Genetic Diversity and Antifungal Susceptibility of Candida Albicans Strains Isolated from Lranian HIV+ Patients With Oral Candidiasis

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Research note

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Abstract

Objective: The objectives of this study were to investigate the antifungal susceptibility and genetic diversity of oral Candida albicans strains isolated from HIV+ patients with oropharyngeal candidiasis. A total of 50 C. albicans isolates were cultured on Sabouraud glucose agar containing chloramphenicol. The antifungal susceptibility of C. albicans against fluconazole, clotrimazole, nystatin, amphotericin B, ketoconazole and flucytosine was assessed using disc diffusion method. The genetic diversity of different C. albicans strains was determined using random amplified polymorphic DNA technique.

Results: The inhibition zones ranged from 4±1.8 to 40±3.8 mm for fluconazole, 7±1.0 to 37±1.8 mm for ketoconazole, 14±0.8 to 24±0.8 mm for amphotericin B, 25±0.0 to 33±0.0 mm for nystatin and 7±4.2 to 40±0.0 mm for clotrimazole. At 90% similarity, three distinct groups were observed. The smallest cluster composed of 3 of 50 C. albicans isolates, whereas the largest cluster composed of 17 of 50 isolates. Of 50 C. albicans isolates, 32%, 28% and 14% were resistant to fluconazole, ketoconazole and clotrimazole, respectively. There were no significant differences among antifungal susceptibility of different C. albicans strains from three genotype clusters.

Introduction

Candida albicans (C. albicans) has been known as one of the most prominent clinical pathogen, as well as in primary and secondary immunocompromised patients(1). Oropharyngeal candidiasis (OPC) is frequently complicated situation of predisposing factors such as hematologic malignancy, acquired immunodeficiency syndrome (AIDS), Nezelof syndrome, zink and iron deficiency. OPC, as the independent predictor of immunodeficiency in AIDS patients, increases the mortality and morbidity among these patients; consequently, it requires prompt therapy and precise diagnosis (2). C. albicans, only species recovered from up to 70% of HIV-infected individuals, is one of the most common cause of mucosal yeast infection in human. Totally, 93% of untreated HIV patients harbour OPC at least with one frequent recurrences per year (3). Recently, the prevalence of oral Candida infections in HIV patients has been decreased. Two factors have been described this phenomenon. First, overuse of antifungal agents, particularly the azole antibiotics. Second, the introduction of highly active antiretroviral therapy has resulted in a significant decrease in the incidence of a number of opportunistic diseases and the mortality of AIDS(4). Resistance of Candida species to the azole antifungals (as the main challenge antibiotic susceptibility) is the most prevalent type of resistance to antifungals in these patients. Since azoles, particularly fluconazole, have been used for prophylaxis or treatment of AIDS patients, resistance to this antifungal agent is common during AIDS-related complex(5). Resistance can be caused by an alteration of the target enzyme, the cytochrome P-450 lanosterol 14 α-demethylase, mediated by ERG11 gene, or the failure of azole antifungal agents to accumulate inside the yeast cell as a consequence of enhanced drug efflux, mediated by MDR and CDR genes(6).

In recent years, RAPD analysis has been increasingly used as molecular method for population genetics and genotyping of different organisms(7). The purposes of the current study were to determine the
antifungal susceptibility and the genetic diversity of *C. albicans* isolates collected from oral cavity of Iranian HIV+ patients with OPC.

**Main Text**

**Microorganisms**

This study was performed on 50 *C. albicans* isolates obtained from oral cavity of HIV+ patients, from October to November 2011 at the AIDS Research and Training Center of Imam Khomeini Hospital, Tehran, Iran. We used a wet mount with 10% KOH preparation and Giemsa stain for microscopic examination of pseudo-hyphae and yeast cell forms. In addition, all samples were cultured on Sabouraud glucose agar (SGA; 20 g/l glucose, 10 g/l peptone, 20 g/l agar, pH: 5.6) containing 0.05% chloramphenicol (Merck Co., Darmstadt, Germany). The cultures were incubated at 37 oC and examined daily for one week. Identification of *C. albicans* isolates were performed on the basis of germ tube test, colony color on Chrom agar (Paris, France Company), sugar fermentation and assimilation tests by RAPID yeast plus system (Remel Inc., USA) and internal transcribed spacer (ITS) primer pairs (CALB1 and CALB2).

**Antifungal drugs**

All standard antifungal discs including fluconazole (25 µg/disc), clotrimazole (10 µg/disc), nystatin (50 µg/disc), amphotericin B (20 µg/disc), ketoconazole (10 µg/disc) and flucytosine (1 µg/disc) were obtained from Oxoid (Hampshire, UK). The agar disc diffusion method was employed for the determination of anti-*C. albicans* activity as described by the National Committee for Clinical Laboratory Standard(8). Briefly, a suspension of *C. albicans* (106 cell/ml) was spread on Muller-Hinton agar containing 2% glucose and 0.5 µg/ml methylene blue dye. Standard antifungal discs were placed on the inoculated plates. These plates were incubated at 37 oC for 48 h. The diameter of the inhibition zones was measured in millimeter (mm) and the results were interpreted based on comparison to standards. Antifungal susceptibility assay was performed in duplicate.

**DNA extraction**

All samples were cultured on SGA at 37 oC for 48 h. Genomic DNA was extracted as previously described (9) and purified using a commercial DNA purification kit (Ultraclean Microbial DNA Isolation kit, MO BIO, USA) according to manufacturer’s structures. DNA concentration and purity were determined by optical density at 260 nm and ratio OD 260 /280 nm determinations, respectively.

**PCR assay and RAPD analysis**

Diagnostic PCR analysis was performed with the oligonucleotide primers CALB1: TTTATCAACTTGTACACACCAGA and CALB2: ATCCCGCCTTACCATACCG (The GenBank accession number refers to primers L47111, L28817) with amplify at 450 bp fragment within the 5.8 s ribosomal RNA gene(10). Amplification reactions were done in a final volume of 25 µl containing 2.5 µl of reaction buffer (10X), 1.5 mM MgCl2 (50 mM), 0.2 mM dNTP (10 mM), 0.5 µM (each) primers, 0.5 u of Taq DNA
polymerase and 2 µl of genomic DNA template. Amplification was carried out using a Techne Tc-512 thermo cycler (Techne, UK), Initial denaturation was at 96 °C for 5 min, as follows: 35 cycles of 30 s for denaturation at 94 °C, 30 s for annealing at 55 °C and 30 s primer extension at 72 °C, followed by a terminal extension at 72 °C for 15 min. The PCR products were electrophoresed on 1.5% agarose gel for 1 h at 80v and stained with ethidium bromide (2 µg/ml) to visualize fluorescent band while using UV in the gel document system (Biorad, UK).

RAPD-PCR was performed with RSD12: 5′- GGTCCGTGTTCAGACG-3′ primer(11). Each reaction mixture contained 2.5 µl of reaction buffer (10X), 2.5 mM Mgcl2, 200 mM dNTPs mix,1.25 µM of primer RSD12, 1U of Taq DNA polymerase and 100–400 ng of *C. albicans* DNA as template in final volume of 25 ml. PCR amplification program for RSD12 primer involved 1 cycle at 95 °C for 5 min, then 40 cycles as follows: 30 s for denaturation at 94 °C, 2 min for annealing at 57 °C and a final extension step 72 °C for 2 min in a TC-512 thermocycler (Techne, Cambridge, UK). The PCR products were analysed by electrophoresis on 1.5% agarose gel at 70 V for 80 min in TBE buffer (1x) and stained in a 0.5 mg/ml ethidium bromide solution for 15 min and photographed by CCD Video Camera. DNA banding patterns were analysed using the GelCompar software package, version 6 (Applied Math, Belgium).

**Statistical analysis**

The chi-square test and t-test using SPSS software version 12.0 (SPSS Inc., Chicago, IL, USA) were performed to statistical analysis. A phenogram was constructed by the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) after determining the association coefficients by the simple matching method.

**Results**

Antifungal susceptibility and genetic diversity of 50 clinical isolate of *C. albicans* were evaluated by disc diffusion methods and RAPD analysis, respectively. The antifungal susceptibilities of *C. albicans* strains from oral cavity of understudy Iranian HIV+ patients were shown in Tables 1 and 2. Results of susceptibility to standard antifungal drugs were as follows: fluconazole: 29 isolates (58%) susceptible, 5 isolates (10%) susceptible-dose dependent or intermediate and 16 isolates (32%) resistance; ketoconazole: 31 isolates (62%) susceptible, 5 isolates (10%) susceptible-dose dependent or intermediate and 14 isolates (28%) resistance; amphotericin B: 48 isolates (96%) susceptible, 2 isolates (4%) susceptible-dose dependent or intermediate; clotrimazole: 35 isolates (70%) susceptible, 8 isolates (16%) susceptible-dose dependent or intermediate and 7 isolates (14%) resistance; flucytosine: 50 isolates (100%) resistance and nystatin: 50 isolates (100%) susceptible.

The inhibition zone diameters of fluconazole ranged from 4±1.8 to 40±3.8 mm (mean value: 23.0±11.7 mm), 7±1.0 to 37±1.8 mm (mean value: 24.8±9.4 mm) for ketoconazole, 14±08 to 24±08 mm (mean value: 17.2±2.1 mm) for amphotericin B, 7±4.2 to 40.0 mm (mean value: 24.8±8.7 mm) for clotrimazole and 25±0.0 to 33±0.0 mm (mean value: 26.6±2.0 mm) for nystatin. In addition, all isolates were resistant to flucytosine (no inhibition zone was observed).
Using the primer CALB, *C. albicans* isolates yielded RAPD profiles with one strong band, with molecular size of 273 bp. On the basis of RAPD-PCR profiles (Fig. 1) and similarity coefficient ≥ 90%, genotypes containing from 3 to 17 isolates, which encompassed 30 (58.82%) isolates and 13 (25.49%) genotypic particular strains. The first cluster, which was the smallest one, composed of ramification a (ATCC strain) and a’ (C32 and C35 strains), the second cluster composed of ramification b and b’ as shown in Figure 1, and the third one (the largest cluster) composed of 17 of 50 isolates which distributed in c and c’ ramification. There were no significant differences among the anti- *C. albicans* susceptibility of different genotype clusters.

*C. albicans* has been known as one of the most frequently yeasts obtained from HIV-infected in Iran and the world(12). The findings of the current study revealed genetic diversity and antifungal susceptibility in the genotype groups of *C. albicans* strains isolated from oral cavity of HIV+ patients. Genetic finger typing of *C. albicans* has been studied in different countries(13). But there is no information about the correlation between the antifungal susceptibility and genetic diversity of *C. albicans* isolated from HIV+ individuals(14). Regarding the specific primer pair for *C. albicans* CALB1 and CALB2, it yielded approximately molecular size of 273 bp. The molecular size of this study is same with the results of previous study that CALB1 and CALB2 produced amplicon size of approximately 273 bp. These results are in line with that of Sharifzadeh et al. (2013) as well(15). The results of this study using with primer RSD12 indicated different genetic profiles between *C. albicans* strains with various antifungal susceptibility patterns. Whereas it was not possible in our study to show that different susceptibility to various antifungal agents is attributable to the genetic diversity of the understudy strains. Regarding the RSD12 primer profile and similarity coefficient ≥ 90%, genotypes consisted of 3 to 17 isolates, which were encompassed 30 (58.82%) isolates and 13 (25.49%) genotypic particular strains. Hamzehee et al. (2019) carried out RAPD-PCR method for estimating the strains of *C. albicans*, 46 genotypes were defined including 11 cluster with 80% similarly coefficient(16). Sun et al. (2009) used primer RSD6 and also the same RSD12 assess genetic diversity of *C. albicans* isolates from root canal infection and found 31 genotypes among the 37 isolates(17). RSD10 and RSD12 primers to were used to determine the clonal viability of 443 *C. albicans* strains obtained from 16 HIV-infected individuals. These isolates formed clusters comprising 2 or more strains at value of a similarity coefficient ≥ 80%(18). Our results are in agreement with mentioned previous reports as well. Considering to our results, amphotericin B and nystatin were the most effective antifungal drugs, and fluconazole had the poorest activity. Fluconazole, in spite of using drug potency up to 25 mg per disc, had poor activity on isolates tested. There are many studies indicating that fluconazole had less activity against *Candida* species(19). Over all, fluconazole resistance, in spite of using drug potency up to 25 µg per disc, was 32%. These higher rates of resistance are not in accordance with those observed in Mexico, Brazil, United Kingdom and other studies which reported lower rates of resistance to the antifungal(20). The reason for higher fluconazole resistance could be explained by the fact that azoles, especially fluconazole, have been used for prophylaxis or incompletely treatment of oral candidiasis in HIV-infected patients and resistance to fluconazole are common during AIDS related complex(21).
Limitations

- Despite all efforts, 50 isolates as the sample size is not efficient to study the genetic relatedness and anti-fungal susceptibility of C. albicans isolated from HIV+ patients.
- RAPD is a strong genotyping method; however, it is not precise enough because of lack of reproducibility. Other genotyping methods are suggested to be implemented.

Abbreviations

OPC: Oropharyngeal candidiasis; AIDS: Acquired ImmunoDeficiency Syndrome; MDR: MultiDrug Resistant; RAPD: Randomly Amplified Polymorphic DNA; HIV: Human Immunodeficiency Virus; ITS: Internal Transcribed Spacer;

Declarations

Ethics approval and consent to participate

This study was reviewed and approved by the ethical committee board, Faculty of medicine, Tehran University of Medical Science, Tehran, Iran (2010/06/12/006). Authorization to perform this investigation at the Imam Khomeini hospital was obtained from the Infectious diseases department of Imam Khomeini hospital, Tehran Univesity of Medical Science. Before taking samples, written informed consent was obtained from each patient at the present study.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interest.

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This study was self-funded.

Authors’ contributions

IAT design the research and did the exeriments; BP wrote the first draft and reviewed the manuscript; BNF analyzed the data and supervised the research.

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Tables

Table 1.

Antifungal susceptibility of oral Candida albicans isolates from HIV-infected patients.
| Antifungal agents  | Drug concentration | Resistance | Intermediate | Susceptible |
|-------------------|--------------------|------------|--------------|-------------|
| Fluconazole (FCN) | 25 μg              | 32%        | 10%          | 58%         |
| Clotrimazole (CTM)| 10 μg              | 14%        | 16%          | 70%         |
| Nystatin (NY)     | 100 units          | 0          | 0            | 100%        |
| Amphotericin B (AMB)| 20 μg         | 0          | 4%           | 96%         |
| Ketoconazole (KCA)| 10 μg              | 28%        | 10%          | 62%         |
| Flucytosine (FY)  | 1 μg               | 100%       | 0            | 0           |

Table 2.

Antifungal susceptibility of the standard antifungal agents against *Candida albicans* isolates (mean±standard deviation, Millimeter).
| Clotrimazole | Nystatin | Amphotericin B | Ketoconazole | Fluconazole | Genotype |
|--------------|----------|----------------|--------------|-------------|----------|
| 11±1.4       | 26±0.2   | 20±1.8         | 8±1.8        | 9±1.2       | C1       |
| 19±2.2       | 25±0.0   | 17±2.0         | 11±2.4       | 11±0.8      | C2       |
| 36±0.8       | 30±0.0   | 20±3.2         | 25±0.8       | 18±0.0      | C3       |
| 32±0.8       | 27±0.0   | 14±0.8         | 32±2.0       | 30±2.2      | C4       |
| 33±1.0       | 26±0.0   | 18±0.0         | 30±0.4       | 35±2.6      | C5       |
| 10±2.2       | 25±0.8   | 17±1.0         | 7±1.0        | 9±1.4       | C6       |
| 18±1.4       | 25±0.4   | 17±2.8         | 28±2.2       | 12±1.8      | C7       |
| 33±4.2       | 25±0.0   | 18±1.4         | 31±3.6       | 33±1.2      | C8       |
| 25±2.6       | 25±0.0   | 18±4.2         | 32±0.8       | 29±2.4      | C9       |
| 17±1.8       | 27±0.0   | 18±2.0         | 28±0.0       | 12±1.4      | C10      |
| 9±2.6        | 25±0.4   | 23±1.8         | 11±0.2       | 5±0.8       | C11      |
| 30±0.8       | 25±0.0   | 17±1.4         | 31±1.2       | 37±1.8      | C12      |
| 8±3.4        | 28±0.0   | 16±2.2         | 28±0.6       | 9±0.8       | C13      |
| 35±0.8       | 25±0.0   | 18±0.0         | 33±0.2       | 35±1.0      | C14      |
| 12±2.4       | 26±0.8   | 16±3.3         | 10±0.8       | 8±2.6       | C15      |
| 28±0.0       | 25±0.6   | 17±1.0         | 28±0.0       | 18±1.0      | C16      |
| 26±0.8       | 25±0.0   | 15±5.0         | 34±0.0       | 35±3.2      | C17      |
| 25±0.6       | 26±1.2   | 16±0.8         | 35±2.8       | 39±0.8      | C18      |
| 9±3.8        | 25±0.0   | 15±2.0         | 15±0.0       | 6±0.0       | C19      |
| 30±2.6       | 26±0.0   | 16±3.2         | 35±0.8       | 40±3.8      | C20      |
| 28±6.2       | 25±0.0   | 15±1.0         | 32±2.4       | 32±2.0      | C21      |
| 17±0.8       | 26±0.0   | 16±0.8         | 7±3.4        | 4±1.8       | C22      |
| 25±1.6       | 25±0.8   | 16±3.4         | 25±0.8       | 17±0.0      | C23      |
| 30±2.4       | 25±0.8   | 15±3.2         | 21±2.8       | 29±2.2      | C24      |
| 40±0.0       | 27±0.0   | 15±1.8         | 25±3.2       | 18±1.0      | C25      |
| 23±3.6       | 27±0.0   | 15±3.6         | 32±2.0       | 35±0.8      | C26      |
| 7±4.2        | 25±1.2   | 16±1.8         | 8±4.8        | 5±3.8       | C27      |
| 30±1.6       | 27±0.8   | 17±0.8         | 35±0.8       | 31±0.6      | C28      |
|      |      |      |      |      |      |      |
|------|------|------|------|------|------|------|
| 25±2.6 | 25±0.0 | 16±0.0 | 31±2.0 | 30±1.9 | C29  |
| 13±0.8 | 28±1.6 | 18±1.0 | 29±1.8 | 5±4.0  | C30  |
| 25±1.2 | 25±0.0 | 16±4.0 | 17±1.6 | 10±1.2 | C31  |
| 27±3.8 | 25±0.0 | 16±2.0 | 28±4.2 | 28±3.0 | C32  |
| 24±2.2 | 25±0.0 | 17±1.4 | 14±2.0 | 21±2.8 | C33  |
| 25±2.8 | 23±0.0 | 16±0.6 | 15±0.0 | 18±0.0 | C34  |
| 35±0.8 | 26±0.8 | 16±0.0 | 31±0.8 | 27±1.4 | C35  |
| 17±4.2 | 26±0.0 | 15±3.4 | 9±0.8  | 7±1.6  | C36  |
| 26±3.2 | 26±0.0 | 17±2.4 | 37±1.8 | 33±4.8 | C37  |
| 19±1.4 | 28±0.0 | 18±2.8 | 10±1.0 | 4±3.6  | C38  |
| 32±4.2 | 28±0.0 | 18±1.4 | 30±2.8 | 31±0.8 | C39  |
| 35±0.8 | 26±1.4 | 16±4.2 | 30±0.0 | 29±2.4 | C40  |
| 29±3.2 | 26±1.2 | 17±2.4 | 31±0.6 | 34±1.8 | C41  |
| 31±2.8 | 29±0.0 | 20±1.0 | 34±2.0 | 32±2.8 | C42  |
| 29±1.0 | 33±0.0 | 18±3.2 | 30±1.4 | 37±2.4 | C43  |
| 29±0.0 | 31±0.0 | 22±2.0 | 31±4.0 | 29±3.8 | C44  |
| 33±0.8 | 30±0.2 | 24±0.8 | 29±3.2 | 37±2.0 | C45  |
| 31±2.0 | 27±0.0 | 17±2.8 | 21±1.6 | 30±0.0 | C46  |
| 8±1.6  | 31±1.4 | 14±6.0 | 9±2.4  | 8±2.8  | C47  |
| 30±0.8 | 25±1.0 | 18±3.2 | 33±1.8 | 31±0.8 | C48  |
| 31±2.0 | 30±0.0 | 17±2.6 | 30±0.8 | 29±3.4 | C49  |
| 30±0.0 | 31±0.0 | 22±1.8 | 33±3.2 | 34±2.2 | C50  |
| 35±0.8 | 27±0.0 | 19±0.8 | 30±2.8 | 30±1.8 | ATCC |