

ARTIGO ORIGINAL

Biofilm antifungal susceptibility of *Candida* urine isolated from ambulatory patients

Susceptibilidade antifúngica em biofilme de *Candida* isolada a partir da urina pacientes ambulatoriais

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DESCRITORES

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KEYWORDS

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Disease Resistance;
Antifungal Agents.

RESUMO

Justificativa e Objetivos: A associação entre a formação de biofilme e resistência antifúngica foi sugerida por ser um fator importante na patogênese de diversas espécies de *Candida*. Além disso, estudos têm incluído candidíase invasiva de pacientes hospitalizados; no entanto, existem poucos estudos que avaliaram a distribuição das espécies, susceptibilidade aos antifúngicos e formação de biofilme de espécies de *Candida* isoladas de pacientes ambulatoriais. Assim, o objetivo deste estudo foi avaliar se a produção de biofilme contribui para a resistência antifúngica em *Candida* isoladas de amostras de urina obtidas de pacientes de ambulatório. **Métodos:** Durante um ano, 25 amostras de urina positivas para leveduras, foram coletadas, armazenadas e semeadas em ágar *Sabouraud* suplementado com cloranfenicol e deixadas a temperatura ambiente por 5 dias, para posterior identificação: 52% (13/25) foram *C. albicans*, 36% (9/25) *C. tropicalis*, 8% (2/25) *C. krusei* e 4% (1/25) *C. parapsilosis*. **Resultados:** A capacidade de formar biofilme foi detectada em 23 (92%) leveduras estudadas. 15,4% (2/13) de *C. albicans* foram fluconazol (FLU) e cetoconazol (PET) resistente, enquanto que 11,1% (1/9) de *C. tropicalis* foram resistentes cetoconazol e foram anidulafungina (ANI) não-suscetíveis. **Conclusão:** Nossos resultados mostraram a alta capacidade de formação de biofilme entre espécies de *Candida* isoladas de pacientes ambulatoriais.

ABSTRACT

Background and Objectives: the association between the biofilm formations and antifungal resistance has been suggested to be an important factor in the pathogenesis of several *Candida* species. Besides, studies have included invasive candidiasis from hospitalized patients; however there are few studies that evaluated the species distribution, antifungal susceptibility and biofilm formation of *Candida* species isolated from ambulatory patients. Thus, the aim of this study was to evaluate whether biofilm producing contributes to antifungal resistance in *Candida* isolates from urine sample obtained from ambulatory patients. **Methods:** During one year, 25 urine samples positive for yeast were collected, stored and plated on agar supplemented with chloramphenicol and Sabouraud left at room temperature for 5 days for subsequent: 52% (13/25) were *C. albicans*, 36% (9/25) *C. tropicalis*, 8% (2/25) *C. krusei* and 4% (1/25) *C. parapsilosis*. **Results:** The ability to form biofilm was detected in 23 (92%) of the yeast studied and 15.4% (2/13) of *C. albicans* were fluconazole (FLU) and ketoconazole (KET) resistant, while 11.1% (1/9) of *C. tropicalis* were ketoconazole resistant and were anidulafungin (ANI) non-susceptible. **Conclusion:** our results showed the high capacity for biofilm formation among *Candida* isolates from ambulatory patients.

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INTRODUCTION

Candida biofilm is a yeasts population, as hyphae and pseudohyphae, growing on a biotic or abiotic surface, such as mucosal and medical devices, which is enclosed in an extracellular polymeric matrix forming a three-dimensional structure.¹⁻³ Biofilms have been suggested to be an important factor in the pathogenesis of several *Candida* species. Due to the biofilms thickness and density, and likely due to the metabolic state of the yeast, antifungal drugs are not very effective in eradicating yeast contained therein.⁴ Several other mechanisms are also proposed for increasing of antifungal resistance when the yeasts are in biofilm form such as: altered growth; presence of extracellular matrix with delayed penetration of the antimicrobial agent; expression of resistance genes and presence of persisters cells.^{5,6}

The planktonic cell form is rarely found in human tissue, they are generally found in biofilm form in tissue, mucosal, implants, catheters and other surfaces, though.⁷ Besides, it has recently been shown that the cells that detach from the biofilm have a greater association with mortality than equivalent planktonic yeasts.⁸

In addition, in clinical laboratory routine, tools that allows biofilm cells antifungal resistance evaluation are not available, as a result only resistance profile of planktonic forms are accessible to most laboratories. Therefore, studies involving both planktonic and biofilm forms, as well as, antifungal resistance are necessary to allow the use of direct therapy with greater specificity when the *Candida* isolates are capable of producing biofilms.⁹

Candiduria is rarely present in healthy individuals; on the other hand it is a common clinical finding in hospitalized patients, especially those in intensive care units (ICU), which often have multiple predisposing factors such as diabetes mellitus, prolonged exposure to antibiotics and the use of urinary catheters.¹⁰ Currently, there are no specific methods for differentiation between *Candida* infection and *Candida* colonization.^{11,12} In consequence, there are few studies that evaluated the *Candida* biofilm production and antifungal resistance from urine sample obtained from ambulatory patients, thus the pathogenic potential of yeast obtained from ambulatory patients remain unclear.

Fluconazole is preferred for the treatment of *Candida* urinary tract infections (UTIs), because of its safety and achievement of high concentrations in the urine. Furthermore, it is less toxic than amphotericin B for the treatment of opportunistic infections by *Candida*. The main problem of the use of this drug is related to the intrinsic resistance of some *Candida* species such as *C. glabrata* and *C. krusei*, which limits its use in infections caused by these pathogens. The role of echinocandins and azoles that do not achieve measurable concentrations in the urine is not clear; however there may be an alternative treatment.^{13,14}

The antifungal resistance has emerged as an important phenomenon becoming a therapeutic challenge worldwide. Besides, the limited numbers of clinical antifungal agents can difficult candidiasis treatment. The

increase of antifungal resistance has been associated to use of selective therapies with inadequate doses or the increasing use of prophylaxis drugs for yeast infections, which is likely to lead to the selection of clinical resistance.¹⁵⁻¹⁷ Therefore, the aim of this study is to evaluate whether biofilm producing contributes to antifungal resistance in *Candida* isolates from urine sample obtained from ambulatory patients.

METHODS

Isolates. A total of 25 yeasts were obtained from urine samples during a 1-year period (2011-2012). They were collected, consecutively, at the Laboratório de Análises Clínicas e Toxicológicas at Faculdade de Farmácia and registered in the mycology collection of human pathogenic fungi of Federal University of Rio Grande do Sul and stored at -20°C until they were used.

This study was approved in the ethical committee of Federal University of Rio Grande do Sul number 18923 (UFRGS/Brazil).

Isolation and identification. The yeasts were isolated from urine samples when were seeded on the surface of Sabouraud agar with 40 mg chloramphenicol I⁻¹ (SCA) and incubated at 37°C in aerobic atmosphere until 5 days. The colonies with yeast typical morphology in the SCA were identified at the species level using CHROMagar *Candida*[®] medium, microculture and by automated systems using Vitek2 (BioMérieux, France).

Antifungal agents and susceptibility test in planktonic cells. The following antifungal agents were tested: Fluconazole (FLU); Ketoconazole (KET); Miconazole (MCZ); Anidulafungin (ANI) and Nystatin (NST). Stocks solutions were prepared and stored at -20°C until they were used. Working solutions were prepared in RPMI-1640 with L-glutamine, without bicarbonate (Gibco Invitrogen Corporation, USA) buffered with morpholinopropanosulfonic acid (MOPS) 0.165 M at pH 7.0 (RPMI-MOPS) at 128 mg I⁻¹ to FLU, 32 mg I⁻¹ for KET and MCZ, 16 mg I⁻¹ to ANI and 512 mg I⁻¹ to NST.

The Minimal Inhibitory Concentration (MIC) values were determined in microplates by broth microdilution using the twofold dilution method according to the guidelines of the CLSI (Clinical and Laboratory Standards Institute, 2008), document M27-A3. The antifungal concentrations tested ranged from 0.125 to 64 µg I⁻¹ for FLU, from 0.03 to 16 µg I⁻¹ for KET and MCZ, from 0.016 to 8 µg I⁻¹ for ANI and from 0.5 to 256 µg I⁻¹ for NST. Microplates were incubated at 37°C, and the results were read after 24-48h. Minimal Inhibitory Concentrations (MICs) were established for NST as the lowest concentration of antifungal agent which resulted in a complete inhibition of visible yeast growth, while for the azoles and ANI they were defined as the lowest concentration of antifungal agent that produced a 50% reduction in fungal growth when compared to the drug-free growth control one.

The MIC was interpreted according to the breakpoints suggested by document M27-A3 for FLU and KET. The breakpoints for MYC, ANI and NST were adapted

from Espinel-Ingroff and Cantón (2011), Ferrazza et al. (2005) and Isham and Ghannoum (2009).

Control strains. Four strains from the American Type Culture Collection (ATCC) were used as controls: *Candida albicans* (24433), *C. tropicalis* (750), *C. krusei* (6258) and *C. parapsilosis* (22019).

Biofilm formation assay on polystyrene microplate. The initial screening of yeast biofilm producers were performed according to Crystal Violet method as previously described by Stepanović et al. (2007), with adaptations in the yeast growing time. Briefly, 20 µL of a suspension containing 1.5×10^6 cells ml^{-1} in sterile saline (0.85%) and 180 µL trypticase soy broth (TSB) medium (Oxoid; Basingstoke, UK) supplemented with 1% glucose added in flat-bottom 96-well microplates and incubated for 48h at 37°C. To remove non-adherent cells, the wells were rinsed three times with sterile saline. The attached fungal were fixed with methanol for 20 min and dried for 30 min at room temperature. Crystal violet (0.5%) was used to stain the fungal for 15 min and dried for 15 min at room temperature. The biofilm was resuspended with ethanol for 30 min. The absorbance was measured at 570 nm in a microtitre plate reader (Envision, Perkin Elmer). TSB was used as a negative control and *Staphylococcus epidermidis* (ATCC 35984) was used as a positive control, because it is considered to be a strong biofilm producer. The mean absorbance values and their standard deviations (SD) were calculated. The average optical density (OD₄₇₀) values were calculated for all tested yeast and negative controls, performed in quadruplicate and repeated three times. All isolates were separated into categories using the O.D. measurement of bacterial films, as commonly used by Stepanovic et al., as follows: O.D. \leq O.D.c = no production of biofilm, O.D.c $<$ O.D. \leq (2x O.D.c) = weak production of biofilm, (2x O.D.c) $<$ O.D. \leq (4x O.D.c) = moderate production of biofilm and (4x O.D.c) $<$ O.D. = strong production of biofilm. The cut-off O.D. (O.D.c) was defined as three standard deviations above the mean O.D. of the negative control.

Biofilm formation assay in catheter. The yeasts that were strong biofilm producers in the biofilm formation assay on polystyrene microplate were used to determine its biofilm formation capacity in catheter. Firstly, the yeasts were growth in Sabouraud agar for 24h at 37°C, to obtain pure young colonies. After that, seven colonies were added to 2 ml of TSB and incubated for 24h at 37°C. The catheters (Foley Siliconized Latex - Rusch) were added to 99 ml of peptone water containing 1ml on the inoculum of TSB colonies and incubated for 96h at 37°C. Subsequent to incubation period, the catheters were washed three times with peptone water to remove the poorly adhered cells and were added 50 ml of peptone water. The adhered cells were release of catheter by sonication with frequency of 40 KHz, for 10min. Water containing each sonicated catheter was submitted to decimal dilutions (10^{-1} , 10^{-2} and 10^{-3}) and 20 µl of each dilution were seeded in Sabouraud agar. The plates were incubated for 48h at 37°C and the numbers of c.f.u (cm^2)⁻¹ were determined. As an experiment control, the

same procedure was performed with a catheter at zero time and the value of c.f.u (cm^2)⁻¹ was compared to the test value. All counts were made in duplicate and each experiment was repeated twice.

Susceptibility test in recently detaches cells and post-biofilm cells. The susceptibility profile of yeasts recently detach from the biofilm and susceptibility profile of planktonic yeasts post-biofilm cells were performed as describe above to planktonic cells. For these assays were also only used strong biofilm producers isolates.

Minimal biofilm eradication concentration (MBEC). For the strong biofilm yeasts it was determined the as described by Moskowitz et al. (2004) with modifications in the incubation time. The isolates were cultured in agar Sabouraud Dextrose for 24h at 37°C and biofilm formation was performed as described above in materials and methods. The microplates were incubated for 48h at 37°C and cells not adhered were removed by washing with sterile saline. This procedure was preceded by the antifungal susceptibility test as described above by microdilution broth. The antifungal agent was removed and the plates were washed three times with sterile saline. Subsequent steps of fixation, staining and elution were performed as described in biofilm formation assay on polystyrene microplates. The minimum concentration of antifungal agent required to eradicate the biofilm was defined as MBEC.

Statistical Analysis. The differences in the frequency of the distinct genotypes to biofilm production and antimicrobial resistance were analysed using the χ^2 test and Person's correlations coefficients. All statistical analysis were performed in SPSS 18.0 (SPSS Inc., Chicago, IL, USA), and a p value <0.05 was considered statistically significant.

RESULTS

To establish the resistance profile of planktonic yeast obtained from urine sample from ambulatory patients twenty five *Candida* spp. isolated in the one year period of study were evaluated. The result of the analysis of the qualitative urine test showed the presence of leukocytes in all samples, while 32% (8/25) samples had counts above the reference value and 68% (17/25) had counts within the value considered normal. These isolates were identified at species level and then antifungal resistance and the biofilm formation ability were evaluated. Of these 52% (13/25) were *C. albicans*, 36% (9/25) *C. tropicalis*, 8% (2/25) *C. krusei* and 4% (1/25) *C. parapsilosis*.

The susceptibility profiles of planktonic cells of 25 yeasts isolated in this study are shown in Table 1. All *C. krusei* isolates were fluconazole and ketoconazole resistant. When overall antifungal resistant was evaluated we observed that non-albicans *Candida* species were more resistant than *C. albicans* species with 50% (6/12) and 30.8% (4/13), respectively. The high rate of antifungal resistant obtained in this study was related to azoles classes, when compared with others antifungal classes tested here, 32% (8/25) of azoles, 4% (1/25) of echinocandin and

Table 1. Susceptibility profile of 25 different yeast antifungal.

Isolates (25)	Fluconazole			Ketoconazole			Miconazole			Anidulafungin		Nystatin		
	S	SDD	R	S	SDD	R	S	SDD	R	S	NS	S	SDD	R
<i>C. albicans</i> (13)	10	1	2	10	1	2	13	0	0	13	0	13	0	0
<i>C. tropicalis</i> (9)	9	0	0	7	1	1	8	1	0	8	1	9	0	0
<i>C. krusei</i> (2)	0	0	2	1	1	0	0	0	2	2	0	2	0	0
<i>C. parapsilosis</i> (1)	1	0	0	1	0	0	0	1	0	1	0	1	0	0

S: Susceptible, SDD: Susceptible dose-dependent, R: Resistant, NS: non-susceptible

none isolate was polyene class resistant.

The MIC_{50%} from FLU, KET, MCZ, ANI and NST to *C. albicans* were 1 µg ml⁻¹, 0.0313 µg ml⁻¹, 0.125 µg ml⁻¹, 0.0313 µg ml⁻¹ and 2 µg ml⁻¹, respectively and to *C. tropicalis* isolates were 2 µg ml⁻¹, 0.0313 µg ml⁻¹, 2 µg ml⁻¹, 0.0313 µg ml⁻¹ and 2 µg ml⁻¹, respectively. Of the 25 yeast studied herein, 92% (23/25) were biofilm producer and among these 34.8% (8/23) were strong, 39.1% (9/23) were moderate and 26.1% (6/23) were weak biofilm producer. No association was detected between biofilm production and antifungal resistance (*p*=0.529). Among strong biofilm producer 75% (6/8) were *C. tropicalis* and 12.5% (1/8) were *C. albicans* and *C. parapsilosis*.

Biofilm produced in Foley urinary catheters were evaluated in all strong biofilm producers of *Candida* isolates and the results obtained showed that all *Candida* isolates were able to produce biofilm in urinary catheters

(Fig. 1). In addition, to determine whether there are differences between antifungal concentration to eradicate planktonic cells and antifungal concentration to eradicate biofilm cells MIC was determined to planktonic cells and MBEC to cells in biofilm. These assays showed that the antifungal concentration to eradicate cells in biofilm is higher when compared with the MIC from planktonic cell. Besides, the MBEC was higher than all antifungal concentration tested in all drugs studied.

Some differences were observed in antifungal susceptibility from post-biofilm cells, 8 isolates had high MIC value when compared with the MIC value of planktonic cells (Table 2). Furthermore, two isolates recently detached from biofilm (*C. parapsilosis* and *C. tropicalis*) became fluconazole resistant. Interestingly, after successive cultivation, these isolates lowered the MIC value and became fluconazole sensitive again.

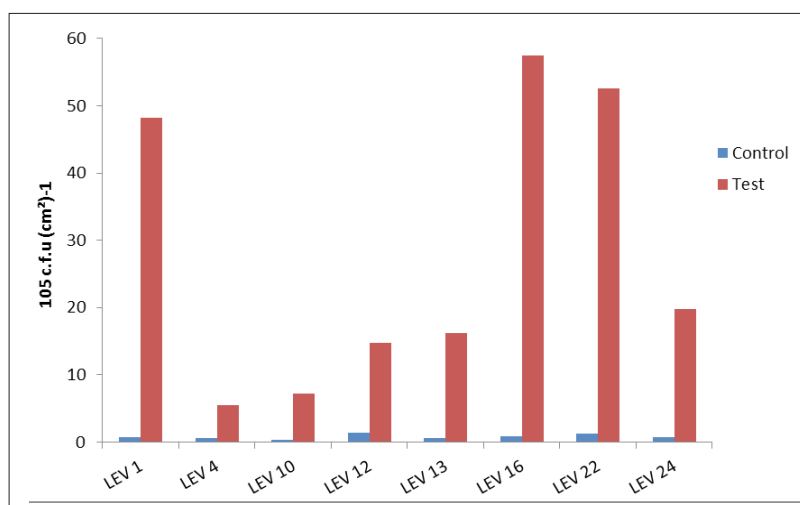


Figure 1. Biofilm formation of *Candida* spp. isolates in urinary catheter.

Table 2. Susceptibility profile of planktonic cells, biofilm cells and post-biofilm cells from 8 isolates of *Candida* spp.

Isolates (25)	Nystatin			Anidulafungin			Fluconazole			Miconazole			Ketoconazole		
	MIC*	MBEC*	PBC*	MIC*	MBEC*	PBC*	MIC*	MBEC*	PBC*	MIC*	MBEC*	PBC*	MIC*	MBEC*	PBC*
<i>C. tropicalis</i> (LEV1)	2	> 256	4	0.016	> 8	0.016	2	> 64	4	0.0313	> 16	0.5	0.0313	> 16	0.0313
<i>C. tropicalis</i> (LEV4)	2	> 256	2	1	> 8	1	0.5	> 64	0.5	4	> 16	4	0.0313	> 16	0.0313
<i>C. tropicalis</i> (LEV10)	2	> 256	2	0.016	> 8	0.016	4	> 64	4	8	> 16	8	0.0313	> 16	0.0313
<i>C. parapsilosis</i> (LEV12)	1	> 256	2	0.016	> 8	0.016	2	> 64	> 64	8	> 16	8	0.0313	> 16	0.0313
<i>C. tropicalis</i> (LEV13)	2	> 256	4	0.016	> 8	0.016	0.5	> 64	> 64	4	> 16	4	0.0625	> 16	0.0625
<i>C. tropicalis</i> (LEV16)	2	> 256	2	2	> 8	2	2	> 64	4	2	> 16	2	0.0313	> 16	0.0313
<i>C. albicans</i> (LEV22)	2	> 256	2	0.016	> 8	0.016	0.125	> 64	0.125	0.0313	> 16	0.0313	0.0313	> 16	0.0313
<i>C. tropicalis</i> (LEV24)	2	> 256	2	0.0313	> 8	0.0313	2	> 64	2	2	> 16	2	0.0313	> 16	0.0313

*The results are demonstrate in µg ml⁻¹; MIC = Minimal Inhibitory Concentration; MBEC = Minimal Biofilm Eradication Concentration; PBC = Post-biofilm Cell.

DISCUSSION

In this study were identified yeasts at specie level, analyzed the ability to form biofilm and the antifungal resistance profile from planktonic and biofilm cells of *Candida* isolated from urine sample from ambulatory patients. The identification at the specie level showed that more prevalent species were *C. albicans* followed of *C. tropicalis*; in others studies the *C. albicans* has also been described as more prevalent specie isolated from urine sample.^{18,19} Besides, *C. albicans* was the most prevalent in candiduria other non-*albicans Candida* species have become emerging isolates.²⁰ We also found some non-*albicans Candida* species in the urine sample from ambulatory patients, these data are in agreement with previous studies that described the high rate of non-*albicans Candida* invasive in patients from critical units. However, the epidemiological changes have also been detected in patients from community this phenomenon is important because the ambulatory patient, with superficial infections, may represent reservoirs of invasive fungal infections. Thus, fungal infections from ambulatory patients could be underestimated especially because sometimes there are no specific methods for differentiation between yeast infection and yeast colonization. Besides, some authors have described the importance of monitoring fungal infection in ambulatory patients which may lead to an invasive infection if opportunistic infection conditions occur.²¹

There are not specific methods for differentiation between *Candida* infection and *Candida* colonization we studied urine sample from ambulatory patients and it was difficult to determinate whether the patient was colonized or infected by yeasts. Then, we tried to analyze the number of leukocytes in the urine, however the results were inconclusive.²²

When we evaluated the susceptibility profile of planktonic yeast we found low rates of antifungal resistance among twenty five isolates studied. These results agreed with rates previously published about candiduria nosocomial, when was found low rates of antifungal resistance to amphotericin B, fluconazole, voriconazole and caspofungin.²³ In opposite, others studies found high rate of antifungal resistant to *Candida* species obtained from urine samples from patients in intensive care unit and patients with candiduria.^{24,25} In our study, when we tested the azole compounds we found two *C. albicans* fluconazole and ketoconazole resistant. Two *C. krusei* isolates, as expected, were resistant to fluconazole, its isolates were also miconazole resistant. One *C. tropicalis* isolate was ketoconazole resistant and one was anidulafungin non susceptible. On the other hand, all isolates tested were sensitive to nystatin. Consequently, we obtained a high rate of antifungal resistant to azoles classes, when compared with others antifungal classes we tested in accordance to previous data published that also described high level of resistance to azoles in patients with candiduria.²⁴ In this context, resistance to azoles could represent ambulatory reservoir of invasive fungal infection and resistant isolates. In addition, azole resistance is

a potential issue with *C. glabrata* and *C. krusei*, because these species are intrinsically more resistant and develop further resistance during prolonged azole therapy, especially to fluconazole. This fact may be a problem for therapeutic and prophylactic strategies.²⁵

Echinocandins have remarkable in vitro activity against *Candida* spp., including azole-resistant isolates, in our results it was also observed, because all *Candida* isolates azole resistant were anidulafungin sensitive. Although some reports the reduced susceptibility or resistance to these *Candida* isolates antifungal.^{2,3,25} In this study, both *C. albicans* and non-*albicans Candida* species were susceptible to nystatin *in vitro*, which supports the use of nystatin for the treatment of *Candida* infections in the genitourinary tract. Additionally, when we compared the rates of resistance between *C. albicans* and non-*albicans Candida* species, we also observed these rates were higher among non-*albicans Candida* species, this fact is a common characteristic among *Candida* infections.²⁵ In this study, biofilm formation appears as a characteristic present in different yeasts species clinical isolates from urine sample of ambulatory patients. We found high number of isolates with capacity to form biofilm, 92% (23/25) of isolates were biofilm producer. These results are in accordance with previous studies which reported that many yeasts species were capable of producing biofilms.¹⁶ When we classified the ability to form biofilm by species we found that *C. tropicalis* were strong biofilms producer, besides that, previous published results also demonstrate non-*albicans Candida* higher ability to form biofilm than *C. albicans* isolates.⁸

Many authors have showed that there is a relation between biofilm formation and antifungal resistance, however when we analyzed the correlation between biofilm production from planktonic cells and antifungal resistance no significance difference was found.¹⁷ When we analyzed eight yeast isolates, which were strong biofilm producer, in assay to biofilm producer in catheter, all isolates were able to produce biofilm in urinary catheter. Moreover the cells in biofilm, on microplates, were increasing MIC, thus the biofilm is an important factor in antifungal resistance, because when the yeast was in biofilm the antifungal drugs are not very effective in eradicating yeast contained therein as previously reported.⁴

To try elucidating to role of biofilm in antifungal resistance we compared the results obtained to MIC and MBEC, although we found no association between antifungal resistance and biofilm formation, MBEC was higher than MIC in planktonic cells. In consequence, these results suggest that biofilm could be an important factor in the antifungal resistance.¹⁷

The post-biofilm cells were tested and it was possible to observe that the MIC was higher in two species non-*albicans Candida* (*C. tropicalis* and *C. parapsilosis*) than planktonic cells to fluconazole. Other interesting results observed were that cells recently detached from biofilm showed higher MIC value, however after some passages in culture medium the MIC returned to value obtained when the planktonic cells were tested. Recent

studies reported a more considerable association between the cells that detach from biofilm and mortality than equivalent planktonic yeasts.¹² These results confirm that biofilm formation is a complex system and when the cells are in biofilm they became high pathogenic than planktonic cells, thus is necessary to evaluate mainly antifungal resistance of biofilm forms.

Biofilms of *Candida* can be formed quickly on plastic surfaces as Foley catheters and intrauterine devices.¹⁶ Previous studies have been shown that the use of Foley catheter is associated with candiduria, once the catheter serves as an entry of microorganisms in the urinary systems.^{9,10} Among patients undergoing catheterization in the short term, i.e up to seven days, 50% of them may developed infections; while all patients undergoing long-term catheterization, longer than 28 days, will be likely to develop urinary infection.¹¹ Our results showed that all tested yeasts are able to biofilm production in urinary catheter in a period of 96h. The high ability of biofilm formation of this genus is confirmed by both the data observed in this study and the literature.

In conclusion, our results showed the high capacity for biofilm formation among *Candida* isolates from ambulatory patients. When we analyzed the relation between antifungal resistance and biofilm formation from planktonic cells we did not find any significant association. However, our results point to the relation of biofilm in higher rates of MBEC and higher MIC value from post-biofilm cells, these results suggest that cells in biofilm are resistant to high dose of antifungal i.e above the therapeutic dose usually used. Thus, our results demonstrate the importance of a correct choice of antifungal therapy for the treatment of biofilm infections, as well as, the need, in routine clinical laboratory, of tools that determine specific susceptibility tests for cells in biofilm, as the susceptibility test based on MIC values alone cannot accurately determine the exact susceptibility of fungal in biofilm.

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