

Comparative Study of Esterase and Hemolytic Activities in Clinically Important *Candida* Species, Isolated From Oral Cavity of Diabetic and Non-diabetic Individuals

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Background: Diabetes mellitus as a chronic metabolic disease occurs in patients with partial or complete deficiency of insulin secretion or disorder in action of insulin on tissue. The disease is known to provide conditions for overgrowth of *Candida* species. *Candida* spp. cause candidiasis by many virulence factors such as esterase, hemolysin and phospholipase.

Objectives: This study aimed to compare esterase and hemolytic activity in various *Candida* species isolated from oral cavity of diabetic and non-diabetic individuals.

Patients and Methods: Swab samples were taken from 95 patients with diabetes (35 men and 60 women) and 95 normal persons (42 men and 53 women) and cultured on Sabouraud dextrose agar. Identification of isolated yeasts was performed by germ tube test, morphology on CHROMagar *Candida* medium, corn meal agar and ability to grow at 45°C. Hemolysin activity was evaluated using blood plate assay and esterase activity was determined using the Tween 80 opacity test.

Results: Different *Candida* species were isolated from 57 (60%) diabetic and 24 (25%) non-diabetic individuals. Esterase activity was detected in all *Candida* isolates. Only 21.6% of *C. albicans* from patients with diabetes had esterase activity as +3, while it ranged from +1 to +2 in others. Hemolytic activity was determined in *C. albicans*, *C. dubliniensis*, *C. glabrata* and *C. krusei* as 0.79, 0.58, 0.66 and 0.74, respectively. Hemolytic activity was significantly different in the two groups of diabetics and non-diabetics.

Conclusions: Oral carriage of *C. albicans* in the diabetic group (n = 42; 66.7%) was significantly greater than the control group (n = 16; 57.1%). Esterase activity of *C. albicans* in diabetic group was higher than non-diabetic group. Although *C. albicans* remains the most frequently pathogenic yeast for human, but other species are increasing.

Keywords: Esterase; Hemolysin Factor; *Candida albicans*; Diabetes Mellitus

1. Background

Diabetes is one of the global problems, especially in developing countries. Diabetes mellitus (DM) as a chronic metabolic disease is one of the most prevalent causes of mortality in low-income and middle-income countries (1). Patients with diabetes are more sensitive to certain infections. Today, it has been proven that oral cavity can act as a primary site for dissemination of microorganisms including *Candida* species (2). Oral candidiasis is one of the most common opportunistic infections in patients with diabetes and immunodeficiency (3). According to a report by Mohammadi, et al. in Iran, from 855 yeast strains isolated from different clinical specimens, *Candida albicans* was the predominant species (58.6%) followed by *C. parapsilosis* (11.0%), *C. glabrata* (8.3%), *C. tropicalis* (7.0%), *C. kefyr* (5.8%), *C. krusei* (4.4%), *C. orthopsilosis* (2.1%) and *C. guilliermondii* (0.6%). A few strains of *C. lusitanae*, *C. rugosa*, *C. intermedia*, *C. inconspicua*, *Cryptococcus neoformans* and *Saccharomyces cerevisiae* were isolated (4).

Although *C. albicans* is the most common cause of candidiasis, but 12 to 14 species of non-*albicans* *Candida* are known to cause such infections. Recent epidemiological studies have shown that non-*C. albicans* *Candida* species (NACs) such as *C. tropicalis*, *C. parapsilosis*, *C. krusei* and *C. glabrata* have repeatedly emerged as human pathogens (4). *Candida* species, especially *C. albicans* tend to colonize oral cavities, secrete hydrolytic enzymes and cause inflammation and hyperplasia of the gums and mouth, which can lead to discomfort, pain and disseminated infection in patients with impaired immune system. *Candida* species secrete different lytic enzymes such as phospholipases, esterases and hemolysin factor which digest surface membrane of the host cells, facilitate binding and penetration of microorganisms into the host tissue and contribute to tissue invasion, thus play important roles in *Candida* pathogenicity (5). They also attack host immune cells and molecules and prevent their antimicrobial activity.

Hemolysin of *Candida* species is amidst the most frequently studied enzymes produced by fungi, which is released during iron acquisition and facilitates the hyphal invasion in disseminated candidiasis (6). To our knowledge, secretion of hemolysin by *Candida* isolates from patients with diabetes had not been studied before. Detection of secretory enzymes in *Candida* strains isolated from DM can be an important step for management of these patients. Additionally, most previous researches focused on *C. albicans*.

2. Objectives

We aimed to:

- 1) Isolate, identify and compare enzymatic activities in *Candida* species isolated from diabetic and non-diabetic patients referred to Imam Sajjad Diabetes Center
- 2) Evaluate enzymatic activity in both *C. albicans* and NACs, especially *C. glabrata*, *C. tropicalis* and *C. krusei*.

3. Patients and Methods

3.1. Patients

Ninety-five patients with diabetes mellitus (35 men and 60 women) and 95 healthy volunteers (42 men and 53 women) were included in the study and matched for age, sex, dental status and smoking habits. The study protocol was approved by the research ethics committee (Ahvaz Jundishapur University of Medical Sciences). Selected patients from local outpatient diabetes clinics aged 10 to 85 years. After getting informed consent, patients referred to Imam Sajjad Diabetes Center (Dehdasht-Iran, 2013) were sampled using sterile swabs.

3.2. *Candida* Isolates

Swab samples were dispensed onto CHROMagar media (CHROMagar *Candida*, Paris, France) and incubated at 37°C for 48 hours. *Candida* species were determined by colony color on CHROMagar medium, germ tube test, growth at 45°C and microscopic morphology after growing on corn meal agar medium.

3.3. Esterase Assay

Esterase activity was measured using the Tween 80 opacity test medium, which was prepared with 10 g of bacteriological peptone (Merck, Germany), 5 g of sodium chloride, 0.1 g of calcium chloride, 15 g of agar and 1000 mL of distilled water. After, the medium was autoclaved; it was cooled to about 50°C and was added 5 mL of autoclaved Tween 80 (Merck, Germany). The inoculated agar plates were incubated at 30°C and examined daily for 10 days. All inoculations were performed in duplicate. The colony diameter (a) and the diameter of colony plus precipitation zone (b) were measured. The esterase activities were expressed as Ez Value (a/b) as described by Price et al. (7).

3.4. Hemolysin Assay

Hemolysin production was evaluated using a modification of the plate assay described by Manns et al. (8). In brief, the isolates cultured on Sabouraud glucose agar were spot inoculated on a sugar-enriched sheep blood agar medium to yield a circular inoculation site of about 5 mm in diameter. The latter medium was prepared by adding 5 - 7 mL of fresh sheep blood (Darvash Co. Iran) to 100 mL of Sabouraud glucose agar supplemented with 3% glucose (Merck, Germany). The plates were incubated at 37°C in 5% CO₂ for 48 hours. The ratio of the diameter of the colony to that of the translucent zone of hemolysis (in mm) was used as the hemolytic index (Hz Value) (9).

4. Results

Candida spp. were isolated from 57 patients with diabetes (60%) and 24 non-diabetic persons (25.5%). The oral carriage of *C. albicans* in the diabetic group (n = 42; 66.7%) was significantly greater than that in the control group (n = 16; 57.1%) (Chi-square test, P = 0.05). Other *Candida* species isolated included *C. glabrata* (9 isolates from DM and four from control groups), *C. dubliniensis* (eight isolates from DM and four from control groups), *C. krusei* (four isolate from DM and the control group) (Table 1).

Table 1. Prevalence of *Candida* Species in Diabetic and Non-Diabetic Groups^a

Variable	<i>C. albicans</i>	<i>C. dubliniensis</i>	<i>C. krusei</i>	<i>C. glabrata</i>
Diabetic	42 (66.7)	8 (12.7)	9 (14.3)	4 (6.4)
Non-Diabetic	16 (57.1)	4 (14.3)	4 (14.3)	4 (14.3)

^a Data are presented No. (%).

Hemolytic activity was detected in 100% and 52% of the isolates from diabetic and non-diabetic groups. Although 75% of *C. albicans* isolates from non-diabetic group produced hemolysin, the total hemolysin activity of *Candida* species was significantly higher in diabetic group than non-diabetic group (Table 2).

Table 2. Hemolytic Activity (Hz Value) of Oral *Candida* Species Isolates From Diabetic and Non-Diabetic Subjects

Variable	<i>C. albicans</i>	<i>C. dubliniensis</i>	<i>C. krusei</i>	<i>C. glabrata</i>	Total
Diabetic	0.72	0.59	0.65	0.72	0.67
Non-Diabetic	0.78	0.93	1	0.94	0.91

After grading esterase activity in various *Candida* species, it was found that only *C. glabrata* and *C. albicans* were able to demonstrate esterase activity at + 3 level. In non-diabetic, 50% of *C. glabrata* and *C. krusei* produced esterase at + 1 level, although all of *C. dubliniensis* and 87.5% of *C. albicans* produced it at + 2 level (Figure 1 and Table 3).

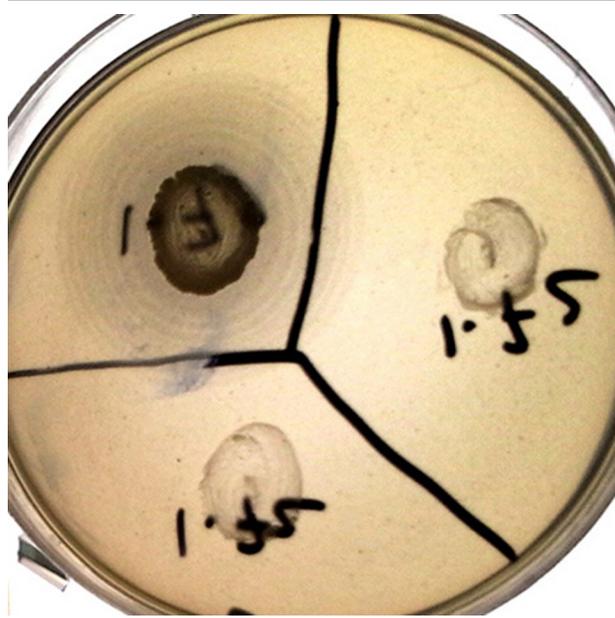


Figure 1. Esterase Activity of *Candida* Species

Table 3. Esterase Activity (Ez Value) of Oral *Candida* Species Isolates From Diabetic and Non-Diabetic Subjects ^{a, b}

Variables	Diabetic	Non-Diabetic
<i>C. krusei</i>		
1	-	50
2	100	50
3	-	-
<i>C. glabrata</i>		
1	-	50
2	66.7	50
3	33.3	-
<i>C. dubliniensis</i>		
1	25	-
2	75	100
3	-	-
<i>C. albicans</i>		
1	8.1	12.5
2	70.3	87.5
3	21.6	-

^a Score of esterase: +1 = 0.7 - 0.99; +2 = 0.4 - 0.69; +3 = 0.1 - 0.39.

^b Data are presented as %.

5. Discussion

The purpose of this research was to identify and assess various species of *Candida* in oral cavity of diabetic and non-diabetic individuals and to compare their virulence factors such as esterase and hemolysin enzymes with the assumption that virulence factors of *Candida* species iso-

lated from diabetic patients are higher than the control group. In this research, *Candida* was isolated from 60% of diabetic patients versus 25.5% of normal subjects. In patients with DM, due to reduced salivary flow, increased blood glucose concentration and decreased saliva pH the possibility of adherence and colonization of *Candida* is increased in mouth. The results of this study confirmed previous studies (2, 10, 11).

The most frequent oral yeast isolates from the study subjects was *C. albicans*. 66.7% of diabetic patients and 57.1% of normal subjects had *C. albicans*. Based on previous studies, the most common pathogen obtained in adults with oral mucosal was *C. albicans* (11). The oral carriage of *C. albicans* in the diabetic group (n = 42; 66.7%) was significantly greater than the control group (n = 16; 57.1%) (Chi-square test, P, 0.05). Manfredi et al. reported that *Candida* grew out from the oral of diabetic subjects and non-diabetics by 60.5% and 57.3%, respectively (11). The highest growth rates related to *C. albicans* from the oral cavity of diabetic subjects (83.3%) and normal group (76.9%).

In our study, the percentage of *C. albicans* isolates from both groups was lower than report of Manfredi et al. (11). These results may be due to lower average age of our subjects, living conditions and some host dietary habits. However, Soysa et al. in a study on diabetic patients reported isolation of *C. albicans* between 18 - 80%, which is in accordance with our results (12). In this research, *C. dubliniensis* was isolated from eight of 95 patients with diabetes (8.4%). Willis et al. in a study isolated 18.2% *C. dubliniensis* from insulin-dependent diabetics (13), while Manfredi (11), showed it 3.5%. Previously, it was thought that this species of *Candida* is only separated from patients with HIV.

Four *C. glabrata* isolates were isolated from each of the two groups of patients and controls. Isolation of these species was higher in healthy subjects than patients with diabetes (Table 1). Studies on hemolysin activity of *Candida* species isolated from patients with diabetes are limited. Hemolysin activity in our study was significantly higher in the diabetic group than the control group. Hemolytic activity was assayed in *C. albicans*, *C. dubliniensis*, *C. glabrata* and *C. krusei* as 0.72, 0.59, 0.65 and 0.79, respectively. Hemolytic activity was detected in 100% of the *Candida* species in diabetic group, while only 52% of *Candida* isolates in non-diabetic group showed hemolytic activity. Of course, 75% of *C. albicans* isolated from non-diabetic group produced hemolysin factor. The hemolytic activity of *C. albicans* was significantly higher than that of non-*C. albicans* *Candida* isolates (P = 0.0001) in control group, but was not in the diabetic group (Table 2).

Our findings indicated that non-*C. albicans* isolates in patients with diabetes may possess greater virulence potential than non-*albicans* species in the control group. Manns et al. found that *C. albicans* does not show any hemolytic activity when there is no glucose available in the medium. They stated that adding glucose to the culture medium increases the hemolytic activity. Therefore, it

can be predicted that increasing glucose concentrations in saliva would enhance hemolysin production, adhesion and colonization of *Candida* species in oral cavity of diabetic subjects (8). In this study, each of the *Candida* species in diabetic group significantly showed higher hemolytic activity the same as normal subjects. The differences in the hemolytic activities of non-*albicans* species were further. Perhaps these species in abnormal conditions such as diabetes had more pathogenicity. Shimizu et al. injected them into mice and examined the virulence of different strains and confirmed that production of extracellular enzymes by *Candida* isolated from patients with diabetes is higher than normal individuals (14). Luo et al. examined the hemolytic activity of 14 species of *Candida* separately. They showed the ability of *Candida* species in production of one or both hemolysins, but there was no quantitative report regarding the hemolytic activity of *Candida* species in patients with diabetes (15) and our research was the first in this regard. Aktas et al. evaluated esterase activity in different species of *Candida* qualitatively. In this study, almost all species produced sediment in Tween 80 opacity test medium after 2 - 10 days (16). This test is confirmed by other researchers, because it is convenient, economical and easy to perform (17). Kumar et al. qualitatively surveyed the esterase activity of *Candida* species isolated from immunocompromised hosts (18). Slifkin believed that due to lack of esterase production on Tween 80 opacity test medium by *C. dubliniensis* could identify *C. dubliniensis* from *C. albicans*, whereas in our study 100% of *C. dubliniensis* produced esterase (17). In our study, esterase activity was quantitatively evaluated. 21.6% of *C. albicans* strains and 33.3% of *C. glabrata* strains isolated from patients with diabetes produced esterase at level + 3, whereas these two species in the control group showed esterase activity at levels + 1 and + 2 (Table 3).

It is concluded that esterase and hemolytic activities of *Candida* in patients with diabetes are higher than healthy subjects, but some species such as *C. glabrata* act similar to *C. albicans* in diabetes.

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Authors' Contributions

1- Mahnaz Fatahinia (Corresponding Author): Study concept and design, analysis and interpretation of data, drafting of the manuscript, administrative, technical and material supports, study supervision and statistical analysis. 2- Farzad Poormohamadi: Acquisition of data and technical support. 3- Ali Zarei Mahmoudabadi: Criti-

cal revision of the manuscript for important intellectual content and material support.

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