Original Article

Distributions and Antifungal Susceptibility of Candida Species from Mucosal Sites in HIV Positive Patients

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Abstract:

Background: Mucocutaneous candidiasis (almost endogenous) is one of the most common manifestations of human immunodeficiency virus (HIV) infection. The aim of this study was the investigation of colonization patterns of *Candida* species, particularly *C. dubliniensis*, among mucosal sites of HIV-positive patients and determining corresponding *in vitro* susceptibility patterns to the antifungals.

Methods: From July 2006 to May 2008, specimens from the mucosal sites of 273 seropositive HIV patients were collected for *Candida* colonization. All isolates were identified by standard methods and carbohydrate assimilation patterns. Isolates phenotypically identified as *C. albicans* or *C. dubliniensis* were subjected to molecular identification. Susceptibility patterns of the isolated species to seven antifungal agents were determined using the broth microdilution method.

Results: The 359 samples from mucosal sites which consisted of 273 oral and 86 vaginal were collected and evaluated for *Candida* species distributions and their corresponding susceptibility patterns. The most commonly isolated species were: *C. albicans* (50%) followed by *C. glabrata* (21.4%), *C. dubliniensis* (13.3%, reported for the first time in Iran), *C. krusei* (9.8%), C. *kefyr* (3.1%), *C. parapsilosis* (1.6%), *and C. tropicalis* (0.8%). All species were sensitive to amphotencin B, ketoconazole, nystatin, voriconazole, and caspofungin. In some isolates, resistance to fluconazole and itraconazole was noted.

Conclusion: As demonstrated, resistance to fluconazole and itraconazole, the most frequent antifungals in use in the region suggests regular investigation into antifungal resistance in medical centers should be undertaken in order to promote the effective management of invasive candidiasis in HIV/AIDS patients.

Keywords: *C. albicans, C. dubliniensis,* fluconazole, Mucocutaneous candidiasis

Introduction

Mucocutaneous candidiasis is one of the most common manifestations of human immunodeficiency virus (HIV) infection and is clearly related to the development of clinical cellular immunodeficiency. There are three forms of mucocutaneous candidiasis that are common in HIV infection: oropharyngeal, esophageal, and vulvovaginal. The occurrence of mucocutaneous candidiasis is common, with up to 90% of cases with advanced disease that develop oropharyngeal infection,[1,2] 27 to 60% of women develop vaginal candidiasis, and 10 to 20% develop esophageal disease.[3] Oral microbial flora is remarkably stable in healthy subjects, but significant changes may occur during serious systemic disease and its treatment.[4] In fact, oropharyngeal candidiasis is an independent predictor of immunodeficiency in patients with acquired immunodeficiency syndrome (AIDS), which increases the morbidity and mortality among such patients, and therefore,

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requires prompt diagnosis and adequate therapy. Chronic, refractory vaginal candidiasis is common in women with HIV infection. Antifungal agents can effectively treat mucosal candidiasis; however, their use can lead to the colonization with less susceptible species among normally susceptible strains.

In Iran, *in vitro* antifungal testing is not routinely performed. Lack of comprehensive studies on the antifungal susceptibility patterns of *Candida* species from HIV positive patients in the region has prompted this study which aims at characterizing the colonization patterns of *Candida* species, particularly *C. dubliniensis*, among oral and vaginal fungal isolates of HIV-positive patients and determining *in vitro* susceptibility patterns to the antifungal agents.

Patients and Methods

During a cross-sectional study, from July 2006 to May 2008, 273 seropositive HIV patients (diagnosed by ELISA and Western-blot) were identified for *Candida* species colonization, presence of infection and evaluated for *in vitro* susceptibility to seven antifungal agents in the Consultation and Behavioral Modification Center (CBMC), Shiraz University of Medical Sciences, Shiraz, southern Iran. None of the patients received prophylaxis treatment. Demographic data of the patients were obtained through questionnaires.

Upon admission to the study, oral and gynecological examinations were performed by trained clinicians to diagnose oropharyngeal and genital tract conditions. Specimens from the oropharynx and vagina were collected at CBMC by sterile rayon-tipped applicator stick swabs and cultured on sabouraud dextrose agar (MERCK, Darmstadt, Germany) supplemented with chloramphenicol (50 mg/L). Specimens were transferred to the Professor Alborzi Clinical Microbiology Research Center (PACMRC) and incubated at 35°C. After 48 – 72 hours, colonies were re-cultured for purity onto potato dextrose agar (OXOID, Ltd., Hampshire, England) and incubated at 35°C for an additional 48 hours. Different colony morphologies were noted, and single isolates of each colony type were sub-cultured onto sabouraud dextrose agar slants.

Species identification of the isolates was performed by standard methods (germ-tube formation, cornmeal for blastoconidia, pseudohyphae, and true hyphae, as well as clamydospore production and growth on HiCrome Candida agar) and by the API 20 C system (bioMérieux, France) for sugar assimilation. Isolates phenotypically identified as *C. albicans* or *C. dubliniensis* were subjected to molecular identification.[5]

Susceptibility patterns of the isolates to fluconazole, amphotericin B, ketoconazole, and nystatin (SIGMA-Aldrichemie GmbH-Steinheim, Germany), itraconazole (Jenssen Pharmaceutical, Beerse, Belgium), voriconazole (Pfizer, United Kingdom), and caspofungin (Merck & Co., Inc., NJ, USA) were determined by broth microdilution assay according to the Clinical and Laboratory Standard Institute (CLSI).[6] Stock solutions of the drugs were prepared in di-methyl sulfoxide or water.

Two CLSI quality control strains, *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258, were tested each time a set of clinical isolates was evaluated.[7] The yeast suspension and antifungal dilutions were prepared according to CLSI M27-A2 guidelines.[6] Final concentrations of amphotericin B ranged from 8 to 8 to 0.016 µg/mL, fluconazole from 128 to 0.250 µg/mL, nystatin from 37 to 0.07 µg/mL, and itraconazole, ketoconazole, and voriconazole from 16 to 0.032 µg/mL. One control positive with no drugs and one negative control with no fungal suspensions were used in each series. The plates were sealed and incubated at 35°C for 24 and 48 hours. Finally, visual MIC end-points were determined with the aid of a mirror. Visual end-points were determiners as described in the CLSI – M27-A2 that recommended an end-point for azoles as the lowest drug concentration with a prominent decrease in turbidity (inhibitory concentration that gives 50% growth reduction), while for amphotericin B and nystatin the MIC is the drug concentration showing complete inhibition of growth.

The amount of growth in each tube was compared to the growth of the positive control. Antifungal activity was expressed as the MIC of each isolate to the drug. The following resistance breakpoints were used according to CLSI guidelines[6] or based on previous investigations.[8,9,10]

MIC_{50} (the MIC at which 50% of the isolates are inhibited) and MIC_{90} (the MIC at which 90% of the isolates are inhibited) were also calculated. Data were entered into SPSS version 11.5 and were subsequently analyzed using descriptive statistics and

cross tabulation.

Ethical considerations

The Ethics Committee of the Clinical Microbiology Research Center, Shiraz University of Medical Sciences, reviewed and approved the study prior to patient participation. Patients were given the opportunity to sign approved, written consents before participating in the study.

Results

The 359 samples from mucosal sites, 273 oral and 86 vaginal, were collected from 273 HIV-positive patients and were evaluated for *Candida* species distributions and their corresponding susceptibility patterns. The female-to-male ratio was 86:187 and the mean age of the patients was 36.8 years (20 – 71 years). Risk factors for HIV infection were injectable drug use (79% of the cases), heterosexuality (20%) and transfusion (1%). There were 89 (32.6%) single patients, 101 (36.9%) married, and 83 (30.5%) who were separated. Of these patients, 51 were symptomatic, which consisted of 33 patients with thrush and 18 with vaginal candidiasis. The remainder were asymptomatic (Table 1).

Overall, 195 (71.5%) species were isolated from the mouth and 39 (45.5%) from the vagina (total 234 *Candida* species). The median CD4 count was 342 (range: 32 to 1371).

The most abundant species isolated from patients (total 234 isolates) were: *C. albicans* (50%), *C. glabrata* (21.4%), *C. dubliniensis* (13.3%), *C. krusei* (9.8%), C. *kefyr* (3.1%), *C. parapsilosis* (1.6%), and *C. tropicalis* (0.8%) (Table 1). All species were sensitive to amphotencin B, ketoconazole, nystatin, voriconazole, and caspofungin. In some isolates, resistance to fluconazole and itraconazole were evident. MICs of the species are shown in Table 2. Our results show that 10% of *C. albicans,* 17.4% of *C. krusei* and 4.5% of *C. dubliniensis* isolates were resistant to fluconazole (MICs ≥64 μg/mL). A total of 8% of *C. albicans*, 60% of *C. glabrata*, 39% of *C. krusei*, and 9.5% of *C. dubliniensis* were resistant to itraconazole (MIC_{90} itraconazole ≥1 μg/mL).

Discussion

Candida is cited as an important cause of morbidity and mortality among patients with AIDS, those undergoing bone marrow transplantation, and those who have received aggressive anti-neoplastic therapy.[11] Oral candidiasis occurs in more than 95% of AIDS patients and is considered to be an important marker of the disease and its progression.[1,2] Severity of cellular immunodeficiency in HIV infection is closely correlated with *Candida* colonization of the oral and vagina mucosal surfaces and the development of symptomatic candidiasis.[12]

Culture media used for the differentiation between *C. albicans* and *C. dubliniensis* are useful for phenotypical screening, but definitive identification still requires genotyping techniques.[13] Accordingly, the present study used a molecular method (PCR) to identify *C. dubliniensis*. Some studies have revealed the presence of *C. dubliniensis* in several parts of the world. Milan et al. (2001) evaluated 108 Brazilian patients with AIDS that had oropharyngeal candidiasis and reported a total of three patients were positive for *C. dubliniensis*.[14] Chavasco et al. (2006) observed approximately 5.4% of individuals positive for this species amongst HIV-negative and HIV-positive patients with erythematous oral candidiasis.[15] To the best of our knowledge, the pres-

Table 1. Yeasts isolated from mucosal sites of HIV-positive patients

Yeast species	Oral Number (%)		Positive symptoms	Vaginal Number (%)	Positive symptoms
	M	F			
C. albicans	58	38	22	21 (53.9)	13
C. glabrata	42	3	4	4 (10.1)	1
C. krusei	22	1	2	0	0
C. dubliniensis	8	11	3	10 (25.6)	3
C. kefyr	3	2	1	2 (5.1)	0
C. parapsilosis	2	1	1	2 (5.1)	1
C. tropicalis	1	1	0	0	0
Total	195 (100)		33	39 (100)	18
F=Female; M=Male					

Table 2. Antifungal susceptibilities of clinical yeast isolates as determined by NCCLS microdilution reference broth method

Organisms	Antifungal agent	Frequency		MIC (μg/mL)		
		R*	S*	Range	50%	90%
C. albicans (117)	Amphotericin B	0	117	0.032–0.250	0.032	0.13
	Fluconazole	12	105	0.132–32	0.50	4
	Itraconazole	10	107	0.125–2	0.50	1
	Voriconazole	0	117	0.032–0.250	0.125	0.250
	Ketoconazole	0	117	0.032–0.5	0.032	0.064
	Nystatin	0	117	0.14–18.5	0.58	1.15
	Caspofungin	0	117	0.032–0.250	0.032	0.132
C. krusei (23)	Amphotericin B	0	23	0.032–0.250	0.032	0.50
	Fluconazole	4	19	4–64	32	64
	Itraconazole	9	14	0.032–4	0.125	1
	Voriconazole	0	23	0.032–0.25	0.125	0.250
	Ketoconazole	0	23	0.032–0.132	0.032	0.064
	Nystatin	0	23	0.290–18.5	0.29	1.15
	Caspofungin	0	23	0.032–0.250	0.064	0.132
C. glabrata (49)	Amphotericin B	0	49	0.125–1	0.125	0.250
	Fluconazole	0	49	8–64	32	64
	Itraconazole	29	20	0.064–16	0.5	1
	Voriconazole	0	49	0.032–1	0.132	0.250
	Ketoconazole	0	49	0.032–0.5	0.032	0.132
	Nystatin	0	49	0.14–2.30	0.29	1.15
	Caspofungin	0	49	0.032–0.500	0.125	0.125
C. kefyr (7)	Amphotericin B	0	7	0.125–0.50	0.125	0.250
	Fluconazole	0	7	0.250–1	0.250	0.50
	Itraconazole	0	7	0.064–0.50	0.250	0.500
	Voriconazole	0	7	0.032–0.064	0.032	0.064
	Ketoconazole	0	7	0.032–0.064	0.032	0.064
	Nystatin	0	7	0.29–0.58	0.29	0.58
	Caspofungin	0	7	0.032–0.064	0.032	0.032
C. dubliniensis (31)	Amphotericin B	0	31	0.125–1	0.250	0.50
	Fluconazole	2	29	0.132–0.500	0.250	0.500
	Itraconazole	3	28	0.064–2	0.250	0.500
	Voriconazole	0	31	0.032–0.125	0.032	0.125
	Ketoconazole	0	31	0.032–0.064	0.032	0.064
	Nystatin	0	31	0.14–1.15	0.58	0.58
	Caspofungin	0	31	0.032–0.250	0.032	0.064
Others *(7)	Amphotericin B	0	7	0.125–0.250	0.0.250	0.250
	Fluconazole	0	7	0.125–0.25	0.125	0.25
	Itraconazole	0	7	0.250–0.500	0.250	0.250
	Voriconazole	0	7	0.032–0.064	0.032	0.064
	Ketoconazole	0	7	0.032–0.064	0.032	0.064
	Nystatin	0	7	0.280–0.560	0.280	0.560
	Caspofungin	0	7	0.032–0.64	0.0.32	0.032
Total (234)	Amphotericin B	0	234	0.032–1	0.125	0.250
	Fluconazole	18	216	0.125–64	16	32
	Itraconazole	51	183	0.032–16	1	2
	Voriconazole	0	234	0.032–1	0.064	0.125
	Ketoconazole	0	234	0.032–0.5	0.064	0.130
	Nystatin	0	234	0.14–2.30	0.56	1.12
	Caspofungin	0	234	0.032–0.50	0.064	0.125

Others : [5] *C. parapsilosis*, [2] *C. tropicalis*; * R=resistant. S=susceptible

ent study is the first one reporting C. *dubliniensis* in HIV-positive patients in Iran.

Research by Sobel et al. (2001) who noted that among oral and vaginal isolates, *C. albicans* was the most frequently identified followed by *C. glabrata*, *C. tropicalis, C. parapsilosis,* and *C. krusei*.[16] Similarly, in a study by Omar et al. (2008), *C. albicans* was the most frequently isolated species in 84.5% of 250 patients, followed by *C. glabrata* (6.8%), and *C. krusei* (3.4%).[16] Differences in species distributions of *Candida* could also have therapeutic implications because species which are less susceptible to fluco-

nazole, such as *C. glabrata* and *C. krusei,* play an important role in the management of AIDS patients.

The prolonged management of mucosal candidiasis might cause the development of drug-resistant infections and there have been reports of the emergence of resistance to antifungal agents in HIV/AIDS patients. Refractory clinical oral and vaginal candidiasis due to azole-resistant *Candida* species has emerged and continues to be a major clinical problem in AIDS sufferers.[16–18]

Fluconazole has been widely used for the treatment of mucosal candidiasis because of its low toxicity and ease of administration. Magaldi et al. (2001) reported that 9.8% and 4.9% of *C. albicans* isolated from patients with no previous treatment were resistant to fluconazole and itraconazole, respectively which increased to 44.7% and 44.15% after treatment with these drugs.[19] These data suggest that continued use of antifungal agents, particularly fluconazole, may lead to clinical treatment failure which is significantly correlated with reduced susceptibility to fluconazole, itraconazole, and other azoles.[20] Fluconazole resistance in *C. albicans* occurs most often in patients with low CD4 counts who are taking fluconazole prophylactically for recurrent thrush.

In immunocompromised patients, non-albicans candidiasis such as *C. glabrata* that are intrinsically resistant to fluconazole are on the rise.[21] This can be considered important since infections caused by *C. albicans* generally have the best prognosis in comparison to those caused by non-albicans species. *C. glabrata* and *C. krusei* remain the least susceptible species to fluconazole and because of cross resistance between azole drugs, they have high MICs to other azoles.[22] It is important for empirical therapy in HIV patients that an anti-fungal with low MIC and high activity for the *Candida* species be considered. According to Table 2, amphotericin B, voriconazale, and caspofungin which have the lowest MICs seem to be suitable drugs for empirical therapy. Nystatin could be an antifungal agent of choice to eradicate the colonization of *Candida* in oral lesions. Based on our data, MIC_{90} in the total isolated *Candida* for voriconazole and caspofungin is 0.125 µg/mL. Therefore, it is advisable to use these drugs as second-line therapy for the mucosal candidiasis which is resistant to fluconazole or for empiric therapy as reported in other studies.[23,24] The second generation of azoles has shown improved activities against fluconazole-resistant *Candida* species. As revealed in the present study, caspofungin was the most active agent against all *Candida* species except for one isolate of *C. parapsilosis,* which had an MIC of 2.0 µg/mL.

Conclusion

In summary, the surveillance of antifungal resistance patterns and the spectrum of *Candida* species in HIV/AIDS patients can provide important information about significant differences in different populations in terms of species distribution and susceptibility patterns. Infections with *Candida* are almost endogenous; therefore, identification of the species and corresponding susceptibility patterns to antifungal agents can be helpful for the management of these infections. As demonstrated, resistance to fluconazole and itraconazole, the most frequent antifungals used in the region, could be suggestive of the need for regular investigations into antifungal resistance in medical centers in order to more efficiently manage invasive candidiasis in the above-mentioned patients, particularly AIDS sufferers.

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