

HAART therapy does not reduce the proteinase and phospholipase secretion by oral *Candida albicans* isolated from HIV-positive patients

A terapia HAART não reduz a secreção de proteinase e fosfolipase por isolados bucais de *Candida albicans* de pacientes HIV positivos

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ABSTRACT

Production of exoenzymes, specifically the proteinase and phospholipase, is considered one of the most important of pathogenicity mechanisms of *C. albicans*, which is crucial for tissue invasion. This study aimed at evaluating the production of these exoenzymes in 50 oral *C. albicans* isolates from HIV-positive (HIV+) patients treated with highly active anti-retroviral therapy (HAART), and from 50 control individuals. For testing the production of phospholipase and proteinase, the culture media containing egg yolk and bovine albumin were used, respectively. The results were obtained by measuring the diameter of the colony and divided by the diameter of colony plus the precipitation zone, defined as Pz. Data were statistically analyzed by Student's t test (5%). Statistically significant difference ($p = 0.001$) was observed between the mean values of Pz for proteinase in isolates from HIV+ patients ($Pz = 0.358 \pm 0.295$) and from control group ($Pz = 0.660 \pm 0.370$). The same results were observed for phospholipase production ($Pz = 0.399 \pm 0.227$ for HIV+ group; $Pz = 0.635 \pm 0.292$ control group). Both enzymes were highly produced by *C. albicans* isolated from HIV+ patients when compared with those secreted by *C. albicans* obtained from control group, suggesting that HAART did not reduce the secretion of these enzymes by this pathogenic fungus infecting HIV+ patients.

Keywords. *Candida albicans*, HIV, proteinase, phospholipase, oral cavity.

RESUMO

A produção de proteinase e fosfolipase é considerada como um dos principais mecanismos de patogenicidade de *C. albicans*, pois essas enzimas são importantes na invasão tecidual. Este estudo avaliou a produção dessas exoenzimas de 50 isolados bucais de *C. albicans* de pacientes HIV positivos (HIV+), sob tratamento com terapia antirretroviral altamente ativo (HAART), e dos isolados de 50 indivíduos controle. Para os testes de fosfolipase e proteinase, foram empregados meios de cultura contendo, respectivamente, gema de ovo e albumina bovina. Os resultados foram obtidos pela medida do diâmetro da colônia dividida pela somatória do diâmetro da colônia e do halo de precipitação, definido como Pz. Os dados foram analisados pelo teste t de Student. Houve diferença estatisticamente significativa ($p = 0.001$) entre os valores médios de Pz para a proteinase obtida do grupo HIV+ ($Pz = 0,358 \pm 0,295$) e controle ($Pz = 0,660 \pm 0,370$). O mesmo foi observado para a produção de fosfolipase ($Pz = 0,399 \pm 0,227$ grupo HIV+; $Pz = 0,635 \pm 0,292$ controle). Os isolados de *C. albicans* provenientes dos pacientes HIV+ apresentaram maior produção de fosfolipase e proteinase em relação ao controle, o que indicou que a terapia HAART não reduziu a secreção dessas enzimas pelos isolados dos pacientes HIV+.

Palavras-chave. *Candida albicans*, HIV, fosfolipase, proteinase, cavidade bucal.

INTRODUCTION

Candida spp. are commensal members of the normal oral microbiota, and can be isolated from around 40% of human healthy population¹⁻³. Hence, isolation of *Candida* in the oral cavity is no confirming evidence of infection and must be taken along with clinical indicators¹.

The transformation of *Candida* from its innocuous into the parasitary form depends on virulence factors of the microorganism, predisposing factors of the host, and of the environment, i.e. the presence of prostheses⁴.

Candida is the main group related to superficial and systemic fungi infections⁵. Oral candidosis occurs among over 95% of patients with AIDS, being recognized as an important marker of that disease and its progression, being a frequent cause of morbidity and mortality in those patients⁶⁻⁸.

Enzymatic activity is considered to be a pathogenicity mechanism of *Candida*, as they are important in tissue invasion. *C. albicans* secretes several enzymes such as phospholipase, lipase, phosphomonoesterases and proteinases^{4,5}.

Proteinase is mainly produced by the most pathogenic species, as *C. albicans*, *C. dubliniensis*, *C. tropicalis* and *C. parapsilosis*, that supports the idea that the secretion of proteinases is a virulence-related factor⁹⁻¹². The secreted aspartyl proteinase (SAPs) degrades many human proteins on the lesion site, as albumin, hemoglobin and secretory immunoglobulin A (IgA-s). The proteolytic activity has been associated with tissue invasion¹³. Naglik et al.¹¹ reported that the expression of Sap-family genes is related to the co-regulation of other virulence factors, such as the biofilm formation, adherence, filamentation, in addition to pervasion, tissue invasion, nutrition and interaction with the host's immune system functions. Samaranyake et al.⁴ reported that *C. albicans* isolates from HIV-positive patients showed greater proteinase activity in rats than isolates from HIV-negative individuals.

Phospholipases act by cleaving the phospholipids, affecting the stability of the membrane and causing cell lysis. They seem to be connected to production of germinative tubes, transition into hyphal forms, and tissue injury. Phospholipase production is concentrated on the tips of the hyphae, and the productive activity is greater when the hypha is in direct contact with the membrane, what shows that the extra-cell phospholipases are relevant in tissue invasion from *C. albicans*¹³⁻¹⁵.

The term anti-retroviral highly active anti-retroviral therapy (HAART) is used when there is an association of three or more anti-retroviral drugs with different mechanisms of action. In the literature, it is reported that with this form of therapy, the oral manifestations have decreased, because this practice promotes inhibition of viral replication, redistribution and restoration of immunity, resulting in an increase in CD4 cells counts^{4,16}.

Considering that *C. albicans* is associated to several facts that demand attention, especially whenever related to AIDS¹⁷, the evaluation of proteinase and phospholipase production is relevant to express pathogenicity. This way, the aim of the present study was two-fold. First, to verify the effect of HAART therapy on exoenzymes production by *Candida albicans* oral isolates. Also, to compare the phospholipase and proteinase production by *C. albicans* oral isolates from HIV-positive patients and control individuals.

MATERIAL AND METHODS

Fifty (n = 50) isolates of *C. albicans* from HIV-positive patients and fifty (n = 50) from control individuals, previously isolated from oral rinses. Forty-five individuals (23 female and 22 male), aged from 22 to 66 years, HIV-positive diagnosed by ELISA and confirmed by Western-blot, in treatment at the Day Hospital of Taubaté Medical School and using a highly active anti-retroviral therapy for at least one year were included in the study. Regarding the treatment, it was not possible to evaluate a fixed therapy protocol for all patients because the treatment strategy changes according to the CD4 lymphocyte level and viral load as well as other relevant clinical variables, such as the occurrence of opportunistic diseases and adverse reactions to medication.

For the control group, 45 healthy individuals aged from 23 to 66 years, and with similar conditions in relation to age, gender, prosthesis use and oral conditions to the HIV-positive individuals were selected among the patients under treatment at São José dos Campos Dental School, São Paulo State University. Patients with diabetes *mellitus* or other systemic diseases, pregnant women, smokers, denture or orthodontic devices users and individuals under treatment with antimicrobials/antifungals during the last 60 days that preceded the sampling or with lesions of oral candidosis were excluded.

Oral rinses samples were collected from HIV-positive patients and control individuals in a previous study¹⁸ approved by the Local Ethics Committee (protocol number 012-PH/CEP) (Local Ethics Committee 012-PH/CEP). Phenotypical identification included germ tube formation in bovine serum, growth in corn meal-Tween 80 agar, fermentation and assimilation of carbohydrates^{19,20}. Isolates identified phenotypically as *C. albicans*/*C. dubliniensis* were submitted to molecular detection of *C. dubliniensis*. These isolates were analyzed by polymerase chain reaction (PCR), according to the methodology proposed by Donnelly et al.²¹ and Mähns et al.²², with modifications. For the present study, only *C. albicans* isolates were included.

For the phospholipase and proteinase testings, culture media containing egg yolk and bovine albumin, respectively, according to Price et al.²³ and Rüchel et al.²⁴ were used. First, the samples were plated onto Sabouraud agar (Difco, Detroit, USA), 24 hours before the experiment and incubated at 37 °C. After growth, the samples were inoculated in the culture media for the testing, by inoculation in five equidistant points. The plates were incubated at 37 °C for 5 days.

Presence of phospholipase production was observed by the formation of an opaque area around the colony (indicating calcium precipitation, so called precipitation zone), and enzymatic activity was measured according to Price et al.²³. These authors define Pz as the colony diameter (dc) divided by the colony diameter plus the precipitation zone (dp) ($Pz = dc/dc+dp$). Presence of proteinase was verified as the formation of a halo around the colony, and enzymatic activity was measured as reported also by this author, through the Pz value. Pz values were classified considering no activity when $Pz = 1.0$; positive activity, when $1.0 < Pz < 0.64$ and strongly positive, when $Pz < 0.64$. Data were statistically analyzed by Student's t test (5%).

RESULTS

There was statistically significant difference ($p = 0.001$) between mean Pz values for proteinase obtained for the HIV group ($Pz = 0.358 \pm 0.295$) and control group ($Pz = 0.660 \pm 0.370$). The same was verified about production of phospholipase (HIV group $Pz = 0.399 \pm 0.227$; control group $Pz = 0.635 \pm 0.292$) ($p = 0.001$) (Table 1).

Comparing the mean Pz values for phospholipase and proteinase enzymes for the control group, the values are

Table 1. Mean values according to the enzymes and studied groups (HIV and control groups)

Enzyme	HIV	Control	p-value
	Mean ± SD	Mean ± SD	
Phospholipase	0.399 ± 0.227	0.635 ± 0.292	$p = 0.001^*$
Proteinase	0.358 ± 0.295	0.660 ± 0.370	$p = 0.001^*$

SD = standard deviation

*The table shows the statistically significant difference according to the enzymes and studied groups (HIV and control groups) by Pz values mean

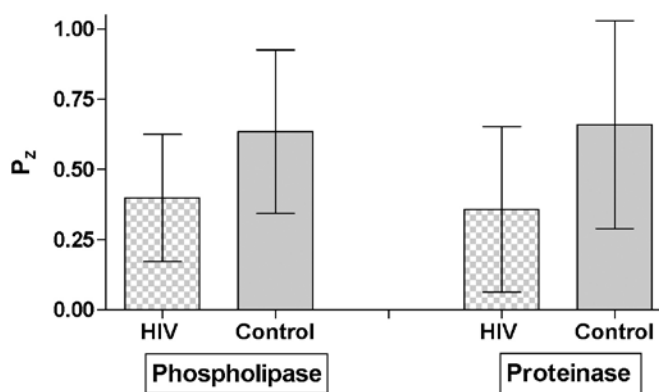


Figure 1. Comparing the mean Pz values for phospholipase and proteinase enzymes for the HIV and control groups

closer to the $\geq 0.64 < 1.00$ interval, that indicates positive enzymatic activity. On the other hand, for the HIV group, the Pz mean figures are lower than 0,64, indicating strongly positive enzymatic activity for both enzymes (Figure 1).

In the HIV group, for proteinase, 82% of the isolates were classified as strongly positive enzymatic activity, and 18% negative; in the control group, 48% were strongly positive, and 52% negative. For phospholipase, in the HIV group, 70% were strongly positive, 16% positive and 14% negative. In the control group, 62% were strongly positive and 38% negative. The Figure 1 shows the mean values of Pz and standard deviation for each studied group.

DISCUSSION

The development process of an infection depends on the relationship between microorganism virulence and the host's ability in preventing or resisting to the microbial invasion/colonization^{9,13}. Virulence factors expressed by *C. albicans* are quite variable and depend

on the infection type, stage and location, as well as on the host's response. *C. albicans* presents high adaptation ability in the host organism and this suggests the presence of virulence factors different from other non-pathogenic fungi⁹. A number of potential virulence factors have been reported, as adherence factors, formation of germ tubes, hyphae/pseudohyphae production and extracellular proteolytic activity^{13-15,25-27}. Phospholipase can play an important role in host's tissue invasion in candidosis lesions, breaking the lipid membrane of epithelial cells and facilitating the hyphae penetration into cytoplasm. Proteinase production increases the ability of the fungus in colonizing and penetrating the host tissue, destroying a number of proteins that are relevant for the host's defence, such as immunoglobulins, complement and cytokines²⁷.

The enzymatic production of *Candida* spp. isolated from several anatomical locations was evaluated by Kantarcioglu e Yücel²⁷, who reported 78.9% enzymatic activity for proteinase and 62.1% for phospholipase in the studied isolates. A total of 93.3% of *C. albicans* isolates produced phospholipase and 95% produced proteinase. In the present study, 82% of the isolates from HIV-positive patients and 48% from control individuals produced proteinase. Phospholipase activity was observed in 86% of the isolates from HIV group and 62% from the control group. In a study including children with AIDS¹⁷, the authors reported that *C. albicans* oral isolates showed higher proteinase and phospholipase production when compared to control group.

Ribeiro et al.²⁸ analyzed *C. albicans* isolates from Down's syndrome children and verified that 96% of the samples produced phospholipase, while 80% of the samples from the control group were positive to the same enzyme. Candido et al.²⁹ analyzed 79 clinical *Candida* oral isolates from patients with clinical evidence of oral candidosis and verified that 83.3% and 66.7%, respectively, produced phospholipase and proteinase enzymes. The same authors analyzed patients without clinical indicators of oral candidosis and verified that 71.9% of isolates were positive for phospholipase and 68.7% for proteinase. Koga-Ito et al.²⁶ reported higher production of proteinase by *C. albicans* isolated from patients with oral candidosis when compared to isolates from control individuals. For phospholipase enzyme production, no statistically significant between the studied groups were detected.

Upon introduction of highly active anti-retroviral therapy (HAART) including protease HIV inhibitor, a

significant reduction of fungal infections incidence in HIV-positive patients has been reported^{16,30}. Previous studies reported that protease-inhibitors drugs showed anti-*Candida* activity as they are able to inhibit proteinases of the fungi by cross-reaction^{4,16,30-34}. Ribeiro et al.³² observed higher production of exoenzymes in oral and vaginal samples of HIV-positive women. However, when isolates from HAART-using patients with protease inhibitors were analyzed, lower levels of proteinase were produced. The levels reported by these authors were similar to those found for the isolates from the control group in our study.

The fact that anti-retroviral agents may reduce exoenzyme production was not confirmed in this study. The same results were verified in previous study¹⁷, that demonstrated proteinase and phospholipase production were strongly positive in the AIDS group when compared to the control group. As the production of these exoenzymes suggests increased pathogenicity of these isolates, great attention should be given for these results.

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