

## OCCURRENCE OF YEASTS, ENTEROCOCCI AND OTHER ENTERIC BACTERIA IN SUBGINGIVAL BIOFILM OF HIV-POSITIVE PATIENTS WITH CHRONIC GINGIVITIS AND NECROTIZING PERIODONTITIS

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### ABSTRACT

The purpose of this study was to determine the prevalence of enteric bacteria and yeasts in biofilm of 80 HIV-positive patients with plaque-associated gingivitis or necrotizing periodontitis. Patients were subjected to extra, intra oral and radiographic examinations. The oral hygiene, bleeding on probing, gingival conditions, and attachment loss were evaluated. Clinical specimens were collected from gingival crevices or periodontal pockets, transferred to VMGA III, diluted and transferred to Sabouraud Dextrose agar with 100 µg/ml of chloramphenicol, peptone water, EVA broth, EMB agar, SS agar, Bile esculin agar and Brilliant green agar. Isolation of yeasts was carried out at room temperature, for 3-7 days; and for the isolation of enteric microorganisms plates were incubated at 37°C, for 24-48 h. The yeasts identification was performed according to the carbon and nitrogen assimilation, fermentation of carbohydrates and germ tube formation. Bacteria were identified according to their colonial and cellular morphologies and biochemical tests. Yeasts were identified as *Candida albicans* and its occurrence was more common in patients with CD<sub>4</sub><sup>+</sup> below 200/mm<sup>3</sup> and was affected by the extension of periodontal involvement ( $P = 0.0345$ ). Enteric bacteria recovered from clinical specimens were identified as *Enterobacter sakazakii*, *Enterobacter cloacae*, *Serratia liquefaciens*, *Klebsiella oxytoca* and *Enterococcus* sp. *Enterobacteriaceae* and enterococci were detected in 32.5% of clinical samples from patients with necrotizing periodontitis. In conclusion, non-oral pathogenic bacteria and *C. albicans* were more prevalent in periodontal sites of HIV-positive patients with necrotizing periodontitis and chronic gingivitis.

**Key-words:** HIV patients, *Candida albicans*, enterococci, enterobacteria.

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### INTRODUCTION

In AIDS patients the firsts clinical opportunistic pathologies are observed in oral cavity, particularly involving periodontal tissues (15). Clinical characteristics of periodontal disease in HIV-patients are closely related to the immunological status. Hence, in symptomatic HIV-patients, whose CD<sub>4</sub><sup>+</sup> cell counts are below 200/mm<sup>3</sup> in the blood, necrotizing and acute involvements of the periodontal tissues are common, frequently

followed by intense malodor, bleeding and pain (15), besides a rapid loss of attachment (3).

Previous studies have shown that microbiology of gingivitis and periodontitis in HIV patients may differ significantly in comparison to these periodontal pathologies in immune-competent individuals (1,16). These peculiarities are not related to the distribution of the strictly anaerobic (14), but probably it can involve superinfecting microorganisms and other

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pathogenic agents normally not involved in periodontal infections, such as fungi and enteric organisms (1,17).

Most of the HIV patients receive medicaments to control the viral infection and opportunistic diseases, this however produces a severe selective process on subgingival biofilm, leading to the elimination of members of the indigenous microbiota and giving conditions to the periodontal colonization by minor components of oral microbiota and superinfecting organisms (16,17).

Bacteria such as *Porphyromonas gingivalis*, *Prevotella intermedia*, *Aggregatibacter (Actinobacillus) actinomycetemcomitans*, *Fusobacterium nucleatum*, *Fusobacterium necrophorum*, *Fusobacterium varium*, spirochetes, *Peptostreptococcus micros*, *Campylobacter rectus*, *Bacteroides fragilis*, *Collinsella (Eubacterium) aerofaciens*, fungi and enterobacteria, may be involved in the pathogenesis of periodontitis in HIV-patients (2,14). Enteric microorganisms frequently act as reservoirs for resistance genes to antimicrobials and may spread them to microbial population in nosocomial infections, since these bacterial groups are commonly involved in systemic infections (5).

In this study, the occurrence of yeasts, enterococci and enterobacteria in clinical samples of subgingival biofilm from chronic gingivitis or necrotizing periodontitis from HIV-positive patients was evaluated.

## MATERIALS AND METHODS

### 1. Patients

Four hundred two HIV-positive patients from the Centro de Atendimento a Pacientes Especiais (CAPE), visiting the clinics of dentistry of the University of São Paulo, SP, Brazil, were initially subjected to intra and extra oral examinations. However, 80 patients, 59 males and 21 females, who presented clinical and radiographic features characteristics of chronic gingivitis (n = 40) or necrotizing periodontitis (n = 40) were selected. HIV-positive patients were confirmed by serologic examination for the HIV specific antibody (ELISA) and Western blot, and all of them were aged between 20 to 43 years old (Mean age 31.5). Medical staff of the CAPE provided data on the systemic conditions and medications used by the patients, as well as the CD<sub>4</sub><sup>+</sup> counts.

### 2. Clinical parameters

The medical and dental histories of the patients were analyzed and patients were submitted to extra and intra oral examinations. The oral hygiene was evaluated by mean of the plaque index of O'Leary. Bleeding on probing and attachment loss were also recorded. Complete radiographic examinations using the parallelism technique with a Kodak film (Ektaspeed plus) were performed. The criteria to determine the presence of periodontal disease were those previously established by

Machtei *et al.* (10). Patients showing more than 5% gingival bleeding, but without clinical attachment loss higher than 6 mm in two or more sites and without one or more sites with probing depth ( $\geq 5$  mm), were diagnosed as having chronic gingivitis. Finally, for the clinical definition of patients who positively and unequivocally exhibited periodontitis, patients showing more than 5% gingival bleeding, with clinical attachment loss higher than 6 mm in two or more sites and with one or more sites with probing depth  $\geq 5$ mm were diagnosed as having periodontitis. Necrotizing periodontitis was identified by the presence necrosis of hard and gingival tissues in the periodontium and rapid tooth loss.

## 3. Microbiological parameters

### 3.1. Clinical Specimens

After probing, clinical specimens were collected from three periodontal sites: from two deepest periodontal sites and from area with the most significant attachment loss among the lower central incisors. Before sample collection, the supragingival biofilm was removed using a scaling (Trinity Ind. Co. Ltd., São Paulo, SP, Brazil). Then, three sterilized paper points were introduced into the apical region of the periodontal pocket or inflamed gingival crevice for 60 seconds. The paper points were then transferred to 2.0 ml of VMGA III (11) and processed within 2 h.

### 3.2. Microorganisms isolation and identification

Specimens were serially diluted and 0.1 ml of each dilution was transferred to Sabouraud dextrose agar with 100 mg/ml of chloramphenicol, peptone water and ethyl violet azide broth (EVA broth). Plates and tubes were incubated at room temperature, for 3 or 7 days. The bacterial growth in peptone water was transferred (0.1 ml) to eosin methylene blue agar (EMB), *Salmonella-Shigella* agar (SS) and brilliant green (BG) agar. Bacterial growth in EVA broth was transferred (0.1 ml) to bile esculin agar. Plates were incubated at 37°C, for 24-48 h.

The yeast identification was performed by the carbon and nitrogen assimilation. Fermentation of dextrose, maltose and sucrose and germ tube formation at 37°C and at 39°C was also performed. Colonial morphology on CHROM agar *Candida* (MastDiagnostica, Paris, France), and growth at 37°C and 42°C were also evaluated. Bacterial species were identified according to their colonial characteristics, Gram-staining, growth at 10% sodium chloride, and biochemical tests using the API-20E commercial kit (bioMérieux).

## 4. Statistical analysis

The design of the study and statistical analysis were carried out using the SPSS-PC/Windows version 12.0 software package (SPSS Inc., Chicago, IL, USA) and the parameters such as sex, periodontal parameters, age and occurrence of the pathogens in the microbiota were evaluated. The relationship between the

occurrence of yeasts and enteric bacteria and periodontal clinical parameters (plaque index, gingival index, bleeding on probing, attachment loss) was determined to all patients. The statistical analyses have included descriptive analysis (median and standard deviation). The Kruskal-Wallis, Mann-Whitney and Chi-square tests were employed to determine the presence of significant differences and correlations between sex, age, periodontal parameters and presence of fungi and enteric bacteria. Statistical significance was defined as  $P < 0.05$

## RESULTS

The results of this study show an evident homogeneity among analyzed groups particularly to the age (Mann-Whitney test,  $P = 0.105$ ). Clinical parameters and the CD<sub>4</sub><sup>+</sup> counts of the patients are showed in Table 1. Gingivitis and periodontitis patients displayed similar scores of CD<sub>4</sub><sup>+</sup>. Patients with necrotizing periodontitis showed statistically significant differences in comparison to patients with gingivitis, particularly with a high deterioration of the periodontal attachment and oral hygiene (Kruskal-Wallis test,  $P = 0.046$ ), probing depth (Chi-square test,  $P = 0.041$ ), gingival index (Kruskal-Wallis test,  $P < 0.001$ ) and bleeding on probing (Mann-Whitney test,  $P = 0.031$ ).

All the yeasts were identified as *Candida albicans* but this yeast was isolated in low number from patients with CD<sub>4</sub><sup>+</sup> > 200/mm<sup>3</sup> when compared with HIV-patients with CD<sub>4</sub><sup>+</sup> < 200/mm<sup>3</sup> (Chi-square test,  $P = 0.0279$ ). The extension of the periodontal damage seems to be statistically related with the occurrence and levels of *C. albicans* in subgingival samples (Chi-square test,  $P = 0.034$ ) (Table 2). This yeast was recovered in higher values from periodontitis (40%) than gingivitis (7.5%) patients. On the other hand, sex ( $P = 0.259$ ) or age ( $P = 0.781$ ) did not influence the occurrence of *C. albicans*. Moreover, the number of *C. albicans* isolated from gingivitis was  $1.3 \times 10 \pm 1.3 \times 10$  CFU, while from necrotizing periodontitis was  $3.8 \times 10 \pm 7.8 \times 10$  CFU.

**Table 1.** Periodontal parameters evaluated in HIV-positive patients with chronic gingivitis or necrotizing periodontitis.

Parameters	Gingivitis	Periodontitis
CD <sub>4</sub> <sup>+</sup>	264.6±243.01	251.7±223.63
Plaque Index	45.0±20.9	88.9±16.68
Bleeding on probing	17.7±12.8	38.7±13.6
Gingival index	1.75±0.36	1.82±0.31
Percentage of sites with:		
Probing depth ≥ 5mm	0.0	4.8±4.7
Attachment loss > 5mm	0.0	2.1±1.7
Dental mobility	0.0	1.4±1.1
Number of teeth	27.0±2.83	24.1±6.18

<sup>1</sup>Standard deviation.

**Table 2.** Occurrence of microorganisms in HIV patients with chronic gingivitis or necrotizing periodontitis.

Microorganism	Chronic gingivitis No. (%)	Necrotizing periodontitis No. (%)
<i>Candida albicans</i>	3 (7.5)	16 (40.0)
<i>Enterobacter sakazakii</i>	0 (0.0)	4 (10.0)
<i>Enterobacter cloacae</i>	0 (0.0)	4 (10.0)
<i>Enterococcus</i> sp.	0 (0.0)	2 (5.0)
<i>Klebsiella oxytoca</i>	0 (0.0)	2 (5.0)
<i>Serratia liquefaciens</i>	0 (0.0)	1 (2.5)
Total enteric bacteria	0 (0.0)	13 (32.5)

*Enterobacteriaceae* and enterococci were isolated only from HIV-patients with necrotizing periodontitis ( $P < 0.001$ ), regardless their immunological status (CD<sub>4</sub><sup>+</sup> counts). Enteric bacteria such as *Enterobacter sakazakii*, *Enterobacter cloacae*, *Serratia liquefaciens*, *Klebsiella oxytoca* and *Enterococcus* sp. (Table 2) were recovered in 32.5% necrotizing periodontitis samples.

## DISCUSSION

Severe periodontal diseases have been associated to alterations in the host' immune system (1,20), which can predispose to gingivitis and periodontitis development. Moreover, the relevance of immune system in the periodontal tissues protection is documented and the impairment of this system could aggravate periodontal status (6). Associations between periodontal status and presence of the opportunistic infections have not been established, and both gingivitis and periodontitis are often found in symptomatic HIV-patients, where may acquire necrotizing or aggressive characteristics. This can be reflects of the role that CD<sub>4</sub><sup>+</sup> cells play in the HIV-infection progression, which are scarce in peripheral-blood of these patients.

The presence of the anaerobic bacteria, enterobacteria and superinfecting microorganisms may be relevant in the gingivitis and periodontitis etiology, especially in immunodeficient patients (1,14,16). However, the role of the enteric bacteria plays in the periodontal diseases etiology remains unclear, and it must be an alert to clinicians who use systemic antibiotics, such as ciprofloxacin as an adjunct in the periodontitis treatment in such patients (18).

In HIV-positive patients, the occurrence of enteric bacteria and other superinfecting microorganisms and the *C. albicans* proliferation may also be related to opportunistic viral infections, such as herpesvirus, which are common in these patients producing immunosuppressive factors and cytokines, leading to overgrowth of pathogenic bacteria and fungi (6).

Certainly, the peculiarities and differences among microbiota associated to gingivitis and necrotizing periodontitis of the HIV-positive patients can be influenced by medications, which are employed in the treatment of several opportunistic infections during the HIV-infection. Since all the patients received daily anti-retroviral or antibiotic therapy, this gives opportunity to colonization and dissemination of superinfecting microorganisms and fungi (1). Moreover, these data can represent an intense selective pressure of drugs on the resident microbiota and host immune response. Antimicrobial drugs and the immune deficiency are a way to the periodontal tissues colonization by non-oral organisms such as enteric rods. Moreover, they also give environmental conditions to the oral opportunistic microbiota, such as *C. albicans* (8).

The occurrence of yeasts and enterobacteria associated to periodontal diseases in HIV-patients (Table 2) is in accordance with Moore *et al.* (12). However, Dios *et al.* (7) isolated yeasts and enterobacteria in 68% of the oral smooth mucosa. A relationship between occurrence of *Candida* spp. and inflammation of the periodontal tissues was observed by Wang *et al.* (21). It is early to associate the presence of *C. albicans* in periodontal HIV-positive patients with gingival or periodontal inflammation, since in these patients, factors such as immune suppression, use of antibiotics and illegal intravenous drugs, may induce the periodontal tissues colonization for this yeast.

In our study, it was verified a close relationship between *C. albicans*, necrotizing periodontitis and CD<sub>4</sub><sup>+</sup> counts. Of the 19 *Candida*-positive patients, 14 were HIV-patients with CD<sub>4</sub><sup>+</sup> < 200 cells/mm<sup>3</sup> and 16 of them displayed necrotizing periodontitis. Of the 16 necrotizing periodontitis patients and harboring *C. albicans*, 11 showed CD<sub>4</sub><sup>+</sup> < 200 cells/mm<sup>3</sup>. The inflammation of the periodontal tissues colonized by *C. albicans* can be associated to the hyphae development invading the periodontal connective tissues and producing several degrees of inflammation (9).

In addition, the presence of the enteric organisms in low numbers, in oral cavity, is relevant since the periodontium may act as a reservoir to opportunistic microorganisms, especially in patients with immunodeficiency. In this study, the occurrence of enterobacteria was high in HIV-patients with necrotizing periodontitis and displaying CD<sub>4</sub><sup>+</sup> < 200/mm<sup>3</sup>. The role of the superinfecting microorganisms in patients with necrotizing periodontitis, remains unclear. Our data showed a correlation between the presence of opportunistic pathogens, attachment loss and presence of necrosis in periodontal tissues ( $P=0.031$ ).

Botero *et al.* (4) detected enteric microorganisms in higher numbers in HIV-positive patients with periodontitis than in HIV-negative patients. However, no ecological relationship between these microbial groups has been established. Since *Enterobacteriaceae* are not considered as important members of the oral microbiota, it is possible that disequilibrium of the oral microbiota collaborate to an establishment in oral biofilm (17).

In HIV patients with periodontitis is often observed an aggressive and necrotizing character, producing bad breath, pain and rapid conjunctive attachment loss (13). Data in literature show that enteric rods and cocci may play a role in the development of these pathologies (4,19), due to their wide scope of virulence factors that confer the ability to adhere and invade the host's tissues, besides the induction of local inflammatory processes in periodontal tissue (13).

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## RESUMO

### **Ocorrência de leveduras, enterococos e outras bactérias entéricas no biofilme subgingival de pacientes HIV-positivos com gengivite crônica e periodontite necrosante**

O objetivo desse estudo foi avaliar a ocorrência de bactérias entéricas e leveduras no biofilme subgingival de pacientes HIV-positivos com gengivite crônica ou periodontite necrosante. Os pacientes foram submetidos a exame clínico e radiográfico e de higiene bucal, sangramento à sondagem, condições gengivais e a perda de inserção. Os espécimes clínicos de sulcos gengivais ou bolsas periodontais foram inoculados em ágar Sabouraud dextrose com 100 mg/ml de cloranfenicol, água peptonada, caldo EVA, ágar EMB, ágar SS, ágar Bile esculina e ágar verde brilhante. O cultivo de leveduras foi realizado à temperatura ambiente, de 3-7 dias; das enterobactérias a 37°C de 24-48 h. A identificação das leveduras foi realizada pela assimilação de carbono e nitrogênio, fermentação de açúcares e formação de tubo germinativo. As bactérias de acordo com a morfologia celular e colonial e testes bioquímicos. Foram identificadas *Candida albicans* e sua prevalência foi maior em pacientes com contagens de CD<sub>4</sub><sup>+</sup> < 200/mm<sup>3</sup>, e sua ocorrência foi afetada pela extensão da destruição periodontal ( $P = 0,0345$ ). *Enterobacteriaceae* e enterococos foram detectados em 32,5% dos pacientes com periodontite necrosante. As enterobactérias foram *Enterobacter sakazakii*, *E. cloacae*, *Serratia liquefaciens*, *Klebsiella oxytoca* e *Enterococcus* sp. Concluiu-se que bactérias patogênicas exógenas à cavidade bucal e *C. albicans* podem ser detectadas no biofilme subgingival de pacientes HIV-positivos com periodontite necrosante e gengivite.

**Palavras-chave:** HIV, gengivite, periodontite necrosante, *Candida albicans*, enterococos, enterobactérias.

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