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Effect of antifungals on itraconazole resistant *Candida glabrata*

Communication

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Abstract: In the last decade, infections caused by *Candida glabrata* have become more serious, particularly due to its decreased susceptibility to azole derivatives and its ability to form biofilm. Here we studied the resistance profile of 42 *C. glabrata* clinical isolates to different azoles, amphotericin B and echinocandins. This work was also focused on the ability to form biofilm which plays a role in the development of antifungal resistance. The minimal inhibitory concentration testing to antifungal agents was performed according to the CLSI (Clinical and Laboratory Standards Institute) M27-A3 protocol. Quantification of biofilm was done by XTT reduction assay. All *C. glabrata* clinical isolates were resistant to itraconazole and sixteen also showed resistance to fluconazole. All isolates remained susceptible to voriconazole. Amphotericin B was efficient in a concentration range of 0.125-1 mg/L. The most effective antifungal agents were micafungin and caspofungin with the MIC_{100} values of ≤0.0313-0.125 mg/L. Low concentrations of these agents reduced biofilm formation as well. Our results show that resistance of different *C. glabrata* strains is azole specific and therefore a single azole resistance cannot be assumed to indicate general azole resistance. Echinocandins proved to have very high efficacy against clinical *C. glabrata* strains including those with ability to form biofilm.

Keywords: *Candida glabrata* • *Antifungal agents* • *Resistance* • *Biofilm*

1. Introduction

Candida species belong to the group of opportunistic pathogens that cause both mucosal and disseminated infections in humans. *Candida albicans* and *Candida glabrata* are responsible for approximately 60% and 15% of candidiasis, respectively [1]. *C. glabrata* is an emerging pathogen and is now reported to be the most common "*non-albicans*" species causing candidaemia [2,3]. This yeast has manifested decreased susceptibility to fluconazole (FLC) and it is often recovered from clinical samples originating from AIDS or cancer patients [3,4]. In comparison with *C. albicans*, *C. glabrata* develops fluconazole resistance more easily following prolonged therapy [5,6]. Azoles, such as itraconazole (ITR) and voriconazole (VOR) are often used in the case of fluconazole resistant *C. glabrata* [7,8]. An alternative option is amphotericin B (AMB), which is considered to be a gold standard treatment for serious, especially life threatening, fungal infections [9,10]. The echinocandins,

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such as micafungin (MICA), caspofungin (CAS) and anidulafungin comprise the latest class of antifungals. Their target - the fungal-specific β-1,3-glucan synthase - results in lower toxicity for the host [6]. Additionally, clinical studies have demonstrated excellent efficacy of echinocandins in the treatment of candidiasis caused by azole resistant fungi or biofilm-associated infections [11]. *C. glabrata*, similarly to *C. albicans* has an ability to adhere to different medical substrates such as polystyrene, polyurethane or silicone, and then initiate biofilm formation. Confocal Laser Scanning Microscopy (CLSM) coupled with COMSTAT software revealed clear differences between *C. albicans* and *C. glabrata* in organization, architecture, kinetics and viability of biofilm [12]. *C. glabrata* biofilm matrix contains a greater amount of protein and carbohydrate [13]. Resistance to azoles in *C. glabrata* biofilm seems to be due to upregulation of *CgCDR1* and *CgCDR2*, two multidrug transporter genes, which is similar to that observed in *C. albicans*. However, no significant upregulation of *CgERG11* was observed during throughout the entire biofilm development [14]. The other important explanation for resistance has been implied by Al-Fattani and Douglas (2004) [15] and Al-Dhaheri and Douglas (2008) [16] who describe slower antifungal penetration through the biofilm. Our work studied the resistance profile of 42 *C. glabrata* clinical isolates to different azoles, amphotericin B and echinocandins. Additionally, in selected strains, the effect of echinocandins on biofilm formation was tested.

2. Experimental Procedures

2.1 Strains and identification

For this study, 42 *C. glabrata* clinical isolates resistant to itraconazole were selected. Thirty nine of the clinical isolates were obtained from Slovak women patients (all from vaginal or cervical tampon), and 3 isolates were obtained from men (2537/1 (from urine sample), 2238/1 (permanent catheter tip) and 8079/1 (from exudates)). *C. glabrata* ATCC 2001 American Type Culture Collection, USA) was used as a standard strain. The strains were identified using the cultivation of primoculture on CHROMagar Candida (Becton Dickinson, USA) overnight at 35°C. Identification was completed with the commercial biochemical set API 20C AUX (BioMérieux, France) according to the manufacturer's protocol.

2.2 Susceptibility testing

The susceptibilities to FLC (Pfizer, Switzerland), ITR (Janssen-Cilag, Beerse, Belgium), VOR (Pfizer, USA), AMB (Bristol-Myers Squibb, USA), MICA (Fijisawa, Japan), and CAS (Merck, USA) were tested by broth microdilution method according to the CLSI M27-A3 reference method [17] in RPMI medium (Applichem, Germany); FLC, MICA and CAS were dissolved in sterile water and ITR, VOR and AMB in dimethylsulfoxide (Sigma, USA, 1% v/v). The different concentrations tested were as follows: for FLC from 0.125 mg/L to 64 mg/L; for ITR and VOR from 0.0625 mg/L to 32 mg/L; for AMB from 0.0313 mg/L to 8 mg/L. The echinocandins MICA and CAS were tested in the concentrations from 0.0313 mg/L to 8 mg/L. The *Candida* inoculum was prepared by growing on Sabouraud dextrose plate and then suspended in RPMI medium to a final density of 1 x 10^3 cell/mL. The 96-well microtiter plates (Sarstedt, Germany) containing inoculum and appropriate concentrations of antifungal drugs were incubated at 35°C for 24 and 48 h, respectively. The growth was evaluated by microplate reader (MRX Microtitre plate absorbance reader, Dynex Technologies, USA) at 595 nm. While azoles have fungistatic effect on growth, AMB is fungicidal, so MIC_{80} and MIC_{100} were determined in three parallel wells, independently in three repeats.

2.3 Biofilm formation

Biofilm assay with selected clinical isolates was done according to Li *et al.* (2003) [18] with minor modifications. Cells were grown overnight at 37°C in YNB (Difco, USA) supplemented with 50 mM glucose. After three washing steps (5000 rpm, 5 min, 22°C) with 1 x PBS, 100 µl of cell suspension (OD_{600} 1.0) was applied into each well on a microtitre plate in triplicate and incubated for 1,5 h at 37°C to permit yeast adherence. After the period of adhesion each well was washed twice with 200 µl of 1 x PBS and then filled with 200 µl of YNB with 50 mM glucose. The plates were incubated at 37°C. After 24 h, non-adherent cells were removed by two washing steps with 200 µl of 1 x PBS and the different concentrations of MICA and CAS prepared in YNB with 50 mM glucose were applied (range from 16 µg/ml to 0.03125 µg/ml) followed by the incubation at 37°C for the next 24 h. Prior to the biofilm quantification, the wells were washed twice with 200 µl of 1 x PBS and the metabolic activity of biofilm was measured by XTT reduction method, where the XTT sodium salt [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] (Sigma, USA) is converted by mitochondrial dehydrogenasis of metabolically active cells to water-soluble formazan measured by spectrophotometer at 490 nm (MRX Microtitre plate absorbance reader, Dynex Technologies, USA) [18]. The statistical significance in the difference among samples was compared by Student's t-test. A P value of <0.05 was considered statistically significant,

Clinical isolates		Fluconazole		Itraconazole		Voriconazole		Amphotericin B		Micafungin		Caspofungin	
		MIC_{80}	MIC_{100}	MIC_{80}	MIC_{100}	MIC_{80}	MIC_{100}	MIC_{80}	MIC_{100}	MIC_{80}	MIC_{100}	MIC_{80}	MIC_{100}
1. *Candida glabrata*	2447/1	32	≥64	≥32	≥32	0.5	0.5	1	1	≤0.0313	≤0.0313	0.0625	0.0625
2. *Candida glabrata*	2633/1	≥64	≥64	≥32	≥32	0.5	0.5	1	1	≤0.0313	≤0.0313	0.0625	0.0625
3. *Candida glabrata*	3483/1	16	≥64	≥32	≥32	0.25	0.25	1	1	≤0.0313	≤0.0313	0.0625	0.0625
4. *Candida glabrata*	3621/1	32	≥64	≥32	≥32	0.5	0.5	0.5	0.5	≤0.0313	≤0.0313	0.0625	0.0625
5. *Candida glabrata*	4559/04	≥64	≥64	≥32	≥32	0.5	0.5	1	1	≤0.0313	≤0.0313	0.125	0.125
6. *Candida glabrata*	719/03	≥64	≥64	≥32	≥32	0.5	0.5	1	1	≤0.0313	≤0.0313	0.0625	0.0625
7. *Candida glabrata*	2338/1	≥64	≥64	8	≥32	0.125	0.125	1	1	≤0.0313	≤0.0313	0.0625	0.0625
8. *Candida glabrata*	2373/1	64	≥64	≥32	≥32	0.5	0.5	1	1	≤0.0313	≤0.0313	0.0625	0.0625
9. *Candida glabrata*	2407/1	32	≥64	≥32	≥32	0.25	0.25	1	1	≤0.0313	≤0.0313	0.0625	0.0625
10. *Candida glabrata*	2410/1	16	32	≥32	≥32	0.25	0.25	1	1	≤0.0313	≤0.0313	0.0625	0.0625
11. *Candida glabrata*	2452/1	16	≥64	32	≥32	0.25	0.25	1	1	≤0.0313	≤0.0313	0.0313	0.0313
12. *Candida glabrata*	2453/1	≥64	≥64	8	≥32	0.25	0.5	1	1	≤0.0313	≤0.0313	0.0313	0.0313
13. *Candida glabrata*	**12 441/1**	8	32	4	8	0.25	0.5	1	1	≤0.0313	0.0313	0.0625	0.0625
14. *Candida glabrata*	12 434/1	8	16	4	4	0.25	0.5	0.5	1	≤0.0313	≤0.0313	0.0625	0.0625
15. *Candida glabrata*	12 145/1	8	16	4	8	0.25	0.5	0.5	1	≤0.0313	≤0.0313	0.0625	0.0625
16. *Candida glabrata*	10 795/2	16	32	4	4	0.125	0.25	0.5	0.5	0.0625	0.0625	0.0313	0.0313
17. *Candida glabrata*	10 910/1	32	32	8	8	0.25	0.5	0.5	0.5	≤0.0313	≤0.0313	0.0625	0.0625
18. *Candida glabrata*	5809/1	8	16	8	16	≤0.0625	≤0.0625	0.5	1	0.0625	0.0625	0.0625	0.125
19. *Candida glabrata*	7443/1	≥64	≥64	8	16	0.5	0.5	0.5	0.5	0.0625	0.0625	0.125	0.125
20. *Candida glabrata*	12 399/1	16	16	4	4	0.25	0.25	0.5	0.5	0.0625	0.0625	0.0313	0.0313
21. *Candida glabrata*	**7738/1**	16	16	4	4	0.25	0.25	0.5	1	≤0.0313	≤0.0313	0.0625	0.0625
22. *Candida glabrata*	12 640/1	16	16	≥32	≥32	0.125	0.125	0.5	0.5	≤0.0313	≤0.0313	0.0625	0.0625
23. *Candida glabrata*	12 851/1	32	32	≥32	≥32	0.25	0.5	0.5	1	≤0.0313	≤0.0313	0.125	0.125
24. *Candida glabrata*	12 871/1	32	32	≥32	≥32	0.25	0.5	0.5	1	≤0.0313	≤0.0313	0.0625	0.0625
25. *Candida glabrata*	12 888/1	8	16	≥32	≥32	0.25	0.5	1	1	≤0.0313	≤0.0313	0.125	0.125
26. *Candida glabrata*	12 889/1	8	16	≥32	≥32	0.125	0.25	0.5	1	≤0.0313	0.0313	0.0625	0.0625
27. *Candida glabrata*	12 901/1	16	16	≥32	≥32	0.125	0.125	0.5	0.5	≤0.0313	≤0.0313	0.125	0.125
28. *Candida glabrata*	12 906/1	8	16	≥32	≥32	0.25	0.25	0,25	0.5	0.0313	0.0625	0.125	0.125
29. *Candida glabrata*	12 913/1	16	16	≥32	≥32	0.125	0.125	1	1	≤0.0313	≤0.0313	0.0625	0.0625
30. *Candida glabrata*	196/1	16	16	≥32	≥32	0.125	0.125	0.5	0.5	≤0.0313	≤0.0313	0.0625	0.0625
31. *Candida glabrata*	290/1	16	16	≥32	≥32	0.125	0.25	0.5	1	≤0.0313	≤0.0313	0.0625	0.0625
32. *Candida glabrata*	1685/1	≥64	≥64	≥32	≥32	0.125	0.125	0.25	0.5	≤0.0313	0.0625	0.125	0.125
33. *Candida glabrata*	2238/1	≥64	≥64	≥32	≥32	0.125	0.125	0.5	1	≤0.0313	≤0.0313	0.0625	0.0625
34. *Candida glabrata*	2315/1	2	4	4	8	≤0.0625	≤0.0625	0.125	0.5	≤0.0313	0.0313	0.0625	0.0625
35. *Candida glabrata*	2537/1	≥64	≥64	4	4	0.125	0.125	0.25	0.5	≤0.0313	≤0.0313	0.0625	0.0625
36. *Candida glabrata*	3569/1	4	4	8	16	≤0.0625	0.125	1	1	≤0.0313	≤0.0313	0.0625	0.0625
37. *Candida glabrata*	7382/1	4	4	≥32	≥32	≤0.0625	≤0.0625	0.5	1	0.0313	0.0625	0.125	0.125
38. *Candida glabrata*	7968/2	2	4	8	≥32	≤0.0625	≤0.0625	1	1	≤0.0313	≤0.0313	0.0625	0.0625
39. *Candida glabrata*	8063/1	4	4	8	≥32	≤0.0625	≤0.0625	0.25	0.5	≤0.0313	≤0.0313	0.0625	0.0625
40. *Candida glabrata*	8079/1	≥64	≥64	≥32	≥32	0.125	0.125	0.5	0.5	≤0.0313	≤0.0313	0.0625	0.0625
41. *Candida glabrata*	8461/1	4	8	≥32	≥32	0.25	0.5	1	1	≤0.0313	≤0.0313	0.0625	0.0625
42. *Candida glabrata*	**8848/3**	4	4	32	≥32	≤0.0625	≤0.0625	0.5	0.5	≤0.0313	0.0313	0.0625	0.0625
***Candida glabrata* ATCC 2001**		8	8	0.25	0.5	0.125	0.25	0.25	0.5	≤0.03125	≤0.0313	≤0.0313	0.0625

Table 1. Efficiency of fluconazole, itraconazole, voriconazole, amphotericin B, micafungin and caspofungin on *C. glabrata* clinical isolates.

MIC – minimal inhibitory concentration
*Breakpoints determining susceptibility/resistance to different antifungals used in this study according to the CLSI document (*S=susceptibility; **R=resistance; ***ND=not determined; ****NS=non-susceptible). For FLC *S≤8 mg/l, **R≥64 mg/l; ITR *S≤0.125 mg/l, **R≥1 mg/l; VOR *S≤1 mg/l, **R≥4 mg/l; AMB *S ***ND, **R ***ND; MICA *S≤2 mg/l, **R ***ND, ****NS≥2 mg/l; CAS *S≤2 mg/l, **R ***ND, ****NS≥2 mg/l)*
The bold signed strains were used for biofilm experiments as well as for the testing of different concentrations of micafungin and caspofungin on biofilm.

P<0.01 very significant and P<0.001 extremely significant.

2.4 Confocal Scanning Laser Microscopy

Mature biofilms formed on polystyrene coverslips in Petri dishes (both from Sarstedt, Germany), prepared according to Li *et al.* (2003) [18] with modifications of Borecká-Melkusová *et al.* (2008) [19] were transferred to a new Petri dish and 20 µL of tetramethyl rhodamine methyl ester, perchlorate (TMRM; Invitrogen, USA; excitation wavelength 549 nm, emission wavelength 573 nm), diluted in distilled deionized water to final concentration of 5 µM, was applied onto each coverslip. Stained biofilms were observed with a LSM 510 META confocal laser scanning microscope head, mounted on an Axiovert 200 M inverted microscope (both Carl Zeiss, Germany). The images were processed with the LSM Image Examiner software. Biofilm images were either displayed individually or reconstructed in three-dimensional (3-D) projections. The thickness was estimated from the outer edges of the area, where the TMRM signal gains intensity above half of its maximum.

3. Results and Discussion

The results of *C. glabrata* susceptibility/resistance testing are summarized in Table 1. All isolates tested were selected on basis of resistance to ITR with MIC_{80} and MIC_{100} ranging from 4 mg/L to ≥32 mg/L. Sixteen isolates out of the total 42 tested also exhibited resistance to FLC (MIC_{100}≥64 mg/L). Despite resistance to ITR, all clinical isolates showed susceptibility to VOR (MIC_{80} and MIC_{100} ranging from ≤0.0625 mg/L to 0.5 mg/L). It is of interest that resistance to ITR dominates over that estimated for FLC despite the fact that in Slovakia, FLC is the preferred choice used in both hospital practice and ambulant-treated recurrent candidiasis. Azole ITR is used only in special cases during hospitalization. As all *C. glabrata* isolates tested were recovered from ambulatory patients, we assume that they were not in contact with ITR therapy. Hence, the resistance mechanism is probably not mutation in *CgERG11* gene or overexpression of *CgCDR1* and *CgCDR2* efflux pumps. The prevalence of ITR resistance could be explained in relation to reduction of drug uptake rather than changes in ergosterol biosynthesis [20,21]. Our results confirmed that resistance of *C. glabrata* to different azoles is apparently independent of each other, so that single azole resistance should not be assumed to indicate general azole resistance. To this point it is important to mention that the determination of susceptibility to different azoles, not only to FLC, in routine clinical microbiological laboratories can prevent the exclusion of all azoles from consideration in treating *C. glabrata* infections.

Amphotericin B proved to be very effective in comparison with azoles: MIC_{80} and MIC_{100} for amphotericin B ranged between 0.125 mg/L and 1 mg/L.

In the treatment of very serious or biofilm-associated infections, echinocandins are recommended [11]. All our tested clinical isolates proved to be highly susceptible to both echinocandins. For CAS the MIC values ranged from 0.125 mg/L to 0.0313 mg/L. Caspofungin is an antifungal agent with high activity against a number of *Candida* species including those that are resistant to azoles [11, S. Kucharíková *et al.*, unpublished data]. In addition to this, it was shown to be better tolerated than amhotericin B deoxycholate [2,22]. So far, no resistance of *C. glabrata* isolate to echinocandins has been documented, but Brzankalski *et al.* (2008) [11] described 11 *C. glabrata* clinical isolates with decreased susceptibility to caspofungin (MIC ≥4 mg/l). In our study, all clinical isolates were also tested for their ability to form biofilm on 96-well polystyrene plates, but no correlation between biofilm-forming capacity and resistance to any antifungal drug was found (data not shown). Antifungal agents were administered prior to development of fully mature biofilm. Compared to *C. albicans*, *C. glabrata* develops biofilms that are usually thinner and patchy, rather than biofilms consisting exclusively of blastospores embedded within an extracellular matrix (Figure 1) [12]. The biofilm thickness was ~37 µm. Low concentrations of both MICA and CAS markedly reduced adherence capability and biofilm formation in *C. glabrata* 12441/1,

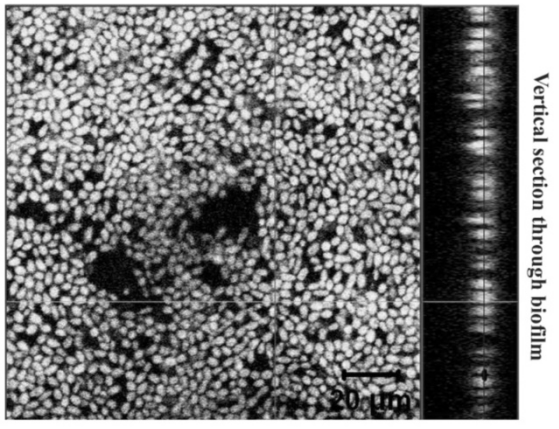

Figure 1. Confocal Scanning Laser Microscopy (CSLM) of mature biofilm formed on polystyrene slides by *C. glabrata* ATCC 2001 *in vitro*. Biofilm architecture is composed mainly of basal layer of yeast cells followed by upper layers covered by extracellular matrix. The biofilm thickness showed to be approximately 37 µm.

8848/3 and 7738/1 clinical isolates with high ability to form biofilm (OD_{490} 0.6-0.8, respectively, using the XTT reduction assay). When subinhibitory concentrations of MICA were added, mature biofilm was reduced by 70-96% compared to control biofilm without antifungal agents ($P<0.001$). Similar results were obtained with CAS (87% reduction of biofilm) ($P<0.001$). The excellent activity of echinocandins, not only during the early stages of biofilm development but also on mature biofilm, may result from the main mechanism of action leading to the inhibition of β-1,3 glucan synthase, which is responsible for forming the glucan polymers in the cell wall [23].

The data presented in this paper shows that resistance to antifungal drugs should not be assumed as a general phenomenon in *C. glabrata* and determination of MIC to different azoles seems to be necessary. Echinocandins proved to have a very high efficiency on itraconazole-resistant *C. glabrata* strains and could be a very good alternative drug for the treatment of *C. glabrata* infections.

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