In vitro antifungal susceptibility of *Candida* spp. oral isolates from HIV-positive patients and control individuals

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Abstract: Oropharyngeal candidiasis is the most common fungal infection among HIV-positive patients. This condition can be treated with either systemic or topical antifungal agents; treatments are usually indicated empirically on the basis of clinical data. The knowledge of *in vitro* antifungal susceptibility is important to determine correct therapeutic guides for the treatment of fungal infections. Therefore, the objective of this study was to determine the antifungal susceptibility profile of oral *Candida* isolates from HIV-positive patients and control individuals. Amphotericin B, fluconazole, flucytosine, nystatin and ketoconazole were tested according to the methodology of microdilution proposed by the Clinical and Laboratory Standards Institute (CLSI); results were recorded in values of minimal inhibitory concentration (MIC). A total of 71 *Candida* isolates from HIV-positive patients were examined with the following species represented: *C. albicans* (59), *C. tropicalis* (9), *C. glabrata* (1), *C. guilliermondii* (1) and *C. krusei* (1). A total of 15 *Candida* isolates were evaluated from control individuals comprised of 11 *C. albicans* and 4 *C. tropicalis* samples. Our results demonstrated that the tested antifungal agents showed good activity for most isolates from both groups; however, variability in MIC values among isolates was observed.

Descriptors: HIV; Antifungal Agents; *Candida*.

Introduction

The oral cavity is inhabited by more than seven hundred microbial species; many intrinsic and extrinsic factors impact the composition, metabolic activity, and pathogenicity of these highly diversified oral microflora.[1,2] Yeasts from the *Candida* genus are commonly found in oral human microflora and are considered to comprise the majority of fungal species present in the oral cavity. Although the growth of *Candida* in the oral cavity is usually controlled, under certain conditions *Candida* species can rapidly multiply resulting in disease and possible tissue invasion. The incidence of oropharyngeal candidosis has increased over the last several decades due to the widespread use of antibiotics, antifungal drugs and immunosuppressive drugs and some conditions, such as HIV-infection.[2,3,4] Oropharyngeal candidosis is the most common fungal infection among patients infected with HIV. Oral candidosis occurs in more than 95% of AIDS patients, and it is considered an important marker of the AIDS disease and its progression. The prevalence of oral candidosis in

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HIV-positive patients appears to be correlated to the severity of immunological disfunction.[5,6] The occurrence of oral candidosis at initial stages of AIDS is common, particularly when the CD4 T cell count is low (400-700 cells/mm^3).[5]

The widespread use of antifungal agents to prevent the occurrence of oral candidosis in HIV-infected patients has been cited as an important factor for the positive selection of non-*albicans* species as opportunistic pathogens; these species have different susceptibilities to the antifungal agents utilized to treat *C. albicans*.[7] Certain non-*albicans* species, such as *C. glabrata* and *C. krusei*, are inherently less susceptible to fluconazole than *C. albicans* and have been isolated with increasing frequency in HIV-infected patients.[7,8,9,10,11]

The increasing resistance to antifungal treatments and expanding drug therapy options has prompted the need for clinically relevant antifungal susceptibility testing; these results could act as a guide in the selection and control of antifungal therapy. For this purpose, the Clinical and Laboratory Standards Institute (CLSI) proposed a reference method for yeast antifungal susceptibility testing using serial dilutions and obtained values of minimal inhibitory concentration.[12,13,14]

Fluconazole is considered to be the drug of choice for the treatment of most common HIV-associated opportunistic yeast infections.[14,15] It has been the most widely used drug because of its good absorption, low toxicity and ability to be administered through both oral and intravenous routes.[16] Fluconazole resistance is associated with prolonged exposure to azoles.[14,17] Further, in 2002 Espinel-Ingroff[18] reported an association between the *in vitro* resistance of *Candida* to fluconazole and clinical failure in the treatment of candidosis. Ketoconazole is the first drug of the azole class of agents capable of achieving therapeutic blood levels when administered orally. While ketoconazole is used to treat immunocompromised patients, its adverse side effects, including nausea and hepatotoxicity, has restricted its use.[19] However, despite the toxicity, it shows several adverse drug interactions, disabling its use in association with some drugs, including antiretrovirals.[16] The antifungal drug amphotericin B decreases the adherence of *Candida* to buccal epithelial cells and can interfere in the pathogenesis of candidosis. The use of amphotericin B in dentistry is common; it is indicated for the treatment of superficial candidosis cases and is used locally. The systemic use is generally indicated for the treatment of disseminated cases and is highly toxic to the patient, causing kidney disorders.[16] The antifungal drug flucytosine has better gastrointestinal absorption compared to amphotericin B. However, flucytosine also has toxicity, negatively impacting metabolic and liver function.[19]

Based on this information, it can be observed that all antifungals have "pros and cons" when used in the treatment of candidosis. Thus, the evaluation of the susceptibility profile of oral *Candida* isolates is important in determining the proper method of treatment in candidosis. The objective of this study was to determine the antifungal susceptibility profile of oral *Candida* isolates from HIV-positive patients and control individuals.

Materials and Methods

Oral rinses and dental biofilm samples were collected from HIV-positive patients and control individuals. The isolates were identified by germ tube test, hyphae/pseudohyphae, chlamydospore formation, assimilation and fermentation of carbohydrates as previously described[20] (Local Ethics Committee 012-PH/CEP).

In total, 59 *C. albicans*, 9 *C. tropicalis*, 1 *C. glabrata*, 1 *C. guilliermondii* and 1 *C. krusei* isolates from HIV-positive patients and 11 *C. albicans* and 4 *C. tropicalis* isolates from control individuals were evaluated.

The isolates were inoculated onto Sabouraud dextrose agar and were incubated for 48 h at 37°C. After this period, the colonies were suspended in sterile saline solution (0.85% NaCl) resulting in an initial concentration of 1.5 x 10^6 cells/ml. Subsequently, the suspension was diluted in 1:2000 in RPMI 1640 medium (Sigma, St. Louis, USA) and buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (Sigma, St. Louis, USA) to obtain a final concentration of 0.5 x 10^3 - 2.5 x 10^3 cells/ml.

The antifungal agents tested were: amphotericin B (Sigma Chemical Company, St. Louis, USA), fluconazole (Galena Chemical and Farmacêutica, São Paulo, Brazil), ketoconazole (Galena Chemical and Farmacêutica, São Paulo, Brazil), nystatin (Galena Chemical and Farmacêutica, São Paulo, Brazil) and flucytosine (Sigma Chemical Company, St. Louis, USA). Amphotericin B and ketoconazole were dissolved in dimethylsulfoxide (Sigma, Irvine, United Kingdom). The other antifungal agents were dissolved in sterile distilled water. The drugs were prepared at the following concentrations: 320 μg/ml for amphotericin B, 1000 μg/ml for flucytosine, 1250 μg/ml for fluconazole, and 640 μg/ml for ketoconazole. The solutions were diluted in RPMI medium and final drug concentrations ranged from 64 to 0.03 μg/ml. Antifungal susceptibility was determined by the microdilution method in 96-well microplates as described by the CLSI.[21] An aliquot of 100 μL of the final concentration of antifungal drug and 100 μL of the inoculum test sample were added to each well. The plates were incubated at 37°C and readings were performed after 48 h. The plates with amphotericin B were covered with aluminum foil to protect against light. Result readings were based on the visual scale of turbidity of control tubes represented by: 0 (completely clear), 1 (slightly cloudy), 2 (intermediate turbidity), 3 (prominent turbidity) and 4 (completely cloudy).

For the members of the azole drug class and flucytosine, the MIC were defined as the lowest drug concentration that resulted in 80% growth inhibition. For amphotericin B, this value was defined as the value in which 100% growth inhibition was observed.

The results are expressed as ranges of minimum and maximum MIC values and the values of MIC_{50} and MIC_{90}. These values represent the drug concentration that inhibits the growth of 50% and 90% of the isolates, respectively. *C. parapsilosis* ATCC 22019 was used as a reference for quality control in all the experiments.

The endpoints for fluconazole, flucytosine and ketoconazole considered for the classification of the isolates followed the values of the CLSI[21] represented in Table 1. There were no CLSI endpoints defined for amphotericin B and nystatin.

Results

The number of isolates from both studied groups according to the MIC value for each tested drug is displayed in Table 2.

The results obtained for *Candida* isolates to the drugs tested are presented in Table 3. The values are

Table 1 - Endpoints for fluconazole, flucytosine and ketoconazole (μg/m).

Antifungal agents	Susceptible	S-DD	Intermediate	Resistant
Fluconazole	≥ 8	16 - 32	–	≥ 64
Flucytosine	≤ 4	–	8 - 16	≥ 32
Ketoconazole	≤ 8	–	-	> 16

S-DD: susceptible dose-dependent.

Table 2 - Distribution of *Candida* spp. according to the MIC of each antifungal agent tested.

	Number of isolates inhibited at concentrations (μg/ml) of:											
	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64
Ampho	1	6	4	12	43	15	3	2	0	0	0	0
Fluco	0	0	7	36	42	1	0	0	0	0	0	0
Flucy	0	9	20	27	10	17	1	2	0	0	0	0
Keto	5	41	15	17	0	6	0	2	0	0	0	0
Nyst	0	0	0	0	0	1	66	18	1	0	0	0

Ampho, amphotericin B; Fluco, fluconazole; Flucy, flucytosine; Keto, ketoconazole; Nyst, nystatin.

Table 3 - MIC_{50}, MIC_{90}, range values (μg/ml) and percentage of resistant isolates obtained.

Antifungal drug	MIC_{90}	MIC_{50}	Minimum-maximum	% resistant isolates
Amph	1	0.5	0.03 - 4	–*
Fluco	0.5	0.25	0.125 - 1	0
Fluky	1	0.25	0.06 - 4	0
Keto	0.25	0.06	0.03 - 4	0
Nyst	4	2	1- 8	–*

Amph B, amphotericin B; Fluco, fluconazole; Flucy, flucytosine; Keto, ketoconazole; Nyst, nystatin; MIC_{50}, minimal inhibitory concentration values for 50% of the isolates; MIC_{90}, minimal inhibitory concentration values for 90% of the isolates; *no endpoints defined by the CLSI.[21]

Table 4 - Values of MIC range (μg/ml) for *Candida* spp. according to antifungal drug.

Species		N	MIC range				
			amph	fluco	flucy	keto	nyst
HIV-infected group	*C. albicans*	59	0.06 - 4	0.125 - 1	0.06 - 4	0.03 - 4	1 - 8
	C. glabrata	1	0.5	0.5	2	0.25	2
	C. guilliermondii	1	0.125	0.25	0.125	0.03	4
	C. krusei	1	0.5	0.25	0.125	0.03	4
	C. tropicalis	9	0.03 - 0.5	0.25 - 0.5	0.125 - 0.5	0.06 - 4	2 - 4
Control group	*C. albicans*	11	0.125 - 2 -	0.125 - 0.5	0.06 - 4	0.06 - 0.25	2
	C. tropicalis	4	0.125 - 0.5 -	0.25 - 0.5	0.06 - 1	0.03 - 0.25	2 - 4

N, number of isolates; Amph, amphotericin B; Fluco, fluconazole; Flucy, flucytosine; Keto, ketoconazole; Nyst, nystatin; MIC, minimal inhibitory concentration.

expressed as ranges of MICs, as well as MIC_{50} and MIC_{90} values. The MIC ranges for all the isolates from the control and HIV-infected groups indicate susceptibility to fluconazole, flucytosine and ketoconazole.

The distribution of the species isolates of each group according to the range concentration of the drugs studies is shown in Table 4.

Discussion

Despite the increasing number of commercially available antifungal drugs in recent years, antifungals are still at a disadvantage when compared to antibacterial drugs. Resistance to antifungal drugs also represents a great clinical challenge. Given the difficulties observed in the treatment of fungal infections in some groups of patients, isolation of the causative infectious agent and identification of the susceptibility profile of the yeast isolate are recommended. In this context, screening studies for antifungal resistance are of the utmost importance.

The incidence of clinical fluconazole resistance varies in the literature from 5 - 15%.[14] Lyon *et al.*[22] observed that 90% of *C. albicans*, *C. parapsilosis* and *C. tropicalis* isolates were inhibited by fluconazole at a concentration of 2.0 μg/ml; further, there was no difference between the samples obtained from denture wearers and from patients with natural teeth. However, in *C. glabrata* isolates, the MIC_{90} value reached 8 μg/ml for samples obtained from denture wearers. Interestingly, this study found that all *C. krusei* isolates were resistant. These results contrast with our findings; we found all isolates to be susceptible to fluconazole.

For ketoconazole, all isolates were susceptible in both groups; isolates from HIV-infected patients showed MIC values between 0.03 - 4.0 μg/ml while the MIC values ranged from 0.03 - 0.25 μg/ml for the isolates from control patients. Ketoconazole has been used in the treatment of superficial infections in dentistry patients. However, its use in dentistry is predominately restricted to local administration, as systemic treatment can be highly toxic to the patient.[23] Similar values for azoles have been reported,

as in the study of Wingeter *et al.*[13] They reported that from oral isolates, 72% were sensitive to ketoconazole (MIC < 1 μg/ml), and 86% of the isolates were susceptible to fluconazole (MIC < 8 μg/ml).

There are no endpoints for amphotericin B defined by the CLSI;[21] therefore, we could not classify our results for this drug as susceptible or resistant. However, Sutton *et al.*[24] suggested the values of < 1 μg/ml for an isolate to be considered susceptible and > 2 μg/ml for resistance to amphotericin B as endpoints. If we consider these values for classification, 94.4% of isolates from the HIV-infected group and 93.3% of isolates from the control group were susceptible. Further, this parameter showed that *C. albicans* was the species with the highest level of resistance. This result is in accordance with results published by Rautemaa *et al.*,[25] who reported that most isolates of *C. albicans* were sensitive to amphotericin B. Further, Wingeter *et al.*[13] reported that despite over 50 years of use of polyene antifungal drugs, resistance to amphotericin B is rare. However, in their study 2 of 57 isolates showed MIC > 2 μg/ml for amphotericin B, indicating resistance. Batista *et al.*[26] studied isolates from patients with total dentures and observed good fungistatic activity of amphotericin B with MIC values ranging from 0.03 - 0.15 μg/ml. Lyon *et al.*[22] reported a MIC_{90} value of 1.0 μg/ml for all isolates, regardless of whether they were taken from denture wearers or individuals with natural teeth.

Alves *et al.*[27] found that among isolates susceptible to fluconazole, the activity of flucytosine was highest against *C. albicans* (84.6%) and *C. glabrata* (94.1%). Additionally, they reported that 40% of *C. krusei* isolates were susceptible to flucytosine. Alves *et al.*[27] have argued that the great potential of flucytosine against *C. glabrata* has not been sufficiently explored and that this antifungal may be used as a treatment option when resistance to azoles is observed. In our study, the *C. glabrata* isolate was susceptible to fluconazole and flucytosine.

In a study evaluating oral isolates from patients with periodontal disease,[12] it was observed that all isolates were susceptible to amphotericin B and fluconazole. However, the same was not observed for ketoconazole; one isolate of *C. albicans* and *C. glabrata* from the periodontitis group and one isolate of *Candida* spp. from the control group were resistant. In the same study, only one isolate of *C. albicans* from the periodontitis group was resistant to flucytosine. In our study, while all isolates were susceptible to the azole drugs, strains of *C. albicans* showed a wide variation in MIC values. This finding was especially prominent in isolates from the HIV-infected group.

The antifungal drug nystatin has been reported in the literature to be widely used in fungal infections in the oral cavity.[28] In this study, the range of MICs for nystatin was similar to those reported by Hamza *et al.*;[29] they reported intervals between 2 - 16 for *C. albicans* and 2 - 4 for *C. glabrata* and *C. tropicalis*. Wingeter *et al.*[13] adopted the following end points: ≤ 4, susceptible; 8 - 32, susceptible dose-dependent; and ≥ 64, resistant. In our study, only one isolate from the HIV-infected group had a MIC value of 8 μg/ml; all other isolates were susceptible to nystatin. This result is in accordance with a previous study that reported that 95% of isolates were susceptible.[13]

Conclusion

Our results demonstrate that the tested antifungal agents showed good activity for most isolates of both groups; however, variability found among isolates and resistance to antifungal agents confirmed the importance of susceptibility tests as a guide to the therapeutic prescription of antimycotics.

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