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Clinical and microbiological assessment of patients with a long-term diagnosis of human immunodeficiency virus infection and *Candida* oral colonization

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Abstract

The objective of this study was to evaluate *Candida* oral colonization in human immunodeficiency virus (HIV)-infected patients undergoing long-term highly active antiretroviral therapy (ARV). The cross-sectional study included 331 HIV patients, diagnosed from 1983 to 2003. Oral swabs were performed, and *Candida* species were determined using ID 32C. Isolates were tested for antifungal susceptibility. Clinical and laboratory data were collected to identify the association with *Candida* colonization. In total, 161 *Candida* isolates were detected among 147 of the 331 patients (44%), independently of the time when HIV infection was diagnosed. *Candida albicans* strains represented 137 (85%) of the isolates, and were susceptible to all of the tested antifungal drugs. Among the non-*C. albicans* strains, six isolates were dose-dependently susceptible to fluconazole, nine to itraconazole, and seven to ketoconazole. The isolation of *Candida* was significantly higher in patients with virological failure (83/147; p 0.0002) and $CD4^+$ T-lymphocyte counts <200 cells/mm^3 (30/83; p 0.0003). Recovery of *Candida* in the oral cavity was independent of protease inhibitor (PI) usage (p 0.60). Colonized patients typically underwent salvage therapy (p 0.003), and had more episodes of opportunistic fungal infections (p 0.046) and malignancies (p 0.004). Oral *Candida* colonization in patients under ARV therapy was associated with the immunosupressed status of HIV-infected patients, i.e. low number of $CD4^+$ T-cells per cubic millimetre, failure of ARV therapy (salvage therapy), and higher number of opportunistic infections and malignancies. Despite the fact that PIs have *in vitro* antifungal activity, the use of this class of antiretroviral agent did not influence the presence of *Candida* in the oral cavity of AIDS patients.

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Introduction

Oropharyngeal candidiasis (OPC) was a frequent disease in human immunodeficiency virus (HIV)-infected patients before the introduction of highly effective antiretroviral therapy (ARV). HIV-infected patients were shown to harbour *Candida* in the oral cavity more frequently than healthy subjects, the proportion varying from 60% [1,2] to 88% when more than one sample was collected [3]. In HIV patients, oral colonization by *Candida* spp. has predicted the subsequent development of OPC [3], and low plasma HIV RNA viral load has been related to low oropharyngeal carriage of *Candida* spp. and a reduced risk of developing symptomatic infections [4]. As compared to other at-risk groups [5], an increased incidence of asymptomatic oral *Candida albicans* carriage has been demonstrated in HIV-infected subjects.

Recent data [6] have shown that some antiretroviral protease inhibitors (PIs) act directly on the secretory aspartyl protease, an important virulence factor of *C. albicans*, and the use of this class of ARV might play a role in reducing *Candida* carriage and recurrence of OPC in HIV patients [7].

Previous studies on this subject [7,8] produced contradictory data regarding the association of the use of PIs, HIV RNA viral count and $CD4^+$ cell count with colonization and the occurrence of OPC. However, as the survival rates of HIV patients increased, some questions arose about the

impact of long-term ARV therapy and *Candida* oral colonization.

This study was conducted in an attempt to evaluate patients undergoing long-term ARV therapy with respect to *Candida* oral colonization, to determine the frequency of HIV-infected patients harbouring *Candida* species in the oral cavity, the susceptibility to antifungal drugs, the genomic DNA of *Candida* species, and the conditions associated with *Candida* oral colonization.

Materials and Methods

Settings

The hospital and clinics of the State University of Campinas constitute a tertiary-care university hospital located in Campinas, Sao Paulo, Brazil; this is the reference hospital for more than 5 000 000 inhabitants, and is responsible for more than 65% of the HIV/AIDS patients in the reference area.

Data collection

A cross-sectional study was conducted at two outpatient units from April 2003 to 2004. The patients were included after they signed a consent form. Patients were eligible for the study if they were HIV-positive, they had been undergoing treatment with three or more antiretroviral agents for at least 1 year, without clinical and symptomatic evidence of OPC, they were aged ≥18 years, and they had not undergone antifungal therapy in the last 3 months. Patient demographic data included: year of HIV diagnosis, history of prior fungal infections, prior use of antifungal therapy, last $CD4^+$ cell count and HIV RNA viral load measurements, detectable or undetectable HIV RNA viral load (undetectable, <50 copies/mL), previous opportunistic infections, classes of ARV agents in use at the time of the study (PIs—indinavir, and ritonavir-boosted PI; nucleoside reverse transcriptase inhibitors (NRTIs)—zidovudine, stavudine, lamivudine, didanosine, tenofovir, and abacavir; and non-nucleoside reverse transcriptase inhibitors—efavirenz and nevirapine). The patients were classified, according to isolation of *Candida* spp. from the oral cavity, as colonized or non-colonized.

Mycological assessment and identification of yeasts

Samples from the oral cavity were obtained by swabbing the oral mucosa of the patients with a sterile cotton swab, plated onto Sabouraud dextrose agar plates supplemented with chloramphenicol 0.5 g/L, and incubated for 48–72 h. Five to ten colonies from the positive plates were then inoculated in CHROMagar Candida plates (CHROMagar Microbiology, Paris, France) and incubated at 30°C for 48–72 h. Only one sample was taken per patient.

Green colonies isolated in CHROMagar were presumptively identified as *C. albicans*, and they were cultured in cornmeal–Tween-80 agar, at 25°C for 7 days, for chlamydospore formation. Additionally, the isolates were tested with the ID 32C system for sugar assimilation (Biomerieux, Marcy l'Etoile, France). Colonies presenting other colours were cultured on new Sabouraud dextrose agar plates, and were identified by standard methods and by ID 32C. All *C. albicans* isolates were serotyped using a Candida Check Kit (Iatron Laboratories Inc., Tokyo, Japan). *C. albicans* ATCC 28367, serotype A, and *C. albicans* ATCC 44373, serotype B, were tested as quality controls.

For presumptive identification of *Candida dubliniensis*, all isolates identified as green colonies in CHROMagar were cultured at 42°C, and growth was observed after 48 h [9]. If growth of the colonies extended into the last three quadrants of the plate, it was considered to be good growth, and if the growth area was observed only in the first quadrant, it was considered to be poor growth. The xylose assimilation test was also performed [10]. *C. albicans* ATCC 76615 and *C. dubliniensis* CBS 7987 reference strains were tested as quality controls.

Candida isolates that did not grow at 45°C and/or those that did not assimilate xylose were submitted to genetic analysis. D1/D2 regions of the large-subunit ribosomal DNA were sequenced. Genomic DNA extraction from yeast was carried out using the Instagene Matrix Kit (Bio Rad, Hercules, CA, USA). D1/D2 regions were amplified according to the method of Kurtzman and Robnett [11], using the following primer set: NL-1, 5′-GCATATCAATAAGCGGAAAAG; and NL-4, 5′-GGTCCGTGTTTCAAGACGG. PCR amplification was performed for 30 cycles, with annealing at 52°C, extension at 72°C for 2 min, and denaturation at 94°C for 1 min. The amplified DNA was purified with SUPREC (Takara Bio Inc., Otsu, Shiga, Japan). The PCR products were directly sequenced in both directions, using a Thermo Sequenase II Dye Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech Inc., NJ, USA) and the ABI 3100 PRIMS sequencer (Applied Biosystems, Carlsbad, CA, USA). To determine the sequences, the two following internal primers were used, together with the above external primers: NL-2, 5′-CTTGTTCGCTATCGGTCTC; and NL-3, 5′-GAGACCGATAGCGAACAAG. The nucleotide sequence data were analysed using BLAST from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

Antifungal susceptibility testing

Antifungal susceptibility tests were performed using the broth microdilution method, according to document M27-A2

of the CLSI [12]. The antifungal drugs fluconazole (Gerbras, Sao Paulo, Brazil), itraconazole (Jansen Pharmaceutics, Titusville, NJ, USA), 5-FC (A4888-1G; Sigma Chemical, St. Louis, MO, USA), ketoconazole (Galera, Campinas, Sao Paulo, Brazil), amphotericin B (Sigma Chemical) and nistatine (141-05381; Wako Pure Chemical Industries, Ltd., Osaka, Japan) were obtained as reagent-grade powders from their respective manufacturers. Briefly, broth microdilution testing was performed in sterile, flat-bottomed 96-well microplates containing RPMI 1640 medium (L-glutamine without bicarbonate) buffered to pH 7.0 with MOPS.

A volume of 100 μL containing yeast cells was added to each well to achieve a final inoculum size of 0.5–2.5 $\times$ 10^3 cells/mL. The plates were incubated at 35°C for 48 h. Quality control strains (*Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258) were included on each day of the assay.

Electrophoretic karyotype (EK) determination

DNA preparation for EK determination was performed according to Branchini *et al.* [13] EK determination was carried out using the CHEF DRIII apparatus (Bio-Rad) under the following electrophoretic conditions: 13°C, 4.5 V, and 120°C, and pulse intervals of 120 s for 18 h and 240 s for 22 h. *Saccharomyces cerevisiae* chromosome DNA size standards (Bio-Rad) were included in each gel as standards.

Analysis of the EK was performed by visual inspection of photographs of ethidium bromide-stained gels. Each major band and each minor band was identified, and the distance from the origin of the gel, relative to those of the molecular mass standards, was measured. Isolates were considered to have different DNA subtypes if EK profiles differed by one or more bands. The gels were also analysed by computer, using the Bio-1D and BioGene V programs in a 95% CI (Vilber Loumat, Marnes la Valle, France).

Statistical analysis

Continuous variables were assessed by means of two-tailed Student's *t*-test for differences in means. Categorical variables in colonized and non-colonized patients were evaluated by chi-square and Fisher tests, and a p-value of <0.05 was considered to be statistically significant. Continuous variables were analysed using the non-parametric Mann–Whitney test (computational SAS system for Windows, version 8.02, Cary, NC, USA).

Results

Overall, 331 patients were enrolled in this study and, at the time of the surveillance, 147 (44%) patients harboured *Candida* spp. in the oral cavity. The patients were diagnosed for HIV infection in different years, and the year of diagnosis of HIV infection was compared with the onset of colonization. Ninety patients were diagnosed as having HIV infection during the period 1983–1995, and 43 (48%) were colonized; 241 patients participating in the study were diagnosed as having HIV infection during the period 1996–2003, and 104 (43%) were colonized (p 0.53) (Fig. 1).

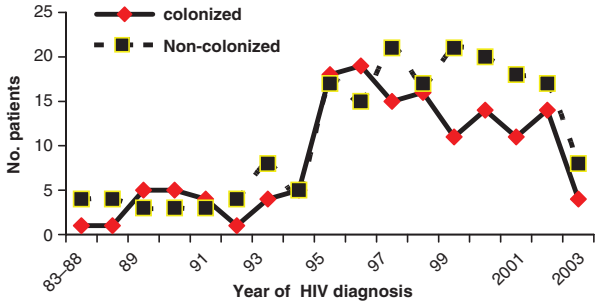

FIG. 1. Number of patients included in the study according to the year of human immunodeficiency virus (HIV) diagnosis and oral cavity *Candida* carriage status.

Among the 331 patients, the mean age was 38.9 years (colonized patients, 39.6 years; non-colonized patients, 38.3 years), 127 (38.4%) were women (colonized, 53; non-colonized, 74), and the majority of patients were white (264/331; colonized, 112; non-colonized, 152) (Table 1).

The number of colonized patients with detectable HIV viral load was significantly higher (83; 58.5%) than the number of non-colonized patients (68; 37%) (p 0.0002), and the mean CD4 cell count in the colonized group was significantly lower (mean, 374.4; standard deviation, 243.5) than in the non-colonized group (mean, 471.5; standard deviation, 306) (p 0.0029). Table 2 shows the number of colonized and non-colonized patients according to HIV RNA viral load and intervals of $CD4^+$ cell count. Patients with detectable HIV viral load and $CD4^+$ cell counts <200/mm^3 were more likely to be colonized (p 0.0003).

Most patients had at least one previous opportunistic infection, and they occurred at similar frequencies in colonized (81%) and non-colonized patients (78%). When the number of episodes of opportunistic infections were compared, fungal infections, including superficial and systemic infections, were the most prevalent; 147 colonized patients and 184 non-colonized patients had 105 and 150 episodes, respectively. However, when the fungal infections were categorized, colonized patients had significantly more previous episodes of oral candidiasis/OPC (55 episodes; p 0.046) and onychomycosis (19 episodes; p 0.03) than non-colonized patients (OPC, 50 episodes; onychomycosis, 11 episodes) (Table 1).

TABLE 1. Characteristics of 331 human immunodeficiency virus (HIV)-1-infected patients participating in this study as correlated with oral *Candida* colonization

Parameter	Colonized *n* = 147 (%)	Non-colonized *n* = 184 (%)	p-Value
Age: mean (years)	39.6	38.3	–
Gender: female	53 (36)	74 (40)	–
Exposure to HIV			
Sexual	118 (80.3)	148 (80.5)	–
Sexual + IDU	29 (19.7)	35 (19)	–
Vertical	–	1 (0.5)	–
HVI RNA viral load	83 (58.5)	68 (37)	0.0002[a]
Detectable			
$CD4^+$ cells/mm^3	326.5	410.5	0.0029[b]
Median			
Opportunistic infections	117 (80)	156 (85)	–
Presence of at least one episode			
Fungal infections			
Pneomocistisjiroveci	25 (17)	29 (15.7)	–
Oral candidiasis	55 (37.4	50 (27.1)	0.046
Cryptococosis	5 (3.4)	11 (5.9)	–
Onychomycosis	19 (13)	11 (5.9)	0.028
Other systemic mycosis	5 (3.4)	5 (2.7)	–
Viral infections			
Herpes zoster	33 (22.4)	21 (11.4)	0.007
Malignancies			
Non-Hodgkin lymphoma	4 (2.7)	0	0.038[c]
Use of protease inhibitor	83 (56.4%)	95 (51.6%)	–
Previous use of antifungal	74 (50.3)	79 (42.9)	–
Fluconazole	32	33	–
Ketoconazole	38	33	–
Itraconazole	1	6	–
Nistatine	48	37	0.009
Amphotericin B	3	10	–

IDU, intravenous drug use.
[a]Chi-square test.
[b]Mann–Whithney test.
[c]Fisher exact test.

TABLE 2. Human immunodeficiency virus (HIV) RNA viral load and intervals of $CD4^+$ cell counts in HIV-infected patients as correlated with oral *Candida* colonization

	Colonized (*n* = 142)		Non colonized (*n* = 182)		Total (*n* = 324)	
Viral load	*n*	%	*n*	%	*n*	p-Value
≥50 copies/mL $CD4^+$ cells/mm^3						
>350	35	25	71	39	106	–
200–350	21	15	31	17	52	–
<200	3	2	12	7	15	–
Total	59	42	114	63	173	–
<50 copies/mL $CD4^+$ cells/mm^3						
>350	26	18	34	19	60	–
200–350	27	19	21	11	48	–
<200	30	21	13	7	43	0.0003
Total	83	58	68	37	151	–

It is of note that herpes zoster was the most frequent viral opportunistic infection, and was significantly more frequent (33 episodes) in colonized than in non-colonized patients (21 episodes) (p 0.007). Bacterial and protozoal infections occurred to a similar extent in colonized and non-colonized patients. Tuberculosis was distinctly prevalent in the HIV population of the present study, i.e. 39 and 36 cases

TABLE 3. Antiretroviral regimen for the 331 human immunodeficiency virus-infected patients participating in this study and *Candida* oral colonization

	Colonized (*n* = 147)		Non-colonized (*n* = 184)		Total (*n* = 331)		
Antiretroviral regimen	*n*	%	*n*	%	*n*	%	p-Value
Two NRTI + PI	53	36	71	38.5	124	37.4	–
Two NRTI + NNRTI	64	43.5	89	48.3	153	46.2	–
Salvage therapy[a]	30	20.4	24	13	54	16.3	0.003

Salvage therapy: Two NRTIs + NNRTI + PI; two NRTIs + NNRTI + two PIs; two NRTIs + two/three PIs; NRTI + NNRTI + PI/NRTI + two PIs; NRTI + PI + FI.
NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; PI, protease inhibitor; FI, fusion inhibitor.
[a]Colonized patients underwent salvage therapy more often than non-colonized patients (p 0.003).

in colonized and non-colonized patients, respectively (data not shown).

ARV regimens containing two NRTIs + one non-nucleoside reverse transcriptase inhibitor and two NRTIs + one PI or ritonavir-boosted PI were more commonly used in 85.6% of the patients in the study (Table 3). At the time of this research, two patients were receiving a fusion inhibitor. The use of PI was not associated with colonization (p 0.44). Nevertheless, patients who harboured *Candida* in the oral cavity (30 patients) underwent salvage ARV therapy (which included one or two PIs) significantly more often than non-colonized patients (p 0.003).

One hundred and sixty-one *Candida* strains were recovered from the oral cavity of 147 colonized patients. *C. albicans* represented 137 (85%) of the isolates; 117 were *C. albicans* serotype A, and 20 strains were serotype B. In total, 24 (15%) non-*C. albicans* species were recovered, including: *Candida glabrata* (seven), *Candida tropicalis* (four), *Candida novergensis* (three), *C. krusei* (two), *C. dubliniensis* (two), *C. parapsilosis* (one), *Candida kefyr* (one), *Candida lipolytica* (one), *Candida guilhermondii* (one), *Candida sake* (one), and *Candida calliculosa* (one). Mixed colonization was detected in 12 patients. The years of diagnosis of HIV infection with serotypes A and B were compared; among patients diagnosed from 1983 to 1990, 83 and 14 were serotype A and serotype B, respectively, and among those diagnosed from 2000 to 2003, 31 and seven were serotype A and serotype B, respectively (p 0.20).

Green colonies in CHROMagar were obtained with 139 *Candida* isolates. All were germ-tube-positive and were tested for growth at 42°C with assimilation of xylose. Ten isolates were xylose assimilation-negative and seven failed to grow, and three isolates grew poorly at 42°C. These ten

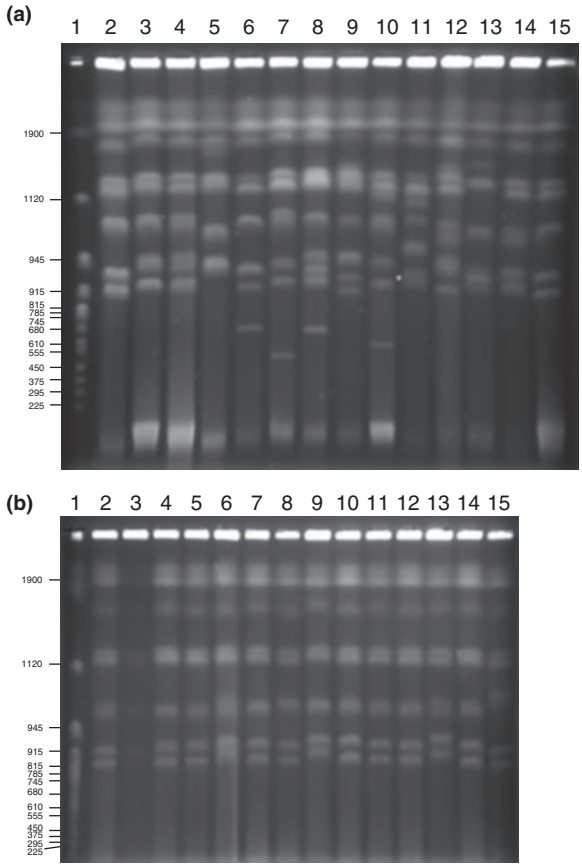

FIG. 2. Electrophoretic karyotypes of *Candida albicans* isolates from the oral cavity of HIV-infected patients. (a) Lanes 2 to 14: *C. albicans* serotype A. Lane 2: *C. albicans* serotype A, genotype A1. Lane 15: *C. albicans* serotype B, genotype B1 identical to profile A1. (b) *C. albicans* serotype B. Lane 1 of each gel: *Saccharomyces cerevisiae* chromosome DNA size standards (kb).

strains were then sequenced, and two of them were 99% compatible with *C. dubliniensis*.

EK determination identified 15 DNA profiles among 117 *C. albicans* serotype A, and profiles A1 and A3 were the most predominant, representing 55.6% and 18%, respectively. *C. albicans* serotype B had only three different EK profiles. It is of note that one of the EK profiles of *C. albicans* serotype B was identical to the A1 profile (Fig. 2). The two *C. dubliniensis* strains had two different profiles, which were also different from all *C. albicans* profiles and from that of the reference strain, *C. dubliniensis* CBS 7987.

Candida isolates mainly exhibited good susceptibility to the tested antifungal drugs (Table 4). *C. albicans* serotypes A and B were susceptible to all tested antifungal drugs. No resistance to azoles was detected among the 159 isolates, including *C. albicans* and non-*C. albicans*. However, isolates of *C. glabrata* were found that were dose-dependently susceptible to fluconazole (three isolates), to ketoconazole (six isolates), and to itraconazole (seven isolates); one isolate of *C. tropicalis* was dose-dependently susceptible to itraconazole, and one isolate of *C. novergensis* was dose-dependently susceptible to fluconazole.

C. krusei isolates were dose-dependently susceptible to all azoles, as this species is intrinsically resistant to this antifungal class. The data for the nine patients with non-*C. albicans* isolates that were dose-dependently susceptible to at least one antifungal drug were analysed, and antifungal agents had previously been used in seven patients (*C. glabrata*, four of seven patients; *C. tropicalis*, one patient; *C. novergensis*, one patient). The previous exposure to antifungal agents did not correlate with higher MIC levels; 74 patients had previously used antifungal agents in the colonized group, and higher MIC levels were detected only for non-*C. albicans* isolates, independently of the use of antifungal agents.

Discussion

The prevalence of HIV patients harbouring *Candida* yeasts in the oral cavity was similar to that found in previous studies [1,14,15]. In the present study, 45% of HIV-infected patients harboured *Candida* in the oral cavity, and 72% of the total isolates were *C. albicans* serotype A. Serotype A represented 85% of all *C. albicans* isolates, which is in contrast to the findings of some studies that found a higher percentage of serotype B isolates [16,17] in AIDS patients and in patients with different degrees of immunosuppression [16]. Whether the serotype was A or B had no relationship with the duration of HIV infection. Other studies, such as the present one, reported serotyping of *C. albicans* and a higher relative frequency of serotype A in clinical samples [18,19]. It is of note that the present EK findings showed surprising genotype diversity in serotype A strains (15 DNA profiles), as compared to serotype B strains (three DNA profiles). Previous studies [20,21] did not correlate serotypes and EK profiles in *C. albicans* isolates from the oral cavity of HIV-infected patients, and the low diversity previously reported might be related to a particular serotype or to geographical distributions.

In the present study population, non-*C. albicans* accounted for 15% of the isolates, suggesting an increasing number of these species in *Candida* oral colonization [15,22]. The identification of *C. dublinensis* in the oral cavity of HIV patients in Brazil has been repeatedly low [23,24] as compared with other studies [25]. *C. dubliniensis* has been cited as having replaced *C. albicans* in patients undergoing fluconazole treatment [26].

All *C. albicans* strains in the present study were susceptible to all tested antifungal agents (Table 4), independently of the

TABLE 4. Susceptibility to antifungal agents of 161 isolates of *Candida* spp. from the oral cavity of human immunodeficiency virus-infected patients

Candida spp. (161)	Range of MIC (mg/L)					
	Fluconazole	Ketoconazole	Itraconazole	Flucytosine	Amphotericin B	Nistatine
Candida albicans (137)						
Serotype A (117)	0.125–2	0.03–0.06	0.01–0.125	<0.125–2	0.125–1	0.50–2
	MIC_{50} 0.5	MIC_{50} 0.03	MIC_{50} 0.03	MIC_{50} <0.125	MIC_{50} 0.5	MIC_{50} 1
	MIC_{90} 1	MIC_{90} 0.03	MIC_{90} 0.06	MIC_{90} 0.125	MIC_{90} 1	MIC_{90} 2
Serotype B (20)	0.125–2	0.03–0.50	0.03–0.125	<0.125–1	0.25–1	1–2
	MIC_{50} 0.5	MIC_{50} 0.03	MIC_{50} 0.06	MIC_{50} 0.5	MIC_{50} 0.5	MIC_{50} 1
	MIC_{90} 1	MIC_{90} 0.06	MIC_{90} 0.125	MIC_{90} 0.5	MIC_{90} 0.5	MIC_{90} 2
Non-*C. albicans* (24)						
Candida glabrata (7)	8–16	0.125–0.50	0.25–0.50	<0.125–0.25	0.25–1	0.50–2
Candida tropicalis (4)	0.25–2	0.03–0.125	0.03–0.50	<0.125–0.125	0.25–1	2
Candida norvegensis (3)	4–16	003–0.125	0.06–0.125	0.25–2	0.50–1	1–2
Candida krusei (2)	16	0.125–0.25	0.125–0.25	1–2	0.50–1	2
Candida dubliniensis (2)	0.25	0.03	0.06	<0.125	0.125–0.25	1
Candida parapsilosis (1)	1	0.03	0.06	<0.125	0.50	2
Candida guilhermondi (1)	4	0.03	0.03	<0.125	0.125	1
Candida kefir (1)	0.50	0.03	0.03	0.125	0.50	0.50
Candida sake (1)	1	0.06	0.06	<0.125	0.06	0.50
Candida calliculosa (1)	–	–	–	–	–	–
Candida lipolytica (1)	–	–	–	–	–	–

use of PIs and previous antifungal agents. Migliorati *et al.* [6] reported *in vitro* results obtained using *Candida* yeast pre-exposed to ritonavir before susceptibility testing, showing that the percentage of resistant isolates decreased considerably. The susceptibility profiles of the *C. albicans* isolates in the present study, indicating susceptibility to the tested antifungal drugs, could not be explained by the use of PIs. It is of note that the number of patients who previously received antifungal treatment was similar in both groups, i.e. colonized (50.3%) and non-colonized (43%) patients, suggesting that previous exposure to antifungal agents did not interfere with antifungal susceptibility. As expected, non-*C. albicans* isolates, i.e. *C. glabrata* and *C. krusei*, were dose-dependently susceptible to azoles, independently of previous use of antifungal drugs.

The present results show that detectable HIV RNA viral load and $CD4^+$ cell counts <200 cells/mm^3 were significantly more frequent in patients harbouring *Candida* in the oral cavity (p 0.003). Other authors have reported similar results [27–29]. A national study conducted by Costa *et al.* [1] showed no relationship between $CD4^+$ cell count, HIV RNA viral load, and oral *Candida* colonization; however, among the patients included in their study, only nine patients had a CD4 cell count <200 cells/mm^3. Gottfredsson *et al.* [4] reported an association between oral colonization and plasma levels of HIV RNA, but they also did not find a correlation between $CD4^+$ cells and oral *Candida* colonization; unlike the present data, the median $CD4^+$ cell counts of the patients were basically identical in both groups, i.e. colonized (382 cells/mL) and non-colonized (381 cells/mL). Considered together, these findings suggest that the different results obtained in the studies might correlate with the patient characteristics in each study population.

The use of PIs in the ARV regimen did not influence the carriage of *Candida* in the oral cavity in the present study. Considering the previous studies on the effect of PIs in reducing the recurrence of oral candidiasis [30,31], it was expected that the use of PIs could decrease *Candida* oral carriage. The present results strongly suggested that the immune status of the patients determined the higher prevalence of yeast carriage, and not the usage of PIs. It is of particular interest that the HIV-infected patients who harboured *Candida* in the oral cavity typically underwent salvage ARV, unlike those who were non-carriers, corroborating the hypothesis that *Candida* colonization was mainly determined by the immune status of the patients.

The study population involved a substantial number of patients as compared with previous studies, and included patients undergoing long-term ARV therapy. When the percentage of oral carriage of *Candida* according to the year of HIV diagnosis was analysed, significant differences were not found between patients diagnosed during the period 1983–1995 and those diagnosed in recent years, independently of the ARV regimen. For instance, five patients were diagnosed as having HIV/AIDS during the period 1983–1988, and only 11 patients started ARV therapy in 2003. Interestingly, the duration of ARV therapy was not correlated with the carriage status. It was expected that patients undergoing prolonged ARV therapy and receiving PIs might harbour *Candida* less frequently, on the basis of the potential antifungal activity of these drugs according to *in vitro* studies [17,32] and clinical data [33].

It is of note that the HIV patients carrying *Candid*a in the oral cavity had significantly more opportunistic infections, e.g. previous oral candidiasis, onychomycosis, and herpes zoster, and four patients had non-Hodgkin lymphoma. As compared to the non-colonized patients, these findings were substantially consistent with laboratory data that showed a significantly lower mean number of of $CD4^+$ cells per cubic millimetre and a detectable HIV RNA viral load. Other authors have also described the correlation between low $CD4^+$ count and occurrence of herpses zoster [34]. Glesby *et al.* [35] found that HIV-infected women with $CD4^+$ counts <200 cells/mm^3 were substantially more likely to develop shingles.

In conclusion, the present data suggest that the immune status of the HIV-infected patients was probably the most important factor for harbouring of *Candid*a spp. in the oral cavity. Colonized patients were significantly more immunosuppressed than non-colonized patients. Effective ARV therapy appeared to be fundamental in determining an undetectable viral load and a higher $CD4^+$ cell count in HIV-infected patients, rather than the use of PIs. These findings underscore the complexity of *Candida* oral colonization in HIV-infected patients undergoing ARV therapy. As new classes of antiretroviral agents are being released, additional studies involving longitudinal follow-up will be required.

Transparency Declaration

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