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Repetitive sequences based on genotyping of *Candida albicans* isolates obtained from Iranian patients with human immunodeficiency virus

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ABSTRACT

Objective(s): Candidiasis infection caused by *Candida albicans* has been known as a major problem in patients with immune disorders. The objective of this study was to genotype the *C. albicans* isolates obtained from oral cavity of patients with positive human immunodeficiency virus (HIV+) with or/and without oropharyngeal candidiasis (OPC).

Materials and Methods: A total of 100 *C. albicans* isolates from Iranian HIV+patients were genotyped using specific PCR primers of the 25S rDNA and RPS genes.

Results: The frequencies of genotypes A, B and C which were achieved using 25S rDNA , were 66, 24 and 10 percent, respectively. In addition, genotypes D and E were not found in this study. Each *C. albicans* genotype was further classified into four subtypes (types 2, 3, 2/3 and 3/4) by PCR amplification targeting RPS sequence.

Conclusion: In general, genotype A3 constituted the majority of understudy clinical isolates obtained from oral cavity of Iranian HIV+ patients.

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Introduction

Candida species have commonly been considered harmless commensals, and are isolated from the vagina, mouth and gastrointestinal tracts. When the host-fungus interaction becomes unbalanced, usually due to a change in the host immune functions, the yeast is able to initiate infection or disease. In the majority of the cases, there are superficial mucosal lesions, but in severely ill patients, it can enter the blood stream and cause a disseminated disease (1). Candidiasis of the oral mucosa, a disease recognized since antiquity, has gained renewed significance more recently as an infection frequently observed in patients with AIDS (Acquired immune deficiency syndrome), and in other immunodeficiency conditions.

Molecular typing of *Candida albicans* isolated from the oral cavity of HIV+ (Human immunodeficiency virus) patients has been established for epidemiological studies and development of appropriate infection control strategies (2-4). In order to surveillance of candidiasis, genotype analysis of clinical isolates, especially in HIV+ individuals, is necessary. For prevention of candidiasis, it is important to identify the route and source of infection by typing the isolates at the strain level. For characterization of *C. albicans* in addition to a deeper comprehension of its epidemiology, new molecular techniques have been developed (5, 6). Genomic sequencing, multilocus enzyme electrophoresis, restriction enzyme digestion, pulsed field gel electrophoresis and randomly amplified polymorphic DNA analysis have been used for strain typing of *C. albicans* (7, 8).

Ribosomal sequence is extensively used for typing of *C. albicans* isolates and differentiation between *C. albicans* and *C. dubliniensis* (9). PCR targeting 25SrDNA, which has been frequently used for genotype analysis of *C. albicans*, allows *C. albicans* to be grouped into five genotypes: A, B, C, D (The last corresponds to *C. dubliniensis)*, and E (10). In addition, it has been demonstrated that genotype A is the most common genotype in different regions (11). Several studies approved that *C. albicans* chromosomes contain

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Table 1. List of PCR primers (25SrDNA and RPS) and expected sizes of PCR products

Primer	Nucleotide sequence (5'-3')	Band size (bp)	25SrDNA type
CA-INT-L CA-INT-R	ATAAGGGAAGTCGGCAAAATAGATCCGTAA CCTTGGCTGTGGTTTCGCTAGATAGTAGAT	450 840 450 & 840 1040 1080	A B C D E
Primer	Nucleotide sequence (5'-3')	Band size (bp)	RPS TYPE
ASDcF Pcscr	TGATGAACCACATGTGCTACAAAG CGCCTCTATTGGTCGAGCAGTAGTC	526 698 870 1042 1214 1396	1 2 3 4 5 6

characteristic repetitive sequence (RPS) with a tandem short repeating unit of 172bp designated ALT (12). The numbers of ALT which leads to variation in molecular characteristics of different size and copy numbers of ALT sequences are attractive targets for genotyping of *C. albicans* (13). Based on our knowledge, no previous attempt has been made to investigate the genotypes of *C. albicans* isolates using 25S rDNA and RPS genes in Iran. The aim of the present study was to genotype the *C. albicans* isolates obtained from oral cavity of Iranian HIV^+ patients using specific primers to RPS sequence.

Materials and Methods

Isolates of Candida albicans

This study was performed on 100 *C. albicans* isolates obtained from 100 HIV^+patients at the Research and Training Center of Imam Khomeini Hospital, Tehran, Iran. All subjects gave informed consent to participate in the study.In order to identify the *C. albicans*; colony color on CHROM agar (Paris, France company), RapID™YeastPlus System *(*Remel. USA*)* and internal transcribed spacer (ITS) primer pairs (CALB1: TTT ATC AAC TTG TCA CAC CAG A and CALB2: ATC CCG CCT TAC CAC TAC CG) were used.

Extraction and purification of genomic DNA

All *C. albicans* isolates were cultured on sabouraud glucose agar plates plus chloramphenicol (Merck Co, Darmastdt, Germany) at 30˚C for 48 hr. Genomic DNA was extracted by vortexing with glass beads and purified using a commercial DNA purification Kit *(*UltraClean Microbial DNA Isolation Kit, MO BIO,USA*).* DNA samples were stored at -20°C until used.

PCR primers

Primers used in this study were on the basis of 25S rDNA including forward primers CA-INT-L: ATA AGG GAA GTC GGC AAA ATA GAT CCG TAA and reverse primer CA-INT-R: CCT TGG CTG TGG TTT CGC TAG ATA GTA GAT. *C. albicans* isolates were divided into 5 genotypes of the size of PCR products (Table1). In order to obtain different *C. albicans* subtyping on the basis of ALT repeats, two primers (ASDcF: TGA TGA ACC ACA TGT GCT ACA AAG and pCSCR: CGC CTC TAT TGG TCG AGC AGT AGT C) were set on the basis of the nucleotide sequences of *C. albicans* repetitive sequence (Table1) (10). These primers can divide the *C. albicans* isolates into 6 subtypes according to the sizes of PCR products.

Conditions for PCR amplification and agarose gel electrophoresis

Clinical isolates are known as *C. albicans*, when a PCR product of 273bp was exclusively amplified (12). For genotyping on the basis of 25S rDNA and RPS sequences, genomic DNA was amplified in a reaction mixture (25 µl) that contained 1.75 mM $MgCl_2$, 0.2 mMdNTPmix, 1U Taq DNA polymerase (Fermentase), 50 pmol of the primers and 2 µl DNA template. The PCR condition was as follows: incubation at 95˚C for 3 min prior to 35 cycles at 94˚C for 30 sec, 59˚C for 30 Sec, 72˚C for 30 sec and 72˚C for 10 min. All reactions were amplified using a thermal cycler (Techne, TC512, England). PCR products were electrophoresed on a 1.2% agarose gel and stained with ethidium bromide for 20 min at 20˚C.

Results

All *C. albicans* isolates were identified by two specific pairs of oligonucleotide primers (Gen Bank accession nos. L47111, L28817) (Figure 1). The genotypes of all understudy isolates of *C. albicans* were analyzed by the PCR method. PCR amplification of 450, 840, and both 450 and 840 bp DNA products which was performed using 25S rDNA, corresponded to genotypes A, B and C, respectively. Results presented in Table 2 revealed that among 100 isolates, 66 were classified as genotype A (66%), 24 were classified as genotype B (24%) and 10 were classified as genotype C (10%). In addition, genotypes D and E were not found in our study (Figure 2).

The RPS based genotyping of the isolates were determined on the basis of the repeated numbers of the ALT sequence in the major product that showed

Table 2. Genotype analysis of *Candida albicans* by 25SrDNA primer

Genotype A	Genotype B	Genotype C	Total
66	24	10	100
66%	24%	10%	100%

Table 3. Subtype analysis of *Candida albicans* by RPS primers

Sub type A				Sub type B				Sub type C		Total
A2	A3	A2/3	A3/4	B2	B3	B2/3	B3/4	C2	C2/3	Subtype
14	30	10	2	4	6	10	4	6	4	100
14%	30%	20%	2%	4%	6%	10%	4%	6%	4%	100%

Figure 1. The presence of *Candida albicans* was confirmed in all isolates. Lane 1: 100bp marker, lane 2: positive control *C. albicans* (ATCC 10231), lanes 3-10: *C. albicans*isolates of HIV+ patients. The presence of genus of *C. albicans* was shown with primers CALB1 and CALB2 in 273 bp, lanes 11-14: negative control (*C. glabrata, C. tropicalis, C. krusei* and *C. stellatoidea*), End lane: 1kb bp marker

the highest intensity. As illustrated in Table 3, genotypes A, B and C of *C. albicans* were classified into 10 genotype sub-groups. A total of 66 A genotypes were further classified into A2 (14%), A3 (30%), A2/3 (20%) and A3/4 (2%), respectively. The 24 B genotype isolates were considered as B2 (4%), B3 (6%), B2/3(10%) and B3/4 (4%), respectively. Among 10 genotype C, the isolates were grouped into genotypes C2 (6%) and C2/3(4%) (Figure 3).

Discussion

C. albicans is a commensal microflora in digestive tract and vaginal mucosa in human and other animals (14). In an immunocompromised host, the yeast can invade mucosal barriers and deep organs and cause life-threatening infections. Recently, the new and aggressive treatment strategies for patients with cancer and the increasing incidence of AIDS have resulted in an increasing in the number of

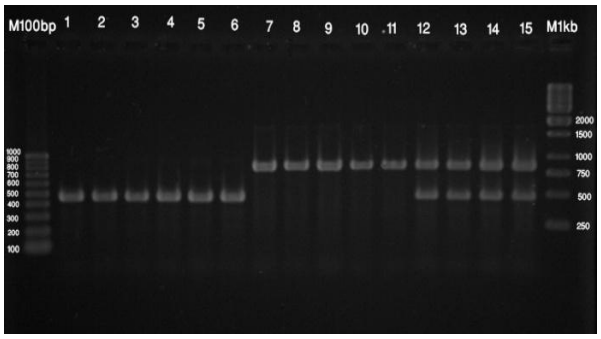

Figure 2. Amplification profiles of PCR products from *Candida albican* isolates from HIV+ patients. Molecular size marker (100bp) is in the line marked M and corresponding sizes as base pairs were given on the left and right. Lanes (1-6) were genotype A (approximately 450 bp), Lanes (7-11) were genotype B (approximately 840 bp) and Lanes (12-15) were genotype C (approximately 450 and 840 bp)

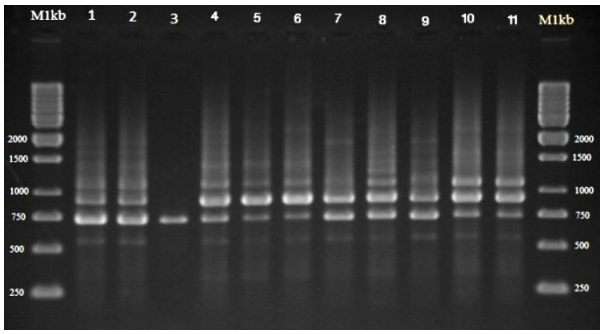

Figure 3. Sub-type variations of *Candida albicans* based on amplification profiles by PCR using RPS. *C. albicans* was classified into 4 sub-types on the basis of the major band number that showed the highest intensity. Molecular size marker (1kb) was in the first and end lines marked M, and corresponding sizes as base pairs were given on the left and right. Lanes (1-3) were subtype 2 (698 bp), lanes (4-6) were subtype 3 (870 bp), lanes (7-9) were subtype 2/3 (698 and 870 bp) and lanes 10 and 11 were subtype 3/4(870 and 1042 bp)

immunocompromised patients prone to opportunistic fungal infections. Different methods such as RFLP, electrophoretic karyotyping, RAPD, MLEE, Microsatellites, MLST and DNA finger typing with *C. albicans* probe have been established to differentiate the isolates of *C. albicans* (15, 16). Moreover, advances in molecular biology have enabled the use of various new molecular biology based genetic methods to answer a variety of epidemiological question regarding to the infection with this organism. In the present study, all yeasts obtained from HIV+ patients with oropharyngeal candidiasis were approved as *C. albicans* using specific *C. albicans* primers.

Previous studies showed that different genotypes of *C. albicans* have various abilities to invade the bloodstream. This difference may be due to the presence or absence of the transposable group I intron in the 25SrDNAof *C. albicans* isolates, which is the basis of this typing system (4). Based on our results, the PCR targeting 25S rDNA approved that genotype A (66%) constituted the majority of the isolates, followed by genotype B (24%) and C (10%). Genotypes D and E were not found in our finding. Tamura *et al* (9) analyzed 301 *C. albicans* isolates in Japan and classified them into 4 genotypes; A (132 isolates), B (66 isolates), C (56 isolates) and D (5 isolates). Karahan *et al* (4) and Hattori *et al* (10) indicated that genotype A had more frequency in invasive and noninvasive *C. albicans* isolates. Contrary to our results, Gurbuz *et al* (17) suggested that genotype A and C were more prevalent in noninvasive and invasive isolates, respectively. Such 25S rDNA-based genotyping has been widely adapted for *C. albicans*, and genotype A *C. albicans*

constituted the majority of isolates in all previous reports (3, 18, 19), although the ratios of genotypes A, B and C *C. albicans* varied among the reports. In a study by Iwata *et al* (8), the ratio of genotype B or C to genotype A *C. albicans* varied in each group of clinical specimens. These findings may be affected by the kinds of clinical specimens colonized by *C. albicans*, geographical location and in different patient populations within time.

It has been accepted that *C. albicans* chromosomes contain characteristic repetitive sequences (RPSs). ALT is defined as the nucleotide sequences of the inner repeats of RPSs (13). The numbers of ALT repeats in the RPS vary in each chromosome, thereby leading to variation in the molecular sizes of RPSs, and these molecular characteristics of the different sizes and copy numbers of the ALT sequence are attractive for the subtyping of *C. albicans*. In this study, each type A, B and C was further grouped into at least 2 subtypes. The majority of subtypes in genotypes A, B and C was A3 (30%), B2/3 (10%) and C2 (6%), respectively. In general, genotype A3 constituted the majority of clinical isolates. These findings were consistent with those of a previous paper, in which *C. albicans* isolated from scales; vaginal secretion, sputum and blood were subjected to RPS-based genotyping (8). In contrast, genotypes A3/4 was found only in a few isolates. This may be due to small number of *C. albicans* investigated in this study. The rare type of *C. albicans* can be useful for identification of source or route of infection rather than the major type A3.

Conclusion

In summary, we demonstrated the evidences that the PCRs targeting the 25S rDNA and the ALT repeats in the RPS sequences were rapid and simple techniques for genotyping of *C. albicans*, and were useful not only for discrimination of *C. albicans* from its related species *C. dubliniensis* and *C. stellatoidea*, but also for management and control of *Candida* infections at the molecular level in dermatological science. In order to genotype the invasive and noninvasive form (commensal) of *C. albicans* isolates and compare them, this study should be continued with a greater population in the future.

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