Original Article

Emergence of azole-resistant *Candida* species in AIDS patients with oropharyngeal candidiasis in Iran

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Abstract

Background and Purpose: Oropharyngeal candidiasis (OPC) and antifungal drug resistance are major health concerns in patients with human immunodeficiency virus (HIV). The increased reports of antifungal resistance and expanding drug therapy options prompted the determination of antifungal susceptibility profile. The present study was performed to determine the antifungal susceptibility of *Candida* species isolated from AIDS patients with OPC in Iran.

Materials and Methods: In total, 100 *Candida* isolates from the oral cavity of patients with OPC (TCD4 < 200 cells/µL) were obtained and cultured on CHROMagar and Sabouraud's dextrose agar. All isolates were identified according to the assimilation profile, colony color and other conventional methods. Broth microdilution of antifungal drugs was carried out, according to the methods described in M27-S4 and M44-A guidelines by the Clinical and Laboratory Standards Institute (CLSI).

Results: Among 60 *Candida albicans* (*C. albicans*) strains, 56.7% were resistant to fluconazole, while 38.3% were resistant to ketoconazole and clotrimazole. The resistance of *C. albicans* isolates against polyene antifungals including amphotericin B was scarce (1.7%). Based on the results, 52.2% of *C. glabrata* strains were resistant to fluconazole, while 47.8% and 30.4% of these isolates were resistant to ketoconazole and clotrimazole, respectively. All *Candida* isolates were susceptible to nystatin and caspofungin.

Conclusion: Based on the findings, it can be concluded that screening of resistant *Candida* isolates by disk diffusion or broth dilution method is essential for the surveillance and prevention of antifungal resistance in patient management. Although nystatin is widely used in clinical practice for HIV patients in Iran, no evidence of enhanced resistance against this agent was found; on the other hand, resistance to azole antifungals, particularly fluconazole, increased. Considering the lack of resistance to caspofungin, administration of this agent is suggested for the treatment of OPC in AIDS patients.

Keywords: Azoles, *CandidA*, Oral candidiasis

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Introduction

Oropharyngeal candidiasis (OPC) is regarded as the most common opportunistic fungal infection in patients with human immunodeficiency virus (HIV) and other immunocompromised hosts. The oropharyngeal cavity, which is the major host of fungal infections in HIV patients, is usually colonized by *Candida* species [1].

Antifungal drug resistance is a major concern in immunocompromised patients. The increased occurrence of antifungal resistance on one hand and the expansion of therapeutic options on the other have promoted the determination of antifungal susceptibility profiles of different fungi. Despite the introduction of various antifungal agents and highly-active antiretroviral therapy (HAART), OPC, particularly the refractory type, is common among HIV patients.

The incidence of OPC is influenced by different factors such as etiological *Candida* species, prevalence of *Candida* resistance, prior experience of antifungal therapies and the status of the host immune system [2-4]. Several studies have assessed the susceptibility of *Candida* species, using samples obtained from the oral cavity of HIV patients, immune-compromised individuals with OPC. Moreover,

the prevalence of resistant *Candida* species resistant against antifungal agents has been studied among Iranian HIV patients. However, in the mentioned studies, HIV-positive patients with and without the clinical symptoms of OPC were included.

It is evident that the susceptibility profile of *Candida* species, isolated from HIV patients with and without the clinical signs of OPC, is invaluable for prophylaxis and treatment. On the other hand, OPC is an independent predictor of immunodeficiency in AIDS patients, which increases the rates of morbidity and mortality among these patients; therefore, prompt diagnosis and adequate therapy are highly required.

The increasing amplitude and expansion of antifungal resistance on one hand and the advent of new antifungal agents on the other have reignited interest in antifungal susceptibility tests. In this regard, simple disk diffusion method has been developed for evaluating the susceptibility of *Candida* isolates against antifungal agents. Despite some difficulties in identifying the exact resistance of antifungal clinical isolates, the disk diffusion method can be a useful means for simple and cost-effective monitoring of antifungal susceptibility in a variety of laboratory settings [5-7].

In vitro antifungal testing is not routinely performed in clinical laboratories of Iran. Considering the scarcity of comprehensive studies on the antifungal susceptibility patterns of *Candida* species from HIV patients, the present study was performed to determine the antifungal susceptibility of *Candida* isolates, obtained from Iranian HIV-positive patients with TCD4 < 200 cells/µl (a diagnostic criterion for AIDS) and oral lesions during 2008-2010. In the present study, for optimized identification of resistance against antifungal agents, disk diffusion and broth microdilution methods were applied.

Material and Methods

Isolates and growth on cultures

In total, 100 *Candida* isolates were obtained from AIDS patients with apparent OPC (HIV-positive patients with TCD4 < 200 cells/µl), referring to Imam Khomeini Hospital, Tehran, Iran. Prior use of antifungal agents was noted in subjects, as patients (70%) had a prior history of fluconazole therapy.

Oral lesions were clinically diagnosed in each subject and oral specimens were obtained by clinicians, using sterile cotton swabs from the lesions, tongue or buccal mucosa. A wet mount with 10% potassium hydroxide was used for the microscopic examination of pseudohyphae and yeast cell forms. The swabs were directly spread on Sabouraud's dextrose agar plates (Merck, Germany) and CHROMagar™ *Candida* (CHROMagar, Paris, France).

The CHROMagar plates were used for primary diagnosis and differentiation of *Candida* isolates. The Sabouraud's dextrose agar plates were used for yeast isolates as they lacked the ability to grow on CHROMagar; therefore, they the isolates were incubated aerobically at 30 °C for 7 days. On the other hand, CHROMagar cultures were incubated at 35 °C for 72 hr in the dark. After the incubation, the yeasts were identified, based on their morphological features (form and color of colony on CHROMagar) and growth parameters such as carbohydrate assimilation were evaluated by Rap ID™ yeast identification system (Remel Inc., USA) [8, 9].

Susceptibility testing

Disk diffusion testing of six antifungal agents including fluconazole (25 µg), ketoconazole (15 µg), clotrimazole (10 µg), nystatin (50 µg), amphotericin B (10 µg) and caspofungin (5 µg) was performed, according to the methods described in Clinical and Laboratory Standards Institute (CLSI) M44-A document [10]. In addition, antifungal disks were obtained from Mast Diagnostics (Mast Group Ltd, UK).

Mueller-Hinton agar plates (150 mm in diameter), supplemented by 2% glucose and methylene blue at a depth of 4.0 mm were used. The agar surface was inoculated, using a swab moistened in a cell suspension, adjusted to 0.5 McFarland turbidity standards by the spectrophotometer at 530 nm. The plates were incubated at 35 °C and read within 24 hours. Zone diameter endpoints were read at

approximately 80% growth inhibition. The published interpretive criteria by CLSI were applied in the disk diffusion method [11].

The minimum inhibitory concentrations (MICs) of fluconazole, ketoconazole, clotrimazole, nystatin, amphotericin B and caspofungin were determined, using the microdilution method, as described by CLSI M27-S4 document via applying morpholine-propanesulfonic acid (MOPS)-buffered RPMI 1640 at pH=7 [12, 13]. The final concentrations of amphotericin B, nystatin and caspofungin ranged from 8 to 0.03µg/ml. Moreover, the final concentrations ranged from 128 to 0.5µg/ml in fluconazole and 16 to 0.03µg/ml in clotrimazoleand ketoconazole.

The broth microdilution plates were sealed and incubated at 35 °C for 24 and 48 hours, respectively. Finally, visible MIC endpoints were determined with the aid of a mirror. The MIC_{90} was defined as the minimum concentration, required to inhibit the growth of 90% of isolates.

CLSI has recommended an interpretive susceptibility criterion. Accordingly, fluconazole resistance was defined as MIC ≥ 8µg/ml. In *C. glabrata* isolates, fluconazole MIC > 64 µg/ml was indicative of resistance. Ketoconazole and clotrimazole resistance was identified as MIC ≥ 1 µg/ml. Moreover, MIC breakpoints ≤ 2.0 µg/ml indicated susceptibility to amphotericin B and nystatin, while MIC > 2.0 µg/ml suggested the resistance of the isolates [14, 15]. With regard to caspofungin, susceptibility and non-susceptibility were defined as MIC breakpoints ≤ 1 µg/ml and >1 µg/ml, respectively, as previously described and approved by CLSI [14-16].

Data were entered into SPSS version 11.5 and were subsequently analyzed, using descriptive tests and cross tabulation.

Results

C. albicans (60%), *C. glabrata* (23%), *C. tropicalis* (5%), *C. dubliniensis* (5%), *C. parapsilosis* (3%), *C. kefyr* and *C. krusei* (2%) were isolated from AIDS patients with OPC. The *in vitro* susceptibilities of 100 oral *Candida* isolates to fluconazole, clotrimazole, ketoconazole, amphotericin B and nystatin are summarized in Table 1.

According to broth microdilution (CLSI M27-S4 document) of 60 tested *C. albicans* isolates, 56.7% were resistant to fluconazole (MIC ≥ 8 µg/ml), while 38.3% were resistant to ketoconazole and clotrimazole, respectively. The MIC values obtained via broth microdilution method in isolates, which were representative of the most frequently isolated *Candida* species, are presented in Table 1. MIC_{50} and MIC_{90} of fluconazole for *C.*

Table 1. Susceptibility and minimum inhibitory concentration (MIC) of *Candida* species obtained from AIDS patients with oropharyngeal candidiasis (OPC) in Iran

Antifungal Drugs	Species (n)	MICs Obtained via broth microdilution method based on M27-S4*					Disk diffusion method based on M44-A* (%)		
		MIC Range (µg/ml)	Geometric Mean (µg/ml)	MIC_{50} (µg/ml)	MIC_{90} (µg/ml)	Resistance (%)	S	S-DD	R
Amphotericin B	*C. albicans* (60)	0.03-4	0.23	0.125	2.0	1.7	96.6	1.7	1.7
	C. glabrata (23)	0.03-1.0	0.13	0.125	0.5	0	91.3	8.7	0
	C. species** (17)	0.03-0.25	0.11	0.125	0.25	0	94.1	5.9	0
Nystatin	*C. albicans* (60)	0.06-1.0	0.19	0.125	1.0	0	98.3	1.7	0
	C. glabrata (23)	0.06-0.250	0.3	0.125	0.5	0	100	0	0
	C. species (17)	0.06-0.250	0.11	0.125	0.25	0	100	0	0
Fluconazole	*C. albicans* (60)	1-128	10.3	8	64	56.7	58.3	8.3	33.4
	C. glabrata (23)	2-128	33.9	64	128	52.2	30.4	8.7	60.9
	C. species (17)	1-128	3.1	2	8	23.5	76.4	5.9	17.7
Ketoconazole	*C. albicans* (60)	0.06-8	0.76	0.5	2.0	38.3	63.3	8.3	28.4
	C. glabrata (23)	0.06-8	0.34	0.125	4.0	47.8	47.8	8.7	43.5
	C. species (17)	0.06-2	0.25	0.125	2.0	23.5	82.3	11.8	5.9
Clotrimazole	*C. albicans* (60)	0.06-8	0.75	0.5	2.0	38.3	65	8.3	26.7
	C. glabrata (23)	0.03-8	0.37	0.25	4.0	30.4	47.8	8.7	43.5
	C. species (17)	0.125-2	0.32	0.125	2.0	29.5	76.5	17.6	5.9
Caspofungin	*C. albicans* (60)	0.06-1.0	0.18	0.125	0.5	0	98.3	1.7	0
	C. glabrata (23)	0.06-0.250	0.14	0.125	0.5	0	100	0	0
	C. species (17)	0.06-0.250	0.11	0.125	0.25	0	100	0	0

S: Susceptible, R: Resistant, S-DD: Dose-dependent susceptibility
*Interpretation of test results is based on M27-S4 and M-44A guidelines broth microdilution and disk diffusion methods, respectively.
**C. dubliniensis (5), C. tropicalis (5), C. parapsilosis (3), C. kefyr (2), C. krusei (2)

albicans isolates were reported to be 8 and 64 µg/ml, respectively. MIC_{50} and MIC_{90} of clotrimazole and ketoconazole for *C. albicans* were 0.5 and 2 µg/ml, respectively. Based on the disk diffusion method (M-44A), 33% of *C. albicans* isolates were resistant against fluconazole (MIC ≥ 64 µg/ml).

The resistance of *C. albicans* isolates against polyene antifungals, including amphotericin B and nystatin, was scarce; overall, 1.7% of the isolates were resistant to amphotericin B. All *C. glabrata* and other *Candida* isolates were sensitive to amphotericin B. Based on the findings, *C. glabrata* was the second most common species isolated in this study. The antifungal susceptibility evaluation of *C. glabrata* showed that 52% of the isolates were resistant to fluconazole (MIC_{50}=64 µg/ml and MIC_{90}=128 µg/ml).

In contrast, 47% and 38% of the isolates were resistant to ketoconazole and clotrimazole, respectively. The resistance of *Candida* species to azoles was significantly different between patients receiving azole antifungal drugs and those not using these agents ($P<0.05$). All *Candida* isolates were sensitive to nystatin and caspofungin.

Discussion

In the present study, the antifungal susceptibility evaluation of AIDS patients with OPC showed that the emergence of antifungal-resistant *Candida* species is of pivotal importance in the epidemiology of OPC in Iran. Considering the treatment challenges, induced by antifungal resistance, the susceptibility profiles and the emerging resistance need to be determined.

In the present study, disk diffusion and broth microdilution methods were applied for 100 *Candida* isolates, obtained from AIDS patients in Iran. This is in fact the most recent research on fungal susceptibility to antifungals, performed on AIDS patients in our country. Previous studies in this field have evaluated HIV-positive patients with different conditions, and a diversity of non-*albicans Candida* species has been reported including *C. tropicalis*, *C. parapsilosis*, *C. krusei*, *C. glabrata* and *C. dubliniensis* [17].

Based on various international studies, *C. albicans* is regarded as the first most common *Candida* species, isolated from AIDS patients. On the other hand, the isolation rate of *C. glabrata* is exceptional. Based on multiple studies in different countries, the prevalence of *C. glabrata* varies in HIV patients. *C. glabrata* has been reported as the first or second most prevalent non-*C. albicans* species in HIV patients [18, 19]. It should be mentioned that colonization in *C. glabrata* increases after fluconazole treatment [20].

Fluconazole resistance, which is associated with prolonged use of azoles, has been well documented by various researchers [13, 21]. Nevertheless, fluconazole was speculated to show poor efficacy against *Candida* isolates, especially *C. albicans* isolates, as 56.7% of these isolates demonstrated resistance against fluconazole; this finding was not in consistence with previous studies. However, many studies have reported a lower incidence of fluconazole resistance, compared to the present study [22, 23]. In this regard, according to a study by Yenisehirli *et al*., the resistance of *C. albicans* isolates to ketoconazole and fluconazole was estimated at 32% and 34%, respectively [13].

Resistance to fluconazole is often observed in patients with a prior history of the use of fluconazole for the prevention of infections or previous episodes of OPC. In this study, the majority of AIDS patients had received previous antifungal agents, especially fluconazole and nystatin. The prolonged management of mucosal candidiasis might lead to the development of drug-resistant infections. In fact, there have been reports on the emergence of resistance against antifungal agents in HIV/AIDS patients. Emergence of clinical refractory OPC, induced by azole-resistant *Candida* species, has been previously reported and introduced as a major clinical problem in AIDS patients [24].

Generally, resistance to amphotericin B is extremely uncommon [25]. In this study, the majority of *Candida* isolates were susceptible to amphotericin B and nystatin; however, a low rate of resistance in *C. albicans* was observed; this finding was consistent with previous studies. The incidence of amphotericin B-

resistant *Candida* isolates was low in many studies. In studies by Blignaut *et al.* [18], Badiee *et al*., [26] and Mokaddas *et al.* [27], 8.4%, <1% and <3% of *C. albicans* isolates were resistant to amphotericin B, respectively.

In contrast with the findings reported in previous studies, although the prescription of nystatin was common in many evaluated patients, no resistance was observed against this agent and nystatin showed good efficacy in the prevention of oral candidiasis. However, local utilization and lack of systemic absorption hampered inhibition of systemic diseases and esophageal disorders.

Unfortunately, considering the challenges hampering the detection of novel antifungal agents, patients have no choice but to use fluconazole therapy. Physicians should pay particular attention to the prescription of novel antifungal agents, and involved organizations should employ different approaches for the production of new antifungal drugs in Iran.

Comparison between disk diffusion and microdilution methods showed acceptable agreement in the results, with no significance difference. It should be mentioned that the results obtained by disk diffusion method are interpreted, based on previous CLSI antifungal clinical breakpoints for resistance against fluconazole.

In the current study, we presented data on species distribution and antifungal susceptibility profiles of *Candida* isolates, obtained from AIDS patients at the Iranian Research Center for HIV/AIDS of Imam Khomeini Hospital, affiliated to Tehran University of Medical Sciences, Iran, within a three-year period. Non-*C. albicans* yeast species constituted 40% of the isolates, which is an alarming rate in epidemiology of OPC in Iranian patients. Differences in *Candida* species distributions could also have therapeutic implications species, which are less susceptible to fluconazole (e.g., *C. glabrata*), play an important role in the management of AIDS patients.

Conclusion

Based on the findings, it can be concluded that screening of resistant *Candida* isolates by disk diffusion or broth dilution method in clinical laboratories is useful and necessary for surveillance and prevention of antifungal resistance to in patient management. Although nystatin is widely used in clinical practice for HIV patients in Iran, no evidence of enhanced resistance was found against this agent; on the other hand, resistance to azole antifungals, especially fluconazole, increased. The second generation of caspofungin was the most active agent against all *Candida* species; therefore, its administration is suggested for the treatment of OPC in AIDS patients.

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Authors' Contributions

F.K. designed and supervised the research, F.K. and M.S. edited the final manuscript and F.T. and F.K. performed the tests.

Conflicts of Interest

The authors declare no conflicts of interest.

Financial Disclosure

The authors declare no financial interests related to the materials of the study.

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