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Oral *Candida* spp carriage and periodontal diseases in HIV-infected patients in *Ribeirão Preto*, Brazil

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ABSTRACT

The majority of HIV-infected patients develop *Candida* spp-associated clinical oral lesions. Studies have shown that asymptomatic oral colonization of *Candida* spp may lead to oral lesions or become a source of disseminated infections. The aim of this study was to verify the effects of periodontal conditions on *Candida* spp prevalence and *Candida* spp carriage in the oral cavity of HIV-infected patients compared to non-infected patients. Twenty-five patients not infected with HIV and 48 HIV-infected patients were classified according to periodontal conditions as being periodontal healthy or with periodontal disease. *Candida* spp carriage and classification were performed in oral rinse samples. Viral load and $CD4^+$ T lymphocyte (CD4+L) counts were performed in blood samples from HIV-infected patients. No differences in *Candida* spp prevalence related to HIV status or periodontal condition were detected. However, *Candida* spp carriage was increased in periodontally affected HIV-infected patients when compared to periodontally healthy HIV-infected patients (p= 0.04). Periodontally healthy HIV-infected patients presented *Candida* spp carriage in similar levels as healthy or periodontally affected non-HIV-infected patients. *Candida* spp carriage was correlated with CD4+L counting in HIV-infected patients. We concluded that periodontal disease is associated with increased *Candida* spp carriage in HIV-infected patients and may be a predisposing factor to clinical manifestations of candidiasis.

KEYWORDS: HIV infection. Oral *Candida* carriage. Periodontal disease. *Candida spp*.

INTRODUCTION

In the last few decades, an increase in the prevalence of fungal oral infections was observed and they seem to be related to the growing antibiotic use and the immunosuppressive condition, especially in AIDS patients[1]. Most of the infections are related to *Candida* spp, which is generally a commensal in the oral cavity, but in immunologically-compromised patients it can assume pathogenic characteristics[2]. Some studies showed that candidiasis comes from commensal strains and that asymptomatic oral colonization can lead to oral lesions or become a source of disseminated infections[3-5].

C. albicans is the most common species isolated in oral candidiasis[6]; however, other species, such as *C. tropicalis, C. glabrata, C. parapsilosis, C. stellatoidea, C. guilliermondii,* and *C. krusei* were also observed[7].

In healthy individuals, the prevalence of *Candida* spp in the oral cavity is 40-60%[8] while in HIV-infected patients, the prevalence increases to 62-93%[9,10]. Oral candidiasis is the most common lesion associated with HIV infection. It is

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considered one of the first clinical signs of HIV-related immunosuppression and it is highly predictable of the disease evolution[11]. Studies have shown that the prevalence of oral candidiasis varies between 20% and 70% in HIV-infected patients[6,11], and although a decline in its prevalence has been observed after the antiretroviral therapy (ART) era[12], it is estimated that 90% of HIV-infected patients will develop oral candidiasis during the evolution of HIV infection[13], emphasizing the importance of studies on this topic. Among the factors that predispose to oral candidiasis are smoking[14], age[3,15] and oral hygiene[16]. According to many studies, the presence of yeasts in the biofilm, mainly *C. albicans*, contribute not only to oral candidiasis, but to dental caries[16,17] and periodontal disease[18-20].

Studies on the correlation between *Candida* spp and the etiology and aggravation of periodontal diseases are important, but it is also essential to verify the effects of periodontal conditions on *Candida* spp carriage, either due to the possible correlation with the development of oral lesions or systemic disseminations[3-5]. Thus, due to the importance and prevalence of *Candida* spp associated with oral diseases in HIV-infected patients, the aim of this study was to identify different species of *Candida* in oral rinses from patients with and without periodontal disease in two population groups, with and without HIV infection. Furthermore, to quantify the number of *Candida* ssp colonies in the oral rinses between not infected and HIV-infected patients with and without periodontal disease.

MATERIALS AND METHODS

The study protocol was approved by the Ethics Committee of the Clinical Hospital of the School of Medicine of *Ribeirão Preto*, University of *São Paulo*, *Ribeirão Preto* (protocol Nº 3621/2011). The procedures were performed in accordance with the ethical standards on human experimentation and the Helsinki Declaration of 1975 and 1983 revision. Prior to selection, oral and written explanations about the research protocol were given to eligible participants. All patients provided a written informed consent before their participation in this study.

Twenty-five patients not infected with HIV and 48 HIV-infected patients were selected from the Clinical Hospital of the School of Medicine of *Ribeirão Preto*, University of *São Paulo*. Patients were over 18 years old, had more than 19 teeth, and had not received any periodontal treatment, antibiotic therapy, anti-inflammatory or antifungal therapy in the three months before the study participation.

All patients had no clinical signs of candidiasis and no history of complete or partial removable prosthesis use with palatal coverage. Participants with other oral lesions associated with HIV were not excluded from the study. Only patients who met the following criteria were selected: no clinical sign of periodontal disease, no probing depth greater than 4 mm, no loss of attachment and less than 20% of sites with bleeding. Patients who presented more than 20% of sites with bleeding and/ or at least 4 sites with loss of clinical attachment and more than 3 mm and/or 4 sites with probing depths than 4 mm were also included.

HIV-infected patients presented HIV seropositivity confirmed by ELISA and Western Blot tests, as well as by a recent viral load and CD-4$^+$ T lymphocyte (CD4+L) count exams (at least three months before the beginning of the study).

Patients' Clinical Evaluation

Patients underwent examination and collection of an oral rinse sample. The periodontal exam was performed after the collection of the oral rinse sample, so bleeding could not possibly interfere with the quality of collected samples. For the periodontal exam, we used a millimeter periodontal probe and all teeth (except third molars) were probed at six sites: mesiobuccal, buccal, distobuccal, mesiolingual, lingual and distolingual. In each site, we performed three different measures: deep probing, loss of attachment and bleeding.

Related to the periodontal condition, patients were classified into periodontally healthy or with periodontal disease. The periodontally healthy were patients who had no clinical sign of periodontal disease, no probing depth greater than 4 mm, no loss of attachment and less than 20% of sites with bleeding. The patients who were periodontally affected presented more than 20% of sites with bleeding and/ or at least 4 sites with loss of clinical attachment and more than 3 mm and/ or 4 sites with probing depths than 4 mm[21].

Biological Sample Collection

Oral Rinse Sample

One day before collection, sterilized bottles with 10 ml of PBS were prepared in autoclaves (pH 7.3, 0.1 M) and conditioned at 4 °C until use. Before oral rinse collection, the subjects were instructed not to eat, drink, smoke or brush their teeth for one hour prior to sample collection to minimize the risk of contamination. Participants were instructed to rinse with 10 ml of PBS for 60 seconds and spit inside a universal collector that was immediately centrifuged (2,000 g per 10 minutes). After centrifugation, supernatant was discarded and solid residue was diluted in 1 ml of PBS. In the obtained solution, *Candida* spp *was*

counted and identified. The count and identification of *Candida* spp were performed in the oral PBS rinse sample by the Samaranayke's technique, described in 1986[22].

Blood Collection

For blood exams, 10 mL of blood were collected in vacuteiner tubes with ethylenediamine tetra-acetic acid (EDTA). Plasma was obtained by centrifugation of total blood (800 x g per 10 min.), divided in tubes and stored at –80 °C in a maximum period of six months, until its use.

Laboratorial Analysis

Viral load and CD4+L count

The HIV-Monitor kit (Roche Diagnostic Systems, Branchburg, NJ, USA) was used to quantify the HIV RNA (viral load) in -blood plasma. The RNA was extracted from the samples using a modified silica protocol (QIAmp RNA viral kit; Qiagen, Chatsworth, CA, USA), and polymerase chain reaction was performed using the HIV-Monitor kit (Roche Diagnostic Systems, Branchburg, NJ, USA). The CD4+L counts in the blood plasma were determined by flow cytometry (FACS), which was performed within three months after sample collection.

Candida spp quantification

The oral rinse samples were diluted in PBS to obtain three different concentrations: pure sample, 10x PBS-diluted sample and 100x PBS-diluted sample. One hundred microliters of each concentration was plated in duplicate on petri dishes with Agar Sabouraud-Dextrose and chloramphenicol and incubated at 37 ºC for 48 hours. The colony forming units per milliliter (CFU/ ml) was obtained by the mean between duplicates of positive culture. Depending on the number of CFU/ mL, counts were categorized into 0 (none), 1 (isolated, <10 CFU/mL), 2 (moderate, 10-10^2 CFU/ mL), 3 (many, 10^2-10^3 CFU/mL) and 4 (massive, >10^3 CFU/ mL)[23].

Candida spp identification

To identify *Candida* spp, 100 microliters of oral rinse 10x PBS-diluted were plated on petri dishes with CHROMagar *Candida*. Later, colonies were plated in tubes with Sabouraud Dextrose Agar (Himedia, India) and incubated at 37 °C per 24 hours. From such inocula in the exponential growth state, the following were performed: fungal microcultivated test[24], germ tube formation[25]; hypertonic Sabouraud broth[26]; reduction of trifeniltetrazole chloride; zimogram; API (ID 32 C); formation of germinate tube, chlamydoconidia production, carbohydrates fermentation and assimilation[24]. The species identification was obtained based on the positivity or negativity of Sandven (1990) tests[27].

Statistical Analyses

Statistical analysis was performed using the Graph Pad Prism software (San Diego, CA, USA). Data were not normally distributed. Fisher's exact test and the Chi-squared test were used to compare the number of patients using ART, the number of patients who smoked and the prevalence of different *Candida* species in the groups. Differences between groups were assessed using Chi-squared test. The Mann-Whitney U test was used to compare two means, and the one-way Kruskal Wallis test was used to compare three or more means. Data are presented as the mean ± standard deviation, and for the analysis, a confidence interval of 95% was used, and p values were considered to be significant when they were equal to or less than 0.05.

RESULTS

Seventy-three patients participated in this study: 25 who were not HIV-infected and 48 who were HIV-infected. Patients were divided into four groups according to serum status and periodontal condition. Group A: 12 not HIV-infected and periodontally healthy patients; Group B: 13 not HIV-infected and periodontally affected patients; Group C: 19 HIV-infected and periodontally healthy patients; Group D: 29 HIV-infected and periodontally affected patients.

The participants of all groups were homogeneous -regarding epidemiologic and clinical characteristics, as described in Table 1.

Oral *Candida* spp counting and identification related to HIV-infected and non-infected patients' periodontal conditions

In general, we identified oral *Candida spp* to have an equivalent distribution in all groups, as described in Table 2.

C. albicans and *C. parapisilosis* were identified in all studied groups. *C. tropicalis* was identified in the C and D groups and *C. dubliniensis,* and *C. glabrata,* in the D group exclusively. Although a major *Candida spp* variety had been verified in the D group, its prevalence was not statistically significant (Table 2).

Candida spp were distributed in a homogeneous way among groups, but despite this, *Candida* spp counting was increased in - patients from the D group. Among non-HIV-infected patients, *Candida* spp counting did not correlate with the periodontal state. In the group A, analysis of the oral rinse revealed that 58.3% (n=7) of the samples were

Table 1 - Demographic Data and Clinical Parameters of Subjects Stratified by HIV Status and Oral Condition

	Group				
	A	B	C	D	*P* value
HIV status	Negative	Negative	Positive	Positive	-
Oral conditions	Healthy	Gingivitis/Periodontitis	Healthy	Gingivitis/Periodontitis	-
Number of subjects	12	13	19	29	-
Mean age (years)	35 (± 9)	37 (±9)	38 (±9)	39 (±5)	0.411[a]
Males (%)	50	54	32	55	0.4157[b]
Smokers (%)	8	23	15	34	0.2473[b]
Mean number of teeth	27 (±4)	27 (±5)	27 (±5)	26 (±5)	0.6106[a]
Sites with BOP (%)	9 (±7)	35 (±21)	5 (±6)	30 (±13)	<0.0001[a]
Sites with PPD> 4 mm (%)	0	1,2 (±2)	0	1,3 (±2)	<0.0001[a]
Use of ART (%)			84	79	1.000[c]
Mean CD-4$^+$ cells			514 (±312)	387 (±276)	0.2254[d]
Median viral load (cop/ml)			<50	<50	0.2365[d]

[a]Kruskal Wallis test; [b]Chi-squared test; [c]Fisher's exact test; [d]Mann Whitney test. Group A: Not HIV-infected and periodontally healthy patients. Group B: Not HIV-infected and periodontally affected patients. Group C: HIV-infected and periodontally healthy patients. Group D: HIV-infected and periodontally affected patients. BOP: bleeding on probing. PPD: probing pocket depth. ART: Antiretroviral therapy.

Table 2 - Identification of the Oral *Candida* spp in Studied Groups

	Group A	Group B	p value[a]	Group C	Group D	p value[a]
Candida spp	8 (67%)	10 (77%)	0.6728	11 (58%)	24 (83%)	0.0959
Candida albicans	7 (58%)	8 (61%)	1.000	11 (58%)	23 (79%)	0.1931
Candida krusei	0 (0%)	0 (0%)	-	0 (0%)	2 (7%)	0.5115
Candida parapisilosis	3 (25%)	2 (15%)	0.6447	1 (5%)	2 (7%)	1.000
Candida tropicalis	0 (0%)	0 (0%)	-	1 (5%)	2 (7%)	1.000
Candida dubliniensis	0 (0%)	0 (0%)	-	0 (0%)	1 (3%)	1.000
Candida glabrata	0 (0%)	0 (0%)	-	0 (0%)	3 (10%)	0.2673

[a]Fisher's exact test. Group A: Not HIV-infected and periodontally healthy patients. Group B: Not HIV-infected and periodontally affected patients. Group C: HIV-infected and periodontally healthy patients. Group D: HIV-infected and periodontally affected patients

Candida carriers, 41.6% (n=5) of the patients showed isolated to moderate numbers of CFU/ mL, whereas 16.6% (n=2) presented many to massive CFU/ mL. Similar results were found in group B: 63.5% (n=8) of the samples were *Candida* carriers; 48.1% (n=6) of the patients showed isolated to moderate numbers of CFU/ mL, whereas 15.4% (n=2) presented many to massive CFU/ mL (p= 0.97), as described in Figure 1A. Differently, among HIV-infected patients, group C presented 36.8% (n=7) of the samples with isolated to moderate numbers of CFU/ mL and 21.1% (n=4) showed many to massive CFU/ mL. High CFU/ mL was noted in the group D, 27.6% (n=8) and 55.2% (n=16) showed isolated to moderate, and many to massive numbers of CFU/ mL, respectively (p= 0.04), as described in Figure 1B.

It is important to note that *Candida* spp counting was similar between non-HIV-infected and infected patients who were periodontally healthy, groups A and C (p=0.94).

Influence of viral load and CD4+L count on the *Candida* spp carriage of HIV-infected patients

Among HIV-infected patients, we verified that *Candida* spp carriage was associated with viral load and CD4+L count. The 11 patients who presented less than 200 cells/mm^3 CD4+L presented 9% (n=1) with none CFU count; 9% (n=1) and 82% (n=9) showed isolated to moderate and many to massive numbers of CFU, respectively. The 20 patients who presented between 200 and 500 CD4+L/mm^3 presented 30% (n=6) with none CFU count, 30% presented isolated to

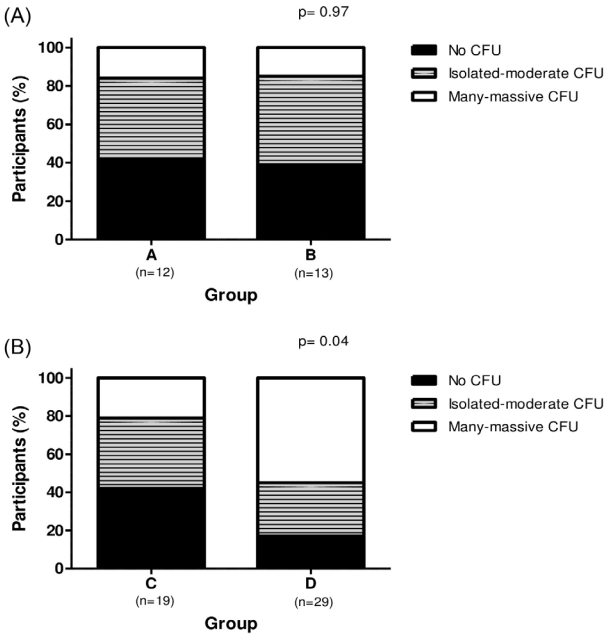

Figure 1 - (A) Relative *Candida* spp carriage in oral rinses of non-HIV-infected patients without (Group A) and with (Group B) periodontal diseases; p = 0.97, Chi-squared test; (B) Relative *Candida* spp carriage in HIV-infected patients without (Group C) and with (Group D) periodontal diseases; p = 0.04, Chi-squared test.

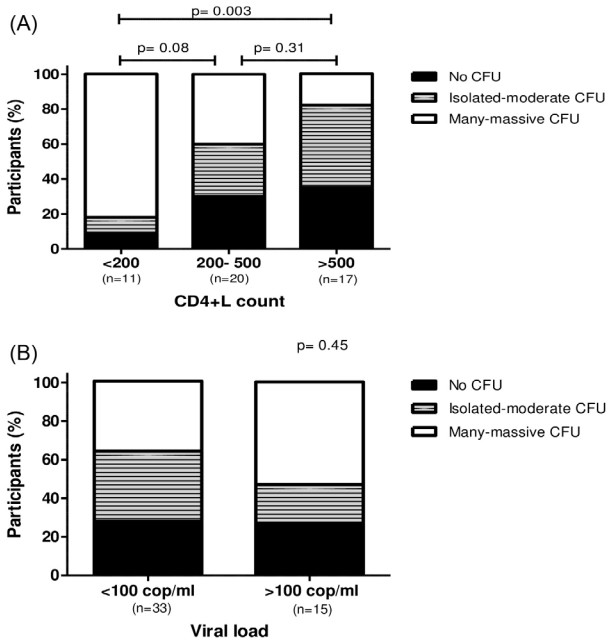

Figure 2 - (A) Relative *Candida* spp carriage in HIV-infected patients with different CD4+T cells count (< 200, 200-500 and > 500 cells/ mm^3). A significant difference was found between patients with less than 200 CD4+T cells/ mm3 and patients with more than 500 CD4+ T cells/ mm^3; p= 0.003, Chi-squared test; (B) Relative *Candida* spp carriage in HIV-infected patients with different viral loads (< 100 and > 100 copies/ mL); p=0.45, Chi-squared test.

moderate CFU count and 40% (n=8) showed many to massive CFU count. *Candida* spp carriage was reduced in the 17 patients with more than 500 CD4+T/ mm^3, when compared to the patients who presented less than 200 cells/mm^3, 35.3% (n=6) presented none CFU count, 47% (n=8) and 17.7% (n=3) showed isolated to moderate and many to massive numbers of CFU, respectively (p= 0,003), as demonstrated in Figure 2A.

No association between viral load and *Candida* spp carriage was detected. Thirty-three patients with viral load less than 100 copies/mL presented 27% (n=9) no CFU count, 36.5% (n=12) with isolated to moderate and 36.5% with many to massive CFU/ mL commensal *Candida* spp, while 15 patients presented a viral load greater than 100 copies/mL showed 26.7% (n=4) of the participants with none CFU/mL count, 20% (n=3) and 53.3% (n=8) with isolated to moderate and many to massive numbers of CFU/mL, respectively (p=0.45), as demonstrated in Figure 2B.

DISCUSSION

The majority of HIV-infected patients develops *Candida* spp-associated clinical lesions. These lesions can increase in frequency and severity according to the progression of HIV infection[11]. Although the clinical manifestations of candidiasis depend on mucosal fungus adhesion and colonization[28], the asymptomatic *Candida* spp in HIV-infected patients with low CD4+L count is also associated with clinical disease[29]. This is the reason why it is of great importance to know the predisposing factors of increased prevalence of commensal *Candida* spp in the oral cavity, especially HIV-positive patients.

In this study, we found similar prevalences of different commensal *Candida* species in the oral rinse from non-HIV-infected and infected patients, regardless of their periodontal state.

The prevalence found in our study was higher than that in other studies. Kamtane *et al.*[30], in 2003, verified salivary *Candida* spp in 15% of non-HIV-infected patients and 55% in HIV-infected patients. Other studies reported fungus colonization in HIV-positive patients between 44% and 62%[9,31-33]. In the present study, we verified the presence of different *Candida* spp in 72% of non-HIV-infected patients and 73% of HIV-infected patients. The variation among the different studies may be due the collection methodology, such as the use of swabs, total saliva collection and oral rinse. According to Samaranayake *et al.*[22], oral rinse is the most sensitive technique for quantifying and detecting *Candida* spp, as used in the present study. No differences in *Candida* spp prevalences were detected in these study groups.

Candida spp carriage was increased in patients with less than 200 CD4+L cells when compared to patients presenting

more than 500 cells/ mm^3 (p= 0.003). These results agree with previous studies that verified more colonization in inferior CD4+L counting, 200 cells/ mm^3 [33-35]. On the other hand, some studies have not found this association[32-36].

The highlight of our work is the high commensal *Candida* spp count in HIV-infected and periodontally affected patients, when compared to HIV-infected patients not affected by periodontal disease. These results address the importance of oral health in oral candidiasis prevention because *Candida* spp density is associated with a higher risk for candidiasis development[29]. On the other hand, periodontal conditions were not observed as an important factor for *Candida* spp carriage in non-HIV-infected patients. Non-HIV-infected patients presented similar *Candida* spp prevalence and counting, regardless of the periodontal state, and these results agree with those of Darwazeh *et al*.[37], who verified a similar prevalence and counting in 149 orally healthy patients. One limitation of this study was the absence of the sample size calculation, therefore, the study may not have enough power to detect the difference between groups.

Among HIV-infected patients, the periodontal state did not influence *Candida* spp presence or absence, but it was responsible for the 4.8 fold increase in counting. This increase is epidemiologically important as demonstrated by Fong *et al*.[29], in 1997, who showed that candidiasis only developed in patients with persistent asymptomatic carriage of *C. albicans.*

The predisposal of HIV-infected patients to more oral *C. albicans* carriage, associated with the influence of oral hygiene on *Candida* spp carriage, are responsible for the high values of *Candida* spp in HIV-infected patients who have periodontal disease, as shown in this present study. It is important to emphasize that HIV-infected patients who presented good periodontal conditions had *Candida* spp levels similar to those of non-HIV-infected patients, renforcing the affirmation that the periodontal condition may be considered a factor -of increases in the count of *Candida* spp in HIV-infected patients.

Many studies have shown *C. albicans* to be an etiologic and worsening factor of periodontal disease[19,20,38]. This study shows that periodontal disease increases with *Candida* spp carriage, acting as a vicious cycle in HIV-infected patients because in this condition, periodontal disease increases *Candida* spp counts, which worsen periodontal conditions.

In conclusion, periodontal disease may be a factor responsible for the increase in commensal *Candida* spp count in HIV-infected patients, and based on current medical literature, it is reasonable to affirm that this increase may predispose the patient to the development of the clinical manifestations of candidiasis.

CONFLICT OF INTERESTS

The authors confirm that this article content has no conflict of interests.

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