Mycopathologia (2013) 175:91–98
DOI 10.1007/s11046-012-9593-0

Pseudomembranous Candidiasis in HIV/AIDS Patients in Cali, Colombia

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Received: 14 April 2012 / Accepted: 9 October 2012 / Published online: 21 October 2012

Abstract *Candida albicans* is the most frequently isolated yeast from the oral cavity of HIV/AIDS individuals. The use of fluconazole has increased the number of resistant or less-sensitive *Candida* species different than *C. albicans*. The purpose of this study was to identify the *Candida* species producing pseudomembranous candidiasis in patients suffering from AIDS, their relationship with $CD4^+$ counts and their sensitivity to fluconazole and itraconazole. We studied 71 patients at a hospital in the city of Cali. Samples of white plaque were seeded on CHROMagar *Candida*, yeast identification was done with API 20C Aux, and susceptibility testing was determined by *E* test. Ninety-three yeast isolates were obtained, 52 single and 41 mixed. *C. albicans* was the most isolated, followed by *C. glabrata*. An increased frequency of isolates and variety of *Candida* species occurred in patients with a $CD4^+$ cell count $\leq$100 cells/mm^3 without significant differences ($p = 0.29$). The susceptibility study showed that 8 (8.6 %) isolates were resistant to fluconazole and 11 (11.8 %) to itraconazole, while 6 (8.8 %) *C. albicans* were simultaneously resistant. No association was found between the isolates of *C. albicans* or *Candida* species different than *C. albicans* and the use of fluconazole ($p = 0.21$). The results of this study indicate that in the tested population, fluconazole continues to be the best treatment option for oropharyngeal candidiasis in patients suffering from AIDS (HIV/AIDS); however, susceptibility tests are necessary in patients who present therapeutic failure.

Keywords Oropharyngeal candidiasis · *Candida albicans* · *Candida* species different than *C. albicans* · HIV · $CD4^+$ Lymphocytes · Fluconazole

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Introduction

Oropharyngeal candidiasis is the most common opportunistic fungal infection in the oral cavity of individuals with human immunodeficiency virus (HIV); at some point in the development of the disease, it infects more than 90 % of them [1, 2]. The described clinical forms include pseudomembranous candidiasis, erythematous candidiasis and angular cheilitis. These lesions are usually associated with $CD4^+$ T lymphocyte counts of <200 cells/mm^3 [3, 4].

Pseudomembranous oral candidiasis is the most frequent clinical form characterized by presenting

creamy white patches, especially on the tongue and palate [3]. A study carried out in Cali, Colombia, with 319 HIV subjects showed that oral candidiasis was the second most common oral pathology. The most abundant form was pseudomembranous (18.3 %) followed by erythematous (13.9 %), angular cheilitis (13.3 %) and lastly by hyperplastic candidiasis (0.3 %) [1]. Although the incidence of oral candidiasis has been reduced with the use of antiretroviral therapy, it remains a common disease in HIV patients [5].

This disease is mainly caused by *Candida albicans,* but in the last decade, other species, such as *C. glabrata, C. krusei, C. tropicalis, C. parapsilosis* and *C. dubliniensis* [6], have increased as single isolates or in combination with *C. albicans* [6–9]. The presence of *Candida* species different than *C. albicans* is related to the use of fluconazole, an effective low-toxicity antifungal drug that is widely used in the prophylaxis and treatment for candidiasis. Exposure to this azole has led to resistance in *C. albicans* and to the selection of less-susceptible *Candida* species, such as *C. glabrata* and *C. krusei* [10, 11]. In Colombia, oropharyngeal candidiasis in HIV/AIDS patients has been little studied [12, 13]. The purpose of this study was to establish the *Candida* species implicated in pseudomembranous candidiasis, its relationship to the $CD4^+$ T lymphocyte count and the in vitro sensitivity profile for fluconazole and itraconazole in HIV/AIDS patients who consulted at the University Hospital of Valle (HUV), Cali, Colombia.

Materials and Methods

A prospective descriptive study was conducted between February and December 2008 at the HUV of Cali, Colombia. Seventy-one patients with AIDS that were diagnosed by enzyme-linked immunosorbent assay (ELISA) and confirmed by Western blot and in turn presented creamy, white patches in the oral cavity were selected. These individuals were included whether or not they were receiving antifungal therapy. All patients participated voluntarily and signed informed consents. Data concerning age, sex, time of HIV diagnosis and whether or not they had received antiretroviral treatment or fluconazole were collected for each participant. The study was approved by the Institutional Research Ethics Committees of the School of Health Sciences of the Universidad del Valle and the HUV.

Samples of the lesions were taken with sterile swabs (Culturette, Becton, Dickinson, France) which were cultured in CHROMagar *Candida* (CHROMagar Company, Paris, France) and incubated at 37 °C for 48 h. Identification of the yeast began with the observation of the color that the colonies presented [14]. Isolates with colors different from green hues underwent API 20C Aux (bioMerieux, Marcy l'Etoile, France). Colonies presumptive for *C. albicans*/*C. dubliniensis* underwent phenotypic testing which included germ tube formation, chlamydospore production on corn meal Tween 80 agar (Oxoid, UK), growth on Sabouraud dextrose agar 2 % (Merck, Germany) at 42 and 45 °C and growth at 25 °C in Sabouraud agar with sodium chloride 6.5 % [15]. Isolates in which tests were suggestive for *C. dubliniensis* also underwent API 20C Aux [16]. *C. albicans* ATCC 14053 and a known *C. dubliniensis* isolate were used as references for the various tests.

The minimum inhibitory concentration (MIC) to fluconazole and itraconazole of all *Candida* isolates was determined by the *E* test® technique (AB Biodisk, Solna, Sweden) following manufacturer's instructions. Cutoff points used for these azoles were based on a document prepared by the CLSI (Clinical Laboratory Standards Institute) [17]. The minimum inhibitory concentration capable of inhibiting 50 and 90 % of the isolates (MIC_{50} and MIC_{90}, respectively) was also determined. *C. parapsilosis* ATCC 22019 was used as a control strain. Additionally, since new clinical breakpoints (CBPs) for fluconazole were recently proposed [18], we also analyzed fluconazole susceptibility of *C. albicans, C. tropicalis, C. parapsilosis and C. glabrata* using these new definitions. For *C. albicans, C. tropicalis* and *C. parapsilosis,* they were $\leq$2 μg/ml susceptible, 4 μg/ml susceptible dose-dependent (SDD) and $\geq$8 μg/ml resistant, whereas $\leq$32 μg/ml was SDD and $\geq$64 μg/ml was resistant for *C. glabrata* isolates. Furthermore, the percentage of the wild-type isolates of *Candida* (those that do not harbor any acquired resistance) was established to separate them from the non-wild types (organisms with acquired or mutational resistance mechanisms) for fluconazole and itraconazole, according to epidemiological cutoff values (ECV) recently proposed. These values correspond to *C. albicans* $\leq$0.5 and 0.12 μg/ml, *C. parapsilosis* and *C. tropicalis* $\leq$2 and $\leq$0.5 μg/ml, *C. glabrata* $\leq$32 and $\leq$2 μg/ml and *C. krusei* $\leq$64 and $\leq$1 μg/ml for fluconazole and itraconazole, respectively [19, 20].

The $CD4^+$ T lymphocyte count was carried out in a venous blood sample taken with a Vacutainer tube with EDTA (ethylenediaminetetraacetic acid) using a FACSCalibur™ flow cytometer (Becton–Dickinson, San Jose, California, USA).

Statistical Analysis

The SPSS statistical program version 15.0 (SPSS Inc., Chicago IL, USA) was use for data analyses. Demographic and clinical information were presented as measures of central tendency and dispersion with absolute frequencies and percentages. The frequency of *Candida* species and its relationship to $CD4^+$ T lymphocytes was established; patients were stratified into four groups, depending on the cell counts per mm^3 as follows: ≤100, 101–200, 201–350 and >350 cells/mm^3. We determined the percentage of S, SDD and R for the two antifungals. The Pearson's chi-squared test was used, and a p value < 0.05 was considered statistically significant.

Results

Of the 71 patients, 53 (74.6 %) were males and 18 (25.4 %) were females with a range of ages from 18 to 60 years and a mean of 37 ± 10 years; Table 1 presents the demographic and clinical characteristics of studied subjects. Ninety-three yeast isolates were obtained from the 71 patients, 70 were green colonies, of these, two were phenotypically identified as possibly *C. dubliniensis*. However, in this study, these isolates were included as *C. albicans* (see Table 2).

Candida albicans was the most commonly isolated species, followed by *C. glabrata*. The relationship between the isolates of *C. albicans* or *Candida* species different than *C. albicans* and the use of fluconazole showed no statistically significant differences ($p = 0.21$). $CD4^+$ T lymphocytes cells/mm^3 ranged between 5 and 844 cells/mm^3 with a mean of 156 cells/mm^3.

Figure 1 shows the distribution of *Candida* species in relation to the number of $CD4^+$ T lymphocytes and the greater frequency of isolates and variety of species presented. In patients with $CD4^+$counts ≤100 cells/mm^3, and only in this range, *C. tropicalis* and *C. parapsilosis* were isolated. Statistical analysis did not show any significant differences between $CD4^+$ count ≤200 cells/mm^3 and the frequency of *Candida* species ($p = 0.61$), nor with $CD4^+$ ≤100 cells/mm^3 ($p = 0.29$).

Table 1 Demographic and clinical characteristics of HIV/AIDS patients with pseudomembranous candidiasis

Variables	Frequency n (%)
Age (years)	
18–28	18 (25.4)
29–39	24 (33.8)
40–50	24 (33.8)
51–60	5 (7.0)
Antiretroviral therapy	
Yes	13 (18.3)
No	58 (81.7)
Fluconazole	
Yes	9 (12.7)
No	62 (87.3)
Time of HIV diagnosis	
< 5 months	37 (52.1)
6–11 months	7 (9.9)
1–3 years	16 (22.5)
>3 years	11 (15.5)
$CD4^+$ lymphocyte count (cells/mm^3)	
≤100	41 (57.7)
101–200	13 (18.3)
201–350	6 (8.5)
>350	11 (15.5)

Sixteen isolates were obtained from the 13 patients undergoing antiretroviral therapy (ART), of which 12 (75 %) were *C. albicans*. Among *Candida* species different than *C. albicans*, *C. tropicalis* 1 (6.2 %), *C. glabrata* 1 (6.2 %) and *C. krusei* 2 (12.5 %) were found. The relationship between *Candida* species and antiretroviral therapy showed no statistical association ($p = 0.85$).

The study of in vitro susceptibility to the two triazoles in the 93 isolates showed that 81 (87.1 %) were susceptible to fluconazole, 4 (4.3 %) had a dose-dependent susceptibility, and 8 (8.6 %) were resistant. With itraconazole, 76 (81.7 %) were sensitive, 6 (6.5 %) were susceptible dose-dependent, and 11 (11.8 %) were resistant. The MIC range and MICs at which 50 % (MIC_{50}) and 90 % (MIC_{90}) of the strains were inhibited are presented in Table 3. MIC_{90} was not determined in species with <10 isolates. Six isolates of *C. albicans* showed simultaneous resistance to the two triazoles but none corresponded to patients

Table 2 Distribution of isolates of *Candida* obtained from 71 HIV/AIDS patients with pseudomembranous candidiasis

Candida species (No. of isolates)	Patients		$CD4^+$ range (cell/mm^3)	Total patients n (%)
	Without FLC* n (%)	With FLC n (%)		
C. albicans (55)[a]	48 (77.4)	5 (55.6)	7–844	53 (74.7)
C. parapsilosis (1)	–	1 (11.1)	99	1 (1.4)
C. albicans + *C. glabrata* (14)	6 (9.7)	1 (11.1)	16–389[b]	7 (9.9)
C. albicans + *C. tropicalis* (8)	4 (6.5)	–	6–100	4 (5.6)
C. albicans + *C. krusei* (2)	–	1 (11.1)	309	1 (1.4)
C. glabrata + *C. krusei* (2)	–	1 (11.1)	7	1 (1.4)
C. glabrata + *C. tropicalis* (2)	1 (1.6)	–	39	1 (1.4)
C. albicans + *C. glabrata* + *C. tropicalis* (3)	1 (1.6)	–	5	1 (1.4)
C. albicans + *C. glabrata* + *C. krusei* (3)	1 (1.6)	–	113	1 (1.4)
C. albicans + *C. krusei* + *C. tropicalis* (3)	1 (1.6)	–	46	1 (1.4)
Total (93)	62 (100)	9 (100)		71 (100)

* *FLC* Fluconazole

[a] Two presumptive isolates of *C. dubliniensis*

[b] In this range five patients with counts <200 cells/mm^3

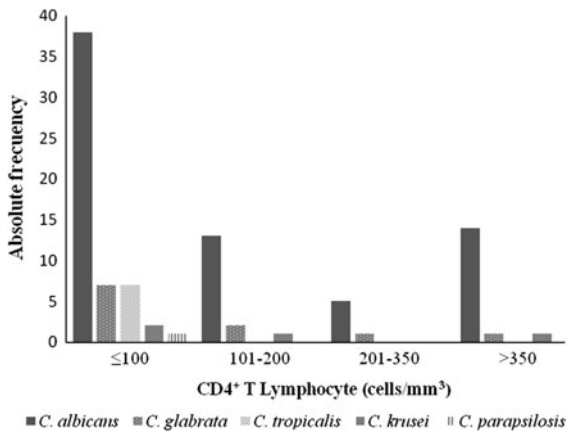

Fig. 1 Distribution of *Candida* species by the number of lymphocyte T $CD4^+$ cells in HIV/AIDS patients with pseudomembranous candidiasis

with exposure to these antifungals. The ECVs for fluconazole and the five species of *Candida* show that ≥75 % of them include a MIC from within the wild-type population, and for itraconazole ≥25 % (Table 3). Applying the species-specific CBPs proposed for fluconazole, we observed that 10 isolates previously sensible (8 of *C. glabrata*, one of *C. albicans* and one of *C. tropicalis*) became SDD.

Discussion

The diagnosis of oral candidiasis is usually based on clinical manifestations; however, the isolation and specific identification of the responsible yeast are necessary and important steps for choosing the appropriate therapy [3].

Candida albicans is the most common pathogen implicated in oral candidiasis. It was confirmed in this study where 75.3 % of the isolates corresponded to this species. These data are very similar to that found by several researchers [21–25], although other studies report prevalence rates of 91–100 % [8, 13, 26]. This study included in this classification possible isolates of *C. dubliniensis* which are phylogenetically related to *C. albicans* and require the use of molecular techniques for accurate identification [27]. However, according to some authors, the use of more than one phenotypic test is a valid option for the identification of *C. dubliniensis* [28]. It must be noted that *C. albicans* is part of the normal microbiota of the oral cavity in approximately 30–50 % of healthy adults [3].

Candida species different than *C. albicans* accounted for 26.9 % (25/93) of the isolates. Other studies have reported frequencies of 22.6–35 % [24–28]. The increase in these species has been attributed to the widespread use of fluconazole which may affect colonization by these yeasts [10]. However, in this study, only 5 (20 %) of the 25 isolates were from patients who had been exposed to fluconazole, and this drug was the only azole used as prophylactic. This suggests that the presence of *Candida* species different than *C. albicans* in the oral cavity could be due to other factors, such as immunosuppression, xerostomia, impaired production of secretory IgA and antifungal constituents of saliva, as well as oral hygiene, use of prosthetic dentures and smoking [29]. It is also important to note that the use of chromogenic media, such as CHROMagar *Candida*, increases the possibilities for detecting differing species of *Candida* in clinical specimens [14]. In this study, the majority of *Candida* species, other than *C. albicans*, were found mixed with other yeasts (Table 2) that

Table 3 Susceptibility profile and proposed ECVs for five *Candida* species isolated from AIDS patients with pseudomembranous candidiasis

Species (No. of isolates) and antifungal agent	MIC (μg/ml)			No. of isolates (%)			ECV (%)[a]
	Range	50 %	90 %	S	SDD	R	
C. albicans (70)							
Fluconazol	0.023–>256	0.094	2	64 (91.4)	0 (0)	6 (8.6)	0.5 (87.1)
Itraconazol	0.002–>32	0.008	0.5	62 (88.6)	1 (1.4)	7 (10)	0.12 (57.1)
C. glabrata (11)							
Fluconazol	0.047–>256	1.5	32	8 (72.7)	2 (18.2)	1 (9.1)	32 (90.9)
Itraconazol	0.008–>32	2	32	6 (54.5)	5 (45.5)	0 (0)	2 (54.6)
C. tropicalis (7)							
Fluconazol	0.064–3	0.25	–	7 (100)	0 (0)	0 (0)	2 (85.7)
Itraconazol	0.08–2	0.016	–	6 (85.7)	0 (0)	1 (14.3)	0.5 (85.7)
C. krusei (4)							
Fluconazol	0.25–>256	24	–	1 (25)	2 (50)	1 (25)	64 (75)
Itraconazol	0.012–32	2	–	1 (25)	0 (0)	3 (75)	1 (25)
C. parapsilosis (1)							
Fluconazol	0.047	–	–	1 (100)	0 (0)	0 (0)	2 (100)
Itraconazol	0.002	–	–	1 (100)	0 (0)	0 (0)	0.5 (100)

MIC_{50}: Minimum inhibitory concentration capable of inhibiting 50 % of the isolates

MIC_{90}: minimum inhibitory concentration to inhibit 90 % of the isolates

[a] Percentage of isolates for which MICs are ≤ ECV (epidemiologic cutoff values; μg/ml)

S susceptible, *SDD* Susceptible dose-dependent, *R* resistant

would not been displayed without the use of this medium.

The most frequent isolated *Candida* species different than *C. albicans* was *C. glabrata*, similar to what was reported by Sant'Ana et al. [8]. Isolation of this yeast has increased in immunocompromised patients and generally affects the oral mucosa. It is reported as the second or third most frequent species of *Candida* isolated from oropharyngeal candidiasis as a single isolate or accompanied by *C. albicans* [6, 30]. In this study, it was the second most important species and in the majority of isolates coexisted with *C. albicans*. The risk factor that has contributed to the growth of this yeast is the degree of immunosuppression [6, 31]. In this study, we observed that 81.8 % of patients with *C. glabrata* had $CD4^+$ counts of <153 cells/mm^3. Several researchers report *C. tropicalis* as the *Candida* species different than *C. albicans* species that is most frequently isolated [21, 22, 25]. This study found that *C. tropicalis* was second with a prevalence of 7.5 %. This species is rare in patients with oropharyngeal candidiasis and has been reported in approximately 3–8 % of cases of HIV/AIDS [32].

The present study showed that 19 of 71 individuals (26.7 %) had mixed cultures. Sant'Ana et al. [8] commented that the presence of two or more species in the same subject predisposes to recurrent candidiasis mainly with yeasts intrinsically resistant to fluconazole or with reduced sensitivity, such as *C. krusei* and *C. glabrata*. Although this study did not follow patients to determine whether oropharyngeal candidiasis was recurrent, it did show that 13 of the 19 patients presented combinations that included them (Table 2). However, Dronda et al. [33] have described more than 15 % of mixed cultures in the oral cavity of HIV-infected patients and contend that presence of additional species other than *C. albicans* does not seem to play an important role in pathogenesis.

Oropharyngeal candidiasis caused exclusively by *Candida* species different than *C. albicans* is uncommon. Yet, this study found 3/71 (4.2 %) patients, of whom one presented *C. parapsilosis* and others a combination of two species (*C. glabrata* + *C. krusei* and *C. glabrata* + *C. tropicalis*). Few researchers have reported oropharyngeal candidiasis caused by a *Candida* species different than *C. albicans* [34].

A $CD4^+$ lymphocytes count of <200 cells/mm^3 is associated with the appearance of pseudomembranous candidiasis in HIV/AIDS patients [35], which suggests that immunosuppression is a risk factor for the appearance of lesions in the oral cavity. However, recent research considers the viral load as a better marker for predicting the course of HIV/AIDS. This is due to the fact that oropharyngeal candidiasis has also been reported in patients with $CD4^+$ >200 cells/mm^3 [36] as shown in the present study, where 17 (24 %) individuals presented counts above this value.

Of the 19 patients with more than one species of *Candida*, 14 (73.7 %) had $CD4^+$ counts <200 cells/mm^3. It is probable that immunosuppression favors the coexistence of more than one yeast. Sant'Ana et al. [8] suggest that the isolates of *Candida* species different than *C. albicans* in the oral cavity may be associated with advanced HIV.

While fluconazole and itraconazole are active antifungals in the treatment for oropharyngeal candidiasis in HIV/AIDS patients, the prolonged use of fluconazole increases the risk of developing resistant *C. albicans* or of selecting *Candida* species different than *C. albicans* with reduced susceptibility, such as *C. glabrata* and *C. krusei* [5]. In this study, the resistance of *C. albicans* to fluconazole was 8.8 %, lower than that reported by Magaldi et al. [25] who show a prevalence of 31.5 %. Simultaneous resistance to the two triazoles was observed in 6 isolates of *C. albicans*, indicating possible cross-resistance between these two antifungals, similar to that described in other studies [37, 38].

Candida glabrata is considered a yeast with acquired resistance due to the increased use of fluconazole [38]. In this study, although resistance was 9.1 %, it could not be related to the use of this azole because resistance was found in one isolate which had not been exposed to this antifungal. It is also important to note that using the recently proposed CBPs, these isolates are no longer considered susceptible to fluconazole [19]; *Candida tropicalis* has been characterized as being sensitive to fluconazole, but in recent years, resistance in patients with continuous or intermittent exposure to this drug has been reported [32, 39]. In this study, isolates were sensitive but it must be noted that none of the participants had previously received this triazole, unlike the cases reported by Magaldi et al. [25] who found 23.5 % resistance in subjects with exposure to this drug.

Candida krusei is characterized by presenting intrinsic resistance to fluconazole and is associated with treatment failure [5]; therefore, the use of this antifungal in infections caused by this species is not recommended. Similarly, a sensitive isolate should be considered resistant independent of the value of the MIC. It must also be noted that resistance to itraconazole has also been reported for this yeast [5]. In this study, 75 % of the isolates were resistant, while other studies reported values of 55 and 100 % [25, 37].

In spite of *C. albicans* remaining the most common species, currently the *Candida* species different than *C. albicans* have emerged as important pathogens, especially in patients with $CD4^+$ T lymphocytes <200 cells/mm^3 and exposure to fluconazole. In these cases, identification at the species level is necessary due to the fact that some of them may present a varied susceptibility profile. These results show that in the population analyzed, fluconazole is still the best option for the treatment for oropharyngeal candidiasis in HIV/AIDS patients; however, in vitro susceptibility testing of antifungals should be performed in patients with treatment failure to select and ensure the efficacy of the medication. Also, it is important to realize comparative studies of cohorts or controlled clinical trials.

Acknowledgments This research was funded by the Universidad del Valle, project number CI 1584.

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