JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Original Article

Oral *Candida* colonization in HIV-infected patients in Londrina-PR, Brazil: antifungal susceptibility and virulence factors

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Abstract

Introduction: Host colonization by *Candida* species is an important predisposing factor to candidiasis, which seems to be more frequent in human immunodeficiency virus (HIV)-infected patients. Knowledge about the distribution, antifungal susceptibility, and virulence of oral *Candida* isolates is important for effective management of candidiasis.

Methodology: Oral rinses were collected from 242 HIV-infected patients without clinical evidence of candidiasis seen at the AIDS referral center in Londrina, Brazil. Species were identified by standard phenotypic and molecular methods, and characterized *in vitro* according to antifungal susceptibility, cell surface hydrophobicity, biofilm formation, and enzyme activities.

Results: Oral *Candida* colonization was detected in 50.4% of patients and combined use of antiretroviral therapy and protease inhibitor had a protective effect against colonization. *Candida albicans* (75.2%) was the most prevalent species. A high proportion of *Candida* spp. (39.9%) showed decreased susceptibility to fluconazole. Five isolates were resistant to nystatin. Protease and phospholipase activities were detected in 100% and 36.8% of isolates, respectively. Most isolates displayed a hydrophobic property that was associated with biofilm formation ability.

Conclusions: A significant number of oral *Candida* species exhibiting decreased susceptibility to fluconazole were isolated from colonized HIV-infected individuals. Furthermore, all isolates expressed potential virulence attributes *in vitro*. Given the high incidence and severity of fungal infections in HIV-infected individuals, the results of this study reinforce the importance of antifungal susceptibility testing, which contributes to therapeutic strategies and highlights the need for continuous surveillance of *Candida* colonization in this population.

Key words: *Candida*; HIV; biofilm; protease; phospholipase; cell surface hydrophobicity; fluconazole susceptibility.

J Infect Dev Ctries 2015; 9(12):1350-1359. doi:10.3855/jidc.6970

(Received 06 April 2015 – Accepted 21 May 2015)

Introduction

Candida species are harmless colonizers of the gastrointestinal and reproductive tracts in 50% to 60% of healthy people with no symptoms of candidiasis [1,2]. In individuals infected with human immunodeficiency virus (HIV), the prevalence of asymptomatic colonization of oral mucous is higher, approaching 80% [3-11]. Importantly, colonization of skin and mucous membranes with *Candida* species is an important risk factor that predisposes to candidiasis [12], whose clinical manifestations range from mucosal to life-threatening disseminated infections.

The introduction of antiretroviral therapy (ART) notably improved the immune status of HIV-infected individuals, markedly reducing the incidence of several opportunistic infections [10,13,14]. However, invasive fungal infections are a major cause of HIV-related mortality worldwide, and *Candida* species rank among the four most prevalent etiological agents [15-17]. In Brazil, candidiasis is the second cause of deaths in HIV-positive patients due to fungal

infections [15,17]. Moreover, oropharyngeal candidiasis (OPC) remains clinically relevant in these individuals, where treatment is difficult and recurrent episodes are frequent, requiring multiple antifungal treatments, which may lead to resistance selection [18]. Recurrent OPC may increase morbidity and mortality in these individuals [19].

The severity and chronicity of oral lesions due to *Candida* spp. in these individuals have been attributed to progressive HIV-induced T-cell immunodeficiency [9]. On the other hand, as opportunistic pathogens, *Candida* spp. express several virulence factors that contribute to the pathogenesis of candidiasis. These factors include adhesins (host recognition molecules), secreted aspartic proteases, phospholipases, morphogenesis (yeast-hyphal reversible transition), phenotypic switching, and biofilm formation [20-22].

In the study described here, the yeasts were isolated from the oral cavity of HIV-infected individuals who had no clinical evidence of candidiasis. All isolates were identified and tested for protease and phospholipase activities *in vitro*. In addition, we determined the *in vitro* susceptibility pattern of the isolates to fluconazole and nystatin, the hydrophobicity of the cells, and their capacity for biofilm production on an abiotic surface.

Methodology

Study population

A total of 242 HIV-infected patients seen in 2010 at the Centro de Referência Dr. Bruno Piancastelli Filho, a specialized care service in AIDS in Londrina city, were enrolled in this study. This is the major referral center for the management of HIV-related infections in the north of Paraná State in Brazil. All of the patients signed a written informed consent form to participate in this study, agreeing to the publication of this report and any accompanying images. The study protocol was in accordance with the ethics committee of the Universidade Estadual de Londrina (Document No. 036/10). The patients were evaluated regularly by experienced infectious disease physicians. Sexual transmission was the most prevalent pathway of HIV contamination in these populations. During specimen collection, no patient was on antifungal agents, and there was no clinical evidence of oral candidiasis. The most recent $CD4^+$ and $CD8^+$ T lymphocyte count, viral load, and ART prescribed were obtained from the medical records of the patients.

Isolate identification

Samples from the patients were obtained by the oral rinse method described by Samaranayake *et al*. [23], with some modifications. Patients rinsed with sterile distilled water (10 mL) for 30 seconds and spit it out into a sterile container. Each sample was centrifuged at 1,500 *g* at 4°C, the supernatant was discarded, and the pellet was suspended in 1.0 mL of 50 mM sodium phosphate buffer, pH 7.4, containing 0.15M NaCl (PBS). A 100-µL sample was spread on Sabouraud dextrose (SD) agar supplemented with 50 µg/mL chloramphenicol. The cultures were incubated at 37°C for at least seven days under aerobic conditions, and colonies were counted. Results were expressed as number of colony-forming units (CFU) per milliliter. The samples were also cultured on CHROMagar *Candida* medium (Difco, São Paulo, Brazil) for differential growth analysis. For each positive sample, colonies were examined microscopically after Gram staining, and two colonies of each sample were sub-cultured in SD agar for identification. The identity of each yeast isolate was determined by standard mycological methods [24]. Concomitantly, species identification was confirmed by polymerase chain reaction (PCR)-based methods [25-27].

Antifungal susceptibility testing

The minimum inhibitory concentration (MIC) of fluconazole (Sigma Chemical Co, São Paulo, Brazil) and nystatin (Sigma Chemical Co) for all isolates was determined by the broth microdilution assay for yeasts based on the Clinical and Laboratory Standards Institute (CLSI) guidelines [28]. Quality control *C. parapsilosis* ATCC 22019 was included in each experiment. Two wells of each plate served as growth and sterility controls. The interpretative criteria for susceptibility to fluconazole were those published in the CLSI [28]. For nystatin, MIC was defined as the value in which 100% growth inhibition was observed [29].

Cell surface hydrophobicity determination

The hydrophobicity of the yeasts was determined as previously described [30]. Briefly, each *Candida* isolate was grown at 37°C for 24 hours in SD broth. The yeasts were harvested by centrifugation and washed twice, and the cell density was then adjusted to an absorbance of 0.4 at 660 nm in 5 mL of PBS. A volume of 2.5 mL of this yeast suspension was added to two sterile glass tubes, and 0.5 mL of xylene (Merck, São Paulo, Brazil) was added to one of the

tubes. Following 10 minutes of incubation in a water bath at 37°C, the test tube was vigorously mixed for 1 minute and incubated for an additional 30 minutes under the same conditions. The aqueous phase was carefully removed, and the absorbance was determined at 660 nm. The cell surface hydrophobicity (CSH) was expressed as the percentage decrease in optical density of the aqueous phase of the test as compared with the control, where the greater the change in absorbance of the aqueous phase, the more hydrophobic the yeast sample. Each assay was performed on three separate occasions with triplicate determinations each time.

Biofilm production assay

Determination of biofilm production was performed in polystyrene, flat-bottomed 96-well microtiter plates (Techno Plastic Products, Trasadingen, Switzerland) using the procedure previously described [31]. In brief, the yeast isolates were grown at 37°C for 24 hours in RPMI 1640 broth, and the cells were counted. A 20 μL broth suspension of 6 x 10^5 yeasts was placed in each well containing 180 μL of RPMI medium. The plates were incubated at 35°C for 24 hours and washed once with sterile distilled water. Approximately 100 μL of 0.1 mg/mL XTT/1 μM menadione (Sigma Chemical Co) were added to each well, and the plates were incubated in the dark for 2 hours at 37°C. The supernatant was transferred to new wells of microtiter plate before spectrophotometric readings at 490 nm with a microtiter plate reader (Universal Microplate Reader ELx800, Bio-Tek Instruments, Winooski, USA). Experiments were carried out in triplicate on three different occasions.

Determination of protease and phospholipase activities

Enzyme activity was assayed on SD agar plates containing 0.1% bovine serum albumin (BSA) or 4.0% egg yolk as protease and phospholipase substrate, respectively. For protease activity, the yeast isolates were previously grown at 37°C for 18 hours in minimal medium (MM) broth [32] supplemented with 0.1% BSA, pH 4.0, to induce the secretion of enzymes. To determine phospholipase activity, a cell suspension was obtained from a 24-hour SD broth-yeast culture, and the assay was carried out on SD agar supplemented with 4.0% egg yolk, 350 μM NaCl, and 6.5 μM $CaCl_2$, pH 4.5. For both assays, cells were counted in a Neubauer chamber, and a 10 μL suspension from 10^6 yeasts/mL was placed on the surface of the agar medium. The cultures were incubated at 37°C for 96 hours, after which the diameter of the degradation (protease activity) or precipitation (phospholipase activity) zone around the colony was determined. Enzyme activity was determined by calculating the ratio between colony diameter and colony diameter plus degradation/precipitation zone (Dz/Pz values of 1 indicated no detectable protease or phospholipase activity, respectively) [33]. Each isolate was tested in triplicate, and the experiments were carried out on three different occasions.

Statistical analyses

Qualitative variables associated with *Candida* spp. colonization were analyzed using the Chi-square test or Fisher's exact test where appropriate. Continuous variables were analyzed using the Mann-Whitney test, because these do not show normal distribution. Spearman's rank correlation was determined to compare the degree of association between virulence factors. All analyses were performed using the Statistical Package for Social Sciences (SPSS) software version 20.0, and p value less than 0.05 was considered significant.

Results

Patients and yeast identification

A total of 242 HIV-infected patients were enrolled in this study. Of these, 159 were males and 83 were females, and their mean age was 40.4 years (ranging from 17 to 78 years). Among all HIV-infected individuals, there was a viral load of < 50 copies/mL for 144 patients and > 500,000 copies/mL for one patient. For the other 97 patients, the mean viral load was 43,593 ± 81,467 (ranging from 61 to 353,456 copies/mL). The mean $CD4^+$ and $CD8^+$ lymphocyte counts were 510.20 ± 287.55 cells/mm^3 (ranging from 2 to 1,493) and 1,105 ± 565.33 cells/mm^3 (ranging from 108 to 4,185), respectively (Table 1).

Two hundred and two (83.5%) patients were on ART, and most subjects (141/202, 69.8%) were taking at least one protease inhibitor (PI) in combination with other drugs. The most frequent treatment regimen was one NRTI (nucleoside reverse transcriptase inhibitor, apricitabine) plus one PI (lopinavir) (71/202, 34.1%), followed by one NRTI (apricitabine) plus one NNRTI (non-nucleoside transcriptase inhibitor, efavirenz) (51/202, 25.2%), one NRTI (apricitabine) plus two PI (atazanavir and ritonavir) (20/202, 9.9%), and one NRTI (apricitabine) plus one PI (atazanavir) (16/202, 7.9%).

Table 1. Demographic characteristics, laboratory findings, and medication histories of HIV-infected patients enrolled in this study

Characteristics	All cases (n = 242), n (%)	Yeast culture	
		Positive (n = 122), n (%)	Negative (n = 120), n (%)
Age, mean ± SD (years)	40.42 ± 11.35	40.45 ± 11.87	40.25 ± 11.25
Gender, male	159 (65.7)	82 (67.2)	77 (64.1)
Gender, female	83 (34.3)	40 (37.9)	43 (35.8)
Modes of HIV transmission			
Sexual	183 (75.6)	96 (78.7)	87 (72.5)
Intravenous drug use	7 (2.9)	2 (1.6)	5 (4.1)
Not identified	52 (21.5)	24 (19.7)	28 (23.3)
Antifungal therapy within the previous 6 months			
Fluconazole	88 (36.4)	49 (40.2)	39 (32.5)
Nystatin	37 (15.3)	21 (17.2)	16 (13.3)
HIV infection			
Duration of HIV infection (years)	6.31 ± 4.85	6.48 ± 4.70	6.14 ± 5.00
HIV viral load			
> 50 and < 500,000 copies/mL	97 (40.1)	57 (46.7)	40 (33.3)
> 500,000 copies/mL	1 (0.4)	None	1 (0.8)
> 50 and < 500,000 copies/mL (mean ± SD)	43,593 ± 81,467	52,787 ± 95,357	30,491 ± 54,597
CD count (cells/mL)			
CD4 count (cells/mL)	510.20 ± 287.55	520.14 ± 295.35	506.97 ± 289.27
CD4 count ≤ 200 cells/mL	31 (12.8)	16 (13.1)	15 (12.5)
CD8 count (cells/mL)	1,105 ± 565.33	1,150 ± 552.27	1,032 ± 574.28
Antiretroviral therapy			
None	40 (16.5)	22 (18.0)	18 (15.0)
1NRTI + 1NNRTI	54 (22.3)	32 (26.2)	22 (18.3)
2NRTIs + 1NNRTI	7 (2.9)	4 (3.3)	3 (2.5)
1NRTI + 1PI	88 (36.4)	35 (28.7)	53 (44.2)
1NRTI + 2PIs	21 (8.7)	11 (9.0)	10 (8.3)
2NRTIs + 1PI	11 (4.5)	8 (6.6)	3 (2.5)
2NRTIs + 2PIs	7 (2.9)	3 (2.5)	4 (3.3)
3NRTIs + 1PI	4 (1.7)	3 (2.5)	1 (0.8)
1NRTI + 1NNRTI + 1PI	1 (0.4)	None	1 (0.8)
2NRTIs + 1NNRTI + 1PI	2 (0.8)	1 (0.8)	1 (0.8)
2NRTIs + 1NNRTI + 3PIs	1 (0.4)	None	1 (0.8)
2NRTIs + 1PI + 1II	2 (0.8)	2 (1.6)	None
2NRTIs + 2PIs + 1II	2 (0.8)	None	2 (1.7)
2NRTIs + 1PI + 1FI	1 (0.4)	None	1 (0.8)
2IPs	1 (0.4)	1 (0.8)	None

SD: standard deviation; NRTI: nucleoside reverse transcriptase inhibitor; NNRTI: non-nucleoside reverse transcriptase inhibitor; PI: protease inhibitor; II: integrase inhibitor; FI: fusion inhibitor

Of the 242 patients, 88 (36.4%) reported previous use of fluconazole, and among them, 27 (11.2%) and one (0.41%) also used nystatin and amphotericin B, respectively, during the six months before sampling. Ten patients out of 242 (4.13%) reported previous use of nystatin alone (Table 1).

It was observed that age, gender, viral load, $CD4^+$ and $CD8^+$ lymphocyte counts, global use of ART, and previous use of antifungals did not influence the *in vitro* isolation of *Candida* spp. However, the combined use of PIs with other ARTs had a protective effect against oral yeast colonization in these patients ($p < 0.05$, Chi-square test).

Of all 242 patients, 122 (50.4%) were colonized with *Candida* spp. and of these, 11 (9%) showed colonization by two species, resulting in 133 isolates. The colony counts ranged from 1 x 10^2 to 6.6 x 10^3 CFU/mL. *C. albicans* was the most frequently isolated species, accounting for 75.2% (100/133) of all isolates. *C. glabrata* was recovered from 18.8% of patients (25/133), followed by *C. tropicalis* (3/133, 2.25%), *C. dubliniensis* (3/133, 2.25%), and *C. krusei* (2/133, 1.5%). Among the patients co-colonized, the combinations were *C. albicans* and *C. glabrata* (n = 9) and *C. albicans* and *C. krusei* (n = 2).

Fluconazole susceptibility pattern

The susceptibility profile of the *Candida* spp. isolates to fluconazole is shown in Table 2. Most isolates (80/133, 60.1%) were susceptible to fluconazole (MICs ranging from 0.25 to 8 μg/mL). Thirty (22.6%) and 23 (17.3%) isolates were susceptible dose-dependent (MIC ranging from 16 to 32 μg/mL) and resistant (MIC ranging from 64 to 128 μg/mL), respectively. The mean MIC for *C. albicans* was 20.19 ± 35.34 μg/mL, whereas for *C.* non-*albicans* isolates, it was 35.47 ± 41.41 μg/mL. Previous use of fluconazole was significantly ($p < 0.001$, Mann-Whitney test) associated with an increased recovery of *Candida* spp. isolates that had reduced susceptibility to this antifungal.

Regarding nystatin, 116 out of 133 (87.2%) isolates were considered susceptible (MIC ≤ 4 μg/mL)

Table 2. Antifungal minimum inhibitory concentration (MIC) distribution of *Candida* spp. isolated from oral cavities of HIV-infected patients

***Candida* species**	**Number of isolates (%)**	
	Fluconazole[a]	**Nystatin[a]**
C. albicans (n = 100)		
Range of MIC (μg/mL)	0.25–128	1–128
Susceptible	65 (65.0)	90 (90.0)
Susceptible dose-dependent	22 (22.0)	7 (7.0)
Resistant	13 (13.0)	3 (3.0)
C. dubliniensis (n = 3)		
Range of MIC (μg/mL)	4–8	4–4
Susceptible	3 (100.0)	3 (100.0)
Susceptible dose-dependent	0	0
Resistant	0	0
C. glabrata (n = 25)		
Range of MIC (μg/mL)	0.5–128	2–128
Susceptible	10 (40.0)	20 (80.0)
Susceptible dose-dependent	6 (24.0)	4 (16.0)
Resistant	9 (36.0)	1 (4.0)
C. krusei (n = 2)		
Range of MIC (μg/mL)	4–32	4–128
Susceptible	1 (50.0)	1 (50.0)
Susceptible dose-dependent	1 (50.0)	0
Resistant	0	1 (50.0)
C. tropicalis (n = 3)		
Range of MIC (μg/mL)	8–64	4–8
Susceptible	1 (33.3)	2 (66.7)
Susceptible dose-dependent	1 (33.3)	1 (33.3)
Resistant	1 (33.3)	0

[a] The interpretative criteria for susceptibility to fluconazole and nystatin were those published in the CLSI [28] and Wingeter *et al*. [29], respectively; Fluconazole: MIC < 8 μg/mL, susceptible; MIC = 16–32 μg/mL, susceptible dose-dependent; MIC > 64 μg/mL, resistant; Nystatin: MIC ≤ 4 μg/mL, susceptible; MIC = 8–32 μg/mL, susceptible dose-dependent; MIC > 64 μg/mL, resistant

to this antifungal, and resistance (MIC ≥ 64 µg/mL) was observed only in five (3.7%) isolates, where three isolates of *C. albicans* and one each of *C. glabrata* and *C. krusei* showed a MIC ≥ 128 µg/mL. Twelve out of 133 (9.0%) isolates presented MICs of 8 µg/mL and were classified as susceptible dose-dependent.

Cell surface hydrophobicity and biofilm formation

Overall, the mean relative CSH of *Candida* isolates was 63.57 ± 21.59. The mean relative CSH of *C. albicans* was 64.71 ± 20.87, ranging from 4.22 ± 0.15 to 84.92 ± 0.71. The corresponding mean value for *C.* non-*albicans* species was 60.12 ± 23.64, with a range of 5.87 ± 0.13 to 85.83 ± 0.42. Seventy-four (57.9%, including 57 *C. albicans* and 17 *C.* non-*albicans*) of 133 isolates displayed CSH values higher than 70% and could be classified as hydrophobic cells (Table 3, Figure 1A).

Candida spp. isolates exhibited variable intensity of metabolic activity after incubation for 24 hours on a polystyrene surface, indicating the capacity of biofilm formation (Figure 1B). Most isolates (101/133, 75.9%) showed high metabolic activity (optical density $[OD]_{490nm} > 0.5$) after 24 hours of incubation, with the highest activities detected for *C. tropicalis* isolates (Table 3). A significant correlation was observed between CSH and biofilm formation ($p < 0.01$, Spearman test).

Figure 1. Cell surface hydrophobicity (A) and metabolic activity of biofilm formed on polystyrene surface (B) distribution of *Candida* species isolated from oral cavities of HIV-infected patients

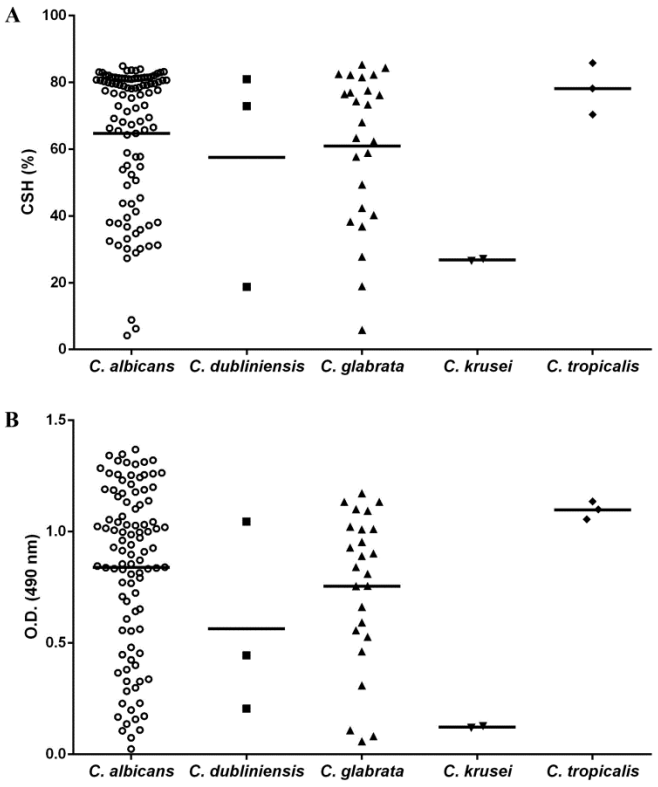

Table 3. Cell surface hydrophobicity (CSH) index and metabolic activity of biofilm of *Candida* species isolated from oral cavities of HIV-infected patients

***Candida* species**	**CSH (%)***	**Metabolic activity ($OD_{490\ nm}$)***
C. albicans	4.22 ± 0.15–84.92 ± 0.71	0.02 ± 0.00–1.37 ± 0.10
C. dubliniensis	18.75 ± 0.49–81.00 ± 0.14	0.20 ± 0.01–1.04 ± 0.02
C. glabrata	5.87 ± 0.13–85.29 ± 0.42	0.06 ± 0.01–1.17 ± 0.07
C. krusei	26.57 ± 0.42–27.13 ± 0.80	0.12 ± 0.03–0.13 ± 0.03
C. tropicalis	70.38 ± 0.07–85.83 ± 0.42	1.05 ± 0.11–1.13 ± 0.05

* Significant correlation between CSH and biofilm formation ($p < 0.01$, Spearman test); OD: optical density

Table 4. Protease and phospholipase activity of *Candida* species isolated from oral cavities of HIV-infected patients

***Candida* species**	**Number of isolates (%)**							
	Scoring of protease activity*				**Scoring of phospholipase activity***			
	Negative	**1 +**	**2 +**	**3 +**	**Negative**	**1 +**	**2 +**	**3 +**
C. albicans (n = 100)	0	8 (8)	27 (27)	65 (65)	59 (59)	41 (41)	0	0
C. dubliniensis (n = 3)	0	0	1 (33.3)	2 (66.7)	3 (100)	0	0	0
C. glabrata (n = 25)	0	1 (4)	7 (28)	17 (68)	18 (72)	6 (24)	1 (4)	0
C. krusei (n = 2)	0	0	1 (50)	1 (50)	1 (50)	1 (50)	0	0
C. tropicalis (n = 3)	0	1 (33.3)	1 (33.3)	1 (33.3)	3 (100)	0	0	0

* The protease and phospholipase activities were determined by calculating the ratio between colony diameter and colony diameter plus degradation/precipitation zone (Dz/Pz) as previously described by Price *et al*. [33]. The enzymes activities were scored into four categories: negative, Dz/Pz of 1.0; low activity (1+) for 0.64 <Dz/Pz< 1.0; intermediate activity (2+) for 0.30 <Dz/Pz ≤ 0.64; high activity (3+) for Dz/Pz ≤ 0.30.

Enzyme activities

All the isolates in this study demonstrated the secretion of protease activity on BSA, and 86 (64.7%) isolates showed high protease activity. The enzyme activity of 37 (27.8%) isolates was classified as intermediate. Low enzyme activity was observed in 10 (7.5%) isolates (Table 4). The mean Dz value observed for all species was 0.34 ± 0.20, and the mean Dz values of 0.34 ± 0.21 and 0.35 ± 0.20 were observed for *C. albicans* and *C.* non-*albicans* isolates, respectively. Protease production by the isolates from individuals undergoing treatment with PI inhibitors, except for nelfinavir, was significantly lower than in isolates from subjects who did not receive PI ($p < 0.001$, Mann-Whitney test).

Phospholipase activity was detected in 49 isolates (36.8%) with a mean Pz value of 0.80 ± 0.08. The mean Pz values of 0.80 ± 0.07 and 0.82 ± 0.13 were observed for *C. albicans* and *C.* non-*albicans* isolates, respectively. Forty-eight (36.1%) isolates showed low phospholipase activity. Only one (0.8%) isolate of *C. glabrata* exhibited intermediate phospholipase activity on egg yolk. No *C. tropicalis* and *C. dubliniensis* isolates were positive for phospholipase under the conditions analyzed here (Table 4).

Discussion

Several studies have shown a high prevalence of oral *Candida* carriage in HIV-infected individuals [5,6,8-10], and this can vary according to sampling method and geographical location [5,8,9]. By using oral rinse for yeast isolation, other Brazilian surveys detected oropharyngeal *Candida* carriage in approximately 60% to 70% of HIV-infected individuals [6,8]. In this study, 50.4% of HIV-infected individuals' oral cavities were colonized by *Candida* species, and the therapeutic combination of ART with PI exerted a protective effect against oropharyngeal yeast colonization in this population. The effect of highly active ART on the risk of *Candida* colonization in HIV-infected has been described elsewhere. While a slight decrease [34] or no effect [5,10] on oropharyngeal yeast colonization in patients receiving ART has been reported, other authors also observed a protective effect of ART treatment regimens [7,11]. On the other hand, higher oropharyngeal *Candida* carriage has been detected in ART-treated HIV-infected individuals [2].

C. albicans was the most frequently isolated species from the oral cavity of HIV-infected patients examined in this study, including co-colonization cases, accounting for around 75% of all yeast isolates. In fact, this is by far the most prevalent commensal and pathogenic of the *Candida* species [3,5,6,8-10,17,35]. However, increasing rates of colonization and even infections with other species of *Candida* in HIV-infected individuals have been observed elsewhere [2-4,8].

One important finding of this study was the high proportion of fluconazole-resistant and susceptible dose-dependent isolates, including *C. albicans*, which accounted for 39.9% of all *Candida* isolates. As observed by others [36], the previous use of azole agents was strongly associated with higher MIC for fluconazole against yeast isolates, making it more difficult to select an empiric therapy during the development of candidiasis in these patients.

The mechanisms by which commensal *Candida* species cause diseases are not completely understood. It is well know that besides the dysfunction of the host immune system or an imbalance of the normal microbiota, the potential virulence of these yeasts is associated with the ability to adhere, invade, and damage host cells [21]. CSH is considered an important nonspecific factor that contributes to adherence of *Candida* spp. on different surfaces. The hydrophobic cells exhibit greater adherence to acrylic surfaces and host cells and tissues, and decreased susceptibility to being killed by polymorphonuclear neutrophils, which can contribute to the colonization and dissemination of the yeast [37]. Most *Candida* isolates exhibited hydrophobic property in this study, and as previously observed [38], this characteristic was strongly associated with biofilm formation on polystyrene surfaces. The formation of biofilm by *Candida* spp. has been demonstrated on a number of non-biological surfaces [22,31,39], and the structure of this cellular community shares similarities with mucosal-biofilm architecture [40]. Biofilms are inherently resistant to antimicrobial agents [22,31,39] and to host immune defenses [41]. Clinically, this mode of growth is a major cause of persistent infection and is associated with high mortality rates [42]. On the other hand, biofilms are also established during host colonization, enabling the yeast to withstand removal by mechanical processes. The ability of oropharyngeal *Candida* isolates from HIV-infected individuals to form biofilm was described previously, and this characteristic was associated with higher virulence in experimental infection models [43,44].

Most *Candida* isolates displayed intermediate to high protease activity in this study, and this finding was also previously observed by other authors [35,45,46]. Except for nelfinavir, other ART treatment

regimens of the patients analyzed here appeared to decrease the secretion of proteases. Similarly, Ribeiro *et al*. [45] reported that *Candida* isolates from HIV-infected individuals receiving combined ART and PI showed lower levels of these enzymes. Secreted aspartic proteases, encoded by a multigene family, have been associated with the virulence of *Candida* species [20]. These enzymes can facilitate adhesion to and penetration into host cells [47,48] and can counteract host defense [49]. In contrast to proteases, most *Candida* isolates were negative for phospholipase activity. Only 36.1% of all isolates secreted low levels of phospholipase, and this result differed from those of others who have shown high enzymatic activity in oropharyngeal *Candida* isolates from HIV-infected individuals [35,45,46]. Several lines of evidence correlate phospholipases with the virulence of *Candida* spp., which target membrane phospholipids, leading to cell lysis and host damage [50].

Conclusions

Oropharyngeal *Candida* colonization remains common in HIV-infected individuals, even with ART. Studies monitoring the distribution and antifungal susceptibility of *Candida* isolates from these populations have crucial importance for choosing the correct antifungal therapy during candidiasis. This study showed a significant number of *Candida* spp. isolated from oropharyngeal colonized HIV-infected individuals, exhibiting decreased susceptibility to fluconazole, the first-line antifungal for treatment of candidiasis. Most importantly, this scenario is strongly associated with previous use of this antifungals. In addition, all isolates expressed potential virulence attributes, supporting the need for continuous surveillance of *Candida* colonization in this population.

Acknowledgements

We thank Dr. Sueli Galhardi and all staff from Centro de Referência Dr. Bruno Piancastelli Filho for permission/help with the collection of samples. We also thank Dr. A. Leyva for English language editing of the manuscript and João Paulo Manfre dos Santos for helping in statistical analyses. This work was supported by grants from Programa de Pesquisa para o SUS: Gestão Compartilhada em Saúde (PPSUS)/Fundação Araucária/SESA-PR/MS/CNPq, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and Pro-Reitoria de Pesquisa e Pós Graduação (PROPPG) of the Universidade Estadual de Londrina (UEL).

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Conflict of interests: No conflict of interests is declared.