

***In vitro* Evaluation of Phospholipase and Proteinase of *Candida albicans* Isolated From Oral Cavity of Diabetic Patients**

Avaliação “*in vitro*” da Atividade de Fosfolipase e Proteinase de Cepas de *Candida albicans* Isolada da Cavidade Bucal de Pacientes Diabéticos

Janaina de Cássia Orlandi Sardi^{a*}; Patricia Vilas-Boas^b; Natalia Aguiar^b; Mariana Fonseca^b; Jéssica Theobaldo^b; José Francisco Höfling^b; Daniel Saito^c

^aState University Paulista Júlio de Mesquita Filho - Faculty of Pharmaceutical Sciences of Araraquara, SP, Brazil

^bUniversity of Campinas, Piracicaba Dental School, Department of Oral Diagnosis, SP, Brazil

^cAmazonas State University, School of Health Sciences, Microbiology and Immunology Division, AM, Brazil

*E-mail: janaina-sardi@uol.com.br

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Resumo

Leveduras do gênero *Candida* são comuns na cavidade bucal e podem causar candidose na presença de fatores predisponentes, especialmente em paciente diabético, o qual é caracterizado por um aumento anormal da glicose no sangue. A manifestação da doença está relacionada a este conjunto de fatores locais, tais como a presença de próteses dentárias, pH salivar, fluxo salivar e tabaco. A redução da saliva é um dos principais fatores de risco para o aparecimento de infecção e o controle glicêmico inadequado causado por diabetes, em associação com todos estes fatores, pode aumentar a incidência de infecções. Os objetivos deste estudo foram: 1) isolar e identificar cepas de *Candida albicans* da mucosa bucal de pacientes diabéticos 2) avaliar os fatores de virulência: proteinase e fosfolipase. Amostras microbiológicas foram coletadas a partir de locais da mucosa bucal e semeadas em CHROMagar para posterior identificação de *C. albicans* por PCR. Foram realizados testes da atividade de proteinase e fosfolipase para todos os isolados de *C. albicans*. Neste estudo, 22 isolados foram identificados como *C. albicans*. Em relação às atividades de proteinases, todas as cepas de *C. albicans* foram capazes de produzir proteinase, enquanto que para fosfolipase, apenas 4,5% dos isolados não produziram esta exoenzima. Portanto, *C. albicans* presente na cavidade bucal de pacientes diabéticos tem potencial patogênico e pode participar de processos infecciosos e inflamatórios, causando lesões e invadindo os tecidos orais.

Palavras-chave: *Candida albicans*. Fatores de Virulência. Boca. *Diabetes mellitus*.

Abstract

Candida yeasts are common in the oral cavity and can cause candidosis in the presence of predisposing factors, especially diabetes, which is characterized by an abnormal increasing in blood glucose concentration. The manifestation of the disease is related to a set of local factors such as the presence of dental prostheses, salivary pH, salivary flow and tobacco. The reduction in saliva is a major risk factor for the onset of infection and poor glycemic control caused by diabetes in association with all these factors further increases the incidence of candidosis. The objectives of this study were: 1) to isolate and identify Candida albicans strains from oral mucosa sites of diabetic patients 2) to evaluate the virulence factors: proteinase and phospholipase. Thus, microbial samples were collected from oral mucosa sites and seeded in CHROMagar for subsequent identification of C. albicans by PCR. For the phenotypic tests, all strains of C. albicans were evaluated for their proteinase and phospholipase productions. In this study, 22 isolates were identified as C. albicans. In regard to the proteinase activities, all strains of C. albicans were able to produce proteinase, while only 4.5% from those isolates were not able to produce phospholipase activity. In conclusion, C. albicans present in the oral cavity of diabetic patients is potentially pathogenic and can participate in infectious and inflammatory processes, causing injury and invading oral tissues.

Keywords: *Candida albicans*. Virulence Factors. Mouth. *Diabetes mellitus*.

1 Introduction

Candida spp. are commensal yeasts that inhabit different sites of the oral cavity. However, given the immunosuppressive conditions, these yeasts can become more virulent and express pathogenicity. *Candida* species have different virulence factors, including mechanisms of cell adhesion and invasion associated with the production of enzymes that aid in tissue degradation and facilitate their proliferation in the oral mucosa¹. *Diabetes mellitus* is a metabolic disease characterized by hyperglycemia due to defects in insulin production, insulin action, or both. Numerous oral complications, such as decreased function of salivary glands, burning sensation, periodontal disease and

yeast colonization have been related to diabetes mellitus²⁻⁴. Species of *Candida* have been frequently isolated from the oral cavities of diabetic patients and the highest rate of colonization occurs in patients with poor glycemic control^{5,6}.

Extracellular hydrolytic enzymes seem to play an important role in the adherence and tissue penetration, invasion and destruction of host tissues^{7,8}. The most important hydrolytic enzymes are proteinases and phospholipases. Ten aspartic proteinases (Sap isoenzymes) are responsible for proteinase activity of *Candida albicans*. These proteins have the molecular weights between 35 and 50 kDa, encoded by the genes Sap1–10⁹. These

exoenzymes are able to degrade immunoglobulins and proteins from the extracellular matrix, inhibit phagocytosis of polymorphonuclear neutrophils and induce inflammatory reactions^{10,11}. Several studies have demonstrated the relationship between the increase in the synthesis and activity of extracellular hydrolytic enzymes, with the increase of the pathogenic potential of yeasts, leading to clinical signs of severe candidiasis¹². The aims of this study were to identify by PCR *C. albicans* isolated from oral mucosa from diabetic patients and evaluated some virulence factors of this fungus.

2 Material and Methods

This research was approved by the ethical committee for research (062/2008) of the Piracicaba Dental School, University of Campinas, SP, Brazil.

2.1 Inclusion and exclusion criteria

Patients resident in Piracicaba, São Paulo, Brazil, ranging from age 31 to 68 and with medical diagnosis of type 2 *Diabetes mellitus* were included in the study. Glycemic control with insulin supplementation was confirmed by an endocrinologist. Exclusion criteria were: use of antibiotics and periodontal treatment during the previous 6 months, pregnancy, smoking, systemic disease, immunodeficiency, use of partial and/or total prosthesis, use of orthodontic apparatus.

2.2 Patients Selection

A total of 22 isolates of *Candida spp.* were obtained from oral cavity of 5 volunteers from the Faculty Clinic. The samples were collected by subjects rinsing with sterile water for 1 minute and immediately after collecting the samples from each volunteers were diluted and plated onto a Sabouraud Dextrose Agar (SDA) with Chloramphenicol and chromogenic medium (CHROMagar Candida®, Biomerieux, Paris, France) and incubated at 37 °C for 48 h in aerobic conditions. The green colonies grown on the agar plate were randomly selected and cultures stored in glycerol stock at -20 °C for later identification by PCR.

2.3 PCR (polymerase chain reaction)

DNA from the *Candida* isolates was extracted using a protocol described by Nascimento *et al.*¹³ and quantified in a spectrophotometer at 260 nm (Genesys 10UV, Rochester, NY, USA) to obtain a standard concentration of 100 ng/mL and stored at -20 °C for subsequent PCR reactions. DNA samples were identified by PCR using specific primers for the portion corresponding to the gene AAT1a (ID 3643468) (F: 5 'ACT GCT CAA ACC ATC TCT GG -3 ' and R: 5 'CAC AAG GCA AAT GAA GGA AT - 3 with fragment size of 472bp) of *C. albicans*. Purified DNA from *C. albicans* (ATCC 90028) was used as a positive control. This primer was designed specifically for *C. albicans*. The

molecular mass ladder (100 bp DNA ladder, Gibco, Grand Island, NY, USA) was included for running in the agarose gel. PCR amplification was performed with a GeneAmp PCR system 2400 (Perkin-Elmer-Applied Biosystems) under the following thermal conditions: 72 °C for 5 min, 38 cycles of 95 °C for 30 s, 55 °C for 45s and 72 °C for 30s and extension at 72 °C for 5 min. The PCR products were separated by electrophoresis in 2% agarose gels and Tris-borate-EDTA running buffer (pH.8.0). The DNA was stained with 0.5 ug ethidium bromide/mL and visualized under UV illumination (Pharmacia LKB-MacroVue, San Gabriel, CA, USA).

2.4 Proteinase and phospholipase activity determination by the agar plate method.

All *C. albicans* isolates were tested in triplicate during three independent experiments to verify the enzymatic activity of proteinases (SAPs) and phospholipases^{14,15}. The test medium for proteinases was a BSA (bovine serum albumin) agar medium containing 2g BSA, 145g YNB (Yeast Nitrogen Base - Difco Laboratories, Detroit), 20g glucose and 20g agar per liter of distilled water. The test medium for phospholipases consisted of 10g peptone, 57.3g sodium chloride, 0.55g calcium chloride, 30g glucose, 20g agar, and 100 mL 50 % sterile egg yolk (egg yolk enrichment) per liter of distilled water. Test isolates were grown on SDA for 24 h and an inoculum of 10⁸ CFU/mL in sterile saline solution (absorbance 0.5 at 600nm) was transferred to the test medium. The plates were incubated at 37 °C in aerobic conditions for 48h and 72h to examine the proteinases and phospholipases, respectively. The enzymatic activity was determined by the formation of a halo around the yeast colony and expressed in terms of the ratio of the diameter of the colony to the total diameter of the colony plus the zone of precipitation (Pz), according to the method described by Price *et al.*¹⁵. According to this system, Pz = 1.0 indicated that the test strain was negative for proteinase/phospholipase, while a value of Pz ≤ 0.63 signified that the test strain was releasing large amounts of proteinases/phospholipases (strongly positive). Values of Pz between 0.64 and 0.99 signified that the test strain was releasing small amounts of proteinases/phospholipases (positive).

3 Results and Discussion

All isolates from section 2.2 were identified by PCR as *C. albicans*.

All *C. albicans* isolates showed proteinase activity. Pz values for the proteinase tests ranged from 0.29 to 0.58 for the isolates and were strongly positive for the production of proteinase. *C. albicans* showed different activities of phospholipases with Pz ranging from 0.41 to 1.0, of which 4.50% of the strains without phospholipase activity (negative), 27.30% having low activity (positive) and 68.20% high activity (strongly positive) (Table 1).

Table 1: Frequency of virulence factors of *Candida albicans* isolated from oral mucosa of diabetic patients

Virulence factor	Value	Results (%)
Proteinase activity	Negative – Pz = 1	0
	Positive – Pz = 0.99- 0.63	0
	Strongly positive - Pz < 0,63	100
Phospholipase activity	Negative – Pz = 1	4.5
	Positive – Pz = 0.99- 0.63	27.30
	Strongly positive - Pz < 0.63	68.20

Candida is the most frequent etiologic agent in fungal infections. It causes opportunistic infections ranging from simple mucocutaneous to invasive infections in patients with immunocompromising conditions. Diseases such as diabetes mellitus, renal failure, organ transplantation, and neutropenia are the main predisposing factors for *Candida* infections. Hematogenous dissemination due to candidemia or direct inoculation followed by any trauma can cause development of soft tissue infections due to *Candida* spp¹⁶. The prevalence of *Candida* species in the oral cavity of immunosuppressed individuals has been found to be higher when compared to the healthy population. Peterson *et al.*¹⁷ observed 55 % prevalence of oral yeasts from saliva of hospitalized patients. In patients with advanced cancer, this prevalence ranged between 47% and 87%. In diabetic patients, the presence of *Candida* spp. in the oral mucosa reached up to 80%¹⁸. In the present study, *C. albicans* strains were identified in 100 % of oral mucosa from patients with diabetes mellitus. *Candida* spp. has developed several virulence traits that facilitate invasion of host tissues and evasion of host defense mechanisms². Studies have reported that 30 to 100% of the oral isolates of *C. albicans* produce phospholipases with variable degrees of enzymatic activity^{19,20}. In the current study, phospholipase activity was detected in 95.5 % and proteinase production in 100 % of the *C. albicans* isolates. Tsang *et al.*²¹ found a high proteinase activity in type 2 *Diabetes mellitus* patients. Manfredi *et al.*² has demonstrated that proteinase expression is not significantly higher in *Candida* isolates from patients with diabetes when compared to healthy patients and that type 2 *Diabetes mellitus* patients have higher proteinase levels than type 1 DM patients. Another study has demonstrated that *C. albicans* strains produced high levels of phospholipase and proteinase²², which are considered important pathogenic factor, because they act enhancing the process of adhesion and invasion by hydrolyzing the peptide and phosphoglyceride bonds, respectively. Mane *et al.*²³ evaluated the production of proteinase and phospholipase enzymes of *C. albicans* isolates and the results were 89.7% and 59.0% respectively.

4 Conclusion

C. albicans present in the oral cavity of diabetic patients is potentially pathogenic and can participate in infectious and inflammatory processes, causing injury and invading oral tissues.

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