Original Article

Identification and Genotyping of *Candida* Species Involved in Oral Candidiasis among Diabetic Patients

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Abstract

Objective: Oral candidiasis is more prevalent among diabetic patients than non-diabetics due to the factors that promote *Candida* oral carriage. This study aimed to isolate and identify *Candida* species involved in oral candidiasis of patients with diabetes mellitus in Sulaymaniyah city.

Methods: The study was performed from August 2021 to March 2022 on two diabetic patients, Type 1 and Type 2 (n=150) and non-diabetics (n=50), as a control group. In Iraq's Kurdistan region, oral swabs were taken from 200 participants at the Sulaymaniyah governorate's Diabetic and Endocrine Center and Shar hospital. Sabouraud dextrose agar (SDA) medium was used to culture the swabs. *Candida* isolates were identified using HiCrome™ Candida Differential agar, then confirmed using polymerase chain reaction based on the ITS region and CHS1 gene detection. Using the CA25S and CA-INT primers, all *C. Albicans* isolates were genotyped based on the transposable intron in 25S rDNA. ITS1 and ITS4 primers were used to sequence the 18S region of ribosomal DNA (rDNA). Descriptive statistics were used for summaries and to describe data.

Results: From the samples of 150 diabetes patients and 50 controls, 64 (42.6%) and 12 (24%) were positive for *Candida* spp. In the diabetic patients, 34 (53.1%) of the 64 isolated *Candida* spp. were identified as *C. Albicans*, while 6 (50%) of the healthy subjects had *C. albicans*. The genotypes A (450 bp), B (840 bp), C (450 and 840 bp) of *C. Albicans and* D (1040 bp) that belongs to *C. dubliniensis* were detected. Genotype A (54.69%) was the most frequent.

Conclusions: This study concluded that there was a difference in the proportion of *Candida* spp. colonization in the oral cavity of diabetic patients compared to the healthy group; also, we found that *C. Albicans* with Genotype A was the most prevalent species among all other species in both groups.

Keywords: *Oral candidiasis, Diabetic, C. Albicans, Genotype A, Phylogenetic tree.*

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Introduction

Fungal pathogens can cause life-threatening invasive infections, chronic conditions, and recurring superficial infections[1]. *Candida* is the main human fungal pathogen that causes candidiasis infections[2]. Even while *Candida* spp. is a part of the oral microbiota in symbiosis, under specific conditions, it may induce microbial imbalance leading to dysbiosis, resulting in oral diseases[3]. Candidiasis generally manifests as a mild disease of the oral mucous membranes but occasionally may be resistant to therapy or become relapsing or recurring[4]. Carriage rates in the general population have been observed to range between 20% and 75% without causing any symptoms[5].

Diabetes mellitus (DM) is a long-term metabolic disorder marked by persistent hyperglycemia. It might be caused to a lack of insulin secretion, resistance to insulin's peripheral activities, or both[6]. Several causes are related to greater *Candida* spp. propensity among DM patients depending on the local or systemic infection. The identified host factors for candidal colonization and eventual infection include yeast attachment to epithelial cell surfaces, greater salivary glucose levels, lower salivary flow[7], microvascular degeneration, and diminished candidacidal activity of neutrophils[8]. These situations are especially dangerous if the patient has hyperglycemia, which causes the production of many degradative enzymes, or if the patient has a widespread immunosuppressive state. These factors greatly influence the host-yeast balance, favoring *Candida* spp. The transformation from commensal to pathogen and infection[9]. Only 40 species of *Candida* are capable of causing illness out of the over 200 that have been identified. In both symptomatic and asymptomatic candidiasis infections, *C. albicans* is the most frequently isolated species[10]. *C. krusei, C. glabrata, C. tropicalis, C. kefyr, C. parapsilosis, C. lusitaniae, C. dubliniensis, C. guilliermondii,* and *C. stellatoidea* are a few more species that have been discovered to be pathogenic to humans[11]. Heterogeneity of virulence features in clinical *C. albicans* isolates is frequently found. Hence, molecular subtyping provides a helpful technique to detect pathogenic subpopulations within this species[12] *C. albicans* can be divided into four subtypes depending on the existence and size of transposable intron region in the large ribosomal subunit 25S rDNA, namely genotype A (450 bp), genotype B (840 bp), genotype C (450- and 840-bp), and genotype E (1080 bp)) as well as also genotype D (1040 bp) belong to *C. dubliniensis*[13].

To identify yeast species isolated from clinical samples, phenotypic methods are used. They are divided into two parts: macroscopic of the colony on a plate surface and microscopic observation of fungal structures[14]. To quickly identify Candida spp. chromogenic medium is required. In mixed cultures of *Candida* on Sabouraud's Dextrose Agar based on colony color, these media contain chromogenic substrates that react with enzymes secreted by microorganisms generating colonies with various pigmentation; these enzymes are species-specific, allowing organisms to be identified at the species level by their color and colony features[15,16]. Polymerase chain reaction-based techniques for detection and identification of *Candida* spp. have also been reported; a single-step PCR using universal primers [internal transcribed spacer (ITS)1 and ITS4] to amplify the ITS1-5.8S-ITS2 region of ribosomal DNA (rDNA) (genes encoding for ribosomal RNA), the most extensively used genetic marker in identifying species[17,18]. Thus, this study aimed to isolate and identify *Candida* spp. involved in oral candidiasis in diabetic patients.

Patients and methods

Sample collection

Oral swabs were collected from 200 participants in the Diabetic and Endocrine center and Shar hospital of Sulaymaniyah governorate, Kurdistan region-Iraq, from August 2021 to January 2022, on two groups of diabetic patients (n=150) and non-diabetics (n=50) as a control group. The samples were collected from different ages ranging from 16 years to 95 years old. The samples included both sexes, males 49 (32.66%) and females 101 (67.33%) from different types of diabetes (Type 1 and Type 2), with males 21 (42%) and females 29 (58%) in the control group. Before collecting the samples, ethical approval was obtained from the Research Protocol Ethics Committee of the College of the Science/ University of Sulaimani, and informed consent was obtained from all study participants.

Culturing and isolating candida species

Swabs were cultured on a Sabouraud dextrose agar (SDA) medium [Mumbai, India]. Supplemented with chloramphenicol [0.5 mg/mL, Sina Darou_Tehran-IRAN] and incubated at 37^0C for 48 hours. As preliminary identification, pure colonies were transferred on HiCrome™ *Candida* Differential agar [Mumbai, India] and incubated at 37ºC. The growth was checked after 48 hours of incubation; this method is used to distinguish distinct *Candida* spp. based on color and morphology[19,20].

Molecular identification of *Candida spp.*

The PCR was performed to amplify intergenic spacer regions (ITS) of the gene encoding 5.8 S rDNA using forward ITS1-F (5'-TCCGTAGGTGAACCTGCG-3') and reverse ITS4-R (5'-TCCTCCGCTTATTGATATGC -3') primers[17]. A single colony was directly used as a template for PCR without the extraction of pure DNA (colony PCR). A large amount of material and the time-consuming process of extracting genomic DNA can be saved using colony PCR. A single colony was taken from the overnight culture and resuspended in 40µl of ddH2O. The DNA was released after a 20-minute incubation at 95°C, according to Shekhany (2021)[21]. The DNA was purified by centrifugation at 12000 rpm for 2 minutes; then, the supernatant was used as a PCR template. The PCR was performed using [Taq Master (2x conc.) / addbio. South Korea] master mix according to manual instructions. The method was started with one cycle of initial denaturation at 95ºC for 5min, followed by 40 amplification cycles of denaturation at 95ºC for 30sec, annealing at 57ºC for 30sec, and extension at 72ºC for 40sec, then finalized with the final extension at 72ºC for 5min. The amplicon was separated by 2% gel electrophoresis and then visualized by ethidium bromide.

Confirmation of the *Candida spp. by* detection of CHS1 gene

CHS1 gene is regarded as a virulence gene present in four *Candida* species (*C. albicans, C. glabrata, C. tropicalis, and C. parapsilosis*), A portion of the chitin synthase gene (CHS1) from *Candida* spp. (122bp) can be amplified using conventional PCR using *C. krusei* as negative control[22]. The colony PCR was carried out with the same DNA polymerase kit as previously mentioned using forward CHS1-F (5'-CGCCTCTGATGGTGATGAT-3') and revers CHSI-R (5'-TCCGGTATCACCTGGCTC-3') primers[23]. The samples were placed in a thermocycler. The amplification programs were configured as follows: one cycle of the initial denaturation at 95ºC for 5min, then 40 cycles of denaturation at 95ºC for 30sec, annealing at 52ºC for 30sec, and extension at 72ºC for 30sec were applied. The PCR was finished with one cycle of final extension at 72ºC for 7min. PCR products were resolved on 2% agarose gel.

Genotypes detection by Nested PCR

Genotype determination was based on the transposable intron in 25S rDNA. The nested PCR technique was used in the first round of PCR, CA25S primers were used Forward CA25S-F (5'-CGATTCAGGGGAGGTAGTGAC-3') and revers CA25S-R(5'-GGTTCGCCATAAATGGCTACCAG-3')[12]. The PCR program was as follows: one cycle of the initial denaturation at 95ºC for 5min. Then, the PCR was continued with the 35 cycles of denaturation at 95ºC for 30sec and annealing at 64ºC for 30sec and extension at 72ºC for 40sec, then the final extension at 72ºC for 5min. The first round's amplicon was utilized as a template for the second PCR, which was performed using the same program with CA-INT primers forward CA-INT-F (5'-ATAAGGGAAGTCGGCAAAATAGATCCGTAA-3') and revers CA-INT-R (5'-CCTTGGCTGTGGGTTTCGCTAGATAGTAGAT-3') and 70°C annealing temperature[12].

Sequencing

Sequencing was performed on 22 samples that were amplified using ITS1-F and ITS4-R forward and reversed primers(10pmol) (Sanger sequencing/ ABI 3500, Macrogen Genome Center, Republic of Korea).

Phylogenetic analysis

Aligned sequences were used for phylogenetic analysis performed with Mega 7 software. The Neighbor-Joining method was used to infer the evolutionary history[24]. The optimal tree is shown, with a branch length sum of 1.74846739. The confidence probability (multiplied by 100) that the interior branch length is greater than 0, as estimated using the bootstrap test (100 replicates are shown next to the branches[25,26]. The branch lengths are in the same units as the evolutionary distances used to infer the phylogenetic tree, and the tree is drawn to scale. The Poisson correction method was used to calculate the evolutionary distances[27], and are in the units of the number of amino acid substitutions per site. A gamma distribution (shape parameter = 1) was used to model rate variation between sites. A total of 40 amino acid sequences were examined. The standard genetic code table was used to translate the coding data. Gaps and missing data were removed from all positions. The final dataset contained a total of 81 positions[28].

Statistical analysis

In this study, descriptive statistics were used to describe the basic features of the data.

Results

The current study was conducted by involving 200 participants in two groups of diabetic patients (n=150) and non-diabetics (n=50) as a control group. Among the samples of 150 diabetes patients and 50 controls, 64 (42.6%) and 12 (24%) were positive for *Candida* species. All isolates were identified on HiCrome™ Candida Differential agar (Table, Figure 1), then confirmed with PCR based on the ITS region (Table, Figure 2). In the DM patients, 34 (53.1%) of the 64 isolated *Candida* spp. were identified as *C. albicans*, while 6 (50%) of the healthy subjects had been identified as *C. albicans*. The current result revealed that oral *Candida* colonization was more common in diabetes patients than in the non-diabetic group.

PCR amplification of the internal transcribed spacer (ITS) regions and genotyping analysis

Positive cultures of samples taken from both groups revealed the following bands after performing PCR amplification, as mentioned in Table 1 and Figure 2. In this study, the results of the molecular technique and the colony features of the isolates on HiCrome™ *Candida* Differential agar exhibited perfect Identity. Furthermore, amplicons of the CHS1 gene (122 bp) were observed in *C. albicans*, *C. glabrata*, *C. parapsilosis*, and *C. tropicalis* and not in the negative control *C. krusei* (Figure 3).

Then all *C. albicans* and *C. dubliniensis* isolates were then submitted to genotypic analysis based on the transposable intron in 25S rDNA using CA25S and CA-INT primers. It has been observed that *C. albicans* isolates belong to three genotypes where are genotype A (450 bp), genotype B (840 bp), genotype C (450 and 840 bp), and *C. dubliniensis* belongs to genotype D (1040 bp) (Table 2).

Results showed that out of 34 isolates from the diabetic group; Genotype A was the most abundant with (26) isolates, followed by Genotype B with (6) isolates and Genotype C (2) isolates. In comparison, all six isolates of *C. albicans* from the control group belonged to genotype A. furthermore, in the current study (4), isolates of genotype D were found, as shown in (Table 2). None of the PCR products was 1080 bp long in our study, which relates to genotype E.

Table 1: Identification and distribution of Candida spp. isolated in the oral cavity of the diabetic and control.

Candida Species	Diabetic group No. (%)	Non-diabetic group No. (%)	Color on HiCrome agar	Fragment size found by PCR with ITS1-ITS4 primers
C. albicans	34 (53.1%)	6 (50%)	Light green	532 bp
C. kefyr	13 (20.3%)	-	pink	722 bp
C. glabrata	12 (18.7%)	4 (33.4%)	Cream to white	874 bp
C. dubliniensis	3 (4.7%)	1 (8.3%)	Pale green	540 bp
C. tropicalis	1 (1.6%)	-	blue	521 bp
C. parapsilosis	-	1 (8.3%)	Cream to white	516 bp
C. krusei	1 (1.6%)	-	Purple, fuzzy	500 bp
Total	64 (100%)	12 (100%)	-	-

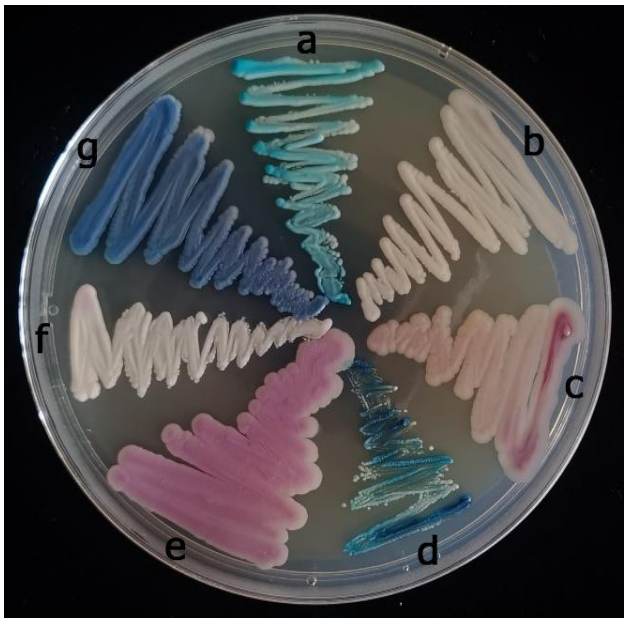

Figure 1: Different colony colors and morphologies of various Candida spp. on HiCrome™ Candida Differential Agar a/ C. Albicans, b/ C. glabrata, c/ C. kefyr, d/ C. dubliniensis, e/ C. krusei, f/ C. parapsilosis, g/ C. tropicalis.

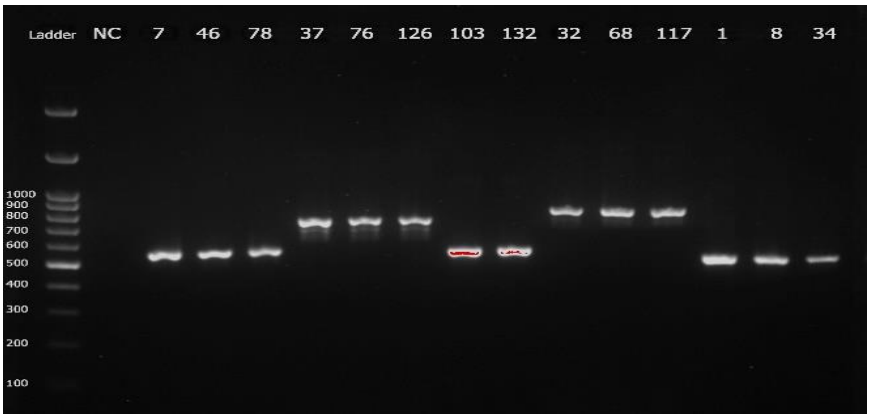

Figure 2: Candida spp. identification showing seven species, from left the DNA marker 100bp [GeneDireX, Inc. United States], negative control, C. albicans (532bp), C. kefyr (722bp), C. dubliniensis (540bp), C. glabrata (874bp), C. tropicalis (521bp), C. parapsilosis (516bp) and krusei (500bp).

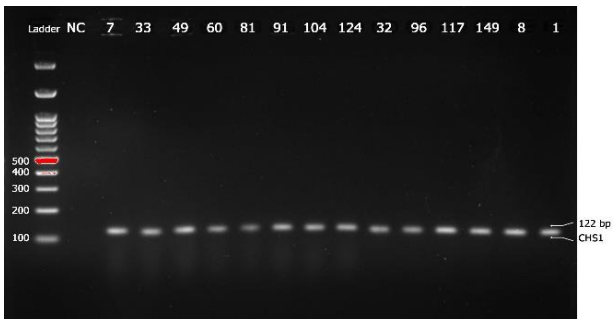

Figure 3: Confirmation of the *C. albicans, C. glabrata, C. parapsilosis*, and *C. tropicalis* identification with CHS1 primers (122bp). The marker was 100bp DNA [GeneDireX, Inc. United States].

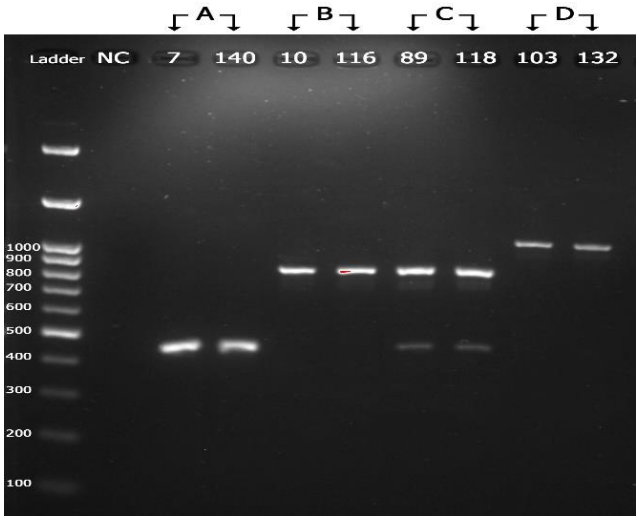

Figure 4: Detection of three genotypes of *C. albicans*, and genotype D of *C. dubliniensis.* The marker was 100bp DNA [GeneDireX, Inc. United States].

Table 2: Frequency of *C. albicans and C. dubliniensis* genotypes in diabetic and control groups for 44 isolates.

Source	No. (%) of strains of the following genotype:				
	Total	Genotype A	Genotype B	Genotype C	Genotype D
Diabetic group	37	26	6	2	3
Control group	7	6	0	0	1
Total	44 (100%)	32 (72.7%)	6 (13.6%)	2 (4.6%)	4 (9.1%)_

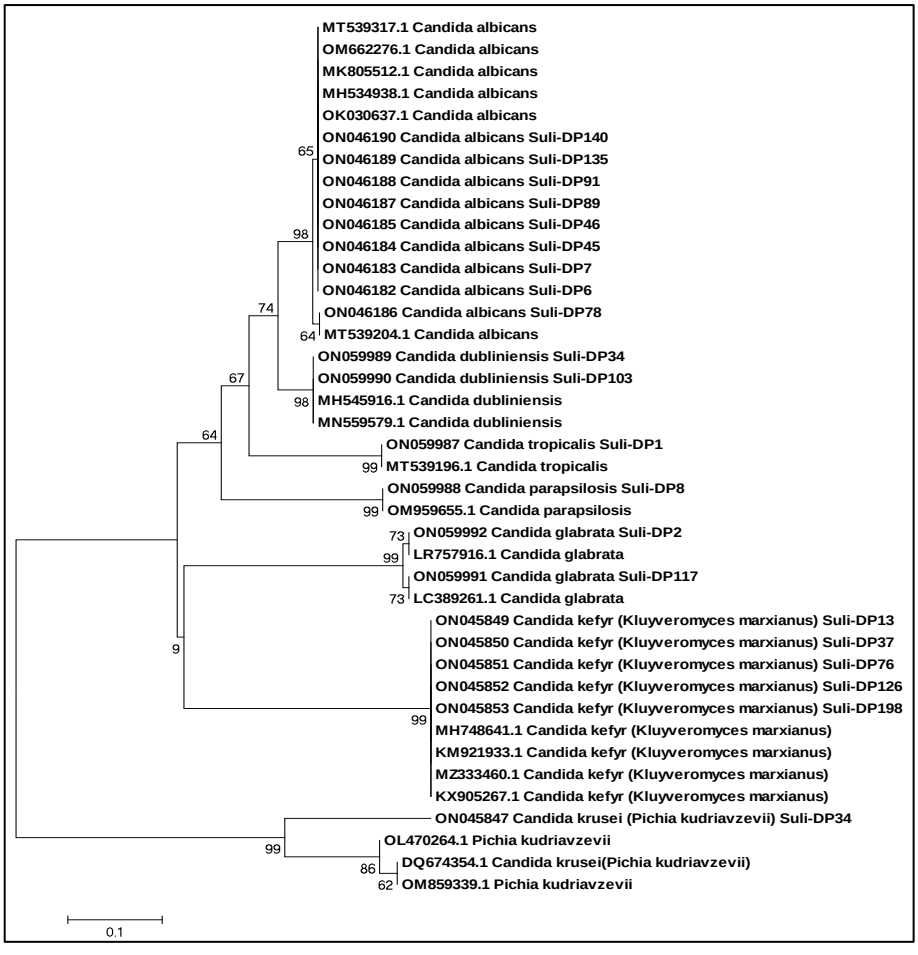

Figure 5: Phylogenetic tree based on ITS1-5.8S-ITS4 region sequences for 21 isolated *Candida* spp. with reference strains.

Discussion

Candidiasis is the most common opportunistic yeast infection globally[29]. Diabetic patients are more predisposed to oral candidiasis than non-diabetics because of the conditions favoring oral carriage of *Candida*[30]. High salivary glucose levels enhance Candida adhesion to buccal epithelial cells. Salivary glucose produces chemically reversible glycosylation products with proteins in tissues during hyperglycemic episodes, which leads to a buildup of glycosylation products on buccal epithelial cells, potentially increasing the number of *Candida*-accessible receptors[31]. Also, poor oral hygiene in diabetes patients may raise *Candida* spp. as part of the oral flora and might alter the superficial and systemic fungal infections compared to healthy individuals[8].

In the current study, the incidence of *Candida* spp. from the oral cavity of the diabetic patient was (42.6%) and (24%) in the control group; this result for the diabetic group was lower than that reported in Baghdad/ Iraq (77.3%)[32], and Iran (55%)[8], but it was more than that reported in Jordan (8.3%)[33]. In the control group, the result was higher compared to Al-Badri *et al.* (18.7%)[32] and Abu-Elteen *et al.* (0%)[33], but lower than those of the Iran study (35.5%)[8]. *C. albicans* was the most predominant species among all other isolated species (53.1%), followed by other species of *Candida;* these results are compatible with all above previous studies[8,32,33]. Gomes *et al.* demonstrated that the Strains of *C. albicans* isolated from diabetic patients increased virulence compared to normoglycemic patients. Several researches studied the association between *C. albicans* different genotypes and pathogenicity of subpopulation within this species, thereby assist in creating effective therapeutic options to combat the disease[34,35]. In another study that done in Diyala province 25(83.3%) of isolates belonged to the genotype A and 5 (16.6%) to genotype C of the *C. albicans*. There were no results for genotype B[36]. These results are inconsistent with our findings in that in total number of isolate in diabetics group 37 the genotype A was the most abundant with 26 isolates (70.3%) followed by genotype B with 6 isolates (16.2%) and genotype C 2 isolates (5.4%), also in current study 3 isolates of genotype D (8.1%) in diabetic groups that are not reported by previous studies.

Notably, there is limited information concerning the phylogeny and genetic diversity of *Candida* spp. Strains isolated in the oral cavity of a diabetic patient in Iraq. In this study, the phylogenetic analyses showed that there are two main clades and ten different subclades. Furthermore, about 95.2% of the studied isolates were placed in clade I, and 4.8% were in clade II (Figure 5). Therefore, it is clear that some isolates were 100% identical and also identical to the reference sequence representing *C. albicans*, *C. dubliniensis*, and *C. kefyr*. Also, we had variation in one isolate of *C. albicans* (Suli-PD78) that was more similar to the *C. albicans* strain from Iran (MT539204.1) and *C. glabrata* which are located in different subclades, notably found that *C. glabrata* (Suli-PD2) was more similar to strain from Kuwait (LR757916.1) and *C. glabrata* (Suli-PD117) was more similar to strain from Iran (LC389261.1).

Conclusions

This study concluded that *Candida* spp. colonization was more common in diabetes patients than in the non-diabetic group. Also, the current study found that *C. albicans* with Genotype A was the most prevalent species among all other species in both groups. The study will continue to determine the capabilities of *Candida albicans* that are isolated from diabetic patients to produce biofilm and study the relation between biofilm production and the prevalence of virulence gene markers.

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