Original Article

Prevalence, Molecular Identification, and Genotyping of *Candida* Species Recovered from Oral Cavity among Patients with Diabetes Mellitus from Tehran, Iran

Narges Zarei, Maryam Roudbary[1], Shahla Roudbar Mohammadi, André Luis Souza dos Santos[2], Fatemeh Nikoomanesh[3], Rasoul Mohammadi[4], Bahador Nikoueian Shirvan, Sanaz Yaalimadad

Department of Mycology, Faculty of Medical Sciences, Tarbiat Modares University, [1]Department of Parasitology and Mycology, School of Medicine, Iran University of Medical Sciences, Tehran, [3]Infectious Disease Research Center, Birjand University of Medical Sciences, Birjand, [4]Department of Medical Parasitology and Mycology, Infectious Diseases and Tropical Medicine Research Center, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran, [2]Department of General Microbiology, Microbiology Institute, Federal University of Rio de Janeiro, Brazil

Address for correspondence:
Dr. Shahla Roudbar Mohammadi, Department of Mycology, Faculty of Medical Science, Tarbiat Modares University, Tehran, Iran. E-mail: sh.mohammadi@modares.ac.ir

Abstract

Background: Oral candidiasis (OC) has been noticed as a common mucous membrane infection in immunocompromised patients such as that diabetes. This study, focused on the genotyping of *Candida albicans* and enzymatic activities of *Candida* species recovered from oral mucosa among diabetes patients and healthy individuals. **Materials and Methods:** Specimens were obtained from oral mucosa of One-hundred and sixty patients with type 2 diabetic and 108 healthy individuals. All isolates were definitely identified by ribosomal DNA (rDNA) gene sequencinghHydrophobicity, hemolytic activities of *Candida* species and genotypes of *C. albicans* were determined through polymerase chain reaction (CA-INT). **Results:**, Eighty eight (55%) samples out of 160, were positive for *Candida* species in diabetic patients. Moreover, 79.5% (70/88) and 20.5% (18/88) isolates belonged to the *C. albicans* and non-*albicans Candida* species respectively. Three genotypes of *C. albicans* have recovered in diabetic patients: genotype A (71.42%), B (21.42%), and C (7.14%). In healthy individuals, 42.6% (46/102) *Candida* species recovered from oral cavity, with the highest prevalence of genotype A (76.6% of *C. albicans*). Additionally, hydrophobicity and hemolytic activities from *Candida* species were significantly greater in diabetes patients than healthy nondiabetic subjects. **Conclusion:** Collectively, *C. albicans* was the most causative agent isolated from diabetes patients and non-diabetes healthy individuals. Genotype A, as the most remarkable genotype, should be mentioned in both groups. Higher potential hydrophobicity and hemolytic activities of *Candida* species in diabetic patients compared to healthy cases suggest these features triggering pathogenicity of OC in diabetes patients.

Keywords: *Candida species, Candidiasis, Diabetes mellitus, Genotype*

Introduction

In recent decades, the rate of opportunistic fungal infections is incredibly increasing worldwide. Accordingly, among them, oral candidiasis (OC) is one of the most common mucous membrane complications in people with the impaired immune system.[1]

Candida albicans (C. albicans) is a normal microbiota of the skin and mucous membranes causing a wide range of superficial infections to fatal systemic infections, especially among immunocompromised subjects such as patients with HIV/AIDS, diabetes, hospitalized subjects receiving long-term antibiotics, patients under organ transplant, chemotherapy or radiotherapy.[2-4] Significantly, diabetes causes one of the largest emerging threats to public health in the 21st century.[5,6] Correspondingly, the World Health Organization and the International Diabetes Federation predicted that the number of adults with diabetes will rise near 629 million by the year 2045 in worldwide.[7] Approximately 4.5 million adult people were living with diabetes in Iran in 2011, it is expected that by the year 2030, 9.2 million Iranian persons will have diabetes. This continuous and noteworthy rise in the prevalence of diabetes reveals the high burden of disease in Iran, particularly diabetes-associated problems.[8]

Previous reports have widely demonstrated that the rate of *Candida* carriage rises in diabetic subjects suffering from oral infections compared to non-diabetic individuals.[9,10] A high glucose level in salivary, uncontrolled hyperglycemia,[11-14] lower salivary pH,[14,15] oral epithelium

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alteration,[16] and impaired neutrophils' response[16-18] are associated with physiopathology and etiology of occurrence of OC in diabetic patients. Notably, *C. albicans* was the most common species isolated from patients; however, the prevalence of non-*albicans Candida* species (NACs) are increasing dramatically.[19,20]

Various potential properties of *Candida* species especially cell surface hydrophobicity (CSH) accelerate the adherence of *Candida* to host mucous membranes of oral cavity. Adhesion to the tissue resulting in biofilm formation, disperse of the infection, invasion, and finally leading to emerging antifungal resistant species. As a result, *Candida* adherence to epithelial cells of the oral cavity is a key initial step in the pathogenicity of OC infection.[21-23] Surprisingly, enzymatic activity, particularly hemolytic ability of *Candida* species, gives an opportunity to acquire iron of host tissue and to enhance the virulence toward the progression of infection.[24-26]

To realize the source of infection, the identification of a genetic link between strains based on typing methods is highly noticed.[27] For this purpose, a large number of molecular typing methods such as random amplified polymorphic DNA (RAPD) fingerprinting,[20] multilocus sequence typing (MLST),[28] electrophoretic karyotyping,[29] and ABC genotyping,[30] with the matter technique based on the presence or absence of an intron in the 25S ribosomal DNA (rDNA), are used to determine the typing of clinical isolates of *Candida* strains. In Iran as a developing country, improvement and use of rapid, cost-benefit and reliable methods for genotyping of fungal isolates attract many attentions due to the limited available sources.

Although previous studies have reported the prevalence of *Candida* species in oral mucosa of diabetic patients in different geographic regions of Iran, genotyping pattern of isolates is not well-studied yet.[31-33] To address this question, in the current study, we isolated and identified the *Candida* species of oral cavity of both diabetic patients and healthy nondiabetic subjects, which have mainly focused on the determination of genotyping of *C. albicans* (25S rDNA region) through polymerase chain reaction (PCR) (CA-INT) as well as on the production of enzymatic activities of *Candida* species.

Materials and Methods

Clinical specimens, culture condition

In the period (October 2018 to November 2019), 160 patients with type 2 diabetes, who were attended at the Diabetic centers (Tehran, Iran), have been recruited in this cross-section study. Besides, 108 non-diabetic individuals were also included in this study. All the participants had assigned a consent form at the beginning of sampling and subsequently, they were identified by numerical codes. All demographic data of patients including age, gender, clinical sign of OC, and hemoglobin A1c (HbA1c) was recorded in questionnaires' form.

Sterile cotton swab samples were taken from the tongue and buccal mucosal lesions of patients, which were immediately transported to Falcone contains sterile phosphate buffer saline (PBS). All specimens were tested for direct microscopic examination, to detect probable yeast and pseudohyphae elements. Subsequently, specimens were cultured on Sabouraud dextrose agar medium (SDA, Sigma Aldrich-USA) containing chloramphenicol, and then incubated for 24–48 h at 35°C–37°C. Moreover, *Candida* isolates were presumptively identified using chromogenic medium (CHROMagar*Candida*, France), as well as the production of chlamydospores on cornmeal agar for morphological identification. In addition, all the fungal isolates were definitely confirmed by PCR and sequencing as described below.

Identification of *Candida* species, DNA amplification, and sequencing

Genomic DNA was extracted from *Candida* species using glass beads and lysis buffer as described earlier.[34,35] Finally, for the precise identification of *Candida* species, PCR-sequencing of the internal transcribed spacer (ITS) region (ITS 1-5.8s-ITS2 rDNA region) was carried out and the obtained data were then analyzed in NCBI gene bank and blasted based on similarity of Nucleotide Blast database. Universal primers were also used for the ITS gene related to fungal *rDNA* genes. Nucleotide sequences of *Candida* species are available on NCBI website.

Genotyping of *Candida albicans*

Genomic DNA of *C. albicans* isolates was extracted as previously described with minor modification.[34] Then, the quality of DNA was checked out using electrophoresis on agarose gel 1.5%. Subsequently, PCR assay was performed as the following process: 12.5 µl of Master mix PCR (Amplicone, Korea) was added to 10 picomole from each specific primers (Forward 5'-CCT TGG CTG TGG TTT CGC TAG ATA GTA GAT-**3')** and (Reveres5'-ATA AGG GAA GTC GGC AAA ATA GAT CCG TAA-3'), then 2 µl DNA from each isolate was added to the mixture, and the final volume adjusted to 25 µl by distilled water. Afterward, the tube was placed in a Thermal Cycler (PreQLab, Germany). PCR program was adjusted 30 cycles containing denaturation for 5 min at 95°C, annealing for 1 min at 65°C, extension for 1 min at 72°C, and the final extension for 7 min at 95°C. Subsequently, the amplified products were analyzed by electrophoresing on 1.5% gel agarose in TAE (Trisbase, acetic acid, EDTA, Sigma-Aldrich, USA) buffer and then stained with 0.5 µg/ml SYBR safe-DNA Gel stain (Takara, Japan), to visualize the bands. Based on the molecular size of different genotypes, we categorized them into three groups as follows: genotype A exhibiting one band size of

450 base pair (bp), genotype representing one band size of 650 bp, and genotype C exhibiting two band sizes of 450 and 650 bp.[36]

Hemolytic assay

Hemolytic activity of *Candida* isolates was evaluated using a modification of the plate assay described by Sardi *et al*.[29] Briefly, the isolates were cultured on Sabouraud dextrose agar (Merck, Germany) containing chloramphenicol for 24 hat 35°C–37°C. In the next step, Sabourauddextrose agar medium, which was enriched with fresh human blood red cells (RBC) and glucose in the final concentration 3% w/v, was prepared. For this purpose, small wells (size 2–3 mm) were created in the medium, and 10 µL equal to 1.5×10^3 yeasts per ml of fresh yeast isolates was individually grown on SDA and then inoculated onto each well of blood agar. Notably, a reference strain of *C. albicans* (ATCC 90028) was used as a positive control. In addition, one standard strain of *Staphylococcus aureus* and one strain of *Streptococcus mutans*, inducing beta and alpha hemolysis were used as positive controls, respectively. Then, the plates were incubated for 48 hat 37°C in 5% CO_2. Alpha-hemolytic (α-hemolytic) strains produced a green halo and beta-hemolytic (β-hemolytic) strains produced a yellow halo. The experiments were performed in triplicate in three independent experiments for each one of the tested yeast isolates. Next, hemolysis and/or non-hemolysis caused by isolates were measured using the following formula: areola diameter = total circle diameter created-diameter of the hole created in the plate.

Hydrophobicity assay

Candida isolates were cultured on SDA medium and then incubated for 24 hat 35°C–37°C. Yeast suspension was prepared (1.5×10^3 yeast per ml), transferred to microtube containing 0.5 ml sterile YNB medium (yeast nitrogen base, Sigma-Aldrich, USA) for 24 h. Then, YNB medium was discarded and the sediment was then transferred to micro tubes containing 1 ml sterile PBS. Optical density (OD) of the samples was measured using spectra photometer with a wavelength of 520 nm. Subsequently, n-octan 25 (TITRACHEM company) was added to yeast suspension and this mixture was incubated for 30 min at 37°C and finally, two phases were observed. Subsequently, the upper phase was discarded and OD of lower-phase was measured for hydrophobicity activity. The OD values of strains in the YNB broth without n-octane overlay were used as negative controls. In addition, the relative CSH was calculated according to the following formula: [(OD570 of the control-OD570 after n-octane overlay)/ OD570 of the control] x 100. The results were expressed as a percentage (%).[21]

Statistical analysis

The data were first analyzed using SPSS software, version 20 (SPSS, Chicago, IL, USA), and subsequently analyzed using descriptive statistics, Fisher's, and Pearson Chi-square tests to determine differences in the ratio of the categorized variables. Afterward, continuous variables with an approximately normal distribution were tested using the Student's test. Furthermore, $P < 0.05$ was considered as statistically significant.

Results

Patients'data, *Candida* species, and enzymatic activity

Of 160 diabetic patients, 69 (43.12%) were men (mean age of 65 years old) and 91 (56.87%) were women (mean age of 68 years old) while out of 108 healthy subjects, (n = 17, 16%) were men (mean age of 60 years old) and (n = 91, 84%) were women (mean age of 64 years old). The mean HbA1c of the diabetes patients was determined to be 9% and 9.5% in men and women, respectively (Normal range of HbA1c is below 6%) whereas the mean of HbA1c level was determined as 4% for both of gender. The demographic data of the patients are summarized in Table 1. Thrush was the most frequent clinical signs of oral lesions in the diabetic patients (n = 50, 56.8%) followed by pseudomembranous plaques (n = 20, 22.7%) and perleche (n = 18, 20.5%).

A total of 88 (55%) patients with diabetes were positive for *Candida* species on SDA medium. Using CHROMagar *Candida* medium, 79.5% of the isolates (70/88) were presumptively identified as *C. albicans,* whereas 18/88 (20.5%) isolates were identified as NACs. Totally, 46 (42.6%) *Candida* species recovered from healthy individuals, among the positive species, 43 (93.5%) were identified as *C. albicans,* and only (n = 3, 6.5%) were NACs, including *C. glabrata* (n = 2) and *Candida dublinensis* (n = 1) [Table 2].

Subsequently, all the *Candida* isolates were definitely identified by sequencing of *ITS 1-5.8s-ITS2* rDNA region and then matched to the reference documents in the GenBank database using BLAST to distinguish species' level. The accession numbers of *Candida* species are represented in (http://www.ncbi.nlm.nih.gov/BLAST) as follows: MG821633, MG821632.1, MG821630.1, MG821628.1, MG821625.1, MG812312.1, KY773288, and MG812310.

In diabetes patients, *C. albicans* (n = 70, 79.5%) was the most frequent species followed by NACs: *Candida krusei* (n = 10; 11.4%), *C. dublinensis* (n = 5; 5.7%), and *C. kefyr* (n = 3; 3.4%) [Table 2].

In patients with diabetes, the majority of *Candida* species (64/88, 72.72%) presented hydrophobicity >88% (high hydrophobicity is considered above 60%). In healthy non-diabetes subjects, hydrophobicity of *C. albicans* was 60%. Remarkably, the hydrophobicity of *Candida* species was significantly greater in patients with diabetes compared to the

Table 1: Demographic characteristic, enzymatic activity and genotyping of *Candida* species in diabetic patients compared to health subjects

Characteristic	Groups		*P*
	Diabetes (*n*=160), *n* (%)	Control (*n*=108), *n* (%)	
Gender			
Male	69 (43.1)	17 (16)	0.76
Female	91 (56.8)	91 (84)	
Age (mean±SD)			
Men	65±10	60±10	0.86
Female	68±8.6	64±9.6	
Recovery of *Candida* spp. on culture	88 (55)	46 (42.6)	
C. albicans	70 (80)	43 (93.5)	
Non *albicans Candida*	18 (20)	3 (6.5)	
HbA1c			
Men	9±2	4±1	0.7
Female	9.5±2	4±1	
Hydrophobicity activity of *Candida* spp. (%)	≥88	60	0.03*
Hemolytic activity of *Candida* spp. (%)	19.5	10	0.04*
Genotyping of *C. albicans* isolates			
A	50 (71.42)	33 (76.7)	
B	15 (21.43)	8 (18.6)	
C	5 (7.15)	2 (4.7)	

*Indicated statically significant difference. SD: Standard deviation, HbA1c: Hemoglobin A1c, *C. albicans*: *Candida albicans*

Table 2: Frequency of *Candida* species identified from oral cavity of diabetes patients and control group

Candida species	Group	
	Diabetes patients, *n* (%)	Healthy non-diabetes individuals, *n* (%)
C. albicans	70 (79.5)	43 (93.47)
C. glabrata	-	2.(4.34)
C. krusei	10 (11.4)	-
C. dublianensis	5 (5.7)	1 (2.17)
C. kefyr	3 (3.4)	-

C. albicans: Candida albicans, C. glabrata: Candida glabrata, C. krusei: Candida krusei, C. dublianensis: Candida dubliniensis, C. kefyr: Candida kefyr

control group (P = 0.03). Similarly, in diabetic patients, *C. albicans* species showed a significantly higher level of hydrophobicity compared to NACs ($P < 0.05$) isolates.

In diabetes patients, hemolytic activities of the majority of both *C. albicans* (82.1%) and NACs (17.9%) were more than 19.5%, whereas in healthy individuals, hemolytic activity of *C. albicans* was detected 10%.(P = 0.04). Interestingly, a statistically significant difference was observed in terms of hemolytic activity of *Candida* species between the healthy controls and diabetes patients ($P < 0.05$). However, no significant association was detected between hemolytic activity and *Candida* species in both diabetic patients and healthy subjects (P = 0.56).

Genotyping of *Candida albicans* isolates

Genotyping pattern of *C. albicans* were determined using amplification of 25S rDNA region through PCR (CA-INT). Three major genotyping patterns were detected in *C. albicans* isolates recovered from diabetic cases as follows: genotype A was the most predominant one among the isolates (71.42%, n = 50) followed by genotype B (21.43%, n = 15) and genotype C (7.15%, n = 5). No statistically significant association was found between different genotype and hemolytic and hydrophobicity activities of *Candida* species (P = 0.7). In addition, no statistically significant correlation was observed among genotyping patterns and age and HbA1c level (P = 0.7) in diabetes patients. Genotype A of *C. albicans* was detected in 26% of diabetic women compared to diabetic men (10%). In healthy controls among *C. albicans* species (n = 43), genotype A (n = 33, 76.7%) was frequently prevalent followed by genotype B (n = 8, 18.6%), and genotype C (n = 2, 4.7%). The frequency of genotypes of *C. albicans* in patients with diabetes compared to healthy individuals is shown in Figure 1. PCR pattern of different genotypes of gel electrophoresis is shown in Figure 2.

Discussion

The obtained findings showed that the *Candida* carriage rate was statistically higher in patients with diabetes than healthy individuals, which is in agreement with previous reports.[33,37] It is well known that immunosuppression condition such diabetes mellitus as a major health challenging, can affect *Candida* colonization in a high rate and consequently progress the infection in host tissue.[1,9]

In our study, *C. albicans* was the most predominantly species of oral cavity in the two groups followed by NACs, which have consistently been studied previously.[33,38,39]

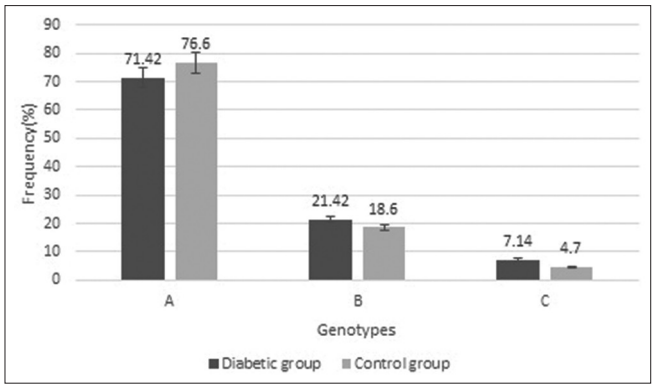

Figure 1: Frequency of genotyping patterns of *Candida albicans* isolates from diabetic patients and healthy, nondiabetes individuals

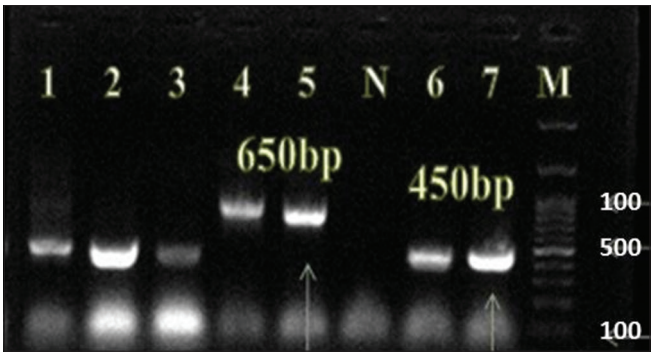

Figure 2: Genotypes of *Candida albicans* representing different product size of CA-INT amplification on agarose gel electrophoresis. Line 1, 2, 3 and 6,7 genotype A (450 bp), N; Negative control, Line 4 and 5 genotype B (650 bp), M; DNA Marker100 bp

Remarkably, *C. krusei* was the most frequently NACs isolated from oral cavity of diabetes patients. It is noteworthy that the number of NACs has significantly increased over the last decades accompanied by relevant growth in morbidity and mortality rates.[40]

Our finding indicated that hydrophobicity of *Candida* species was significantly higher in diabetes patients compared to healthy individuals. Moreover, hydrophobicity ability of *C. albicans* species was significantly greater than NACs. Surprisingly, previous reports support our findings, demonstrating that *C. albicans* through CSH adhered to epithelial surface of host tissue such as oral mucosa causing enhancing virulence and pathogenesis by increasing the adhesion and prompting biofilm formation in infected tissue.[41-43] On the other hand, previous studies indicated a high concentration of glucose in individuals with diabetes serve as a nutritional source to maintain the polysaccharide matrix of *Candida* biofilm to protect this structure from environmental changes.[44]

Biofilm formation[44,45] and enzymatic activity,[25,46,47] serve as two important features of pathogenic ability of *Candia* species. These factors have a main impact on the balance between host and *Candida* sp., favoring the transition of *Candida* from commensal to pathogen and leading to infection.[1] Adherence of *Candida* in oral mucosa can also exhibit antifungal resistance significantly and persistent infection could reach deep tissue of patients and causing disseminated and life threatens candidiasis like Candidemia in immunosuppressive patients with damaged cellular immunity.[45,48] Therefore, appropriate strategies to the management of OC in the high-risk patients seems fundamental to control of disseminated infections.

Hemolytic analysis of *Candida* species revealed a statistically significant difference between patients with diabetes and nondiabetic healthy participants. Unlikely, this activity showed no significant changes between *C. albicans* and NACs in the two studied group. Studies conducted on the activity of hemolysis in *Candida* species are limited. Fatahinia *et al*. demonstrated significantly different hemolytic activity of *Candida* species isolated from oral mucosa of diabetes patients and healthy, nondiabetic controls.[25] In another study, all *C. albicans* strains that were isolated from oral cavity of HIV-infected patients produced hemolysins, as a virulence factor, while 86% NACs revealed hemolytic activity.[49]

It was previously shown that *Candida* isolates among patients with diabetes due to a higher blood glucose concentration, represented a higher pathogenic potential for adhesion and hemolytic activity, which may usually contribute to pathogenesis positively.[46,50,51] In another study by Tsang, significantly higher hemolytic activity was proven in *Candida* species from oral cavity of type 2 diabetes patients compared to the control group.[47] This finding has been supported by the higher blood glucose concentration and HbA1c in patients with diabetes. Hence, Luo *et al*. defined that the increased glucose concentration in culture medium directly or indirectly enhance hemolysin activity of *Candida* species isolated from diabetic patients.[52]

According to our findings, genotype A was the most common genotypes among *C. albicas* species isolated from both diabetes patients and healthy individuals. In this context, we hypothesized that pathogenicity of OC in patients with diabetes is associated with genotype A, as a predominant type. Nevertheless, genotypes B and C with less frequency increased in *C. albicans* species and must be noted in the diabetic patients. Several factors including gene mutations may contribute to the diversity of genotyping patterns in our study.[53-55] In spite of genotype A was the most frequent genotype in two populations, no significant association between genotype A, hydrophobicity, and hemolytic of *C. albicans* species were observed in our study. Sardietal. determined the genetic pattern of *C. albicans* isolated from diabetic patients with chronic periodontitis. The findings indicated two main genotypes A and B in *C. albicans* isolates, which was in contrast with our study, and they also reported a positive association between genotyping A and a higher

hydrophobicity in *Candida* isolates.[29] In consistent with our finding, Bii *et al*.[36] demonstrated different genotypes of *C. albicans* isolated from blood, sputum, urine, and catheters using primer pairs, which span the site of transposable intron 25S rDNA with the highest frequency of genotype A followed by genotypes B, C, and BC. In a study by Menezes *et al*., different genotypes of *Candida* species of the hospitalized patients have been indicated in Brazil using RAPD-PCR.[53]

It seems that strains from different geographical regions have been known to display slight differences in genotypic features.

In conclusion, we reported three genotypes patterns of *C. albicans* isolates from oral cavity of patients with diabetes and healthy individuals in Iran using Amplification of 25S rDNA region. Notably, the low number of isolates was one of the limitations of this study. By selecting large sample size, we might be able to obtain the possible association between different clinical parameters and virulence factors of *Candida* species. Amplification of 25S rDNA region through PCR (CA-INT) suggests high levels of reproducibility and discrimination, which facilitate the description of *C. albicans* genotypes while being easily performed in a laboratory.[56] Definitely, the application of the other confirmatory molecular methods such as Saturn Blatting Hybridization, MLST, and DNA microsatellite analysis would be desired.[29,57]

Further studies on the various extracellular enzymes responsible for virulence are urgently needed to uncover host-pathogen interaction in connection with mucosal *Candida* infections in different types of immunosuppressive populations.

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Ethical approval

This study was approved by the research committee of Iran University of Medical Sciences with ethical code (IR. IUMS. REC.573).

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Conflicts of interest

There are no conflicts of interest.

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