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In vitro Antifungal Susceptibility of *Candida albicans* Isolates from Patients with Chronic Periodontitis and Diabetes

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Abstract

Alterations that lead to deficiency of the immune system, such as diabetes mellitus, may promote proliferation of *Candida albicans* and selection of strains which have greater ability to adhere and to penetrate the host tissues. Recent studies indicate an increase of the antifungal resistance of *C. albicans* isolates in periodontal pockets, suggesting that the oral cavity could be a reservoir of resistant yeast to antifungal agents. Moreover, oral cavity can act as a reservoir of certain pathogens that may cause systemic infections. The periodontal pocket is an ecological niche suitable to host microorganisms that could act as opportunistic pathogens. The aim of this study is to contribute to the understanding of resistance to conventional antifungal against *C. albicans* isolates from patients with periodontitis and diabetes. The determination of the minimal inhibitory concentrations (MIC) was evaluated according to M27S3 of the CLSI (2008), with modifications. The results showed that 48.8% of the studied strains were resistant to one or more antifungals and 6.6% were resistant to fluconazole and voriconazole. These results suggest an increasing resistance to conventional antifungal agents among *Candida* species, suggesting that the oral cavity could host pathogen fungi.

Keywords: *Candida albicans*; Diabetes; antifungal; Minimal inhibitory concentrations (MIC); Resistance

Introduction

A large proportion of healthy adult population holds yeast *Candida* genus in the oral cavity [1]. The mucosa is considered the main reservoir, but studies have shown that *Candida* species can be co-aggregated with bacteria in biofilm and that may be an important factor for manifestations of candidiasis and for colonization of cavities of caries and periodontal pockets [2]. According to some authors, the presence of yeast in subgingival regions may contribute to the pathogenesis of periodontal disease or increase the probability of candidemia, especially in cases of immunocompromised patients [3-5]. Periodontal disease is mainly caused by Gram negative anaerobic species, but has been reported in the literature that the proportion of yeast in the periodontal pockets is similar to some of periodontal bacteria [6], thus suggesting the possible role of *Candida* sp. in disease pathogenesis [2,4,7,8], but it is not clear, in the literature, whether this would continue after periodontal therapeutic antibacterial successive. Moreover, its permanence in these sites can be characterized as potential sources of dissemination, especially in immune compromised individuals [9]. Sardi et al. [10] found a higher prevalence of *C. albicans* in periodontal pockets of diabetic patients compared with non diabetic. Super infection by *Candida* can be refractory to conventional periodontal treatments in specific situations, such as in immune compromised patients. In these cases, the systemic therapy with antifungal drugs could be indicated. Few studies have reported the use of antifungals in *Candida* isolates in periodontal pockets. The presence of *Candida* in periodontal pockets has been investigated a little time and very little is known about the importance of this fungus in this pathology. Oral candidiasis used to be treated with polyenes amphotericin B or nistatin and the discovery of antifungal activity of azolic compounds represented an important advance in the treatment of superficial and systemic fungal infections Moreover, they inhibit germ tube formation, reducing the adherence to oral epithelial cells and acrylic surfaces in dentistry [11]. Caspofungin, an echinocandin, which has demonstrated activity against *Candida* species both *in vitro* and *in vivo* for systemic infections [12,13]. The aim of this study was to analyze antifungal susceptibility of *C. albicans* strains isolated from chronic periodontitis patients and diabetes.

Methods

Microorganisms

We used 45 clinical isolates of *Candida albicans* isolated from subgingival sites of patients with generalized chronic periodontitis and diabetes mellitus type II. These fungi were isolated from patients with age ranging between 31 to 68 years attending in clinic of Periodontics, Piracicaba Dental School, State University of Campinas (UNICAMP). Exclusion criteria were used: use of antibiotics and periodontal treatment during the previous 6 months. As control was used strain ATCC 90028 of *Candida albicans*. All microorganisms belong to the mycology collection of the Laboratory of Clinical Mycology, Department of Clinical Analysis, Faculty of Pharmaceutical Sciences, UNESP, Araraquara.

Preparation of antifungal drugs

Conventional antifungal agents indicated for the treatment of candidiasis were used: amphotericin B, fluconazole, voriconazole and caspofungin. The preparation of the drugs was performed according to M27 S3 of the CLSI (Clinical and Laboratory Standards Institute)

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(2008). Amphotericin B (Sigma Chemical Company, St. Louis, MO, USA) and voriconazole (Sigma Chemical Company, St. Louis, MO, USA) are water-insoluble drugs, therefore, were solubilized in DMSO for the preparation the ten stock solutions with concentrations ranging from 0.0313 to 16 µg/mL. Fluconazole (Sigma Chemical Company, St. Louis, MO, USA) and caspofungin (Sigma Chemical Company, St. Louis, MO, USA) are water-soluble drugs therefore were solubilized in sterile water and ten stock solutions were prepared with a concentration range varying from 0125 to 64 µg/mL. All stock solutions were diluted in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) with L-glutamine, without sodium bicarbonate, supplemented with 2% glucose, and buffered to a pH of 7.0 using 0.165M 3-(N-Morpholino) (propanesulfonic acid) (MOPS), (Sigma-Aldrich, St. Louis, MO, USA) to preparation the working solutions. The range concentration for amphotericin B and voriconazole was 0.01565 to 8 µg/ml and for drugs fluconazole and caspofungin was 0.0625 to 32 µg/mL.

Determination of minimum inhibitory concentration (MIC)

The sensibility of the clinical isolates to conventional antifungal agents was evaluated according to M27 S3 of the CLSI (2008), with modifications. From 24 hours cultivation of clinical isolates of *C. albicans* on Sabouraud dextrose, the inocula were prepared in RPMI-1640 to the final concentration of 1.0×10^4 CFU/mL and added in 96-well microplates (Difco, Detroit, USA) previously prepared with antifungal drugs. The plates were incubated in a shaker at 37°C/150 rpm for 24 hours. The reading of MIC was performed in a spectrophotometry at 490 nm. Through this test, the 45 clinical isolates were classified as sensitive (S), sensitive dose dependent (S-DD) and resistant (R), according with the breakpoints recommended by the CLSI (2008) (Table 1).

	Fluconazole	Voriconazole	Anfotericin B	Caspofungin
S*	≤ 8 µg/mL	≤ 0,125 µg/mL	≤ 1 µg/mL	≤ 2 µg/mL
S-DD*	16 – 32 µg/mL	0,25 – 0,5 µg/mL	-	-
R*	≥ 64 µg/mL	≥ 1 µg/mL	≥ 2 µg/mL	> 2 µg/mL

S - sensitive / DD - dose dependent / R – resistant

Table 1:. Values of MIC for the classification of susceptibility to fluconazole, voriconazole, amphotericin B and Caspofungin in *Candida* species.

Results

Through the determination of MIC was observed that from 45 clinical isolates of *C. albicans*, 48.8% were resistant to one or more antifungals. This percentage was determined by comparison between the MIC values of isolates with cutoff values found in the literature. Our results showed that 6.6% of clinical isolates were resistant to the azole class, being resistant to fluconazole as voriconazole. Figure 1 shows the percentage of clinical isolates sensitive, dose-dependent sensitive and resistant to azoles. Among the azoles, the fluconazole showed greater activity against clinical isolates with only five (11.2%) isolates resistant to this drug (Figure 1-A), differently from the voriconazole, in which 19 (42.2%) of the isolates were resistant (Figure 1-B).

We observed that a large percentage of clinical isolates were sensitive to amphotericin B, and only four (8.9%) classified as resistant (Figure 1-C). It was also observed that the antifungal caspofungin showed only two (4.4%) considered resistant isolates (Figure 1-D). Thus, the clinical isolates of *C. albicans* tested with these two drugs showed no fungal resistance, but showed significant resistance to azole antifungal class.

Discussion

Candida species inhabit diverse ecosystems and are present in the genitourinary and gastrointestinal tracts, nail, skin, bronchus and oral cavity where they can establish themselves as regular commensal microbiota without causing harm to the host [14]. However, systemic diseases such as diabetes mellitus and AIDS, physiological conditions such as pregnancy, infancy or old age, nutritional factors, treatment with broad-spectrum antibiotics, immune suppressants and corticosteroids, in addition to local factors such as xerostomia and use of prosthetic devices are conditions that predispose to the development of *Candida* infections [2,10,15-19]. The use of broad-spectrum antimicrobial, such as tetracycline and metronidazole as an aid in periodontal treatment has also been an important factor for the development of super infections by resistant bacteria and *Candida* species, including patients

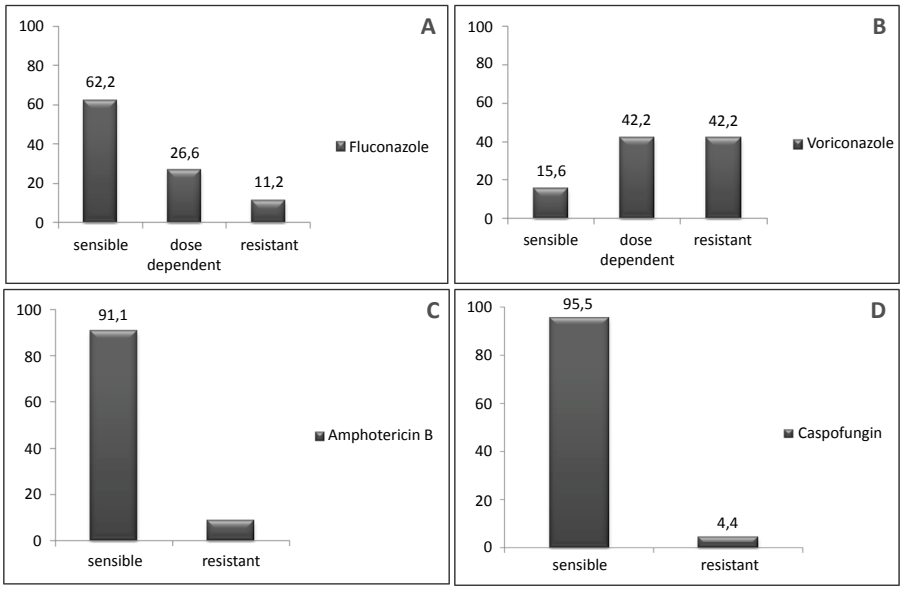

Figure 1: Relation of 45 clinical isolates of *Candida albicans* with antifungal fluconazole (A), voriconazole (B), amphotericin B (C) and Caspofungin (D).

with HIV [6,20]. Resistance to fluconazole has been reported by several authors [21-26]. In the present study, 62.2% of the *C. albicans* isolates tested were susceptible to fluconazole, 15.6% to voriconazole, 91.1% to amphotericin B and 95.5% to caspofungin. The intrinsic resistance of *Candida* species to fluconazole, an agent commonly used for treating antifungal and greater resistance to amphotericin B has been reported [27]. *Candida albicans* appears co-aggregate contributing to bacterial biofilm formation on this structural and impairing penetration of certain antimicrobial drugs [28]. Also, for these researchers *C. albicans* was found typically in the outer layers of the biofilm, and seemed to act, according to the authors, as a barrier that protected the microorganisms from the action of immune mechanismsby assisting the resistance of subgingival microbiota in the face of host defenses, and contributing to persistence of inflammation in the surrounding tissues.

Waltimo et al. [29] evaluated the antifungal susceptibility among isolates of *C. albicans* from periodontal pockets and showed that 100% of these isolates were sensitive to amphotericin B and 5 - flucytosine. However, susceptibility to azole antifungals proved variable cross-resistance occurring in relation to them. Dumitru et al. [30], studied isolates of *C. albicans* and found strains resistant to amphotericin B and four class azole antifungals. Furlletti et al. [31] showed that *C. albicans* isolated from periodontal pockets were resistant to amphotericin B and fluconazole-sensitive. Jewtuchowicz et al. [7] studied isolates of *C. dubliniensis* and *C. albicans* from periodontitis patients and healthy systematically and found that only one isolate was resistant to fluconazole and voriconazole. Junqueira et al. [32] demonstrated that isolates of *Candida* sp. oral cavity of HIV patients are resistant to fluconazole. In our study we found some isolates resistant to fluconazole (11.2%), voriconazole (42.2%), caspofungin (4.4%) and amphotericin B (8.9%), although the highest resistance occurred with the antifungal voriconazole. Some research has shown the presence of different species of *Candida* isolated from different anatomical sites resistant to voriconazole in diabetics patients [33-36]. Antifungal agents such as amphotericin B, 5-fluorocytosine, voriconazole and terbinafine are not usually used in the treatment of oral candidiasis, however, also deserve attention. Although such antifungals are only available for systemic use and are recommended for the treatment of disseminated infections, determination of minimum inhibitory concentration relative to oral cavity isolates obtained from patients with immune suppression, especially in cases of periodontitis is important to obtain epidemiological data and the possibility of this site being the original focus of disseminated fungal infections [37,38]. The results of this study suggest that clinical isolates of diabetic patients have a certain resistance to azoles, perhaps because these patients have already contacted and antifungal therapy with azoles may no longer be as effective in treating periodontal *Candida* super infections that are refractory to conventional treatments. Furthermore, studies on the correlation between clinical results and *in vitro* are needed to establish a better antifungal.

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