Original Contribution

OROPHARYNGEAL CANDIDIASIS AND ANTIFUNGAL ASSESSMENT OF CANDIDA GLABRATA IN PATIENTS WITH HIV INFECTION

A. Sharifzadeh[1], H. Shokri[2*]

[1]Mycology Research Center, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran
[2]Faculty of Veterinary Medicine, Amol University of Special Modern Technologies, Amol, Iran

ABSTRACT

The purposes of this study were to determine the prevalence of oral mycoflora in HIV[+] patients and to assay antifungal susceptibility profile of *Candida glabrata* (*C. glabrata*) isolates to standard antifungal agents. A total of 100 HIV-infected patients with oropharyngeal candidiasis (OPC) were selected in this study. Susceptibility of *C. glabrata* isolates to antifungals was determined using the disc diffusion method. A total of 254 yeasts were isolated from the patients. Forty percent (40%) of the patients had angular cheilitis as the most frequent clinical variant. *Candida* species (94.4%) were the most frequently obtained genera (P<0.05), followed by *Saccharomyces* (2.4%), *Kluyveromyces* and *Cryptococcus* (1.6%) species. *C. albicans* (37.2%) was the most common species isolated from HIV[+] patients with OPC and its frequency was significantly higher than that of other *Candida* species (P<0.05). Among non-*C. albicans* species, *C. glabrata* was the most frequent species. Highest sensitivity of *C. glabrata* to antifungal drugs was seen against polyene drugs such as nystatin and amphotericin B. This study revealed huge diversity of *Candida* species and oral isolates of *C. glabrata* were most sensitive to polyene drugs such as nystatin and amphotericin B and least sensitive to fluconazole.

Key words: Oral cavity, candidiasis, *Candida glabrata,* HIV infection, nystatin, amphotericin B.

INTRODUCTION

Candida species are the most common opportunistic fungal pathogens in humans, with *Candida albicans* (*C. albicans*) being the most prevalent pathogen in mucosal and systemic fungal infections (1, 2). For decades, *C. glabrata* was considered as a non-pathogen, but recent reports suggest that the antibiotic resistance mechanisms and evolutionary pressure has led to the emergence of *C. glabrata* variants which are highly pathogenic and drug-resistant (3). Moreover, therapeutic strategies, like of immunosuppressive drug therapy, anti-mycotic therapies and the emergence of the acquired immunodeficiency syndrome (AIDS), have drastically increased *C. glabrata* infections (4). Indeed, depending on the site of infection, *C. glabrata* currently ranks as the second or third most frequently isolated *Candida* species from all reported

cases of candidiasis (5, 6). Recently a shift has been noted from fungal disease caused by *C. albicans* to that of non-*albicans* species of *Candida*, such as *glabrata*, especially in HIV patients (7, 8). In addition, *C. glabrata*-associated oropharyngeal candidiasis infections in HIV patients tend to be more severe and more difficult to treat than infections due solely to *C. albicans* (7, 9). Despite the fact that oral *Candida* infections are not associated with mortality, they are a significant source of morbidity, and trigger chronic pain or discomfort upon mastication, which may limit nutrition intake in immunocompromised or elderly individuals (10).

Erroneous diagnosis and false-positive interpretations have rigorous impact on drug selection for antifungal chemotheraphy. Therefore, the selection of antifungal agents for treatment of *C. glabrata* infections can be somewhat problematic, especially in critically ill patients. In such cases, the availability of susceptibility testing results may facilitate therapeutic decision-making. This study aimed to determine the prevalence of oral mycoflora in HIV[+] patients and to assay antifungal

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*Correspondence to: *Dr. Hojjatollah Shokri, Faculty of Veterinary Medicine, Amol University of Special Modern Technologies, Imam Khomeini Street, 24th aftab, Amol, Iran. Phone No.: +98 21 61117151, Fax No.: +98 21 66933222, Email: hshokri@ausmt.ac.ir*

susceptibility profile of *C. glabrata* isolates to fluconazole, ketoconazole, nystatin, clotrimazole, amphotericin B and flucytosine.

MATERIALS AND METHODS
Patients
The patients for this study were Iranian HIV$^+$ men and women in Iranian Research Center for HIV/AIDS, Imam Khomeini Hospital, Tehran, Iran (IRCHA). They were outpatients who came to receive treatment. The HIV infection was confirmed by ELISA and western blot techniques. The patients were enrolled after providing informed verbal consent. A standardized data collection form was used for retrieving information such as sex, age, HAART, smoking, marriage, addiction, intravenous drug abuse, Hepatitis B, Hepatitis C and having dentures. Clinical information and data regarding any history of OPC or other opportunistic infection was received. Pregnant women and individuals under treatment with antifungals were excluded.

Specimen collection
Oral lesions were clinically diagnosed for each individual and the specimens were taken by clinician using sterile cotton stick swab from lesions, tongue and buccal mucosa.

Isolation and identification of yeasts
Specimens for fungal evaluation obtained from the oral cavity were cultured on sabouraud's dextrose agar plates (*Merck Co., Darmastdt, Germany*) and CHROM agar TM *Candida* (*Paris, France Company*) directly. CHROM agar TM plates were cultured for primary diagnosis and differentiation of *Candida* isolates. A wet mount with 10% KOH was used for microscopic examination of pseudo-hyphae and yeast cell forms. The sabouraud's dextrose agar plates were incubated aerobically at 30°C for 7 days. CHROM agar TM culture for identification of colony form and color, were incubated at 35°C for 72 h. After incubation period, the yeasts were identified based on morphological features and growth parameters (11). Germ tube test was performed with fresh rabbit serum and fresh yeast colony and incubated at 37°C for 3 h. For evaluation of Chlamydospore and filamentous forms production, isolates were cultured on Dalmau plates (cornmeal-Tween 80 agar) for 48 h at 30°C (12). The ability of the isolates to assimilate carbohydrate sources was determined with Rap IDTM yeast identification system (*Remel, USA*) according to the manufacture's instruction (13). Isolates identified as *C. glabrata* were submitted to molecular identification. The isolates of *C. glabrata* were analyzed by a polymerase chain reaction (PCR) procedure with a specific primer.

DNA extraction
Portion of suspicious colony were washed with PBS containing 0.5% SDS and 50 mM EDTA, cells were disrupted with Freeze-Thawing method and glass beads, and centrifuged at 10000 g for 2 min. Five hundred micro liter of lysis buffer were added to precipitated material and maintained at room temperature for 10 min. After adding 150 mL potassium acetate buffer pH 4.8 (60 mL of 5 M potassium acetate, 11.5 mL glacial acetic acid, 28.5 mL distilled water), the tube was vortexed briefly and cell debris and precipitated proteins were removed by centrifugation at 12000 g for 2 min. The supernatant was transferred to another Eppendorf tube and centrifuged as mentioned above. Then the supernatant was transferred to a new 1.5 mL Eppendorf tube, an equal volume of isopropyl alcohol was added. The tube was mixed briefly by inversion, centrifuged at 12000 g for 2 min and the supernatant was discarded. The resultant DNA pellet was washed three times in 300 mL of 70% (V/V) ethanol. After centrifuging at 12000 g for 1 min, the supernatant was discarded. The DNA was dried and dissolved in 30 mL distilled water. In order to measure of concentration and purification of DNA, optical density (OD) was read and run in the agarose gel.

PCR reaction
All putative isolates of *C. glabrata* were final identified by PCR method. Two pairs of oligonucleotide primers were used in this method. The forward primer CGL1 (5´- TTA TCA CAC GAC TCG ACA CT-3´) and rivers primer CGL2 (5´- CCC ACA TAC TGA TAT GGC CTA CAA-3´) were specific for *C. glabrata* (GenBank accession nos. AB032177, AF167993) and amplified a 423 bp DNA fragment from 5.8S rDNA gene. For PCR with individual primer pairs, each reaction mixture contained 2 μL (1 ng) of diluted genomic DNA template or 0.5 μL of yeast cell suspension, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl$_2$, 0.2 mM (each of the four) deoxyribonucleotide triphosphates, 0.5 μM (each) primer, and 0.5 U of *Taq* DNA polymerase (Cinacolon) in a totalvolume of 25 μL. PCR amplification conditions were 5 min of denaturation at 95°C, followed by 40 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s and a final extension step of 72°C for 10 min (*Techne TC512, England*). A sample of 10 μl of product from each PCR was electrophoresed in a 1.5% agarose gel with 0.5 μg of ethidium bromide/ml and 1X Tris-

acetate-EDTA buffer for 1 to 2 h. DNA bands were visualized on a UV transilluminator.

Antifungal assay

All antifungal discs were obtained from Oxoid (*Hampshire, UK*). The determination and interpretation of anti- *C. glabrata* activity of the drugs were carried out by agar disc diffusion method, according to the protocol in M44-A for yeasts (14). Briefly, a suspension of *C. glabrata* (0.1 ml of 10^6 cells/ml) was spread on sabouraud's dextrose agar plate. The filter paper discs of reference antifungals were placed on the inoculated plates. These plates, after standing at 4^oC for 2 h, were incubated at 37^oC for 48 h. The positive and negative controls were maintained with filter paper discs dipped in reference antifungals and sterile distilled water, respectively. The diameters of the inhibition zones were measured in millimeters (mm). Antifungal assay was performed in duplicate. The test was performed with reference antifungals including fluconazole (25 µg/disc), ketoconazole (15 µg/disc), nystatin (50 µg/disc), clotrimazole (30 µg/disc), amphotericin B (10 µg/disc) and flucytosine (1 µg/disc).

Statistical analysis

The statistical analysis was performed using Statistical Program for Social Science (SPSS) 11.0 for windows. Chi-Square test was used to determine differences in proportion of categorized variables. Continuous variables with an approximately normal distribution were tested using the Student's test. A *P* value less than 0.05 was considered statistically significant.

RESULTS

A total of 100 HIV$^+$ individuals with and without signs or symptoms of OPC were recruited into this study at Imam Khomeini Hospital, Tehran, Iran. The demographic characteristics of the study population were as follows. As illustrated in **Table 1**, all patients ranged in age from 5 to 72 years old, with a mean age of 32.3 years old. The majority of patients (60%) were 31-50, 23% in 11-30, 15% in >50 and 2% in 0-10 year's groups. Seventy-eight subjects were male, and 22 were female. Fifty-six patients were married and 44 were unmarried.

Table 1. Demographic data of 100 HIV$^+$ patients included in this study.

Age (year)	Sex (%)		Underlying factor (%)					Signs and symptoms (%)			
	Female	Male	Addiction	AD[†]	Smoking	IDU[‡]	Jail	AC[*]	EC[**]	HC[***]	PC[****]
0-10	0	2	0	0	0	0	0	0	0	0	3
11-30	6	17	12	10	14	7	5	21	24	12	23
31-50	11	49	36	33	45	30	41	32	17	8	54
> 50	5	10	8	7	11	6	8	22	18	6	21
Total	22	78	56	50	70	43	54	75	59	26	101

†AD: Artificial denture; ‡IDU: Intravenous drug user; [*] AC: Angular cheilitis; [**] EC: Erythematose candidiasis [***] HC: Hyperplastic candidiasis, [****] PC: Pseudomemberanous candidiasis

The patients had a history of smoking (70%), HAART (65%), addiction (56%), jail (54%), denture wearing (50%) and intravenous drug user (43%). Out of 100 patients presented a clinically detectable OPC, angular cheilitis (40%) was the most frequent clinical variant, pseudomembranous candidiasis in 28% patients, thrush in 22% patients and erythematous and hyperplastic candidiasis in 5% patients **(Table 1).**

A total of 254 yeast colonies were isolated from the patients understudy, representing 4 different genera in the tested samples. Among these samples, *Candida* species (94.4%) were the most frequently obtained genera isolated from all study participants (P<0.05), followed by *Saccharomyces* (2.4%), *Kluyveromyces* and *Cryptococcus* (1.6%). *C. albicans* (37.2%) was

the most common species isolated from HIV$^+$ patients with OPC and its frequency was significantly higher than that of other *Candida* species (P<0.05). Of these patients, *C. glabrata*, *C. dubliniensis*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, *C. lusitaniae*, *C. guilliermondii* and *C. norvegensis* were also identified.

As shown in **Figure 1**, the RAPD banding pattern of different clinical isolates using the one specific primer for *C. glabrata*. Using the primer CGL, *C. glabrata* isolates yielded RAPD profiles with one strong band, with molecular size of 423 bp.

In this study, a total of 20 *C. glabrata* isolates were identified and selected for antifungal susceptibility testing. Test results of the susceptibility to antifungal drugs were as

follows: ketoconazole: 17 isolates (85%) susceptible, 2 (10%) susceptible-dose dependent and 1 (5%) resistant; amphotericin B: 20 isolates (100%) susceptible; nystatin: 20 isolates (100%) susceptible; clotrimazole: 12 isolates (60%) susceptible, 8 (40%) susceptible-dose dependent; flucytosine: 11 isolates (55%) susceptible, 7 (35%) susceptible-dose dependent and 2 (10%) resistant; fluconazole: 11 isolates (55%) susceptible, 2 (10%) susceptible-dose dependent and 7 (35%) resistant. The inhibition zones were ranged from 19 to 52 mm (mean value: 35.45 mm) for ketoconazole, 18 to 32 mm (mean value: 23.3 mm) for amphotericin B, 20 to 30 mm (mean value: 23.2 mm) for nystatin, 15 to 35 mm (mean value: 21.45 mm) for clotrimazole, 5 to 32 mm (mean value: 21.35 mm) for flucytosine and 5 to 32 mm (mean value: 17.3 mm) for fluconazole **(Table 2).** Regarding the data, it was revealed that nystatin and amphotericin B were the most effective antifungal drugs and fluconazole and flucytosine had the poorest activity.
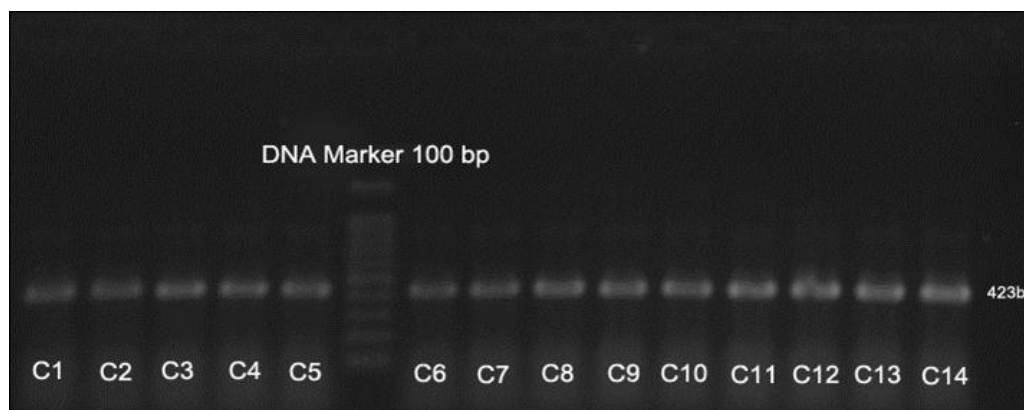

Figure 1. Primer pair designed to amplify DNA specifically from the Candida glabrata strains (C_1-C_{14}) isolated from oral cavity of HIV^+ patients.

Table 2. *Antifungal susceptibility of the reference antifungals against Candida glabrata isolates (C_1-C_{20}) (mm).*

Isolate	Fluconazole	Ketoconazole	Clotrimazole	Nystatin	Amphotericin B	Flucytosine
C_1	32	52	30	26	27	19
C_2	19	31	22	23	25	30
C_3	20	37	20	20	21	30
C_4	10	35	16	22	25	10
C_5	7	34	15	20	20	14
C_6	5	36	17	28	18	28
C_7	20	22	16	25	22	30
C_8	21	40	25	24	24	21
C_9	31	48	35	24	25	13
C_{10}	13	29	25	22	23	32
C_{11}	20	33	15	23	23	5
C_{12}	10	32	15	23	20	16
C_{13}	21	43	31	30	32	18
C_{14}	11	35	17	24	26	14
C_{15}	21	39	22	24	24	19
C_{16}	18	28	24	20	23	26
C_{17}	7	19	22	21	22	27
C_{18}	23	45	23	24	21	24
C_{19}	19	27	22	21	25	31
C_{20}	18	35	17	20	20	20

DISCUSSION

The present study investigated 100 HIV^+ patients with OPC (one of the major markers of HIV disease progression) and included the HIV^+ groups regarding some important variables such as age, smoking, addiction and oral conditions (use of artificial dentures). The results showed that OPC in males (78%) was more frequent than females (22%), which were in accordance with other investigators (15,16).

It seems that Iranian HIV^+ male patients have risk factors such as addiction, jail, intravenous drug user and smoking. These predisposing agents could also be important factors influencing the distribution frequency of *Candida* species among different age groups as well as the incidence of HIV infection (17). The most affected patients had 31-50 years old (60%). Although different clinical signs of OPC appear in newborn and old ages, but in

HIV patients because of decreasing CD_4^+ lymphocytes, every patient with HIV infection has a risk of the infection.

We also found a higher frequency of angular cheilitis (40%) in HIV$^+$ patients regardless of underlying factors. In contrast, Katiraee *et al.* (18) observed thrush (38%) as the most frequent clinical symptom in Iranian HIV-infected patients. In accordance with our findings, oral yeasts carriage, in particular *Candida* species has been demonstrated in asymptomatic HIV$^+$ patients (17), and an increased incidence of asymptomatic oral *Candida* carriage in HIV$^+$ patients compared to that in other at-risk groups has also been noted (18). Thus, a higher prevalence of oral *C. albicans* colonization may be a predisposing factor for the subsequent development of clinical candidiasis.

With respect to the *Candida* species identified in this study, *C. albicans* was isolated from 37.2% of HIV$^+$ patients. In accordance with our finding, the isolation of this species was reported by by Mousavi *et al.* (19), Katiraee *et al.* (16) and Shokohi *et al.* (17) in the oral cavity of HIV$^+$ patients. Even though *C. albicans* was the most common species recovered, the non-*albicans Candida* species (53.8%) have become more recognized as a major source of infection. Recently, several reports have demonstrated a change in the oral *Candida* flora of HIV-infected patients. The use of various medicines such as antiretroviral agents, antibiotics and antifungal agents has been pointed as a reason for this change (21,22). Among non-*albicans Candida* species, *C. glabrata* was the most frequently isolated species from the HIV$^+$ patients with OPC. In a study conducted by Redding *et al.* (23) study, *C. glabrata* has emerged as a notable pathogenic agent in oral mucosa, either as a co-infecting agent with *C. albicans* or as the sole detectable species from oral lesions. In addition, *C. glabrata*-associated OPC in HIV$^+$ patients tend to be more severe and more difficult to treat than infections due solely to *C. albicans*. The comparison of frequency of non-*albicans* isolates in the present study with previous studies is complicated due to different inclusion and exclusion criteria and sampling methods (24).

C. glabrata is naturally resistant to a wide variety of pharmacological and host-derived molecules. The resistance of *C. glabrata* to antifungals continues to be a significant problem in fungal infections. In this study, the susceptibility of *C. glabrata* isolates to different chemical antifungals such as, fluconazole, ketoconazole, nystatin, clotrimazole, amphotericin B and flucytosine were compared. Highest sensitivity of *C. glabrata* to antifungal drugs was seen against polyene drugs such as, nystatin and amphotericin B (20 of 20), followed by ketoconazole (17 of 20) and clotrimazole (12 of 20). The resistance to fluconazole was observed in 7 isolates of *C. glabrata*. In test with flucytosine, the surprisingly wide inhibition zones obtained were probably due to the low molecular weight and high aqueous solubility of the molecule (25). Our results showed that nystatin and amphotericin B were the most effective antifungal drugs and fluconazole had the poorest activity. There are many studies indicating that fluconazole had less activity against *Candida* species (26,27). Our data are in agreement with previous reports as well. In view of the resistance of *C. glabrata* to antifungal agents, it is interesting that this organism is innately resistant to azole antifungals, in particular fluconazole. Recent studies have demonstrated that, in addition to having innate resistance, *C. glabrata* can also acquire drug resistance and become more resistant after selection in the presence of fluconazole. One key mechanism used by *C. glabrata* to develop acquired resistance is to increase azole efflux from the yeast cell through overexpression of two ATP-binding cassette transporters, cdr1 and cdr2 (28). Moreover, *C. glabrata* can upregulate the expression of the CgERG11 gene, which encodes lanosterol 14- demethylase (29). Unlike the azole drugs that exert their effect by inhibition of fungal cytochrome P450 enzymes, the polyene antifungals such as, nystatin and amphotericin B, act by binding to ergosterol, in the fungal cell membrane. Resistance to the polyene antifungals remains an uncommon event among *Candida* isolates. The polyenes still have reliable activity against most of the *Candida* species, except *C lusitaniae,* which is often intrinsically resistant (30, 31).

CONCLUSIONS

In summary, an investigation of HIV$^+$ patients allowed us to monitor the dynamics of fungal oral colonization with particular reference to *Candida* species and its evolution toward OPC and to evaluate the prognostic value of *Candida* carriage in the development of AIDS. Oral colonization and infection by yeasts in Iranian HIV$^+$ patients is composed of different species, including *C. albicans* and non-*albicans Candida* species, in particular *C. glabrata*. Antifungal sensitivity testing revealed that oral isolates of *C. glabrata* were

most sensitive to polyene drugs such as, nystatin and amphotericin B, and least sensitive to fluconazole.

CONFLICT OF INTEREST
No conflict of interest was declared by the authors.

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