Multicenter Brazilian Study of Oral *Candida S*pecies Isolated from Aids Patients

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Oropharyngeal candidiasis continues to be considered the most common opportunistic disease in Aids patients. This study was designed to investigate species distribution, serotype and antifungal susceptibility profile among Candida *spp. isolated from the oral cavity of Aids patients recruited from six Brazilian university centers. Oral swabs from 130 Aids patients were plated onto CHROMagar Candida medium and 142 isolates were recovered. Yeast isolates were identified by classical methods and serotyped using the Candida Check® system-Iatron. Antifungal susceptibility testing was performed according to the NCCLS microbroth assay.* C. albicans *was the most frequently isolated species (91%), and 70% of the isolates belonged to serotype A. We detected 12 episodes of co-infection (9%), including co-infection with both serotypes of* C. albicans. *Non-albicans species were isolated from 12 episodes, 50% of them exhibited DDS or resistance to azoles. Otherwise, only 8 out 130 isolates of* C. albicans *exhibited DDS or resistance to azoles. Brazilian Aids patients are infected mainly by* C. albicans *serotype A, most of them susceptible to all antifungal drugs.*

Key words: oral candidiasis - *Candida* spp. - Aids - Brazil

Oropharyngeal candidiasis (OPC) is the most common opportunistic infection observed in Aids patients, occurring in an estimated 80 to 95% of these patients, when the CD4 T-lymphocyte counts are below 200 cells/mm^3 (Crowe et al. 1991, Dupont et al. 1994, Calvet et al. 1997). Increased retroviral replication and an associated decline in immune defenses render these patients particularly susceptible to OPC, to the extent that it is consider an early sign of HIV infection (Darouiche 1998). The prolonged nature of Aids predisposes these patients to recurrent episodes of OPC that can increase in frequency and severity with progressive HIV disease. Therefore, the prolonged management of OPC in this patient population causes the development of drug-resistant candidiasis (Powderly et al. 1999). *Candida* resistance to the azoles has been frequently attributed to a selective pressure caused by the use of these antifungal drugs as OPC prophylaxis or treatment (Barchiesi et al. 1996, Dronda et al. 1996). Many studies have estimated the incidence of clinical fluconazole resistance to be from 6 to 36%, depending on the patient group studied and the case definition used (Baily et al. 1994, Chavanet et al. 1994, Johnson et al. 1995).

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The advent of highly active antiretroviral therapy (HAART) has permitted suppression of viral replication to very low levels and a partial recovery of CD4 T cell count in HIV infected patients. Consequently, the incidence of opportunistic infections has declined, changing the natural history of HIV infection. However, opportunistic infections remain a problem among patients with a delayed diagnosis of infection and among non-responders to HAART (Brodt et al. 1997, Palella et al. 1998, Patton et al. 2000).

The aims of this study were to determine the species distribution and *C. albicans* serotypes of yeast isolates from Brazilian Aids patients with OPC and to analyze the in vitro susceptibility pattern of the isolates against fluconazole, itraconazole and ketoconazole.

MATERIALS AND METHODS

Specimen collection and culture of clinical isolates - We conducted a prospective study over a 24-month period, from March 1998 to February 2000, aimed at the investigation of species distribution, serotype and antifungal susceptibility profile among *Candida* spp. isolated from the oral cavity of Aids patients. Six tertiary care medical centers with active HIV patient treatment clinics joined the project at different stages during the two year study period: Universidade Federal de São Paulo, Unifesp, Universidade Federal do Rio Grande do Norte, Universidade de São Paulo, Ribeirão Preto, Universidade Federal do Paraná, Universidade Federal da Bahia and Universidade Federal de Uberlândia. The centers were requested to send sequential samples isolated from the oral cavity of Aids patients.

Samples were obtained by swabbing the oral mucosa of the subjects with sterile cotton swabs, plated onto CHROMagar Candida® (CHOMagar Microbiology, Paris, France) and incubated at 30ºC for a period not exceeding five days. After primary isolation, colonies were subcultured on Sabouraud dextrose agar (SDA, Difco Laboratories, Detroit). The centers were requested to send the isolates recovered to the reference laboratory, at Unifesp, together with case report forms containing clinical and epidemiological information, including data about the use of HAART and CD4 cells count for each patient at the time of collection.

Only one sampling was taken from each patient with OPC. When more than one *Candida* species were isolated from oral cavity of the same patient, all isolates were identified.

Green colonies isolated on CHROMagar Candida® were presumptively identified as *C. albicans* and the identification was confirmed by chlamydoconidia production on cornmeal-Tween 80 agar. Colonies presenting other colours were subcultured on new Sabouraud-dextrose agar plates and were identified according to a standard method. Briefly, isolates were submitted to microscopic morphology observation on cornmeal-Tween 80 agar, and carbohydrate fermentation and assimilation of 7 and 15 sugars, respectively. If necessary, organisms were also checked for urease production, nitrate assimilation, and ascospore formation (Kurtzman & Fell 1998).

Serotyping assay - Strains of *C. albicans* were prepared for slide agglutination serotyping with serodiagnostic reagent number 6 from Candida Check kit (Iatron Laboratories, Inc., Higashi-Kanda, Chiyoda, Tokio, Japan), as suggested in the package insert. *C. albicans* cultures were incubated on Sabouraud-dextrose agar plates for 48 h at 25ºC. Small amounts of specimen yeast cells was inoculated onto a Candida Check test tray and approximately 0.05 ml of specific serum (number 6) was added for testing and physiological saline was added as control. The glass test tray was stirred for about 1-2 min. A positive agglutination reaction was interpreted by the visualization of aggregates considered to be indicative of *C. albicans* serotype A. Negative results in the agglutination reaction with reagent number 6 were considered to be indicative of *C. albicans* serotype B.

Antifungal susceptibility testing - Susceptibility to azole drugs was tested using the broth microdilution method recommended by the National Committee for Clinical Laboratory Standards (NCCLS 1997). Reference grade powders of fluconazole (Pfizer Inc., New York, NY, USA), itraconazole (Janssen Pharmaceutica, Titusville, NJ, USA) and ketoconazole (Janssen Pharmaceutica) were used to obtain final drug concentration ranging from 0.125 to 64 µg/ml, 0.03 to 16 µg/ml and 0.03 to 16 µg/ml respectively. Briefly, broth microdilution testing was performed in sterile, flat-bottom 96-well microplates (Nunclon, Delta, Nunc. InterMed, Denmark), with RPMI-1640 (American Biorganics, Niagara Falls, NY, USA) added with L-glutamine and without bicarbonate, and buffered with MOPS, pH 7.0. The microplates containing double the final concentration of azoles were prepared in advance and stored at -70ºC for no more than three weeks until use. On the day of testing, the turbidity of the inoculum suspension was adjusted by the spectrophotometer to that produced by a 0.5 McFarland standard at 530 nm wavelength. A volume of 100 µl of the adjusted inoculum suspension was added to each well, resulting in the desired final drug concentration and inoculum size of 0.5 to 2.5 X 10^3 cells/ml. The plates were incubated at 35ºC for 48 h. A quality control strain (*C. parapsilosis* ATCC 22019) was included on each day of the assay to check the accuracy of the drug dilutions and the reproducibility of the results.

The minimal inhibitory concentration (MIC) of the azoles was defined as the lowest drug concentration which resulted in a prominent decrease in turbidity, as compared with that in the growth control (drug-free) well (NCCLS 1997).

Breakpoint definitions for fluconazole and itraconazole MICs were recently proposed by the NCCLS M27-A standard guidelines, considering isolates with MICs ≤ 8 mg/ml for fluconazole and ≤ 0.125 mg/ml for itraconazole as susceptible, isolates with MICs between 16 and 32 mg/ml for fluconazole and between 0.25 and 0.5 µg/ml for itraconazole as having dose-dependent susceptibility (DDS), and isolates with MICs ≥ 64 mg/ml for fluconazole and ≥ 1 mg/ml for itraconazole as resistant. Due to the lack of consensual definitions of breakpoints for the MICs of ketoconazole, arbitrary values were established based on those suggested by previous publications: susceptible for isolates with MICs ≤ 0.125 µg/ml, DDS for isolates with MICs between 0.25 and 0.5 µg/ml and resistant for isolates with MICs ≥ 1 mg/ml (Rodriguez-Tudela et al. 1995, St-Germain et al. 1995, Maenza et al. 1996).

RESULTS

A total of 142 isolates were recovered from 130 Aids patients with oral candidiasis enrolled in the present study. It is important to note that 11 patients were harboring more than one specie and/or *C. albicans* serotype in their oral cavities, at the time they were sampled. The age of the patients ranged from 18 to 65 years (median = 34 years) and 94 of them were males (72%). CD4+ lymphocyte counts were obtained from 115 of 130 patients and the values ranged from 0 to 454 cells/mm^3, with a median count of 42 cells/mm^3. Only 10 subjects had values higher than 200 cells/mm^3. Aids had been diagnosed from zero to 60 months before sample collection according to data obtained from 109 of 130 patients (median = 3 months; mean = 7.4 months). Fifty percent of the patients were enrolled in this study during their first episode of oral candidiasis, 35% had less than five episodes and only 15% had five or more episodes. Forty-eight patients (37%) were under HAART at the time of enrollment in the study. *C. albicans* was the most frequently isolated species (91%) and 70% of the strains belonged to serotype A. A total of 12(9%) non-*albicans* species were isolated, including *C. glabrata* (5), *C. tropicalis* (4), *C. parapsilosis* (1), *C. krusei* (1), and *Trichosporon inkin* (1). We detected the presence of 12 co-infection episodes (9%), 3 of them represented by both serotypes of *C. albicans*. Table I illustrates the MIC ranges, MIC_{50} and MIC_{90} of the three antifungal drugs tested against isolates of *C. albicans*

TABLE I

Susceptibility profile of *Candida* spp. isolates against three antifungal drugs

Drug	Species (n)	MICs range (µg/ml)	MIC_{50} (µg/ml)	MIC_{90} (µg/ml)
Fluconazole	*C. albicans* A (90)	0.03-≥64	0.25	0.5
	C. albicans B (40)	0.125-8	0.25	0.5
	C. glabrata (5)	4-8	4	8
	C. tropicalis (4)	0.125-1	0.5	1
	C. krusei (1)	32	_	_
Itraconazole	*C. albicans* A (90)	0.015-0.5	0.03	0.06
	C. albicans B (40)	0.007-0.25	0.015	0.06
	C. glabrata (5)	0.06-1	0.5	1
	C. tropicalis (4)	0.03-0.06	0.03	0.06
	C. krusei (1)	0.5	_	_
Ketoconazole	*C. albicans* A (90)	0.015-0.5	0.015	0.06
	C. albicans B (40)	0.015-0.125	0.015	0.03
	C. glabrata (5)	0.06-0.25	0.125	0.25
	C. tropicalis (4)	0.015-0.125	0.03	0.125
	C. krusei (1)	0.5	_	_

TABLE II

Isolates identified as dose dependent susceptibilidy/resistant to fluconazole, itraconazole and ketoconazole by the microdilution method, and CD4 cell count for the source patient

Code	Species	MIC			CD4 Cells/mm^3	Serotype
		FLZ	ITZ	KTZ		
CBA09A	*Candida albicans*	0.25	0.25	0.03	60	A
J17-D1	*C. albicans*	0.25	0.25	0.03	14	A
CUB02A	*C. albicans*	2	0.5	0.03	ND	A
CUB04A	*C. albicans*	2	0.5	0.06	38	A
CUB09A	*C. albicans*	4	0.5	0.125	8	A
J45-D22	*C. albicans*	8	0.25	0.125	167	B
CSP04A	*C. albicans*	≥64	0.25	0.5	ND	A
CSP09A	*C. albicans*	≥64	0.125	0.125	30	A
CNA03B	*C. glabrata*	8	0.5	0.125	54	-
CNA08B	*C. glabrata*	4	0.5	0.25	40	-
CRP02B	*C. glabrata*	4	1	0.25	67,3	-
CRP08C	*C. glabrata*	4	0.25	0.125	20	-
CNA12B	*C. krusei*	32	0.5	0.5	107	-
CBA16A	*Trichosporon inkin*	2	0.5	0.125	29	-

MIC: minimal inhibitory concentration; FLZ: fluconazole; ITZ: itraconazole; KTZ: ketoconazole; ND: not documented

serotype A (90), *C. albicans* serotype B (40), *C. glabrata* (5), *C. tropicalis* (4), and *C. krusei* (1). Both serotypes of *C. albicans* exhibited the same profile of susceptibility against azole drugs and *C. albicans* isolates were more susceptible than non-*albicans* species. The rank of susceptibility was *C. albicans* > *C. tropicalis* > *C. glabrata* > *C. krusei*. Fourteen of the 130 patients (11%) were harboring in their oral cavities *Candida* spp. isolates DDS or resistant to one or more azole drugs (Table II). The DDS/resistant azole isolates included 8 *C. albicans* and 6 non-*albicans* species.

DISCUSSION

C. albicans is the most pathogenic species of the genus *Candida* and is consistently the most frequently causative agent of candidal infection in humans. In Aids patients with OPC, *C. albicans* is followed in frequency by *C. glabrata*, *C. tropicalis*, *C. krusei*, *C. parapsilosis* and other non-*albicans* species (Hazen 1995, Coleman et al. 1998).

This is the first Brazilian multicenter study addressing *C. albicans* serotypes and antifungal susceptibility profiles (using the NCCLS methodology) of yeasts obtained from Aids patients with oropharyngeal candidiasis. We found a high prevalence of *C. albicans* recovered from HIV-infected and Aids patients with OPC (91%). Non-*albicans* species were isolated from 12 (9%) patients either as the sole isolate (n = 3) or in addition to *C. albicans* (n = 9), these being considered cases of co-infection. *C. glabrata* was the most frequent non-*albicans* species recovered in this series, followed by *C. tropicalis*, *C. parapsilosis*, *C. krusei*, and *T. inkin*. Oropharyngeal can-

didiasis due to *Candida* species other than *C. albicans* is relatively uncommon, although it does occur, especially in HIV-infected individuals, those with Aids and other immunocompromised patients submitted to protracted antifungal therapy. In HIV-infected patients, non-*albicans* species have been isolated from 15 to 20% of patients, and they are represented mostly by *C. tropicalis, C. parapsilosis, C. krusei* and *C. glabrata* (Barchiesi et al. 1993, Milan et al. 1998).

C. albicans may be antigenically divided into two major serotypes on the basis of differences in surface antigens defined as A and B, originally described by Hanseclever and Mitchell (1961). Antigenic expression on the surface of the *C. albicans* cell wall is a very dynamic process, which may reflect the ability of the microorganism to adapt to the host. The variability may be influenced by environmental and nutritional factors, as well as by the strain origin (Barturen et al. 1995). As a consequence, the variability of the incidence of OPC caused by serotypes A and B may be related to geographic diversity, antifungal selective pressure, underlying conditions, host factors and even by differences in the methods employed for *C. albicans* serotyping (Brawner 1991, Torssander et al. 1996). In the literature the distribution of serotypes A and B of *C. albicans* varies widely in different series. One of the reasons for this variability may be the number of the individuals evaluated in each series. In our study, 70% of the *C. albicans* isolates belonged to serotype A. In agreement with the data reported here, Pires et al. (1996) found a 70% frequency of serotype A from the oral cavities of Brazilian HIV patients. Thus, in Brazil, serotype A of *C. albicans* seems to occur at high frequency, particularly in Aids and HIV-infected patients. In contrast, in some studies performed in Europe and the USA, the authors suggested that *C. albicans* serotype B seems to be more prevalent. Velegraki (1995) observed a 70% frequency of *C. albicans* serotype B in 30 Aids patients with OPC in Greece. In the US, a 65% prevalence of serotype B was detected in Aids patient (Brawner & Cutler 1989). In Sweden, Torssander et al. (1996) observed that, among homosexual men, the prevalence of serotype B was 40% in HIV-infected patients and 39% in HIV-seronegative patients, while in control subjects of heterosexual non-HIV men, the prevalence was only 7.5%. In Europe, nevertheless, HIV-seronegative populations seem to have also a greater prevalence of serotype A (McMullan-Vogel et al. 1999, Williams et al. 2000).

In agreement with other authors, we found that both serotypes of *C. albicans* exhibited similar in vitro susceptibility patterns to all azole drugs tested. However, it should be noted that serotype B seems to be significantly more resistant than serotype A to 5-fluorocytosine (Auger et al. 1979, Quindos et al. 1995).

Twelve cases of co-infection (9%) were detected, including three cases of isolation of both serotypes of *C. albicans.* Up to 15% of *Candida* spp. mixed cultures from oral cavities of HIV-infected patients has been described by other authors and the species isolated in addition to *C. albicans* did not seem to play an important role in the pathogenesis (Beighton et al. 1995, Dronda et al. 1996). However, the presence of two or more species in the same patient may predispose to recurrent candidiasis, mainly in the presence of species for which azole MICs are intrinsically high, like *C. glabrata* or *C. krusei* (Millon et al. 1994). Of note, episodes of co-infection were observed in our study among patients with none or low exposure to antifungal drugs, as well as a CD4 count lower than 107/mm^3. These data suggest that co-infections may reflect not only previous exposure to antifungal drugs, but also the severity of the immunosuppression condition of patients and contact with patients harboring different species.

Fourteen of the 130 patients (11%) harbored DDS yeast isolates or isolates resistant to one or more azole drugs in their oral cavities. Eight DDS or resistant isolates were *C. albicans* and 6 were non-*albicans* species represented by *C. glabrata* (4) and *C. krusei* (1) and *T. inkin* (1) isolates. All non-*albicans* species mentioned are usually less susceptible or resistant to azoles. Among the 8 *C. albicans* isolates for which azole MICs were high, 2 isolates were resistant to fluconazole and 6 were DDS to itraconazole. Both resistant *C. albicans* isolates were found among patients with previous exposure to antifungal drugs and a CD4 count lower than 30 cells/mm^3. The risk of developing mucosal candidiasis with reduced susceptibility to azoles has been associated with greater duration of HIV-infection, severe immunosuppression states and cumulative prior exposure to antifungal drugs, mainly fluconazole.

Of note, 11 of 13 patients harboring DDS and/or resistant *Candida* spp. isolates to azoles had CD4 count lower than 67cells/mm^3, and most of them reported a history of recurrent OPC episodes and previous use of antifungal drugs. Both *C. albicans* isolates, resistant to fluconazole were obtained from patients (CSP04 and CSP09) who had a long history of exposure to azoles, due to recurrent episodes of OPC (more than five episodes).

In conclusion, Brazilian Aids patients are infected mainly by *C. albicans* serotype A, most of them susceptible to all antifungal drugs. The occurrence of azole refractory candidiasis and non-*albicans* species isolates are more prevalent among patients in advanced stages of disease, with a history of recurrent oral candidiasis and exposed to intermittent or continuous antifungal therapy. Thus, identification procedures up to the species level, as well as antifungal susceptibility testing should be requested mainly for Aids patients with OPC who exhibit such conditions.

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