Original Article

Identification of *Candida* species in the oral cavity of diabetic patients

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Abstract

Background and Purpose: Diabetic patients are more susceptible to oral candidiasis infection than non-diabetics due to the factors promoting oral carriage of *Candida.* Several factors can increase colonization of *Candida* species in the oral cavity such as xerostomia, which reduces the salivary flow and is a salivary pH disorder. In the current study, we aimed to identify and compare the colonization level of *Candida* spp. in the oral cavity of diabetic and non-diabetic groups.

Materials and Methods: Swabs were taken from the mouth of 106 participants and were cultured on Sabouraud dextrose agar (SDA) medium. Likewise, the saliva samples were collected for salivary glucose and pH measurements. The study was performed during June 2014-September 2015 on two groups of diabetic patients (n=58) and non-diabetics (n=48) as the control group. The *Candida* spp. were identified with PCR-restriction fragment length polymorphism (RFLP) using the restriction enzymes *HinfI* and *MspI* and were differentiated by culture on CHROMagar *Candida* medium.

Results: The frequency of *Candida* spp .was higher in diabetic patients compared to non-diabetics. The most frequent *Candida* spp. in the diabetic patients were *Candida albicans* (%36.2), *C. krusei* (%10.4), *C. glabrata* (%5.1), and *C. tropcalis*.(%3.4)Likewise, *C. albicans* was the most frequent species (%27) in the non-diabetic individuals. In this study, the results of both methods for identification of the isolates were consistent with each other.

Conclusion: Xerostomia and disturbance of physiological factors including pH and glucose can promote overgrowth of *Candida* flora in the oral cavity. These factors are considered important predisposing factors for oral candidiasis in diabetic patients .In the present study, it was observed that application of CHROMagar *Candida* and PCR-RFLP methods at the same time contributes to more accurate identification of isolates.

Keywords: CHROMagar *Candida*, Diabetes mellitus, Oral candidiasis, PCR-RFLP

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Introduction

In the recent decade, growth of the immunocompromised population has led to increased incidence of invasive *Candida* infections [1]. Oral candidiasis is a common opportunistic infection of the oral cavity caused by an overgrowth of *candida* species particularly *Candida albicans* [2]. Numerous risk factors such as age, gender, nutrition, oral hygiene, smoking, dentures, salivary pH disorder, and xerostomia (dry mouth) make diabetic patients more susceptible to oral candidiasis [3, 4].

Diabetes mellitus (DM) is the most common endocrine metabolic disorder [5-7]. Approximately 85-90% of diabetic patients are diagnosed with type 2 diabetes (resulting from insulin resistance); in these patients, salivary dysfunctions such as xerostomia, decreased salivary function, lichen planus, tooth decay, and periodontal diseases are common [6, 8]. Among the reasons making diabetic patients more susceptible to oral candidiasis are high levels of salivary glucose, low secretion of saliva, impaired chemotaxis, and defect of phagocytosis due to polymorphonuclear leukocyte deficiency [9, 10]. The attachment of *C. albicans* to the crystalline hydroxyapatite produces collagenolytic enzyme, which increases crystal solubility and consumes nitrogen of dentin collagen in DM patients [11-15].

Due to the upsurge in the level of non-albicans *Candida* species as well as azole-resistant isolates, finding a reliable diagnostic method is necessary for the treatment of *Candida*-related infections. The CHROMagar *Candida* is a chromogenic culture medium for the isolation and identification of *Candida* species based on colony color [16, 17].

Although CHROMagar *Candida* culture helps with identification of *C. albicans, C. tropicalis, C. krusei,* and *C. glabrata*, all isolates cannot be recognized in this method. Therefore, *Candida albicans* and non-albicans were identified by using PCR-RFLP, which is a rapid and cost-effective molecular method [18, 19].

Herein, we aimed to determine the distribution of *Candida* species in the oral cavity with CHROMagar *candida* and PCR-(restriction fragment length polymorphism) RFLP by the restriction enzymes HinfI and MspI in order to evaluate and compared the amount of yeasts colonized in the oral cavity of diabetic with non-diabetic individuals. Our results can help with improving patient treatment outcomes and reducing healthcare costs.

Materials and Methods

Study group

The samples were collected from patients attending the diabetes clinics of Sannandaj, Iran, during June 2014-September 2015. The study groups consisted of DM patients with fasting serum glucose level of higher than 126 mg/dl and medical records of the glycosylated hemoglobin (HbA1c). A form was designed to record the medical history of patients, type of diabetes, fasting serum glucose level, and demographic data.

The study groups were age and gender matched; and the patient group consisted of 58 patients with type II diabetes mellitus (38 males [65.5%] and 20 females [34.5%]) with the mean age of 50.33±7.63 years. The control group comprised of 48 non-diabetic individuals with the mean age of 49.85±8.71 years. Predisposing factors in the healthy subjects included smoking, wearing dentures, dental caries, and oral lesions.

Ethical considerations

Patient-related data was collected in accordance with the applicable principles of research ethics committees of Isfahan University of Medical Sciences, and written consent was obtained from the participants at the beginning the study.

Sample collection

Swabs were taken from the mouth of 106 subjects and were cultured on Sabouraud dextrose agar (SDA) medium (Merck, Germany). Saliva samples were collected for salivary glucose and pH measurements, as well.

Procedure of culturing and isolating Candida species

The plates were incubated for 72 h at 25°C. Then, pure colonies were transferred on CHROMagar *candida* (HiMedia, Mumbai, India) for the isolation and presumptive identification of *Candida* species. The *Candida* isolates were identified after incubation for 48 h at 37°C [20]. Light green colony color reveals *C. albicans*, steel blue colonies *C. tropicalis,* large, fuzzy, and rose-colored colonies indicated *C. krusei* [16].

DNA extraction

DNA was extracted from each sample as described before [21, 22]. A loop full of fresh colony was harvested and added to 300 µl of lysis buffer (100 mM NaCl, 10 mM Tris-HCl, pH=8, 2% Triton X-100, 1% sodium dodecyl sulfate, 1 mM EDTA, pH=8). Thereafter, the solution was added to 300 µl of phenol: chloroform (1:1) and 200 µl of glass beads, with a diameter of 1 mm. After shaking, the samples were centrifuged for 5 min at 10000 rpm. Then, 400 µl of phenol-chloroform-isoamyl alcohol mixture (25:24:1) was added to the supernatant. In the next step, after centrifuging, 1 volume of cold isopropanol and 3 M of sodium acetate were added to the upper phase and were kept at -20°C for 10 min. The sample was washed by 70% ethanol and preserved at -20 °C until the using time [23].

PCR amplification

The PCR amplification of the ITS1-5.8S-ITS2 region in ribosomal DNA was carried out by forward (5'-TCCGTAGGTGAACCTGCGG-3') and reverse (5'TCCTCCGCTTATTGATATGC-3') primers [24].

Afterwards, for preparing the master mix, 2.5 µl of 10x PCR buffer, 2.5 mM of $MgCl_2$, 0.5 µl of 10 mM dNTPs, 0.5 µl of each primer (15 *pmol*/µl), 1.25 units of Taq polymerase (Sinagene, Iran), 5 µl of template DNA, and molecular grade dH_2O up to 30 µl were used. The temperature cycles were as follows: initial denaturation step at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 1 min, extension at 72°C for 1 min, and final extension step at 72°C for 5 min. All the amplified products were visualized by 1.5% agarose gel electrophoresis.

Restriction enzyme digestion

Following PCR amplification, the ITSI-5.8S-ITSII PCR products were digested with HinfI and MspI restriction enzymes. Digestion was performed

by incubating 10 µl of each PCR product, 1.5 µl of digestion buffer, 5 units of MSP1 and HinfI enzymes (Sinagene, Iran), and dH_2o up to 15 µl at 37°C for 2 h [24]. The restriction fragments were visualized and compared by 2% agarose gel electrophoresis after treating with MSP1 and HinfI enzymes as it shown in Table 1.

Statistical analysis

To analyze the data, Chi-square and Mann-Whitney tests were run, using SPSS version 15.0.

Results

From the samples of 58 diabetic patients and 48 controls, 32 (55%) and 17 (35.5%) were positive for *Candida* species, respectively. Table 1 exhibits identification of *Candida* species by growth on CHROMagar *Candida* medium and molecular methods. Of the 32 isolated *Candida* spp. in DM patients, 25 (43.1%) were identified as *Candida albicans*, whereas *C. dubliniensis*, and *C. parapsilosis* were not isolated from any of the study groups. In the healthy subjects group, 13 (27%) *C. albicans* were identified (Table 2). The current results indicated that colonization of *Candida* in the mouth of diabetic patients was more frequent in comparison with the non-diabetic group. In addition, *C. albicans* was the most prominent species isolated from the oral cavity of both groups.

The mean percentages of candidal load in the oral cavity and the baseline characteristics of the DM patients compared to non-diabetics are demonstrated in Table 3.

PCR amplification of the internal transcribed spacer (ITS) regions

Positive cultures of samples taken from both groups revealed the following bands after performing PCR amplification: 37 isolates at 540 bp, 5 isolates at 900 bp, 2 isolates at 520 bp, 7 isolates at 510 bp, and 2 isolates at 720 bp band (Figure 1). In this study, the results of molecular method and the colony features of the isolates on CHROMagar *Candida* culture medium demonstrated perfect harmony.

Discussion

Poor oral hygiene in diabetic patients may increase the level of *Candida* spp. as part of the oral flora and might affect the superficial and systemic fungal infections compared with healthy individuals [25-27]. In oral candidiasis, biofilm formation and overgrowth of *Candida* species are significantly higher in diabetic patients. A combination of host and fungal risk factors such as increased salivary glucose, decreased salivary pH, salivary flow reduction, advancing age, dentures, smoking habits, irritation, and xerostomia facilitate *Candida* spp. colonization [28].

Table 1. Identification of *Candida species* by growth on CHROMagar *Candida* medium and molecular methods

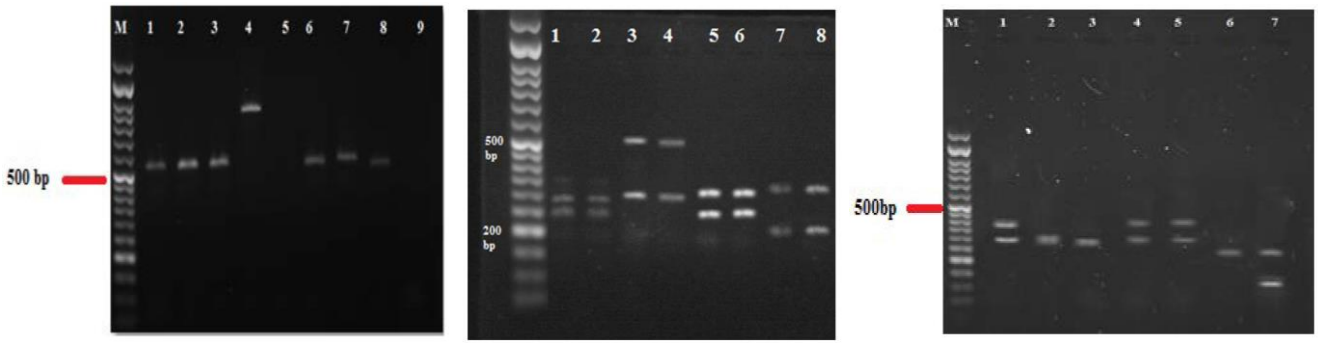
Candida species	Colony color on CHROMagar	Polymerase chain reaction-restriction fragment length polymorphism
C. albicans	Light green(34)	34
C. tropicalis	Blue(2)	2
C. parapsilopsis	-	-
C. dubliniensis	-	-
C. krusei	Pink–purple(5)	5
C. glabrata	Pink–purple(2)	2
C. kefyr	Pink(2)	2

Table 2. Frequency of *Candida* species colonization in the oral cavity of the diabetic patients and controls

Candida spp.	Diabetic patients No.(%)	Healthy subjects No.(%)
C. albicans	21(36.2%)	13(27.08%)
C. krusei	3(5.2%)	2 (4.16%)
C. glabrata	2(3.4%)	-
C. krusei and C. albicans	3(5.2%)	-
C. glabrata and C. albicans	1(1.7%)	-
C. kefyr	-	2(4.16%)
C. tropicalis	2(3.4%)	-
Total	32(55.1%)	17(35.4%)

Table 3. Mean percentages of candidal load in the oral cavity and baseline characteristics of the diabetic patients compared to non-diabetics

Variables	Colony ≥ 50	Colony ≥ 50	*P- value*
Diabetic patients	26(45%)	32(65%)	0.044
Non-diabetic participants	31(64.5%)	17(35.4%)	
Gender			
Male	34(56.6%)	28(57.1%)	0.794
Female	23(40.4%)	21(42.9%)	
Age	48.16±7.81	52.39±7.92	0.009
Salivary pH	6.88±0.36	6.44±0.41	0.001
Salivary glucose	2.25±0.44	2.78±0.54	0.001
Tooth brushing habit (day)			
0	27(47.4%)	34(69.4%)	
1	26(45.6%)	15(30.6%)	
2	4(7%)	0 (0%)	
Dry mouth			
Yes	19(33.3%)	34(69.4%)	<0.001
NO	38(66.7%)	15(30.6%)	



(A) (B) (C)

Figure 1. Gel pictures showing the band patterns: (A) PCR products of ITS1-5.8S-ITS2 in diabetic patients and non-diabetics, 1-3: *C. albicans*, 4: *C. glabrata*, 6, 8: *C. krusei*, 7: *C. tropicalis*, and 9: negative control. (B) MspI restriction fragments for the eight random diabetic samples: 1-2: *C. krusei*, 3-4: *C. glabrata*, 5-6: *C. albicans*, and 7-8: *C. tropicalis.* (C) HinfI restriction fragments on *Candida* spp. for the diabetic and non-diabetic isolates: 1, 4, 5: *C. glabrata*, 2: *C. albicans*, 3: *C. tropicalis*, 6: C. k*rusei*, and 7: *C. kefyr.*

Recently, resistance to antifungal agents has been reported in *Candida* species, especially in strains isolated from immunocompromised patients [29-32]. Our objective was to compare the presence and colonization of *Candida* spp. in the saliva of diabetic patients and non-diabetic controls. We found higher incidence of *Candida* infection in diabetic patients. The increased candidal colonization in diabetic patients could be attributed to the promotion of the binding of *Candida* to epithelial cells and reduction of tissue resistance against infection. Likewise, salivary glucose and pH levels are correlated with the increased carriage rate of *Candida* in diabetic patients [33-36].

In the present study, diabetic patients aged 40-50 years old with high salivary glucose, low salivary pH, history of xerostomia, and low oral hygiene showed more than 50 colonies of *Candida* in their mouth. Our data revealed that 32 (55%) of diabetic patients were found to carry *Candida* spp. in their oral cavity, and *Candida albicans* was the most prominent species (43.1%) isolated from the oral cavity of the diabetic patients compared with the non-diabetic controls (27%), which was in accordance with the findings of the previous studies [28, 37, 38].

Belazi et al. isolated *Candida* spp. from the oral cavity of 64% of diabetic patients and 40% of non-diabetic controls. They evaluated the predisposing factors such as xerostomia, dentures, advanced age, gender, and diabetes for colonization of *Candida* in the oral cavity, which showed the same results as

the current ones [36].

Javed et al. demonstrated high prevalence of oral *C. albicans* carriage associated with denture wearing in males (74%) compared to females (23%) with type 2 diabetes. Dentures may act as an additional reservoir for these organisms through the biofilm formation of *Candida* in the oral cavity [39].

In the present study, the reason for a higher carriage rate of *Candida* in DM patients was the reduction of salivary pH (6.52±0.48) compared to the control groups (6.87±0.29). Eslami et al. reported that the means of salivary pH level in diabetic patients and healthy subjects were 6.96±0.71 and 7.53±0.58, respectively, and revealed a significant difference in salivary pH between the two groups ($P<0.001$) [40].

Kaminishi et al. described that collagenolytic enzyme, which is produced by *C. albicans*, lowers human dentinal collagen and uses the dentinal structure, especially collagen, for growth. The optimal activity of the enzyme is at pH 3.5 to 4.0. Likewise, *C. albicans* produces lactic acid by the fermentation of carbohydrates that leads to reducing the hydroxyapatite structure of the teeth [41], which may be the reason for the low salivary pH level in diabetic patients.

Although, among the *Candida* species, *C. albicans* has the highest frequency in the oral cavity, in the last two decades, the incidence of oral candidiasis with other species such as *C. glabrata* and *C. krusei* that are less sensitive to azole compounds has increased [42]. *C. dubliniensis* is mostly detected in the oral cavity of patients infected with the human immunodeficiency virus (HIV) [43, 44]. Alborzi et al. reported 13.3% *C. dubliniensis* from mucosal sites in HIV positive patients [45]. The differentiation between *C. dubliniensis* and *C. albicans* due to the high degree of phenotypic similarity remains a problem. This species produce a distinctive dark green color on CHROMagar *Candida* compared to *C. albicans* isolates, which are light green [46, 47]. PCR-RFLP is an ideal method for differentiation of *Candida* species from each other [48, 49].

In evaluation of subjects with poorly controlled and well-controlled type 2 diabetes using CHROMagar *Candida*, Melton et al. reported that *C. dubliniensis* was not found in oral samples, which were incubated at 42°C [50] . In the current study, *C. dubliniensis* and *C. parapsilosis* were not detected in oral samples by CHROMagar *candida* and PCR-RFLP methods.

Zahir et al. used PCR amplification and digestion by the restriction enzymes HinfI and MspI for identification of *Candida* species at different sites in the oral cavity. They found that the candidal loads of the saliva, tongue, palate, and buccal mucosa of denture-wearers were significantly higher than those of the control group [24].

Pinto et al. used universal primers ITS1-ITS4 for the amplification of ITS1 and ITS2 regions and they showed fragments ranging in size from 350 to 950 bp. Amplicons were digested with eight restriction enzymes [51].

RFLP-PCR using ITSI and ITS4 primers and restriction enzymes MspI and HinfI is a quick, easy, and reliable method for the identification of clinically important *Candida* spp.

Conclusion

The candidal load of oral mucusa in DM patients were found to be significantly higher than those of the control group. In the present study, the patients with high salivary glucose, low salivary pH, history of xerostomia, and low oral hygiene showed more than 50 colonies of *Candida* in their mouth. Our data revealed that 55% of diabetic patients were found to carry *Candida* spp. in their oral cavity, and *C. albicans* was the most prominent species. Consideration of the possibility of oral *Candida* infections in DM patients is emphasized for improving patient treatment outcomes and reducing healthcare costs.

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Author's contribution

P.D. contributed to study concept, and designed, supervised, and edited the final manuscript. F.M. helped with study design, and analyzed and interpreted the data. R.J. performed sample collection and laboratory examinations and interpreted the data; S.N. carried out RFLP-PCR.

Conflicts of interest

None declared.

Financial disclosure

The authors declare no financial interests related to the materials of the study.

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