Original Contribution

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

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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 chemotherapy. 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
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., Darmstadt, 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 pseudohyphae 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 Chlamydospora 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 GCC 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°C for 2 h, were incubated at 37°C 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 fluacytosine (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* patients 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.**

<table>
<thead>
<tr>
<th>Age (year)</th>
<th>Female</th>
<th>Male</th>
<th>Addiction</th>
<th>AD†</th>
<th>Smoking</th>
<th>IDU†</th>
<th>Jail</th>
<th>AC*</th>
<th>EC**</th>
<th>HC ***</th>
<th>PC ****</th>
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<td>11-30</td>
<td>6</td>
<td>17</td>
<td>12</td>
<td>10</td>
<td>14</td>
<td>7</td>
<td>5</td>
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<td>33</td>
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<td>41</td>
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<td>&gt; 50</td>
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<td>10</td>
<td>8</td>
<td>7</td>
<td>11</td>
<td>6</td>
<td>8</td>
<td>22</td>
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<td>21</td>
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<td>43</td>
<td>54</td>
<td>75</td>
<td>59</td>
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</table>

†AD: Artificial denture; †IDU: Intravenous drug user; * AC: Angular cheilitis; ** EC: Erythematose candidiasis *** HC: Hyperplastic candidiasis **** PC: Pseudomembranous 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. guillermondii* 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.

Table 2. Antifungal susceptibility of the reference antifungals against Candida glabrata isolates (C1-C20) (mm).

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Fluconazole</th>
<th>Ketoconazole</th>
<th>Clotrimazole</th>
<th>Nystatin</th>
<th>Amphotericin B</th>
<th>Flucytosine</th>
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<tr>
<td>C1</td>
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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 CD4+ 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, Katirae 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 Mousavi et al. (19), Katirae 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.

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

REFERENCES


