doi: http://dx.doi.org/10.5892/ruvrv.2012.101.156166

Antifungal susceptibility evaluation of *Candida albicans* isolated from buccal lesions of hiv-positive and HIV-negative patients

Irany Mesquita COELHO[1]
André Luis Ribeiro CLAUDINO[2]
Juliana Moscardini CHAVASCO[3]
Esther Goldenberg BIRMAN[4]
Walderez GAMBALE[5]
Natanael Atilas ALEVA[6]
Amanda Latercia Tranches DIAS[7]
Claudete Rodrigues PAULA[8]
Jorge Kleber CHAVASCO[9]

[1] Mestre em Microbiologia, UNIFAL-MG- iranymcm@yahoo.com.br,
[2] Mestre em Ciências Farmacêuticas,UNIFAL-MG, andrebiofar@yahoo.com.br
[3] Farmacêutica, Professora Titular da UNIVAS e Supervisora do Controle de Qualidade da União Química Farmacêutica Nacional. julianamoscardini@bol.com.br
[4] Livre-docente, Faculdade de Odontologia –USP (Universidade de São Paulo), egbirman@usp.br
[5] Professor Assistente-doutor, Instituto de Ciências Biomédicas (ICB)/USP, vgambale@usp.br
[6] Cirurgião Dentista e Professor de Graduação e Pós Graduação do INAPÓS (Pouso Alegre-MG) natanaelaleva@terra.com.br
[7] Professor adjunto, UNIFAL-MG, amanda.dias@unifal-mg.edu.br
[8] Professor Assistente-doutor, ICB/USP, crpmicol@uol.com.br
[9] Professor associado, Universidade Federal de Alfenas (UNIFAL-MG) (Orientador), jkchavasco@uol.com.br

[*]Autor correspondente:
Prof. Dr. Jorge Kleber Chavasco
Universidade Federal de Alfenas (UNIFAL-MG)
Rua Gabriel Monteiro da Silva 700
37130-000, Alfenas-MG
Telefone: 35-3299-1305
Email: jkchavasco@uol.com.br

ABSTRACT: The antifungal minimal inhibitory concentrations (MIC) were determined to 35 samples of *Candida albicans*; 14 of them were isolated from HIV-positive patients, and 21 from HIV-negative patients with oral erythematous candidosis. The aim of this study was to evaluate the performance of agar dilution method in the determination of susceptibility of *Candida albicans* isolated from buccal lesions of HIV-positive and negative patients to some antifungals and compare the results with the plasmatic concentration reached by each one of these drugs. The samples were evaluated *in vitro* by the agar dilution method and showed higher MIC values to ketoconazole, fluconazole, itraconazole and amphotericin B than the concentrations achieved by these antifungals in plasma. 88.9% of the samples presented *in vitro* resistance to ketoconazole and the plasmatic levels of this antifungal varied from 1 to 8 µg/mL. Regarding fluconazole and itraconazole, most samples presented MIC larger than 128 µg/mL and plasmatic concentration varying from 0.4 to 8 µg/mL. Only 11.9% of the samples were susceptible *in vitro* to fluconazole and 2.7% of them to itraconazol. The usage concentrations prescribed for the topical antifungals nystatin, fenticonazole and miconazole are markedly higher than the values of MIC obtained. Related to nystatin, it was verified that its MIC values were between 1 and 4 µg /mL. The plasmatic levels to this drug are extremely low. Fenticonazole presented a MIC value larger than 128 µg/mL. Relating to miconazole, the plasmatic levels vary from 1 to 8 µg/mL and 11.9% of the samples presented *in vitro* susceptibility to this drug. No significant differences (p<0.05) were found in the susceptibility profiles of the samples obtained from HIV-positive and HIV-negative patients.

Keywords: *Candida albicans.* Antifungal. MIC. Drug plasmatic level. HIV. Agar diffusion test

INTRODUCTION

Candida species are ubiquitous human fungal pathogens that are capable of initiating a variety of recurring superficial diseases especially in the oral and vaginal mucosae (MACPHAIL et al, 1993). The administration of broad-spectrum antibiotics, the extensive use of steroids, immunosuppressive agents in cases of organ transplant and the use of antineoplastic contributing to the increasing morbidity associated with *Candida*. In the last years, mucosal *Candida* infections have received a great attention due to the advance of HIV infection. For instance, it is known that up to 90% of HIV-infected individuals suffer from oropharyngeal candidiasis (SAMARANAYAKE, MACFARLANE, 1990; YUTHIKA et al, 2001).

Oropharyngeal candidiasis, usually diagnosed in more than 75% of the patients with AIDS, during elapsing of their illness, besides relapsing at high frequency, leads to a discomfort during food mastication and may result in great weakness for the organism and alteration of the immunological state and so, an effective antifungical treatment is made necessary and in a fast way (COLOMBO, 1994; DUPONT et al, 1994; IWATA, 1992; KERRIDGE; NICHOLAS, 1986). The frequent use of antifungal therapy in patients with AIDS, due to the constant recurrence of buccal or esophageal candidiasis, is a factor that probably influences on the occurrence of resistance to different drugs (TERREL; HUGHES, 1992).

A number of antifungal agents are available for the management of candidal infections. The major agents that are currently used for oropharyngeal candidiasis are the polyenes: amphotericin B and nystatin, the imidazoles: clotrimazole, econazole, ketoconazole, and miconazole, the triazoles: fluconazole, itraconazole, voriconazole; the echinocandins: caspofungina, anidulafungina, micafungina (MCGINNIS, RINALDI, 1996; JOHNSON and KAUFFMAN, 2003; BORMANN and MORRISON, 2009; MARIO et al., 2012).

The introduction of the imidazole and azole groups of antifungals during the last two decades has modified the management of fungal infections. One of the first effective medicines on the candidiasis treatment was the nystatin. This drug, however, is ideal for topical treatment of oral infections since it is not absorbed from the gastrointestinal tract and the adverse effects are minimal. It presents toxicity only when used by intravenous access. In function of that, new researches continue to be done, proposing the amphotericin B use, that is highly effective but is also endowed of treatment-limiting adverse effects such as nephrotoxicity (MEDOFF; KOBAYASHI, 1980; MEYER, 1994 ;WALSH et al, 1994).

The great number of antifungal drugs, the resistance report verified in *Candida* isolates, mainly in immunocompromised patients, and the need of a fast and effective treatment suscite a great interest in studies capable of standardizing tests of "in vitro"susceptibility for the choice of the appropriate therapy (ALVES, CURY, 1992; COLOMBO, 1994; GALGIANI et al, 1992; KORTING et al, 1988; MERZ,1986; SHADOMY; PFALLER, 1991).

Nowadays, a great part of efforts to develop a routine method for testing susceptibility of yeasts to antifungal agents has employed different species of *Candida* and this development is necessary in order to supply valuable information concerning the research of new substances, resistance to antifungals of frequent use, therapeutic control of infections and characterization of yeast samples (KORTING et al, 1988; MERZ, 1986; SHADOMY; PFALLER, 1991).

Besides the lack of usage of a single method for antimicrobial evaluation, their results can be compared (GALGIANI et al., 1992; LACAZ et al., 1991). By this way, the development of standardized antifungal susceptibility testing methods has been the subject of numerous studies during the last decades (REX et *al*., 2001; CLSI, 2008) and they have been, in their majority, adaptations of those ones proposed to bacteria. The available techniques are based on broth dilution and on agar-dilution test and agar-diffusion test (ALVES, CURY, 1992; CURY, MICHE, 1989; CURY et al., 1989; ESPINELL-INGROFF et al., 1992; MERZ, 1986; PFALLER et al., 1992). In the agar-diffusion test, the disks contain only one concentration of the drug, classifying the microorganism in susceptible, intermediate or resistant to that drug, usually in agreement with the plasmatic concentration reached by the drug (REX et al, 1993).

The aim of this study was to evaluate the performance of agar dilution method in the determination of susceptibility of *Candida albicans* isolated from buccal lesions of HIV-positive and negative patients to some antifungals and compare the results with the plasmatic concentration reached by each one of these drugs.

MATERIALS AND METHODS

Samples. Thirty five samples of *C. albicans*, coming from HIV positive (14) and HIV negative (21) patients with erythematous oral candidiasis. The collection and evaluation were approved by the Research Ethics Committee of the Universidade Vale do Rio Verde (protocol 196/1996). *C. albicans* ATCC 10231 was employed as standard sample. These samples are maintained at the fungus collection of mycology laboratory in the ICB/USP, São Paulo.

Antifungal susceptibility testing. We employed the proposed methodology of agar dilution according to ALVES and CURY, 1992. All drugs were obtained as reagent grade powders and were kindly supplied by the mentioned laboratories being nystatin and amphotericin B from Bristol-Myers Squibb (São Paulo, Brazil); fenticonazole from Asta Doctor (São Paulo, Brazil); fluconazole from Pfizer (São Paulo, Brazil), ketoconazole, itraconazole and miconazole from Jansen-Pharmaceutical Cilag (São Paulo, Brazil).

Antifungal stock solution. 25.6 mg of each antifungal were weighted and dissolved in 1 mL of dimethyl sulphoxide and itraconazol was solely dissolved in polyethyleneglycol. After the preparation in a glass sterile tube, the solution was allowed to rest by 10 minutes for self- sterilization.

Procedures of drug dilutions and culture media preparation. A series of 10 tubes was prepared, from which the first one contained 19 mL of the broth Yeast Nitrogen Base Fosfate (YNBP), and the others, each one contained 7 mL of the same broth. To the first tube, 1 mL of the antifungal stock solution was added followed by homogenization. Starting from this tube, serial dilutions were accomplished in duplicate, reaching up the tube number 10. From each tube of the series, 2 mL were transferred to other 10 tubes containing each one 18 mL of YNBP agar maintained at 45ºC. After homogenization, the medium with the incorporated drug was flowed in sterile Petri plates. After solidification, these were conserved in refrigerator at 4^{o}C for one week. The final concentrations of the drugs ranged between 0.25 µg/mL to 128 µg/mL .

Inoculum preparation. Starting from the 48 h cultivations of the *C. albicans* samples at 25ºC, on Sabouraud-dextrose agar, suspensions in 5 mL of phosphate buffer were made with tween 80. The inoculum was standardized to 10^6 UFC / mL, approximately (GALGIANI et al, 1992; PFALLER et al, 1988; SHADOMY; PFALLER, 1991).

Determination of the minimum inhibitory concentration. The standardized suspensions of *C. albicans* were inoculated in a volume of 10 µL on the welled agar plates containing the antifungal drug. A total of 20 samples were inoculated per plate. After the natural drying of the inoculum, the plates were incubated at 37ºC for 24 h. The reading was accomplished 48 h later by the observation of the presence or absence of yeast growth around the well. The minimum inhibitory concentration was considered the smallest concentration of the drug that hinded the growth of the yeast.

Quality Control. All susceptibility tests were repeated five times. The potency and viability of stock solutions were controlled. Periodic evaluations were realized with standard *C. albicans* sample ATCC 10231, whose antifungal susceptibility profile has been previously established.

Statistical Analysis. The software GraphPad Prism (version 5.0, 2007) was used to compare two independent samples to evaluate the different groups (susceptible strains vs. resistant strains in HIV positive or HIV negative patients).

RESULTS AND DISCUSSION

The MICs for 35 samples of *C. albicans* were ascertained, being 14 of them isolated from HIV positive and 21 from HIV negative patients and 1 standard strain of *C. albicans* ATCC 10231. The samples 03 to 31 correspond to HIV negative patients and the samples 32 to 52 to HIV-positive patients (Table 01).

In the case of amphotericin B, the MIC values were between 2 and 4 μg/mL. The plasmatic levels of this antifungal varied from 1 to 2 μg /mL. By this way, it was verified that a large percentage (61%) of these samples presented MIC values superior to 2 μg /mL and showed themselves an "in vitro" resistance to the drug. SOUZA et al (1990) and MAFFEI (1996) found 100% of the samples susceptible to this drug (Table 01) but HSUEH et al (2005) found 11% (23 of 2007 isolates) of resistance for *C. albicans* isolates. The amphotericin B MIC and plasmatic concentrations results among samples from HIV positive patients didn´t differ (p<0.05) from HIV negative ones.

Regarding Ketoconazole, it was verified the MIC values between 32 and 64 μg/mL for most of the samples. The plasmatic levels of this antifungal varied from 1 to 8 μg/mL, showing, therefore, that most of the samples (88,9%) present "in vitro" resistance to it. There weren´t seen statistical differences (p<0.05) among samples from HIV positive and negative patients. Similar results were obtained by MAFFEI (1996), who applied the same technique that us (Table 01).

Fenticonazole presented a MIC value larger than 128 μg/mL to samples from HIV positive and negative patients without statistical differences (p<0.05) among them. This antifungal is relatively new and its use is restricted to topical applications, being the vaginal cream with a concentration of 2.0% and the vaginal ovule with 600 mg of the drug. These concentrations is much larger than the values of MICs we found (Table 01).

Regarding fluconazole and itraconazole, the results obtained were similar to those of fenticonazole, that is, most samples presented MIC larger than 128 μg/mL (Table 01), and plasmatic concentration varying from 0.4 to 8

μg/mL. By this way, we verified that only 11.9% of the samples were susceptible "in vitro" to fluconazole and 2.7% of them to itraconazol without statistical differences (p<0.05) among samples from HIV positive and negative patients. Similar results were achieved by MAFFEI (1996), who observed a susceptibility to fluconazole for only 4.2% of the tested samples but these results shown discrepancies in relation to the results achieved by MENON et al. (2001) in that out of 16 strains of *C. albicans* isolated from oral lesions, one was resistant to fluconazole where as all were susceptible to itraconazole and in their research the "in vitro" MIC values correlated well with "in vivo" responses in patients. MOHANTY et al. (2007) didn't find complete resistance in any of the *Candida* species evaluated against fluconazole by broth microdilution method.

In the case of miconazole, taking into account that the plasmatic levels vary from 1 to 8 μg/mL, it was verified that only 11.9% of the samples presented "in vitro" susceptibility to the drug. The MIC ranged from 0.25 to 64 μg/mL. No statistical differences (p<0.05) were seen among samples from HIV positive and negative patients. MAFFEI (1996) encountered values different from ours, that is, approximately, 60% of samples were susceptible through "in vitro" evaluations. Miconazole is an antifungal of topical use, and the usage concentration is much higher than its plasmatic concentration (Table 01).

Related to nystatin, it was verified that its MIC values were between 1 and 4 μg /mL. The plasmatic levels to this drug are extremely low by considering that nystatin is not absorbed orally. No statistical differences (p<0.05) were seen among samples from HIV positive and negative patients. The medicines based on nystatin are used broadly for oral candidiasis, and its concentration in those products is approximately 5,000 IU/mg, what corresponds to 1.02 mg of the drug (DEF, 2002). This concentration is higher than the MIC values found in our studies (Table 01).

Concerning the samples collected from HIV-positive patients, one could also observe that, when they were compared to the samples collected from HIV-negative patients, there were no significant differences (p<0.05) in the resistance profile to the evaluated antifungals.

CONCLUSIONS

1 – Most antifungals exhibited MIC values higher than the respective plasmatic concentrations, what suggests that the samples present an "in vitro"resistance.

2 – Despite showing higher MIC values than the values of their plasmatic concentrations, fenticonazole, miconazole and nystatin were

shown to have topical use concentrations higher than the MIC values.

3 – No statistical differences were found in the "in vitro" susceptibility profile of the samples from either HIV positive or HIV negative patients.

Table 01 - Determination of the minimum inhibitory concentration of antifungals on samples of *C. albicans* isolated from HIV-positive patients and HIV-negative patients with erythematous oral candidosis .

Number of the sample	Reference number at ICB/USP	Minimum inhibitory concentration of the antifungals in µg/mL						
		Amphotericin B	Ketoconazole	Fenticonazole	Fluconazole	Itraconazole	Miconazole	Nystatin
Standard	12A	1.0	4.0	>128.0	0,25	0,25	16.0	1.0
3	28P	2.0	32.0	>128.0	4.0	>128.0	32.0	4.0
4	13P	4.0	16.0	>128.0	128.0	>128.0	16.0	2.0
6	20P	4.0	32.0	>128.0	32.0	>128.0	32.0	2.0
7	8P	0.25	4.0	>128.0	>128.0	>128.0	0.25	4.0
11	18P	2.0	32.0	>128.0	>128.0	>128.0	32.0	4.0
12	4P	4.0	4.0	>128.0	>128.0	>128.0	32.0	4.0
13	35P	2.0	32.0	>128.0	>128,0	>128.0	64.0	4.0
14	2P	4.0	32.0	>128.0	>128.0	>128.0	32.0	4.0
15	23P	4.0	32.0	>128.0	>128.0	>128.0	32.0	4.0
16	22P	2.0	32.0	>128.0	>128.0	>128.0	8.0	2.0
20	6P	2.0	32.0	>128.0	>128.0	>128.0	16.0	4.0
21	17P	4.0	32.0	>128.0	>128.0	>128.0	32.0	4.0
22	37P	4.0	32.0	>128.0	>128.0	>128.0	32.0	4.0
23	27P	4.0	32.0	>128.0	>128.0	>128.0	32.0	4.0
24	34P	2.0	64.0	>128.0	>128.0	>128.0	64.0	4.0
25	24P	4.0	32.0	>128.0	>128.0	>128.0	32.0	4.0

Table 01 (Cont.) - Determination of the minimum inhibitory concentration of antifungals on samples of *C. Albicans* isolated from HIV-positive patients and HIV-negative patients with erythematous oral candidosis

Number of the sample	Reference number at ICB/USP	Minimum inhibitory concentration of the antifungals in µg/mL						
		Amphotericin B	Ketoconazole	Fenticonazole	Fluconazole	Itraconazole	Miconazole	Nystatin
26	39P	4.0	32.0	>128.0	>128.0	>128.0	16.0	4.0
28	32P	4.0	32.0	>128.0	>128.0	>128.0	4.0	4.0
29	44P	4.0	32.0	>128.0	>128.0	>128.0	32.0	4.0
30	11P	2.0	32.0	>128.0	>128.0	>128.0	64.0	4.0
31	40P	2.0	32.0	>128.0	>128.0	>128.0	32.0	4.0
32	7H	4.0	32.0	>128.0	>128.0	>128.0	32.0	4.0
33	6H	4.0	32.0	>128.0	>128.0	>128.0	32.0	4.0
34	23PH	4.0	64.0	>128.0	>128.0	>128.0	32.0	4.0
35	17H	4.0	64.0	>128.0	>128.0	>128.0	32.0	4.0
36	23LH	2.0	64.0	>128.0	>128.0	>128.0	32.0	4.0
37	26H	2.0	32.0	>128.0	>128.0	>128.0	16.0	4.0
43	1H	4.0	32.0	>128.0	>128.0	>128.0	32.0	4.0
44	14H	4.0	32.0	>128.0	>128.0	>128.0	16.0	4.0
46	9H	4.0	8.0	>128.0	>128.0	>128.0	4.0	2.0
47	13H	4.0	32.0	>128.0	>128.0	>128.0	32.0	4.0
48	3H	2.0	32.0	>128.0	>128.0	>128.0	32.0	4.0
49	21PH	2.0	64.0	>128.0	>128.0	>128.0	64.0	4.0
51	25H	4.0	64.0	>128.0	>128.0	>128.0	32.0	4.0
52	11H	4.0	32.0	>128.0	2.0	>128.0	32.0	4.0

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