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# Prevalence and antifungal resistance profile of *Candida* spp. oral isolates from patients with type 1 and 2 diabetes mellitus

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ABSTRACT

*Objective:* The goal of the study was to measure the prevalence of *Candida* spp. in the oral cavity of patients with diabetes types 1 and 2 when compared to healthy individuals and to study antifungal resistance profile of the isolates.

*Design:* There were 162 subjects in the study: diabetes type 1 ($n = 39$); control group 1 ($n = 50$): healthy individuals matched in gender, age, and oral conditions to diabetes type 1 patients; diabetes type 2 ($n = 37$); control group 2 ($n = 36$) who were matched to each patient of the diabetes type 2 group. Stimulated saliva was collected and isolates were identified with phenotypic tests. The presence of *C. dubliniensis* was determined by multiplex PCR.

*Results:* There were no statistically significant differences in *Candida* spp. frequency between the diabetes 1 group and its control ($p = 0.443$) nor between the diabetes 2 group and its control ($p = 0.429$). *C. albicans* was the most frequently isolated yeast in all groups. In the diabetes groups, *C. stellatoidea*, *C. parapsilosis*, *C. tropicalis*, *C. lipolytica*, *C. glabrata*, and *C. krusei* were also identified. Additionally, in control groups, *C. kefyr* was also detected. None of the isolates were resistant to amphotericin B and flucytosine. A low percentage of the isolates were resistant to ketoconazole.

*Conclusions:* No differences were detected in colonization of *Candida* spp. oral isolates from type 1 and type 2 diabetes when compared to matched controls. The antifungal resistance of *Candida* spp. isolates for ketoconazole from type 1 diabetes patients was significantly higher than that of its matched control.



## 1. Introduction

The growing importance of fungal infections may be attributed to the use of broad-spectrum antimicrobial drugs, corticosteroids, anti-tumoural agents, and oral contraceptives. An increasing number of immunocompromised patients adds to the problem.[1] In AIDS patients, bone marrow transplant recipients, or those under aggressive antineoplasic chemotherapy, *Candida* spp. are causes of mortality and morbidity.[2,3] Patients with endocrinal or blood diseases are also prone to developing candidosis.[4–6]

Pathogenic mechanisms responsible for the high infection frequency in diabetic patients include decreased humoural and cellular immunity, angiopathy, neuropathy, and a high number of medical interventions.[7]

In particular, diabetic patients are predisposed to fungal infections, which are frequently associated with high glucose levels. The elevated glucose concentration in saliva and low

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salivary secretion may enhance the growth of yeasts and their adherence to oral epithelial cells.[8–11]

Prevalence of diabetes *mellitus* is increasing progressively, mostly in people 60 years and older. The global number of people with diabetes is estimated to rise from 171 million in 2000 to 366 million in 2030.[12] This rise is attributed to population growth, ageing, urbanization, and increasing prevalence of obesity and physical inactivity.[12]

Diabetic patients can be classified in two groups according to insulin need: type 1 or insulin-dependent diabetes *mellitus* (IDDM) constitutes 5% to 10% of the cases and usually starts during childhood or adolescence. It is characterized by total deficiency on insulin production by the organism, caused by auto-immune attack to pancreatic beta cells. Type 2 diabetes or non-insulin-dependent diabetes *mellitus* (NIDDM) comprises 80–90% of diagnosed cases and usually affects middle-aged obese people. This type of diabetes is almost completely determined by genetic factors. Pancreatic beta cells are not destroyed and, thus, insulin is present at low levels.[13]

With the increasing incidence of *Candida* spp. infections, the comprehension of this yeast pathogenicity as well as antifungal susceptibility become important and valuable. The aim of this study was to measure the prevalence of *Candida* spp. on the oral cavity in diabetes types 1 and 2 patients when compared to healthy individuals and to study antifungal resistance profile of the isolates.

## 2. Materials and methods

This study was previously approved by the Human Ethical Committee (Protocol # 094/2003–PH/CEP) and is in accordance with the principles laid down in the Declaration of Helsinki.

### 2.1. Subjects

A convenient sample of 162 subjects was included in the present study and was distributed in the following groups:

- Diabetes type 1 group ($n = 39$): aged 9–27 years, 20 males and 19 females;
- Control group 1 ($n = 50$): healthy individuals matched in gender and age to each patient in diabetes type 1 group;
- Diabetes type 2 group ($n = 37$): aged 37–78 years, 8 males and 29 females;
- Control group 2 ($n = 36$): healthy individuals matched in gender, age, prosthesis use, and oral conditions to each patient in diabetes type 2 group.

Diabetic patients were recruited from primary healthcare units located at the city of São José dos Campos, Brazil. All the diabetic patients who attended lectures about general health, promoted by the care units, were invited to participate in the study. Patients who accepted participation signed a written informed consent. Healthy volunteers were selected from amongst patients under dental treatment at São José dos Campos Dental School.

Patients included in this study had controlled glycaemic levels (glycated haemoglobin lower or equal to 9%)[14,15] and were classified as ''normal'' by the Body Mass Index (18.5–29 kg/m$^2$).[16] Patients selected had 10 years or less of diagnosed diabetes. Healthy individuals included in the control groups had no lesion in the oral cavity.

For both groups – diabetes and controls – individuals who had received antibiotics, steroid therapy, or drugs that could interfere with salivary flow or saliva composition, and those who had been using antiseptic mouthwashes during the six months previous to sampling were excluded from the study. Pregnant women and smokers were not included in the sample.

### 2.2. Isolation and quantification of Candida spp. in saliva

Stimulated saliva was collected from all individuals for 1 min between 9 and 11 a.m. to prevent circadian rhythm variation and at least 2 h after the last meal to reduce the effect of diet on saliva composition.

Microbiological analysis was performed at the microbiology laboratory of São José dos Campos Dental School – Universidade Estadual Paulista (UNESP). Next, dilutions of $10^{-1}$ and $10^{-2}$ in sterile physiologic solution (NaCl 0.9%) were obtained and aliquots of 0.1 mL were plated on Sabouraud Dextrose Agar supplemented with chloramphenicol (0.1 mg/mL). Plates were incubated at 37 °C for 48 h, and colony forming units (CFU) were obtained. Results were expressed as CFU/mL.

### 2.3. Isolates identification

Four colonies were isolated from Sabouraud Dextrose agar and kept at 4 °C until further analysis. A total of 379 isolates were identified by examining colony morphology, germ tube test, microculture on corn-meal agar supplemented with Tween 80 to evaluate hyphal morphology and chlamydospore formation, fermentation and assimilation of carbohydrates according to Sandvén[17] and Williams and Lewis.[18] Isolates phenotypically identified as *C. albicans* or *C. dubliniensis* were submitted to molecular identification. These isolates were submitted to a multiplex polymerase chain reaction (PCR) procedure according to the methodology proposed by Donnelly et al.[19] and Mähnß et al.[20], with modifications.

The isolates were plated on Sabouraud dextrose agar and incubated for 24 h at 37 °C. Then, a single colony was transferred to 75 μL of zymoliase (Sigma, St. Louis, USA) solution (0.5 mg/mL in 1 mol L$^{-1}$ sorbitol buffer). Tubes were maintained at 95 °C for 10 min. After this period, samples were centrifuged (8000 × *g* at 4 °C for 15 min) and the supernatant was kept on ice until the PCR analyses. For PCR, two pairs of primers were used: two universal primers, Uni-f: 5′-GCATATCAATAAGCGGAGGAAAA-3′ and Uni-r: 5′-GGTCCGTGTTTCAAGACG-3′; and two *C. dubliniensis*-specific ones, DUBF Act-f: 5′-GTATTTGTCGTTCCCCTTTC-3′ e DUBR Act-r: 5′-GTGTTGTGTGCACTAACGTC-3′. The amplification was carried out in 10 μL final volume containing 5 μmol L$^{-1}$ of each, 5.0 μL of PCR Master Mix (Promega), 3.2 μL ultra-pure water, and 1 μL of DNA template. Cycling conditions consisted of 3 min at 95 °C followed by 30 cycles of 30 s at 95 °C, 30 s at 58 °C, 60 s at 72 °C, followed by 72 °C for 10 min. In all reactions, *C. albicans* (ATCC 18804) and *C. dubliniensis* (CD33) were included as control. Amplification products were separated by electrophoresis