Biotypes of Candida albicans isolates from the oral mucosa of HIV seropositive and control subjects

Biótipos de amostras de *Candida albicans* isoladas da mucosa oral de sujeitos HIV soropositivos e controles

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Abstract

Purpose: This study evaluated the *Candida albicans* biotypes from oral mucosa according to some host variables, such as HIV infection; medication use – protease inhibitors (PI), non protease inhibitors (NPI) or no medication (NM); dental prosthesis wearing (PW) or not (NPW); and yeast variables (activity levels of protease and phospholipase).

Methods: Samples from the oral mucosa of 193 HIV$^+$ subjects and 205 HIV$^-$ subjects were collected by means of sterile swabs and seeded onto Sabouraud dextrose agar. The isolates were identified by microculture on slide, germ tube formation, auxanogram, and zimogram. Ninety-two isolates were obtained from HIV$^+$ individuals: 49 from patients under PI, 31 from patients under NPI and 12 from patients with no medication. The control group comprised 63 isolates from HIV$^-$ patients.

Results: From the 95 possible *C. albicans* biotypes, 46 were identified in the sample, and the most prevalent were: 10122 (46.4%); 11122 (38.01%); 01031 (42.4%); 00022 (40%); ($P<0.01$ – Spearman correlation test). No difference was detected among PI, NPI, and NM groups. The control group exhibited intermediate enzymatic activity level from dentate isolates, and high protease activity level amongst isolates from prosthesis wearers.

Conclusion: It was not possible to detect any inhibitory action of PI drugs on the enzymatic activity of *C. albicans*.

Key words: *Candida albicans*; AIDS; protease inhibitors; virulence factors

Resumo

Objetivo: Este estudo avaliou biótipos de isolados de *Candida albicans* da mucosa oral utilizando-se variáveis do hospedeiro: infecção pelo HIV; medicação – uso de inibidores de protease (IP), não inibidores (NIP), ausência de medicação (SM); e uso ou não de prótese. Da levedura foram utilizadas as variáveis relativas à produção de protease e fosfolipase (nível de atividade).

Metodologia: O material da mucosa bucal de 193 sujeitos HIV$^+$ e 205 sujeitos HIV$^-$ foi coletado com *swab* estéril e semeado em Agar Sabouraud dextrose. A identificação dos isolados foi obtida pelos testes de micro cultivo em lâmina, tubos germinativos, auxanograma e zimograma. Utilizaram-se 92 amostras isoladas de indivíduos HIV$^+$, sendo 49 de pacientes IP, 31 de pacientes NIP e 12 de pacientes sem uso de medicação (SM). O controle constou de 63 amostras isoladas de indivíduos HIV$^-$.

Resultados: Dos 95 diferentes biótipos possíveis, obteve-se 46, sendo os mais prevalentes: 10122 (46,4%); 11122 (38,01%); 01031 (42,4%); 00022 (40%); ($P<0,01$ – teste de correlação de Spearman). Não houve nenhuma diferença entre os subgrupos IP, NIP e SM. As amostras isoladas dos controles exibiram atividade intermediária de protease (dentados) e alta atividade de protease (portadores de próteses).

Conclusão: Não foi possível constatar interferência dos IP na atividade enzimática de *C. albicans*.

Palavras-chave: *Candida albicans*; AIDS; inibidores de protease; fatores de virulência

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Introduction

Oropharyngeal candidiasis is a common opportunistic infection in subjects with the Human Acquired Immunodeficiency Syndrome (AIDS), and *Candida albicans* is recognized as an important biological marker to evaluate the disease evolution (1-3). The first case of AIDS in the medical literature reported that the patient had oral candidiasis (4). Among the species of *Candida* genus able to colonize the oral mucosa, *C. albicans* is the major etiologic agent of oral candidiasis and a potential source for systemic candidiasis (5,6).

Recent studies have shown that the opportunistic infections still are the main cause of morbidity and mortality in HIV patients despite the remarkable improvement of the therapeutic results with the introduction of the Highly Active Antiretroviral Therapy (HAART), particularly the protease inhibitors (PI) (3,7,8-11). The PI acts directly on the HIV protease to convert it into non-infecting viral particles. This fact may occur due to an increase of the quantity of cd4+ lymphocytes and reduction of the viral load, which leads to an improved specific immunological status and promotes higher resistance against opportunistic infections (6,12,13). Notwithstanding, AIDS patients submitted to HAART therapy demonstrated remission of oral candidiasis signs and symptoms even before the increase of the number of cd4+ cells (12). Various virulence factors may contribute for the pathogenicity of *C. albicans*, including extracellular secreted proteinase and phospholipase. The secreted aspartic protease produced by this yeast belongs to the same class as the protease produced by HIV (5). Some studies have reported an inhibitory action of PI on the protease secretory mechanisms of *C. albicans* and its activity levels (14-18), and the antifungal effect of saquinavir and indinavir also was demonstrated *in vitro* and *in vivo* (11,12,18). On the other hand, species from *Candida* genus can adhere to dental prosthesis and colonize oral mucosa independently from the immunity status of their carriers. Denture stomatitis is frequent among prosthesis wearers and is considered the most common type of oral candidiasis caused by the *albicans* species, although it may be associated with other species from *Candida* genus and other microorganisms, including bacteria (14,15,19).

The aim of the present study was to identify the biotypes of *C. albicans* isolated from the oral mucosa of HIV^+ and HIV^- patients, and to assess any differences in relation to some characteristics of the carriers (HIV infection, dental prosthesis wearing, and treatment with PI, non PI, and no medication) and characteristics of the yeast (production and activity levels of protease and phospholipase enzymes).

Casuistic and Methods

The research protocol was approved by the Institutional Review Board and Ethics Committee of the *Casa da AIDS*, University of São Paulo School of Medicine and School of Dentistry. Males and female seropositive patients under treatment at *Casa da AIDS – Fundação Zerbini*, São Paulo, Brazil, and seronegative patients (with good health status, no use of antibiotics or corticosteroids during the last 30 days) under dental care at the School of Dentistry – University of São Paulo were invited to participate in the present study. After signing an informed consent form, 193 HIV^+ patients and 205 HIV^- patients were recruited.

From the patients' medical charts, data were collected on the prescribed anti-retroviral medication (ARM) and laboratory tests. As the medication administered to seropositive patients is diversified and usually consists of an association of drugs ("cocktail of drugs"), PI group included subjects under PI medication also associated with other non PI drugs.

Clinical examination and material collection

A single examiner with specialty training in Oral Medicine performed the clinical examination of all patients. After intraoral exam, the material was sampled from the oral mucosa with a sterile swab and plated onto Petri dishes containing Sabouraud dextrose Agar (ASD, Difco®, Franklin Lakes, NJ, USA) plus chloramphenicol (Chloromycetin Parke-Davis® Pfizer®, New York, NY, USA – 100 µg/mL). The Petri dishes were incubated at 37 °C to isolate the yeasts. The negative cultures were maintained for 30 days.

Isolation of the cultures and yeast identification

The samples were incubated for 48-96 hours at 25 °C. The positive isolates were identified according to the method by Kreger-Van Rij (20), including macro and micromorphology tests, germ tube and chlamidoconidia production, carbon and nitrogen source assimilation, and sugar fermentation. The different positive isolates were subcultured in ASD and kept at room temperature; if necessary, a new platting was performed every 14 days.

Assessment of protease and phospholipase activity

Protease activity was recorded by calculating the ratio between the colony diameter (CD) and the CD + proteolytic halo (CDH). The activity levels were classified into (21,22): low activity level (0.99-0.70), intermediate activity level (0.69-0.30), high activity level (between 0.29 and >0.0), and no activity (1.00). For incubation of the isolates a protease agar medium was employed (21); an albumin medium was prepared with yeast base medium (Difco, Livonia, MI, USA – 11.7 g); bovine albumin – fraction V (Sigma, St. Louis, Mo, USA – 2.0 g); protovit – 2.5 mL; and distilled water – 100.0 mL. All components were mixed, homogenized, and sterilized by filtration (Millipore filter – 0.22 mm).

Phospholipase activity (23) was measured with the incubation of the isolates in phospholipase agar medium (peptone – 10.0 g; glucose – 20.0 g; sodium chloride – 57.30 g; calcium chloride – 0.55 g; distilled water – 1000 mL) added by egg emulsion (egg yolk – 80.0 g; saline solution – 80.0 mL). The eggs were kept in an alcohol solution (70.0%) during one hour for disinfection. The yolks were separated and placed into a sterile recipient containing glass pearls, loaded, and saline solution was added, under vigorous shaking. The

medium was autoclaved at 120 °C for 15 min. The egg yolk emulsion was added to the agar medium chilled at 50 °C. Aliquots of 20 mL were distributed in Petri dishes, and four *C. albicans* innocula were seeded on each Petri dish. The plates were incubated at 37 °C, and the readings were done after 96 hours.

To validate the results a standard strain (*C. albicans* ICB-2730) was used as a positive control. This isolate produces highly positive halos. The protease and phospholipase activity was confirmed by the presence of an opaque halo around the yeast colony. The obtained indexes were then classified to determine protease and phospholipase production of the isolates (21,23-25). The tests were duplicated and repeated twice in different days. Only the results which were reproduced at least three times were considered.

Biotypes coding

Values were attributed to the host and yeast variables in order to obtain a five-digit code (host: first three digits; yeast: last two digits):

(1) HIV infection: 0 = non infected (HIV^-); 1 = infected (HIV^+).
(2) anti-HIV medication: 0 = No medication (NM); 1 = under protease inhibitors (PI); 2 = under no protease inhibitors (NPI).
(3) Dental prosthesis wearing: 0 = not wearers (NPW); 1 = wearers (PW).
(4) protease activity: 0 = no activity; 1 = low activity; 2 = intermediate activity; 3 = high activity.
(5) phospholipase activity: 0 = no activity; 1 = low activity; 2 = intermediate activity; 3 = high activity.

Data analysis

The homogeneity variance of the quantitative variables was analyzed by the Lèvene test, and the normality of quantitative data was assessed by the Kolmogorov-Smirnov test. Qualitative data were analyzed by Chi-square tests (isolates × medication groups) and Spearman correlation tests (enzymes activity x prosthesis wearing). To verify the differences between isolates from prosthesis wearers and non wearers, data were analyzed by ANOVA and Tukey's test. A significance level of 0.05 was set for all tests.

Results

Tables 1 and 2 show the distribution of *C. albicans* strains isolated from the HIV seropositive and seronegative subjects in relation to medication and prosthesis use. From the 193 HIV^+ subjects, 92 *C. albicans* strains were isolated: 49 from patients under PI, 31 from patients under NPI, and 12 from patients with no medication; 45 in prosthesis wearers (PW) and 23 from non prosthesis wearers (NPW). From the 205 seronegative subjects, 63 *C. albicans* strains were isolated: 30 from NPW and 33 from PW.

Considering the possible 32 biotypes for the isolates from HIV^+ under treatment with PI (Table 3), 14 biotypes were identified. Within the 6 biotypes from NPW, the most prevalent was 10122 (13 isolates), and the 11122 biotype was the most frequent in PW, exhibiting intermediate activity for protease and phospholipase.

For the isolates from HIV^+ under treatment with NPI (Table 4), 10 biotypes were identified, and the 10222 biotype was the most frequent in NPW (7 isolates), showing intermediate activity for protease and phospholipase. In PW, the most prevalent strains were 11222 (6 isolates) and 11230 (5 isolates).

Amongst the isolates from patients under no medication (Table 5), 7 biotypes were identified. The most frequent strains were the 10022 strain in NPW and the 11022 in PW; intermediate enzymatic activity was predominant.

The isolates from the seronegative subjects yielded 15 biotypes, and the 00022 strain was the most prevalent (12 isolates) in NPW, with intermediate activity for both enzymes. Within the strains isolated from PW, biotype 01031 (14 isolates) showed high protease activity and low phospholipase activity (Table 6).

Table 1. Distribution of HIV^+ *C. albicans* isolates by medication group.

C. albicans	PI Group (n=111)	NPI Group (n=56)	NM Group (n=26)	Chi-square Test
Negative	62 (55.8%)	25 (44.6%)	14 (53.8%)	$\chi^2=4.034$ $P=0.133$
Positive	49 (44.2%)	31 (55.4%)	12 (46.2%)	

PI - protease inhibitors; NPI - non protease inhibitors; NM - no medication.

Table 2. Distribution of *C. albicans* isolates by prosthesis wearing* among HIV^+ and HIV^- subjects.

C. albicans	HIV^-		HIV^+		ANOVA
	NPW (n=150)	PW (n=55)	NPW (n=125)	PW (n=68)	
Negative	120 (80.0%)	22 (40.0%)	77 (61.6%)	23(33.8%)	$P< 0.01$
Positive	30 (20.0%)	33 (60.0%)	48 (38.4%)	45(66.2%)	

* NPW - non prosthesis wearers; PW - prosthesis wearers.

Table 3. Biotypes of isolates from HIV+ patients under PI therapy.

No prosthesis			With prosthesis		
Biotype	n	%	Biotype	n	%
10100	0	0	11100	0	0
10101	0	0	11101	1	4.76
10102	4	14.29	11102	3	14.29
10103	0	0	11103	0	0
10110	0	0	11110	0	0
10111	0	0	11111	0	0
10112	0	0	11112	1	4.76
10113	0	0	11113	0	0
10120	3	10.71	11120	3	14.29
10121	2	7.14	11121	0	0
10122	**13**	46.43	**11122**	8	**38.09**
10123	0	0	11123	1	4.76
10130	2	7.14	11130	3	14.29
10131	0	0	11131	1	4.76
10132	4	14.29	11132	0	0
10133	0	0	11133	0	0
Total	28	100.00	Total	21	100.00

Table 4. Biotypes of isolates from HIV+ patients under NPI therapy.

No prosthesis			With prosthesis		
Biotype	n	%	Biotype	n	%
10200	0	0	11200	0	0
10201	0	0	10201	0	0
10202	4	28.58	11202	0	0
10203	0	0	11203	0	0
10210	0	0	11210	0	0
10211	0	0	11211	0	0
10212	0	0	11212	0	0
10213	0	0	11213	0	0
10220	1	7.14	11220	1	5.88
10221	1	7.14	11221	1	5.88
10222	**6**	**42.86**	11222	**7**	**41.18**
10223	0	0	11223	0	0
10230	0	0	11230	5	29.41
10231	0	0	11231	0	0
10232	2	14.28	11232	3	17.65
10233	0	0	11233	0	0
Total	14	100.00	Total	17	100.00

Table 5. Biotypes related to isolates from HIV+ patients with no medication.

No prosthesis			With prosthesis		
Biotype	n	%	Biotype	n	%
10000	0	0	11000	0	
10001	1	16.60	11001	0	
10002	1	16.60	11002	1	16.60
10003	0	0	11003	0	
10010	0	0	11010	0	
10011	0	0	11011	0	
10012	0	0	10012	0	
10013	0	0	11013	0	
10020	0	0	11020	0	
10021	0	0	11021	0	
10022	**3**	**50.00**	**11022**	**4**	**66.60**
10023	0	0	11023	0	
10030	0	0	11030	1	16.00
10031	0	0	11031	0	
10032	1	16.60	11032	0	
10033	0		11033	0	
Total	6	100.00	Total	6	100.00

Table 6. Biotypes of isolates from HIV− patients (control group).

No prosthesis			With prosthesis			*P*
Biotype	n	%	Biotype	n	%	
00000	1	3.4	01000	0	0	
00001	2	6.6	01001	0	0	
00002	0	0	01002	0	0	
00003	0	0	01003	0	0	
00010	0	0	01010	0	0	
00011	3	10.0	01011	0	0	
00012	1	3.4	01012	0	0	
00013	0	0	01013	0	0	
00020	6	20.0	01020	2	6.06	
00021	2	6.6	01021	6	18.19	
00022*	**1222**	**40.0**	01022	2	6.06	$P<0.01$*
00023	0	0	01023	0	0	
00030	0	0	01030	3	9.10	
00031	1	3.4	**01031†**	**14**	**42.40**	$P<0.01$†
00032	2	6.6	01032	6	18.19	
00033	0	0	01033	0	0	
Total	30	100.0	Total	33	100.0	

* Spearman (r=1; df=8); †Spearman (r=1; df=10).

Figure 1 displays the distribution of all biotypes as a function of the study variables.

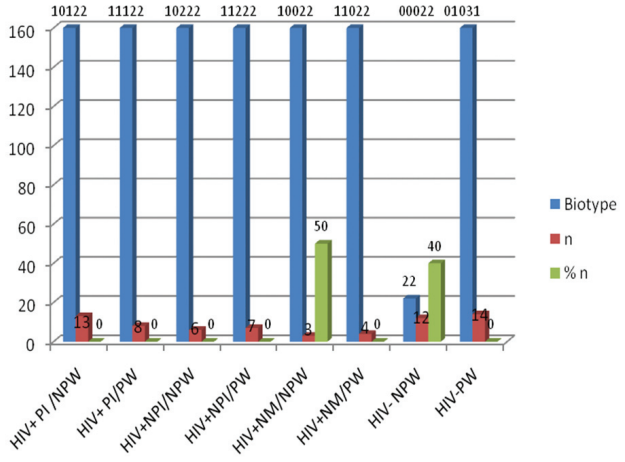

Fig. 1. Most frequent biotypes* related to HIV infection, anti-HIV therapy, and prosthesis wearing. *The last two biotype digits represent, respectively, protease and phospholipase activity levels. Medication: PI - Protease inhibitors; NPI - Non inhibitors; NM - No medication. Prosthesis wearing: NPW - Non prosthesis wearers; PW - Prosthesis wearers.

Discussion

Despite the efficacy of the new anti-AIDS drugs and the last generation of azolic antifungal medication, oral and oropharyngeal candidiasis remain a challenge for clinicians and researchers to better understand the behavioral interaction between AIDS patients and *Candida* yeasts, particularly *albicans* species (3,24). Biotyping methods have been developed to characterize microorganisms, especially in relation to virulence factors. Our research group published an innovative prospective study aggregating host characteristics to biotype assembling, which aimed to provide to practitioners an easy way to understand some aspects of the host/yeast interaction and observe the behavioral changes during the clinical evolution of the disease (25).

The present study aimed to evaluate a possible action of protease inhibitors on *C. albicans* virulence factors, specifically secreted enzymes involved in the pathogenesis mechanism of oral candidiasis. Also, the influence of prosthesis wearing would be important as the yeast adheres to the surface of dental prosthesis, especially in porosities of the resin baseplate. Adhesion is the first step for yeast colonization on the oral epithelium, and dentures may become an important source for reinfection (15,17,18,22).

Previous studies have shown a possible *in vitro* and *in vivo* action of protease inhibitors on the protease secreted by *C. albicans* (18), which belongs to the same class of enzymes as the HIV protease and have similar properties (5). The present investigation assessed some *in vitro* inhibitory effects of the most frequent biotypes identified in the sample, but in HIV^+ patients no significant difference was detected as a function of medication groups as expected a priori (10,12,17,18). However, there were significant differences in relation to dental prosthesis use, which suggest that dental prosthesis hygiene may be inadequate. Moreover, the Oral Medicine clinics usually follow a standard empirical protocol to prescribe antifungal medication without previous antifungal sensitivity testing.

The biotypes from the HIV seronegative subjects with no use of dental prosthesis also showed the same behavior pattern of enzymes with intermediate activity. The isolates from subjects wearing dentures exhibited a high enzyme activity, which confirms the correlation between higher enzymatic activity and dental prosthesis wearing (12,16-18).

One limitation of this study was that the most prevalent biotypes from the isolates of HIV seropositive subjects exhibited intermediate activity for both enzymes. From the clinical point of view, this would lead to absence or reduction of clinical signs of oral candidiasis. Therefore, it is not possible to affirm that there was an inhibitory action of PI on the yeast protease and phospholipase activity based upon the present observations. Furthermore, the more frequent biotypes from the NIP medication group also showed intermediate activity for both enzymes, similarly to the pattern of the biotypes from the group under no medication.

Conclusions

Based upon the methods and results of this study, it may be concluded that:

- No action of PI drugs on *in vitro* activity of protease and phospholipase from the *C. albicans* isolates was found.
- Dental prosthesis wearing was an important factor for the isolation of *C. albicans* from the oral mucosa. Therefore, HIV^+ patients should receive special clinical care in relation to the installation, management, and instructions on adequate hygiene techniques.

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