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RELATED FACTORS FOR COLONIZATION BY *Candida* SPECIES IN THE ORAL CAVITY OF HIV-INFECTED INDIVIDUALS

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SUMMARY

The colonization of the oral cavity is a prerequisite to the development of oropharyngeal candidiasis. **Aims:** The aims of this study were: to evaluate colonization and quantify *Candida* spp. in the oral cavity; to determine the predisposing factors for colonization; and to correlate the levels of CD4+ cells and viral load with the yeast count of colony forming units per milliliter (CFU/mL) in HIV-positive individuals treated at a University Hospital. Saliva samples were collected from 147 HIV patients and were plated on Sabouraud Dextrose Agar (SDA) and chromogenic agar, and incubated at 30 ºC for 72 h. Colonies with similar morphology in both media were counted and the result expressed in CFU/mL. **Results:** Of the 147 HIV patients, 89 had positive cultures for *Candida* spp., with a total of 111 isolates, of which *C. albicans* was the most frequent species (67.6%), and the mean of colonies counted was 8.8×10^3 CFU/mL. The main predisposing factors for oral colonization by *Candida* spp. were the use of antibiotics and oral prostheses. The use of reverse transcriptase inhibitors appears to have a greater protective effect for colonization. A low CD4+ T lymphocyte count is associated with a higher density of yeast in the saliva of HIV patients.

KEYWORDS: *Candida*, HIV; Candidiasis; Oral; Colonization.

INTRODUCTION

Acquired Immunodeficiency Syndrome (AIDS) is characterized by an impaired immune system, resulting in a continuing decrease in the number of $CD4^+$ T lymphocytes. As a result of this, several opportunistic infections may be present in an individual infected with HIV, including cryptococcosis, histoplasmosis, pneumocystosis and candidiasis[5,12,24].

Oropharyngeal candidiasis is the most common fungal infection in individuals with Human Immunodeficiency Virus (HIV), and 90% of them develop this infection at least once during the course of the disease[3,21].

The introduction of highly active antiretroviral therapy (HAART) in the treatment of HIV infection causes a reduction in the occurrence of opportunistic infections. However, oropharyngeal candidiasis is still common among individuals with late diagnosis and those that do not respond successfully to treatment[24,31]. Currently, six classes of antiretroviral drugs are available in Brazil: nucleoside reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, protease inhibitors, integrase inhibitors, fusion inhibitors, and co-receptors[4].

Colonization of the oral cavity does not always culminate in oropharyngeal candidiasis. However, such colonization can be considered a prerequisite for the development of the disease. Before the onset of symptoms of infection, there is usually an intense colonization by *Candida* spp.[8,33]. Other factors that favor the colonization of the oral cavity by *Candida* species include: smoking, diabetes mellitus, oral prostheses, extreme age, antibiotics, decreased salivary flow, food habits, and nutritional status[10,24,29].

In oral fungal microbiota, and in other mucosal microbiota of the body, *Candida* species have been observed to be prevalent, with *C. albicans* being the species most often isolated. Meanwhile, in recent years, there has been an increase in isolation of other species including *C. parapsilosis*, *C. tropicalis*, *C. glabrata*, *C. krusei* and *C. dubliniensis*[19,24,31].

An important marker for the carrier state or infection is the count of colony-forming units (CFUs) of yeast in the oral cavity. Individuals who are only carriers of *Candida* spp. have a colony count of < 1000 CFU/mL of saliva, whereas individuals who have infection present with a colony count exceeding 4000 CFU/mL[9,15].

The aims of this study were: to evaluate colonization and quantify *Candida* spp. in the oral cavity of HIV-positive individuals treated at a University Hospital; to determine the predisposing factors for colonization; and to investigate the correlation between the levels of CD4+ cells and viral load with the yeast count.

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PATIENTS AND METHODS

This study was classified as cross-sectional, which verified the presence of *Candida* spp. only at the time of saliva collection without monitoring the patient after sampling.

Research subjects and sample collection: saliva samples were collected from 147 seropositive HIV individuals in the Outpatient Clinic of Infectious Diseases of the Clinical Hospital of the Federal University of Uberlandia, Uberlandia, Minas Gerais State, Brazil.

During routine consultations in the Infectious Diseases Clinic, patients received guidelines about the study and were invited to participate in the research. This study was approved by the Ethical Committee for Human Research of the Federal University of Uberlandia, protocol 368/11.

Demographic and clinical data for each individual research participant was obtained using a questionnaire, prior to collection of the saliva sample. Medical records were reviewed to obtain data such as age; form of HIV infection; time of diagnosis; use of antifungal agents and/or antibiotics within 30 days and/or seven days prior to collection of the saliva sample; antiretroviral therapy used; values of CD4+ cells; viral load (these values were measured up to two weeks prior to saliva collection); history of oral candidiasis; use of oral prostheses; and smoking status.

After that, patients were requested to collect approximately 2 mL of unstimulated saliva in sterilized tubs. When collecting the saliva sample, no patients had clinically active oral candidiasis.

Sample processing, quantification and identification of yeasts: the saliva sample was homogenized with sterile glass beads, and serial decimal dilutions were made (10^{-1} to 10^{-3}) in sterile saline solution (0.9%). Then, aliquots of 10 µL of pure saliva and of the dilutions were added to plates containing Sabouraud Dextrose Agar (SDA) (Sigma, Rockville, USA) containing chloramphenicol (100 mg/L), and similarly to other plates containing Chromogenic Agar (Conda, Madrid, Spain). The plates were incubated at 30 °C for 72 h. Colonies with similar morphology in both media were counted and the result was expressed in colony-forming units per milliliter of saliva (CFU/mL).

Individual colonies from each sample were identified using the classical methodology and the Auxacolor2® system (Bio-Rad, Marnes-la-Coquette, France), and thereafter stored in BHI-glycerol at −20 ºC, and in sterile distilled water kept at room temperature[22,30].

The samples identified as *C. albicans* were submitted to molecular testing by polymerase chain reaction (PCR) for confirmation of the species or alternatively identification of *C. dubliniensis.* The DNA used in the PCR reactions was obtained according to the proposed by LUO & MITCHELL (2002) modified[20]. Three CFU previously grown in SDA for 24 h were transferred to 50 µL of sterile distilled water and 2 µL of this cellular suspension were directly used in PCR. Specific primers were used for *C. albicans* (*forward*: 3'TTT ATC AAC TTG TCA CAC CAG A5', *reverse*: 5'ATC CCG CCT TAC CAC TAC CG3') and *C. dubliniensis*: (*forward*: 3'AAA TGG GTT TGG TGC CAA ATT A5', *reverse*: 5'GTT GGC ATT GGC AAT AGC TCT A3')[13].

Statistical analysis: patients included in the study were divided into two groups based on the results of the saliva sample culture: (1) colonized and (2) non-colonized. All patients who yielded at least one colony of *Candida* spp. in the culture were included in the first group (Group 1). Group 2 included all individuals with negative culture for *Candida* species.

Qualitative variables associated with either Group 1 or 2 were investigated using *p*-values and the *odds ratio* (*OR*)-values. For analysis of differences in qualitative variables between the two groups of patients, the Mann-Whitney test was used. Comparison of the average CFU/mL with the range of CD4+ T lymphocytes and of viral load was performed using the Kruskal-Wallis test. Non-parametric tests were applied for the analysis of quantitative variables without normal probability distributions. All tests were applied using a significance level of 5%.

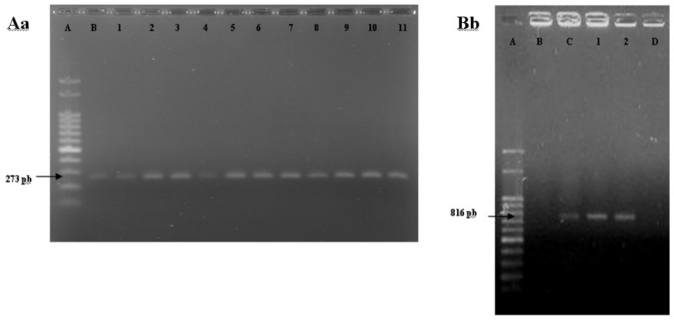

Fig. 1 - Agarose gel electrophoresis of specific primer for *Candida albicans* (Aa) and *Candida dubliniensis* (Bb). Aa: A) Molecular weight, B) *C. albicans* ATCC90028, 1-11) study samples. Bb: A) molecular weight, B) *C. albicans* ATCC90028, C) *C. dubliniensis* INCQS 40172, D) negative control, 1-2) study samples.

RESULTS

Of the 147 patients included in this study, 84 were male and 63 were female. Age ranged from 17-73, with a mean age of 44.7 and a median of 44. Of the 147 patients, 101 (68.7%) acquired HIV by heterosexual contact, 19 (12.9%) by homosexual contact, one (0.7 b%) congenitally, one (0.7%) by a work-related accident, one (0.7%) through a blood transfusion, and 24 patients (16.3%) were unable to inform how they were infected. The mean time for diagnosis of HIV infection was 8.4 years. Within this study group, 13 patients (8.8%) did not use any antiretroviral therapy at the time of collection of saliva sample; 68 (46.3%) used reverse transcriptase inhibitors, 61 (41.5%) used a protease inhibitor associated with a transcriptase inhibitor, and five (3.4%) combined the use of protease inhibitors with antiretroviral transcriptase and integrase inhibitors.

Candida spp. was isolated in 60.5% (89) of the patients, with a total of 111 isolates, and *C. albicans* was the most frequent species (67.6%). *Candida* non-*Candida* species accounted for 32.4% of the total isolates (Table 1). Among the 89 patients with a positive *Candida* species culture, 69 (77.5%) were colonized by only one species of *Candida*, and 20 (22.5%) had a combination of two or more species (Table 1).

The mean count of colonies per milliliter of saliva was 8.8×10^3 CFU/mL, and the median was 8×10^2 CFU/mL (ranging from 10^2 to 5×10^5 CFU/mL).

Table 1
Distribution and frequency of association of *Candida* species isolated from the oral cavity of HIV patients treated at the Outpatient Clinic of Infectious Diseases of the Clinical Hospital of the Federal University of Uberlandia, Minas Gerais, Brazil, in t2012

Species	Frequency of isolates	Percentage (%)	Association of different species	Frequency of isolates	Percentage (%)
C. albicans	75	67.6	*C. albicans* + *C. parapsilosis*	6	30
C. parapsilosis	10	9.0	*C. albicans* + *C. tropicalis*	5	25
C. tropicalis	8	7.2	*C. albicans* + *C. glabrata*	2	10
C. glabrata	5	4.5	*C. albicans* + *C. krusei*	2	10
C. krusei	4	3.6	*C. albicans* + *C. kefyr*	1	5
C. dubliniensis	3	2.7	*C. dubliniensis* + *C. lusitaniae*	1	5
C. kefyr	2	1.8	*C. albicans* + *C. dubliniensis*	1	5
C. famata	1	0.9	*C. albicans* + *C. parapsilosis* + *C. tropicalis*	1	5
C. guilliermondii	1	0.9	*C. albicans* + *C. glabrata* + *C. tropicalis*	1	5
C. lusitaniae	1	0.9			
C. peliculosa	1	0.9			
	111	100		20	100

Predisposing factors for colonization of the oral cavity by *Candida* species are listed in Table 2. There was a significant statistical difference between colonized and non-colonized individuals with respect to the use of antibiotics ($p = 0.0254$, $OR = 3.2$) and the use of dental prostheses ($p = 0.0173$, $OR = 2.46$). However, use of HAART ($p = 0.5671$), use of antifungal agents ($p = 0.7102$), smoking ($p = 0.3868$), gender ($p = 0.4215$), age ($p = 0.2529$), CD4+ cell count ($p = 0.5234$), viral load ($p = 0.2954$), history of candidiasis ($p = 0.8628$), and time of HIV diagnosis ($p = 0.2758$) did not differ significantly between colonized and non-colonized patients.

By analyzing the relationship between HAART (which includes different classes of antiretroviral drugs) and oral colonization by *Candida* spp., it was found that individuals who used a combination of reverse transcriptase inhibitors and protease inhibitors were more likely to be colonized than those using reverse transcriptase inhibitors alone ($p = 0.0315$). When comparing other therapeutic regimens among themselves and with those individuals who were not receiving HAART at the time of collection, no statistical differences were observed ($p > 0.05$). Compared with the other therapeutic schemes, and those who did not use HAART, no statistical difference was observed ($p > 0.05$).

The mean count of CD4+ cells was 551 cells/mm³, and the median was 557 cells/mm³ (range, 3–1619 cells/mm³). There were 25 (17%) patients who had < 200 CD4+ cells/mm³ at the time of collection of the saliva; 108 (73.4%) patients had an undetectable viral load (< 50 copies/mL), whereas 13 (8.8%) had a viral load of > 20,000 copies/mL.

Comparing the CD4+ cell count and the viral load of patients who had at least one isolate of *Candida* spp. in the oral cavity with those who had a negative culture, a very close *p*-value of 0.05 yielded in the range of 0–200 cells/mm³ in the case of CD4+ ($p = 0.0546$) (Table 3). The viral load did not differ significantly between colonized and non-colonized individuals (Table 3).

Comparing the mean of the CFU/mL in saliva for the different ranges of CD4+ T lymphocytes, it was observed that those within the range of 0–200 CD4+ cells/mm³ tended to have a greater CFU/mL than those patients with CD4+ cells > 350 cells/mm³ ($p = 0.0001$) (Table 4). This statistical difference was not observed when comparing the mean CFU/mL between the different ranges of viral load (Table 4).

DISCUSSION

Persistent colonization of the oral cavity by *Candida* spp. can be considered a predisposing factor for the development of oropharyngeal candidiasis[11,16]. In HIV patients, this has been given considerable attention due to the high incidence of oropharyngeal candidiasis and a high density of yeast in the oral cavity may be an important marker for disease progression[1,3].

The frequency of HIV-positive individuals colonized by *Candida* spp. (as well as the species present) varies between different regions of the planet as a result of geographical, climatic and ethnic differences[6,23]. In this study, 60.5% of saliva samples were positive for *Candida* species. Studies show that in Brazil the rate of colonization has ranged from 58 to 62% in patients with HIV[6,21]. However, in countries such as Mexico, Turkey, Argentina and USA, higher rates have been reported[11,24,27,28].

The predominant species found in this study was *C. albicans* (67.6%), corroborating the findings of similar studies in Sao Paulo, Argentina and Nigeria, which yielded a frequency of *C. albicans* of 51.5%, 58.9% and 45%, respectively[21,24,26]. *Candida* non-*albicans* species accounted for 32.4% of the total isolates: *C. parapsilosis*, (followed by *C. tropicalis*), was the most common. This frequency is higher than that reported by LI *et al*. in China[23] (19.8%) and by WINGETER *et al.* in southern Brazil (7%)[34]. However, this increased percentage of *Candida* non-*albicans* species, besides having a major effect on clinical procedures, confirms a trend observed in other similar studies[11,25].

Table 2
Clinical characteristics and rate of *Candida* oral colonization in 147 patients with HIV treated at the Outpatient Clinic of Infectious Diseases of the Clinical Hospital of the Federal University of Uberlandia, Minas Gerais, Brazil, in 2012

Factors predisposing	Colonized (89)	Non-colonized (58)	*p*-value	*OR*
Use of antibiotics	24 (27%)	6 (10.3%)	**0.0254**	3.2000
Use of antifungal	5 (5.6%)	5 (8.6%)	0.7102	0.6310
Smoker			0.3868	
Yes	27 (30.3%)	13 (22.4%)		1.5074
No	62 (69.7%)	45 (77.6%)		0.6634
Use of dental prostheses			**0.0173**	
Yes	45 (50.6%)	17 (29.3%)		2.4666
No	44 (49.4%)	41 (70.7%)		0.4054
Use of HAART	80 (89.9%)	54 (93.1%)	0.5671	1.5146
Sex			0.4215	
Female	41 (46%)	22 (37.9%)		1.3977
Male	48 (54%)	36 (62.1%)		0.7154
Age	45.6	43.5	0.2529	
CD4+	544 ± 331	562 ± 319	0.5234	
Viral load	20215.7 ± 88698	5387.2 ± 21473.9	0.2954	
Historical of oral candidiasis				
Yes	27 (30.4%)	16 (27.6%)	0.8628	1.1431
No	62 (69.6%)	42 (72.4%)		0.8748

Table 3
Frequency of HIV-positive individuals colonized and not colonized by *Candida* spp. according to the CD4+ T lymphocyte cell count and viral load of patients treated at the Outpatient Clinic of Infectious Diseases of the Clinical Hospital of the Federal University of Uberlandia, Minas Gerais, Brazil, in 2012

CD4 (cells/mm³)	Colonized (89)		Not colonized (58)		*p-value*
	No. of individuals	Average (CD4)	No. of individuals	Average (CD4)	
0-200	17 (19.1%)	123.7	8 (13.8%)	70.3	0.0546
201-350	9 (10.1%)	273.2	11 (19%)	271.3	1.0000
>350	63 (70.8%)	694.6	39 (67.2%)	743.5	0.1164
Total	89 (100%)	544.1	58 (100%)	561.1	0.5234
Viral load (CFU/mL)	No. of individuals	Average (viral load)	No. of individuals	Average (viral load)	
<50	62 (69.6%)	–	46 (79.3%)	–	1.0000
51-20000	18 (20.2%)	4317.8	8 (13.8%)	6047.5	0.4899
>20000	9 (10.2%)	191274.8	4 (6.9%)	66021.7	0.1474

Colonization by more than one *Candida* species has been reported previously[11,23,24]. In our study, 22.5% of patients were colonized by at least two species, and association was predominantly between *C. albicans* and *C. parapsilosis* (six of 20). Other studies have showed predominant association between *C. albicans* and *C. glabrata*[11,21,23,24].

Analysis of the concentration of yeast in saliva (8.8×10^3 CFU/mL) was higher than that previously observed in Sao Paulo (Brazil) and in the south of India (5.2×10^2 CFU/mL and 3×10^2 CFU/mL, respectively). However, the technique used for collecting that material was a swab of the oral cavity and rinse in sterile phosphate-buffered saline, respectively[17,21]. There is no consensus on the cut-off level of CFU/mL differentiating individuals colonized by yeast or having oropharyngeal candidiasis, yeast count > 4×10^3 CFU/mL can be considered a sign of

Table 4
Relationship between number of CD4+ cells and concentration of yeasts (CFU/mL) and viral load and concentration of yeasts in the saliva of HIV-positive patients treated at the Outpatient Clinic of Infectious Diseases of the Clinical Hospital of the Federal University of Uberlandia, in 2012

CD4+ (cells/mm³)	Colonized	CFU/mL (average)	*p*-value
0-200[a]	17	7.4×10^3	[ab]0.2631
201-350[b]	9	5.5×10^3	[ac]0.0001
>350[c]	63	9.7×10^3	[bc]0.0948
Viral load (copies/mL)			
<50[d]	62	9.8×10^3	[de]0.2259
51-20000[e]	18	3.6×10^3	[df:]0.4549
>20000[f]	9	8.0×10^3	[ef]0.1270
Total	89	8.8×10^3	

Note: a, b, c: variation ranges of CD4 cells used as a reference at the Outpatient Clinic of Infectious Diseases of the Clinical Hospital of the Federal University of Uberlândia, to begin, maintain or change antiretroviral therapy until the conclusion of this study ab, ac, bc: results from the statistical comparison between the mean of CFU/mL of three tracks CD4+ T lymphocytes. d, e, f: variation ranges of viral load. ab, ac, bc: result of statistical comparison between the mean CFU/mL of three tracks viral load.

oral candidiasis[9,15]. The discrepancy in the average CFU/mL between studies may be explained by the different methodology used for collection of the saliva sample, as some techniques are more sensitive than others[11].

Ways of reducing colonization may contribute to a decreased incidence and severity of oral candidiasis or even to a decreased risk of candidemia, hence the importance of identifying the individual's characteristics and habits that are associated with high oral cavity colonization by *Candida* spp., together with any potential modifications to these characteristics and habits. The use of antibiotics and dental prostheses were considered predisposing factors for colonization of the oral cavity by *Candida* species (and probably for the development of oral candidiasis). A similar study in Taiwan also observed the relationship between these two factors and oral colonization by *Candida* spp[35]. This relationship is explained by the fact that prolonged administration of antibiotics can cause an imbalance in the oral microbiota, thus allowing the proliferation of other microorganisms present there, including *Candida* species[7]. However, the use of an oral prosthesis is an important factor in colonization because of the trauma that the mucosa may suffer if it is not adjusted correctly, and/or due to poor hygiene, that also may occur[14].

In the present study, the frequency of individuals colonized or not by *Candida* spp. was not related to the use of HAART therapy, confirming the results obtained in similar studies[11,18,27]. However, ARRIBAS *et al.*[2], LI *et al.*[23] and YANG *et al.*[36] observed a significant reduction in the rate of oral colonization in individuals who were using HAART. When we analyzed the relationship of a range of therapeutic regimens involving a number of classes of antiretroviral drugs, it was observed that the schema that included reverse transcriptase inhibitors combined with protease inhibitors was related to a greater frequency of colonized individuals compared with individuals whose HAART therapy included only a combination of reverse transcriptase inhibitors. YANG *et al.*[36], observed a significant reduction in the rate of oral colonization, among those individuals included in their studies, that was related to the use of HAART therapy without, however, relating the class of drugs used.

A reduced frequency of individuals with HIV colonized by *Candida* spp. has also been reported by WU *et al.*[35], who demonstrated a reduction of oral colonization by *Candida* yeasts among individuals who used transcriptase inhibitors only. Possible mechanisms for the influence of HAART on oral colonization by *Candida* spp. are unknown. However, an explanation for the relationship between the use of protease inhibitors and an increased incidence of oral colonization by *Candida* species may be the greater immunological impairment and longer HIV infection period of individuals using protease inhibitors at the time of saliva sample collection, since the use of this class of antiretrovirals is only recommended when the combination of other classes of antiretrovirals has been ineffective in combating viral replication and, consequently, controlling HIV infection. Nevertheless, other studies may seek to confirm these findings, and thus contribute to elucidating the possible mechanisms that interfere with the proliferation of microorganisms and their adherence to the surface of the oral cavity.

Previous studies have sought to investigate the relationship between oral cavity colonization by *Candida* species with counts of CD4+ cells and viral load[21,24,27].

When comparing the load of yeast present in the saliva of individuals colonized by *Candida* spp. with CD4+ T lymphocyte count and viral load, it was observed that patients with a lower count of CD4+ cells showed a higher CFU/mL of saliva. This suggests that a low CD4+ cell count may be associated with a higher number of yeast in the saliva of individuals with HIV. This relationship may be explained by the hypothesis that HIV infection may not only compromise the oral mucosal immunity but also stimulate the expression of virulence factors in the yeasts, since it has been observed, *in vitro*, that HIV stimulates the secretion of a proteolitic enzyme by *C. albicans*[32]. However, when the same comparison was made in relation to the viral load of the subjects studied, this was not found to have a statistically significant difference, maybe because the number of subjects studied was insufficient to demonstrate the relationship. To the best of our knowledge, there are no studies in the literature that have set out to examine the relationship of the concentration of yeasts in saliva to the CD4+ cell count and the viral load.

In conclusion, it was observed that despite *C. albicans* being the most frequent species, the *Candida* non-*albicans* species represented a significant percentage of isolates. In addition, the yeast count in the saliva of patients was greater than that observed in previous studies. The use of antibiotics and oral prostheses are directly related to oral colonization by *Candida* spp. However, the use of HAART had no statistically significant influence in reducing the colonization of the site in question, but use of the antiretroviral class of reverse transcriptase inhibitors alone appears to have a greater protective effect against colonization. Finally, a low CD4+ T lymphocyte count seemed to be associated with a higher number of yeast being present in the saliva of HIV patients.

RESUMO

Fatores relacionados a colonização da cavidade bucal de indivíduos portadores do HIV por espécies de *Candida*

A colonização da cavidade oral pode ser considerada um pré-requisito para o desenvolvimento de candidíase orofaríngea. Os objetivos deste estudo foram: avaliar e quantificar espécies de *Candida* isoladas da cavidade oral, para determinar os fatores predisponentes para a colonização, e correlacionar os níveis de células CD4+ e carga viral em indivíduos HIV-positivos atendidos em um hospital universitário. Foram coletadas amostras de saliva de 147 pacientes portadores do HIV, as quais foram semeadas em Ágar Sabouraud Dextrose (ASD) e ágar cromogênico e incubadas a 30 °C por 72 horas. As colônias com morfologia semelhante em ambos os meios foram contadas e o resultado expresso em unidade formadora de colônias por mililitro (UFC/mL). Dos 147 pacientes HIV positivos, 89 apresentaram culturas positivas para *Candida* spp., totalizando 111 isolados, e *C. albicans* foi a espécie mais frequente (67,6%). A contagem média de colônias foi de 8.8×10^3 UFC/mL. Os principais fatores predisponentes para colonização oral por *Candida* spp. foram a utilização de antibióticos e de próteses orais. O uso de antirretroviral da classe de inibidores da transcriptase reversa pareceu ter maior efeito protetor para a colonização. Baixa contagem de linfócitos T CD4+ está relacionada com maior densidade de leveduras na saliva de indivíduos HIV positivos.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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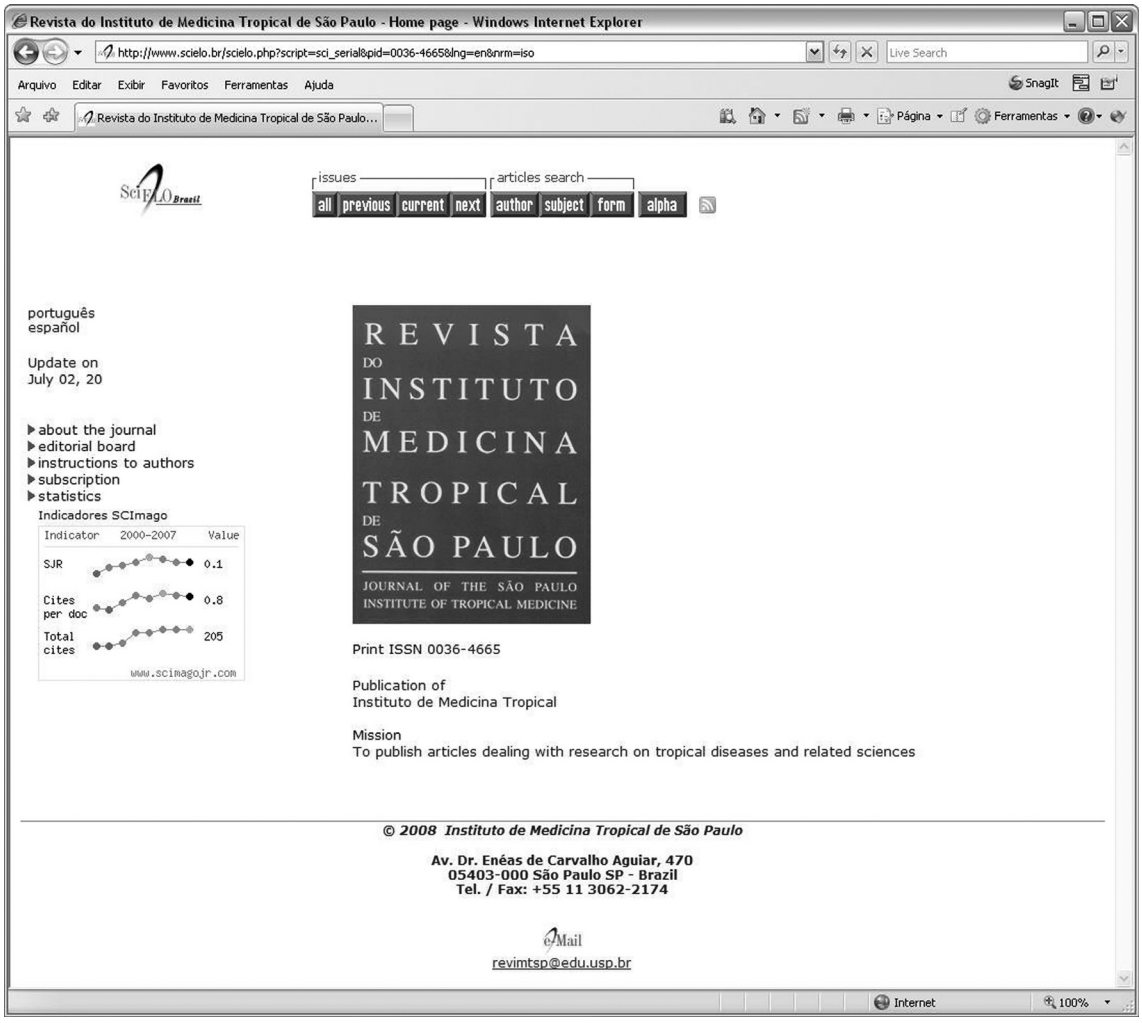

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