofilms of *C. parapsilosis* isolated from blood demonstrated high sMIC, including amphotericin B (1-128 µg/mL) and caspofungin (16- >128 µg/mL). Melo et al (2011) observed resistance of *C. albicans*, *C. tropicalis*, and *C. parapsilosis* complex isolates to fluconazole, but, in general, verified susceptible isolates to amphotericin B, except for six (20%) *C. parapsilosis* strains (minimum biofilm eradication concentration - MBEC $80 \geq 8$ µg/mL). Similar to Ruzicka et al. (2007), the biofilms of all *Candida* species studied in our work were resistant to the antifungal agents: amphotericin B, fluconazole, and voriconazole. Such resistance explains the persistence of many infections,

mainly related to the use of catheters. In our study, caspofungin was effective against the biofilm formed by *C. parapsilosis*, with sMIC 80 of 4 µg/mL for nine strains. However, Ziccardi et al. (2015) showed inefficacy of caspofungin against *C. parapsilosis stricto sensu*, and Kuhn et al. (2002) verified that this antifungal agent presented high sMIC for strains of the same species. These interstudy differences may be due to the differences in *Candida* biofilm models used or to the biofilm-forming abilities of *Candida* isolates tested (Song et al. 2005).

Ruzicka et al. (2007) reported an increase in antifungal resistance during biofilm development, and showed that the progression of drug resistance was associated with increased metabolic activity of the developing biofilm, which indicates that drug resistance develops over time, coinciding with biofilm maturation. Therefore, growth rate is considered an important modulator of drug activity in biofilms (Mahmoudabadi et al. 2014; Prazynska & Gospodarek, 2014).

We used SEM to visualize morphological and structural changes between the three species and verified that *C. parapsilosis* and *C. tropicalis* biofilms are not as large as those generated by *C. albicans*. Similar to results obtained by Junqueira et al. (2011), *C. albicans* strains investigated in this study produced a large number of hyphae and blastoconidia in a thick biofilm mass. An interesting observation verified in our results was the morphology of *C. tropicalis*, which was similar to *C. albicans*, but with lower filamentation. The production of abundant blastospores without filamentation in *C. parapsilosis* samples observed in this study was previously reported by Lattif et al. (2010), which suggests that other factors may be correlated to the biofilm formation besides filamentation.

The ability to form biofilms and the low susceptibility of the three species to the drugs tested confirm the high resistance of *Candida* and non-*C. albicans* species isolated from blood. Reducing the incidence of biofilm related to candidemia in hospitals is required to improve patient care, as biofilm infections have a worse prognosis.

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