

Multidrug-resistant *Candida glabrata* strains obtained by induction of anidulafungin resistance in planktonic and biofilm cells

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Candida glabrata has emerged as a common cause of serious life-threatening fungal infections, largely owing to their low susceptibility to azole antifungals. Recent guidance indicates the use of echinocandins as the first-choice drug for the treatment of systemic infections of *C. glabrata*; however, *C. glabrata* resistance to echinocandins is reportedly increasing. Herein, we present the induction of anidulafungin resistance in planktonic and sessile cells of *C. glabrata* and the development of fluconazole cross-resistance. MICs of 21 clinical *C. glabrata* strains were determined by a broth microdilution method using anidulafungin and fluconazole. Biofilm formation on a tracheal catheter was determined using 1 × 1-cm² polyvinyl polychloride catheter fragments. Induction of anidulafungin resistance in planktonic and sessile cells and evaluation of its stability were performed by exposing the strains to successively higher concentrations of the antifungal. The induction resulted in strains strongly resistant to anidulafungin (MICs: 1–2 µg/mL) and fluconazole (≥64 µg/mL). Most of the sessile cells of *C. glabrata* presented slightly reduced susceptibility compared with the planktonic cells. Clinically, this cross-resistance could lead to therapeutic failure while using fluconazole in patients previously exposed to subinhibitory concentrations of anidulafungin for extended periods.

Keywords: *Candida glabrata*. Biofilm. Anidulafungin. Cross-resistance. Fluconazole.

INTRODUCTION

In recent decades, the frequency of invasive yeast infections has significantly increased with the increase in the number of patients undergoing immunosuppressive therapy, the use of broad-spectrum antimicrobials, and the frequency of organ transplants (Silva *et al.*, 2012). Systemic infections caused by *Candida glabrata* are characterized by a high mortality rate and are difficult to treat due to reduced susceptibility to azole antifungals, particularly fluconazole (McCornack, Perry, 2005; Katiyar *et al.*, 2012; Rodrigues, Silva, Henriques, 2014). This yeast can colonize host tissues and abiotic surfaces (catheters and prostheses) where it develops multi-

layer growth characterized as a biofilm (Katiyar *et al.*, 2012).

Biofilms are communities of microorganisms adhering to a surface and embedded in a polymeric extracellular matrix (Donlan, Costerton, 2002) and considered the most prevalent form of growth in the environment they inhabit. Biofilm formation is an essential virulence factor for *Candida* species, and it confers significant resistance to antifungal therapy by limiting the penetration of substances into the matrix and protecting the cells against the immune response of the host (Mukherjee, Chandra J, 2004).

Considering the importance of invasive mycoses and their current epidemiology, it is necessary to develop multiple therapeutic strategies. A few years ago, therapeutic management was performed with azoles and amphotericin B. However, considering the increasing azole resistance, mainly caused by non-*albicans Candida*

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species and due to renal toxicity of amphotericin B, a new pharmacological group, the echinocandins, emerged as a novel therapeutic option (Pham *et al.*, 2014).

Echinocandins inhibit the 1,3- β -D-glucan synthase (GS) complex that catalyzes the biosynthesis of 1,3- β -D-glucan, the primary glucan component of the fungal cell wall. GS, an enzyme, is complex and has at least two subunits: Fksp and Rho1p. Among these, Fksp, encoded by three paralogous genes (FKS1, FKS2, and FKS3), is the catalytic subunit and target of echinocandins. Resistance to these antifungal agents, resulting in therapeutic failure, has been linked to mutations in the Fksp subunit of GS (Riera *et al.*, 2012).

Recently, the yeast *C. glabrata*, usually commensal, has emerged as a common cause of serious life-threatening fungal infections largely owing to their low susceptibility to azole antifungals widely used in the treatment of these diseases (Silva *et al.*, 2012). Consequently, recent guidance indicates the use of echinocandins as the first-choice drug for the treatment of systemic infections of *C. glabrata* (Silva *et al.*, 2012; Pham *et al.*, 2014). However, studies indicate that *C. glabrata* resistance to echinocandins is increasing (Pham *et al.*, 2014).

Fluconazole-resistant isolates of *C. glabrata* have an increased possibility of being non-susceptible to echinocandins and vice versa. *C. glabrata* can present point mutations and changes in the chromosome structure, possibly related to mechanisms of adaptation to environmental changes. These genomic modifications may be a result of a defense mechanism making this fungus rapidly resistant to multiple drugs after limited exposure. For *C. glabrata* non-sensible to azoles and echinocandins, the only antifungal agent available is amphotericin B. Consequently, a two-fold problem arises because infections increase with aging, and amphotericin B is not well tolerated by elderly patients (Spreghini *et al.*, 2012).

Herein, we present a study of the induction of anidulafungin resistance in planktonic and sessile cells of *C. glabrata* and investigate the development of fluconazole cross-resistance.

MATERIAL AND METHODS

Fungal strains

In total, 21 clinical strains of *C. glabrata* (RL37m, RL03m, RL09m, RL02, RL03, HCCG01, CG08, RL09, CG06, CG03, CG04, CG40039, RL12, CG18S, RL22, RL24, RL25, RL26, RL45, RL49, and RL51) belonging to the mycology collection of the Applied Mycology

Laboratory, UFRGS, were used in this study. The clinical isolates were phenotypically identified by Vitek Yeast Biochemical Card (BioMerieux Vitek, Hazelwood, MO). In some tests, the characterized isolate CG40039 was included as a control.

Antifungal compounds

Stock solutions of anidulafungin (Ecalta®; Pfizer, São Paulo, Brazil) and fluconazole (Cristália®; São Paulo, Brazil) were prepared in sterile distilled water. For the experiments, the antifungal agents were diluted with RPMI 1640.

Biofilm formation assay on tracheal catheter material

The applied methodology is an adaptation of a procedure reported in the literature (Malheiros *et al.*, 2010; Tondo *et al.*, 2010). Initially, the strains were cultured on Sabouraud agar for 24 h at 35 °C. Seven colonies were added to 2 mL TSB followed by incubation for 24 h at 35 °C. Further, 1- × 1-cm² polyvinyl polychloride (PVC, Mark Med, number 12) catheter fragments were added to 9 mL peptone water containing 1 mL standardized inoculum in TSB followed by incubation for 96 h at 35 °C. Then, the catheter fragments were washed thrice with peptone water to remove poorly adhered cells. The fragments were added to other flasks containing 50 mL peptone water, and the adhered cells were released by sonication at 40 KHz for 10 min, the resulting water following sonication was diluted (dilution factor, 10), allowing the determination of colony forming units (CFU), and 20 μ L of each dilution was plated on Sabouraud agar. Next, the plates were incubated for 24 h at 35 °C, and the values of CFU/cm² were determined. All counts were performed in triplicate. The best biofilm-forming strain cells obtained by this test were cultivated on Sabouraud agar and stored for further use.

Planktonic and sessile minimum inhibitory concentration (MIC)

MICs of the planktonic and sessile cells of *C. glabrata* were determined in microplates by a broth microdilution method, as proposed by Clinical and Laboratory Standards Institute (CLSI), in accordance with protocol M27-A3 (CLSI, 2008), following the updates of document M27-S4 (CLSI, 2012). The antifungal was tested at concentrations of 0.0075–4.0 μ g/mL.

Induction of anidulafungin resistance in planktonic and sessile cells and evaluation of its stability

This test was performed by exposing the strains to successively higher concentrations of antifungal, as described by Fekete-forgacs, Gyure, and Lenkey (2000), with slight modifications. One colony of each *C. glabrata* strain, anidulafungin-sensitive [$\leq 0.12 \mu\text{g/mL}$ for anidulafungin was considered as susceptible and $\geq 0.5 \mu\text{g/mL}$ as resistant, M27-S4 (CLSI, 2012)], was incubated with 10 mL RPMI 1640 containing 4% glucose for 24 h at 35 °C. After centrifugation, supernatant was discarded, leaving only the yeast cells in the vials. Further, RPMI 1640 was added until a final absorbance of 0.1 ($\lambda = 640 \text{ nm}$) was obtained. After 10-h incubation, the material was centrifuged, and supernatant was discarded; 10 mL anidulafungin was diluted with RPMI to the desired concentration (MIC/8). After 14-h incubation, the previous procedure was repeated three consecutive times but with a 24-h incubation interval. After the third incubation, 20 μL of each culture was grown on Sabouraud agar for 24 h. A sample of cells was subcultured for MIC determination and stored for further testing. After another round of centrifugation and discarding of supernatant, the antifungal was added at the MIC/8 concentration until the final absorbance of 0.1 was obtained. After 10-h incubation, the culture was centrifuged again, and supernatant was discarded. Then, 10 mL MIC/4 concentration of anidulafungin was added and incubated for 14 h, as described above. The experiment was repeated doubling the concentration of anidulafungin until the final concentration of 0.25 $\mu\text{g/mL}$ was obtained for all strains.

Fluconazole cross-resistance detection

Fluconazole cross-resistance was assessed by MIC determination of anidulafungin-resistant strains and compared with MIC prior to the induction of *in vitro* resistance.

RESULTS

The biofilm formation assay demonstrated that all *C. glabrata* strains could form a biofilm on the tracheal catheter material (Table I). This screening for the identification of optimum biofilm formation indicated seven outstanding biofilm-forming strains (RL12, CG18S, CG40039, RL09m, RL22, RL25, and RL26), which were selected for the experiments of induction of anidulafungin

resistance. These strains were selected based on the results that showed the highest colony count (CFU/cm² and log).

TABLE I - *C. glabrata* biofilm forming strains on tracheal catheter material expressed in colony-forming unit per cm² (CFU/cm²) and logarithmic scale (log)

<i>Candida glabrata</i> Strains	Colony count (CFU/cm ²)	Colony count (log)
RL 37m	1,5x10 ⁴	4,1
RL 03m	2,2x10 ⁴	4,3
RL 09m*	7,5x10 ⁴	4,8
RL 02	0,2x10 ⁴	3,2
RL 03	0,2x10 ⁴	3,2
HCCG 01	1,5x10 ⁴	4,1
CG 08	1,5x10 ⁴	4,1
RL 09	1,5x10 ⁴	4,1
CG 06	0,2x10 ⁴	3,2
CG 03	0,2x10 ⁴	3,2
CG 04	0,2x10 ⁴	3,2
CG 40039*	7,5x10 ⁴	4,8
RL 12*	12,5x10 ⁴	5,1
CG 18S*	12,5x10 ⁴	5,1
RL 22*	7,5x10 ⁴	4,8
RL 24	0,7x10 ⁴	3,8
RL 25*	4,0x10 ⁴	4,6
RL 26*	3,5x10 ⁴	4,5
RL 45	1,5x10 ⁴	4,1
RL 49	2,2x10 ⁴	4,3
RL 51	1,5x10 ⁴	4,1

* Stains selected to the induction experiments of resistance to anidulafungin

MICs of the planktonic and sessile cells of *C. glabrata* determined by a broth microdilution method, as proposed by the CLSI, are presented in Table II. In general, the sessile cells presented slightly reduced susceptibility compared with the planktonic cells. MICs were in the range of <0.0075 – $0.03 \mu\text{g/mL}$ for the planktonic cells and 0.015 – $0.06 \mu\text{g/mL}$ for the sessile cells.

Following the induction of anidulafungin resistance against the sessile and planktonic forms of the *C. glabrata* strains, MICs were determined again. It was observed that six out of seven strains tested (86%) acquired strong anidulafungin resistance (Table II). Differences between MICs of the biofilm and planktonic cells after the induction of *in vitro* resistance were not noted, which was previously

verified. In addition, morphological changes (phenotypic switching) to non-susceptible *C. glabrata* strains were observed. Furthermore, after 30-day subculturing of the resistant strains in anidulafungin-free medium, MICs indicated that resistance was maintained for six strains.

Moreover, the occurrence of cross-resistance to fluconazole was investigated by an MIC assay for anidulafungin-resistant *C. glabrata* strains. MICs were compared with anidulafungin-sensitive strains. Surprisingly, all strains with previous exposure to anidulafungin showed resistance to fluconazole (MIC \geq 64 $\mu\text{g}/\text{mL}$; Table III). For the planktonic and sessile cells, very similar results were obtained.

DISCUSSION

According to protocol M27-A3⁴ and considering the updates of document M27-S4 (CLSI, 2012), anidulafungin-

susceptible isolates present MICs \leq 0.12 $\mu\text{g}/\text{mL}$. Notably, all selected strains were sensitive to the antifungal tested, in both their planktonic and sessile forms (Table II). In previous studies described by Pfaller and collaborators, only 3.8% of the isolates of *C. glabrata* obtained from Asia were resistant to anidulafungin, whereas none of the isolates from Latin America was non-susceptible to this antifungal (Pfaller *et al.*, 2012, 2013). The sessile form of the strains was less or equally sensitive to anidulafungin when compared with the planktonic form (Table II), which corroborates the findings of Kucharikova *et al.* (2011), suggesting that *C. glabrata* biofilms formed *in vitro* or by animal models are resistant to azoles but are sensitive to echinocandins.

The induction of resistance resulted in strains strongly resistant to anidulafungin with MICs in the range of 1–2 $\mu\text{g}/\text{mL}$ (Table II). Bordallo-Cardona and collaborators also obtained resistant *C. glabrata* strains

TABLE II - Minimal inhibitory concentrations (MICs) of anidulafungin and phenotypes (Phen) of sessile and planktonic cells of *C. glabrata* before and after induction of resistance to anidulafungin

<i>Candida glabrata</i> strains	MIC ($\mu\text{g}/\text{mL}$)/Phen*			
	Planktonic cells	Resistant planktonic cells	Sessile cells	Resistant sessile cells
RL 12	0.03/ S	1.0/ R	0.03/ S	1.0/ R
CG 18S	< 0.0075/ S	2.0/ R	0.06/ S	2.0/ R
CG 40039	0.03/ S	2.0/ R	0.03/ S	2.0/ R
RL 09m	0.015/ S	0.03/ S	0.03/ S	0.03/ S
RL 22	< 0.0075/ S	2.0/ R	0.015/ S	2.0/ R
RL 25	0.015/ S	2.0/ R	0.03/ S	2.0/ R
RL 26	< 0.0075/ S	2.0/ R	0.03/ S	2.0/ R

Phen* are expressed as susceptible (S) or resistant (R)

TABLE III - Minimal inhibitory concentrations (MICs) of fluconazole and phenotypes (Phen) of sessile and planktonic cells of *C. glabrata* before and after induction of resistance to anidulafungin

<i>Candida glabrata</i> strain	MIC ($\mu\text{g}/\text{mL}$) / Phen*			
	Planktonic cells control	Resistant planktonic cells	Sessile cells control	Resistant sessile cells
CG40039	16/ SDD	>64/ R	16/ SDD	>64/ R
RL12	32/ SDD	>64/ R	8/ SDD	>64/ R
RL09m	32/ SDD	64/ R	4/ SDD	>64/ R
RL22	1/ SDD	>64/ R	32/ SDD	>64/ R
RL25	32/ SDD	>64/ R	32/ SDD	>64/ R
RL26	4/ SDD	>64/ R	4/ SDD	>64/ R
CG18S	4/ SDD	>64/ R	4/ SDD	>64/ R

Phen* are expressed as susceptible-dose dependent (SDD) or resistant (R).

after *in vitro* exposure to higher concentrations of micafungin and anidulafungin. In their experiment, MICs of Sabouraud agar plates containing these antifungals were 0.06–4 µg/mL (micafungin) and 1–4 µg/mL (anidulafungin) (Bordallo-Cardona *et al.*, 2017).

Biofilm cells have antimicrobial resistance characteristics that confer tolerance to drug concentrations 1000 times higher than the MIC determined for planktonic cells (D'enfert, Janbon, 2016). Thus, as a final result of the induction of *in vitro* resistance, larger MICs were expected for the sessile than for the planktonic cells. Surprisingly, this behavior was not observed in the present experiments (Table II). It was verified that biofilm and planktonic cells present the same MIC, not maintaining their susceptibility observed prior to the induction of *in vitro* resistance.

To analyze the stability of the developed resistance, the strains were harvested for 30 days with antifungal-free Sabouraud agar. The results showed the preservation of resistance for all strains. Previously, Borst and coworkers (2005) also demonstrated the maintenance of acquired resistance to *C. glabrata* for at least 4 months after the removal of fluconazole. This result is clinically relevant and worrying because, once resistant, these cells will continue to express resistance genes for an extended period. Eventually, antifungal treatment becomes ineffective, making the introduction of a second therapeutic drug necessary. In addition, there is a risk of the dissemination of resistance of these isolates to other patients, further limiting therapeutic options.

In addition, morphological changes (phenotypic switching) observed in the *C. glabrata* strains can be related to resistance. Phenotypic switching enables microorganisms to undergo rapid microevolution and to adapt to a constantly changing microenvironment. In addition, it facilitates pathoadaptation into the host (Jain, Hasan, Fries, 2008). Similar to *C. albicans*, *C. glabrata* is capable of expressing different cellular phenotypes in colonizing populations, including phenotype irregular wrinkle (Lachke *et al.*, 2002). To *C. albicans*, this effect was correlated to high-level antifungal resistance, indicating that morphological change may have an effect on drug sensitivity (Vargas *et al.*, 2000).

According to the CLSI, the *C. glabrata* strains having MICs ≥ 64 µg/mL are considered resistant to fluconazole; meanwhile, *C. glabrata* presenting MICs ≤ 32 µg/mL are classified as sensitive in a dose-dependent manner. It was observed that prior to the induction of *in vitro* resistance, the planktonic and sessile forms of the strains were all sensitive to fluconazole in a dose-dependent manner with MICs in the range of 1–32

µg/mL (Table III). Interestingly, after the induction of anidulafungin resistance, this dose-dependent sensitivity to fluconazole was not maintained, making these strains resistant to this antifungal.

Fluconazole resistance has been associated with non-susceptibility to echinocandins in other studies (Pfaller *et al.*, 2012, 2013). Azole resistance often results from the overexpression of efflux pumps, which are not related to echinocandin resistance. However, *C. glabrata* has a unique ability to sequentially acquire and express mutations responsible for conferring resistance. Therefore, it is possible that previous treatments with fluconazole exert selective pressure, resulting in changes in the genome of the pathogen even in the absence of evident fluconazole resistance. These microorganisms may persist as colonizers in patients and emerge as resistant to echinocandins after subsequent exposure to antifungal agents (Vallabhaneni *et al.*, 2015). In our study, the opposite was observed: the emergence of fluconazole-resistant strains after previous exposure to anidulafungin. This observation was also previously reported (Alexander *et al.*, 2013), suggesting that previous therapy with azoles or echinocandins is predictive of resistance to both classes of antifungals.

Finally, most of the sessile cells had slightly reduced susceptibility when compared with the planktonic cells. The strains subjected to the induction test to anidulafungin resistance also developed strong anidulafungin and fluconazole resistances, corroborating reports of cross-resistance between the classes of azoles and echinocandins. Clinically, this cross-resistance could lead to therapeutic failure in fluconazole-treated patients who were previously exposed to subinhibitory concentrations of anidulafungin for extended periods.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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