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RESEARCH ARTICLE

Genetic Identification of Yeast Isolated From Diabetic Patients In Basra Governorate, Iraq

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ABSTRACT

The relationship between diabetes mellitus and oral diseases has received considerable attention in the past few decades. The impaired immune response is the major reason that cause diabetic patients more prone to oral opportunistic infections. Lower respiratory tract infections, urinary tract infections, bacterial and mycotic skin and mucous membrane infections are all found to be increased in diabetic patients. In the current study, twenty-four yeasts were isolated from oral cavity of diabetic patients. All isolates were identified with morphological tests that include culturing onto CHROMagar™ Candida, germ tube formation, chlamydospore formation and culturing on tobacco agar medium. Molecular identification with amplification of ITS1-5.8S-ITS2 rDNA region by means universal primers ITS1 and ITS4 revealed 25 yeasts belong to nine different yeast species, including C. albicans (44%, n=11), C. dubliniensis (28%, n=7), C. tropicalis (8%, n=2), C. aaseri (4%, n=1), Pichia kudriavzevii (8%, n=2) , Pichia fermentans (4%, n=1), Naganishia uzbekistanensis (3.846%, n=1), Kluyveromyces marxianus (4%, n=1), Cutaneotrichosporon terricola (4%, n=1), Saccharomyces cerevisiae (4%, n=1). The current study indicated that C. albicans is the most frequent species in the oral cavity of diabetic patients as the causative agent of candidiasis followed by C. dubliniensis.

INTRODUCTION

Type II diabetes mellitus (DM) is a complex, heritable and heterogeneous condition that occurs in a range of phenotypes (Philipson ,2020) .It is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both which is the most common cause of death among adults with diabetes mellitus [2]. (Abudawood, 2019). The pathogenesis including hyperglycemia, insulin resistance, dyslipidemia, hypertension, and immune dysfunction. These disturbances initiate several damaging processes, such as increased reactive oxygen species (ROS) production, inflammation, and ischemia since the oral cavity is also highly vascularized and innervated, oral complications can be expected as well. The relationship between DM and oral diseases has received considerable attention in the past few decades. However, most studies only focus on periodontitis, and still approach DM from the limited perspective of elevated blood glucose levels only (Verhulst *et al*., 2019).

Immune dysfunction plays a central role in the pathogenesis of diabetes complications. DM can adversely affect several aspects of the immune system. For example, innate (polymorphonuclear neutrophils [PMN], macrophages, and monocytes) and adaptive (T lymphocyte) immune responses are often compromised. More specifically, PMNs exhibit impaired

chemotactic, phagocytic, and bactericidal properties in DM patients Also, adherence of microorganisms to several cell types, such as epithelial and endothelial cells, is increased (Losappio *et al* 2020). The impaired immune response is the major reason why patients with DM are more prone to opportunistic infections. Lower respiratory tract infections, urinary tract infections, bacterial and mycotic skin and mucous membrane infections are all found to be increased in patients with DM (Naser *et al.,* 2023; Talapko *et al*., 2021; Rashid et al., 2023).

Oral candidiasis, commonly referred to as thrush is an opportunistic fungal infection that commonly infects the oral mucosa. The main causative agent, *Candida*, is a highly versatile commensal organism that is well adapted to its human host; however, changes in the host microenvironment can promote the transition from one of commensalism to pathogen. This transition is heavily reliant on an impressive repertoire of virulence factors, most notably cell surface adhesins, proteolytic enzymes, morphologic switching, and the development of drug resistance. In the oral cavity, the co-adhesion of *C. albicans* with bacteria is crucial for its persistence, and a wide range of synergistic interactions with various oral species were described to enhance colonization in the host (Aldossary *et al.,* 2020; Vila *et al*.,2020). *C. albicans* is by far the main causative agent of OC accounting for up to 95% of cases. Although considered a pathogen, *C. albicans* is a ubiquitous commensal organism that commonly colonizes the oral mucosa and is readily isolated from the oral cavities of healthy individuals. In fact, up to 80% of the general population are asymptomatic carriers, and simple carriage does not predictably lead to infection (Williams & Lewis, 2011; Al-Duboon 2010). In order for *Candida* to cause infection, it has to be retained within the mouth. However, removal of loosely attached *Candida* cells from mucosal surfaces via the elects of salivary flow and swallowing is an important factor in host defense against *Candida* overgrowth. Therefore, the ability to circumvent these removal mechanisms can be regarded as a key virulence attribute. Although, during its commensal yeast state, *C. albicans* reversibly adheres to oral epithelial cells through electrostatic interactions, attachment to oral epithelial surfaces is mediated by cell-wall receptors such as the agglutinin-like sequence (ALS) family of glycoproteins (Lewis & Williams, 2011).

There are multiple clinical presentations and several classification systems for OC; however, the most simplistic classification encompasses oral manifestations that can generally be classified into three main broad categories, namely, acute manifestations, chronic manifestations, and chronic mucocutaneous candidiasis syndromes (Vila *et al*., 2020). The genus *Candida* is characterized by large, round, slimy, or dry colonies with white or cream colored. Occasionally they show filamentous mycelial growth in the saprophytic phase, but have typical yeast morphology in the pathogenic phase, in tissue and when grown at 37˚C in the laboratory. They may form pseudohyphae when the buds continue to grow, producing chains of elongated cells that are pinched or constricted at the septations between cells, as well as culturing of Candida at temperatures less than 26˚C in poor nutrient media such as cornmeal agar, several species of *Candida spp*. produce thick-walled resting cells, called chlamydospores (Senanayake *et al*., 2020). However, *Candida* transforms from the commensal form to the pathological form in several cases including: the disturbance of the microbial flora equilibrium such as during long-term antibiotic treatment, and at the compromise of immune system and/or at the damage of epithelial barriers after surgery (Ahmed *et al*., 2022). *Candida* can cause two types of infections: superficial infections of the mucosa, and invasive candidiasis, where the yeast can disseminate throughout the blood and infect every organ (Kanval et al., 2024; Lu, 2021).

Many of *Candida* species have the potential to cause disease. Clinically, the most significant member of the genus is *Candida albicans*, which can cause infections (candidiasis or thrush) in humans and other animals, especially in immunocompromised patients (Villar & Dongari-Bagtzoglou, 2021). According to Hibbett *et al*., (2007) *Candida* species classified in the Family Saccharomycetaceae,

Order Saccharomycetales, Class Saccharomycetes, Sub-phylum Saccharomycotina that belong to Phylum Ascomycota, Sub-kingdom Dikarya in the Kingdom Fungi. The most medically important *Candida spp*. include: *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. guilliermondii*, *C. krusei*, *C. kefyr*, *C. lusitaniae*, *C. lipolytica*, *C. norvegensis*, *C. parapsilosis*, *C. famata*, *C. rugosa*, *C. tropicalis*, *C. stelltoidea*, *C. inconspicua* (Posteraro *et al*., 2015).

2. MATERIAL AND METHODS

2.1. Sample collection

Oral swab samples were collected from patients with diagnosed Diabetes mellitus II, who visited Al-Sadder Teaching Hospital in Basrah province, Iraq, from January 2021 to April 2021, all patients were clinically diagnosed with (Diabetes mellitus II). Patients who having other immune diseases or having antibiotic or antifungal drugs were excluded from the study. Through questionnaire, data of patients were collected briefly information includes (age, sex, serum glucose test, smoking, oral infection and antimicrobial drugs treatment for oral infection).

2.2. Isolation of yeast

Oral swabs were directly transferred to laboratory and cultured on Sabouraud Dextrose Agar plates supplemented with chloramphenicol for fungal recovery. Plates were incubated at 37˚C with daily monitoring for bacterial and fungal growth.

2.3. Identification of yeast

1. Culturing Onto CHROMagar™ Candida

Depending on the enzymatic activity of this medium that cause specific color of yeasts colonies; different colors appears on CHROMagar™ Candida medium that facilitate rapid identification of *Candida spp. (*Tamura *et al*., 2022).

1. Germ Tube

Yeasts were inoculated in serum for 3 hrs. at 37˚C, which identified presumptively as *C. albicans* or *C. dubliniensis (*Al-Ani *et al*., 2023).

2. Chlamydospores

isolates were cultured onto corn meal agar with 1% Tween 80 (CMA) plates at 25° C for 10 days to observe the chlamydospore formation (Prakoeswa *et al*., 2021).

2.4. Molecular Identification

The isolates were subjected to ITS1 and ITS4 sequencing. The extracted DNA performed manually by 0.8 % agarose gel electrophoresis, then the extracted DNA was subjected to amplification by PCR technique. 0.8 % agarose gel electrophoresis analysis of the *Candida spp*. extracted DNA. The size of PCR product of the ITS1, ITS4 region were approximately 400-510 base pair. The sequencing of ITS1 region done with BLAST (https://www.ncbi.nlm.nih.gov/blast).

3. RESULTS

3.1.Morphological Identification

It has been identified the yeasts that were cultured primarily onto SDA medium depending on their phenotypic characters.

3.2.Germ Tube

All yeast isolates were subjected to germ tube test for differentiation between *C. albicans* and *C. dubliniensis* and distinguish them from the rest species which does not form germ tube. yeasts which obtained from diabetic patients that produced germ tube were identified primarily as *C. albicans* or *C. dubliniensis* (Figure 1)

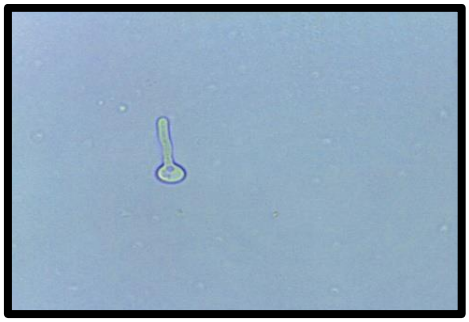

Figure .1 Germ tube formation of C. albicans at human serum at 37˚C for 3hrs. under light microscope(100x)

3.3. Chlamydospore

Yeasts that cultured onto CMA medium to observe the formation of chlamydospores revealed that all yeasts isolates grew well on CMA, but only few of them were capable of producing thick-walled chlamydospores. Therefore, these isolates have been identified as *C. albicans* or *C. dubliniensis* (Figures 2).

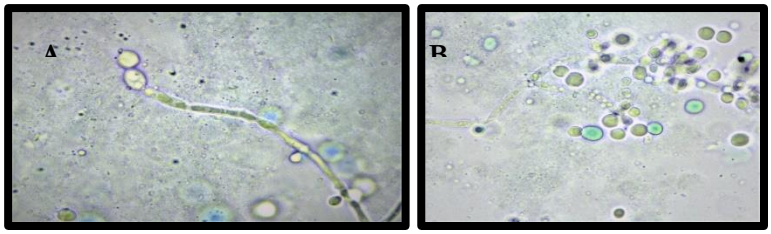

Figure 2. chlamydospores formation, A: chlamydospores clusters of *C. dubliniensis* 100X

B:Single chlamydospores of *C. albicans* 100X

3.4.Culturing onto CHROMagar™ Candida Medium

This medium had allowed the growth of most clinically important yeasts and aids to presumptive identification of yeasts. as well as facilitated recognition of samples containing mixture of *Candida spp*. and other yeasts. (Figure 3; Table 1.).

Figure 3. Growth of Yeasts on CHROMagar™ Medium. Light green represent initial identification as *C. albicans,* dark green represent initial identification as *C. dubliniensis*, metallic blue represent initial identification as *C. tropicalis* and pink colure represent initial identification of other yeasts.

3.5. Culturing onto Tobacco Agar Medium

On this medium, all of *C. dubliniensis* developed rough, yellow to brown colonies with peripheral fringes, and formed chlamydospores on these fringes. On the other hand, all isolates of *C. albicans* developed smooth, white to creamy colonies without fringes or chlamydospores even after being incubated for more than ten days (Figures 4. and Table 1.).

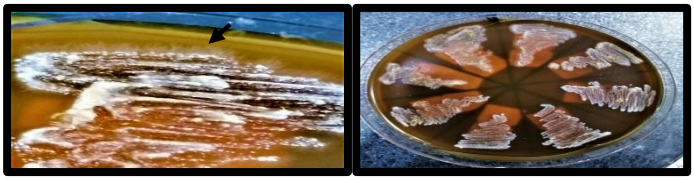

Figure 4. Peripheral hyphal fringes (→) extending from colonies of *C. dubliniensis* on tobacco agar

3.6. Molecular Identification

The isolates were subjected to molecular technique utilizing amplification of ITS1-5.8S-ITS2 rDNA region by means universal primers ITS1 and ITS4 for discrimination and identification these yeast isolates. DNA extraction was performed manually, then genomic DNA was emerged (Figure 5. and Table 2.).

Figure 5. Genomic DNA extracted electrophoresis

3.7. Polymerase Chain Reaction (PCR) Test

PCR products size of the ITS1-5.8S-ITS2 rDNA region for isolates was approximately 400-850 bp., and revealed 25 yeasts belong to 10 different species, including *C. albicans* (44%, n=11), *C. dubliniensis* (28%, n=7), *C. tropicalis* (8%, n=2), *C. aaseri* (4%, n=1), *Pichia kudriavzevii* (8%, n=2) , *Pichia fermentans* (4%, n=1), *Naganishia uzbekistanensis* (3.846%, n=1), *Kluyveromyces marxianus* (4%, n=1), *Cutaneotrichosporon terricola* (4%, n=1), *Saccharomyces cerevisiae* (4%, n=1). (Table 2. & Fig. 7)

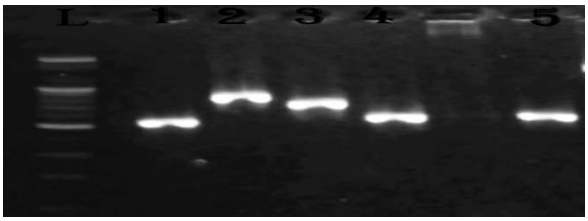

Figure 6. PCR product electrophoresis of six yeast isolates ,L: ladder 1:*C. albi*cans 2: *Pichia fermentans* 3: *Kluyveromyces marxianus* 4: *C. albicans* 5: *C. dubliniensis*

Table 1. Prevalence of oral *Candida* species and other yeasts isolated from DM patients

No.	Chlamido spore	Growth onto Tobacco Medium	Color on CHROMagar Candida	Germ tube	Species
1	+	Smooth	Light green	+	*C. albicans*
2	+	Smooth	Light green	+	*C. albicans*
3	-	Smooth	Blue	-	*C. aaseri*
4	-	Smooth	Pink	-	*Cutaneotrichosporon terricola*
5	+	Smooth	Light green	+	*Candida albicans*
6	-	Smooth	Pink	-	*Kluyveromyces marxianus*
7	+	Smooth	Light green	+	*Candida albicans*
8	+	Rough	Dark green	+	*C. dubliniensis*
9	-	Smooth	Pink	-	*Pichia fermentans*
10	-	Smooth	Blue	-	*C. tropicalis*
11	+	Smooth	Light green	+	*C. albicans*
12	+	Rough	Dark green	+	*C. dubliniensis*
13	+	Rough	Dark green	+	*C. dubliniensis*
14	-	Smooth	Pink	-	*Pichia kudriavzevii*
15	-	Smooth	Blue	+	*C. tropicalis*
16	-	Smooth	Pink	-	*Naganishia uzbekistanensis*
17	+	Rough	Dark green	+	*C. dubliniensis*
18	-	Smooth	Pink	-	*Saccharomyces cerevisiae*
19	-	Smooth	White	-	*Pichia kudriavzevii*
20	+	Rough	Dark green	+	*C. dubliniensis*
21	-	Smooth	Light green	-	*C. albicans*
22	+	Rough	Dark green	+	*C. dubliniensis*
23	-	Smooth	Light green	-	*C. albicans*
24	+	Rough	Dark green	+	*C. dubliniensis*
25	+	Smooth	Light green	+	*Candida albicans*

Table 2. Molecular identification of isolated yeasts

No.	Isolates code	Molecular identification	Identity %
1	C1	*C. albicans*	100
2	C2	*C. albicans*	100
3	C3	*C. aaseri*	100
4	C5	*Cutaneotrichosporon terricola*	98.28
5	C6	*C. albicans*	100
6	C8	*Kluyveromyces marxianus*	100
7	C9	*C. albicans*	96.73
8	C10	*C. dubliniensis*	100
9	C11	*Pichia fermentans*	100

10	C12	*C. tropicalis*	98.12
11	C13	*C. albicans*	100
12	C14	*C. dubliniensis*	97.01
13	C15	*C. dubliniensis*	100
14	C16	*Pichia kudriavzevii*	99.65
15	C17	*C. tropicalis*	99.77
16	C18	*Naganishia uzbekistanensis*	100
17	C19	*C. dubliniensis*	100
18	C20	*Saccharomyces cerevisiae*	94.41
19	C21	*Pichia kudriavzevii*	100
20	C22	*C. dubliniensis*	100
21	C23	*C. albicans*	99.15
22	C24	*C. dubliniensis*	96.83
23	C25	*C. albicans*	100
24	C26	*C. dubliniensis*	100
25	C27	*C. albicans*	100

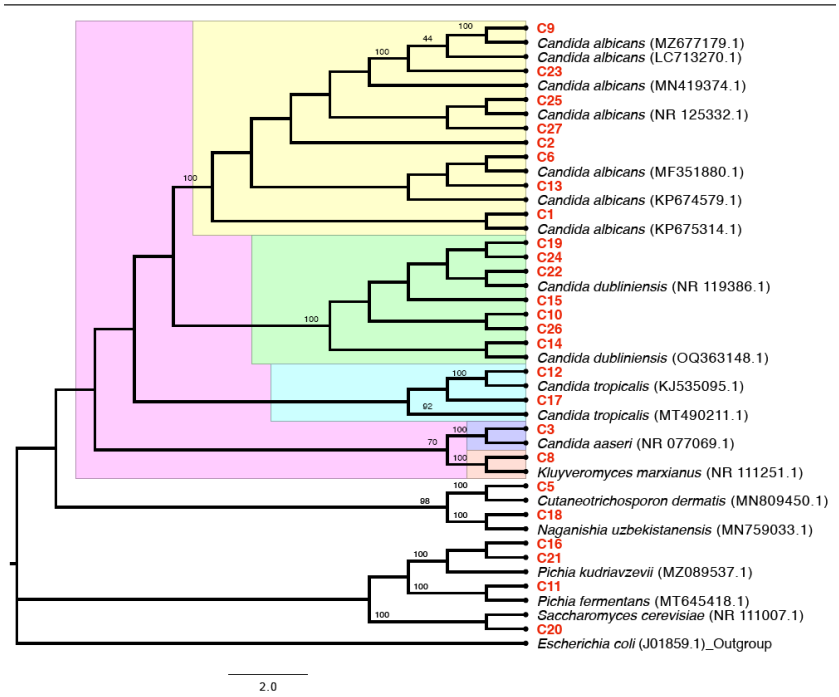

Figure 7. Neighbor-joining phylogenetic reconstruction tree showing evolutionary relationships of Fungal isolates with close relatives available in NCBI GenBank database

4. DISCUSSION

The present study displays considerable elevation of yeasts in the oral cavity of DM patients that indicate increase the frequency of *C. albicans* than other yeast, these results consistent with following study which observed noticeable increase in the frequency of *C. albicans* than non-albicans *Candida*.

(Zarei *et al*., 2022). The variation in *Candida spp.* that isolated in this study due to physiological changes related to age, mucosal surface, natural barriers against yeasts, living environment, habits of individual, changes the ecological environment of the mouth and immune system impairment (Saigal *et al*., 2013; Talapko *et al*., 2021). Identification of *Candida spp*. using CHROMagar *Candida* provides an easy, rapid method for the presumptive identification of Candida species, based on the variation in color and colony morphology with the ability to correctly identify majority of different *Candida spp.* (Sasoni *et al.,* 2022). In the present study *C. albicans* appeared with light green colors, *C. dubliniensis* showed dark green color, *C. tropicalis* colored with metallic blue, and finally non candida species vary from pink to white colure, these results agreed with Shrestha *et al*., (2022). The principle of CHROMagar *Candida* medium depends on the presence of chromogenic substrates which the species specific exoenzymes of different *Candida spp*. react with those substrates and formation colonies with various pigmentation. For instance, *C. albicans* excreted β-N-acetylgalactosaminidase enzyme, which cleavage specific chromogenic substrate in the medium, leads to production light green colonies with the aids of color indicator (Imran and Ali, 2015; Padmapriya *et al*., 2015; Soni *et al*., 2019). Germ tube formation test is the essential test for detection both *C. albicans* and *C. dubliniensis*, this test displays about 95% for identifying *C. albicans* and *C. dubliniensis*, with other morphological tests to confirm the species identification. In this study, all *C. albicans* and *C. dubliniensis* produced germ tubes. Similar results were shown by Al-Ani *et al*., (2023) who revealed germ tube formation in *C. albicans* and *C. dubliniensis*. Corn meal agar with tween-80 is a nutritionally deficient medium that inhibits vegetative growth and promote chlamydospores formation for surviving of the fungus in unsuitable conditions. This feature used for the identification and differentiation of *Candida spp*., because not all species can produce these structures, only *C. albicans* and *C. dubliniensis* have the ability to produce chlamydospores (Prakoeswa *et al.,* 2021). In this study chlamydospores were produced by only *C. albicans* and *C. dubliniensis*. These results were agreed with the results of Deorukhkar and Saini (2014) who displayed high ability to produce chlamydospores by these two species, while the other Candida species does not produce these structures. The reason for the inaccurate identification with conventional methods is due to the high morphological similarity between *Candida spp*., especially between closely related species such as *C. albicans* and *C. dubliniensis*. The accurate identification is important for clinical management of patients because the Candida species have different antifungal susceptibilities, that is why the choice of PCR technique and gene sequencing for discrimination of the species is an important (Aziz et al., 2022; Tay *et al.,* 2022). ITS region important molecular target for taxonomy and identification and it is greater sequence variation than other regions. ITS now perhaps is the most widely sequenced DNA region in fungi and it showed to be beneficial for molecular identification at the species level and even within species (Ali *et al.,* 2015; Aldossary *et al.,* 2016; Hou *et al*., 2016). The high prevalence of *Candida spp*. in the oral cavity in immune-deficient patients is caused by many virulence factors including morphogenesis, adhesion, biofilm formation, phenotypic switching and secretion of hydrolytic enzymes as well as the ability to adapt the surrounding environment changes (Aguilar-Marcelino et al., 2021). Oral symptoms as outlined by AL-kahfaji. (2022) chiefly due to atrophic mucosa and Candida infections included oral mucosa burning sensation, pain, dry mouth, numbness of oral mucosa, recurrent ulcers, bad taste, and dysfunction of taste. Oral candidiasis associated with internal diseases with immunodeficiency, such as diabetes, thymoma, endocrine disorders and HIV infection.

5. CONCLUSION

C. albicans is the most frequent species in the oral cavity of DM patients as the causative agent of candidiasis Followed by *C. dubliniensis*. The best morphological tests to distinguish between are Culturing onto Tobacco agar medium followed by chlamydospore formation test and germ tube formation. using CHROMagar™ Candida provides an easy, rapid method for the presumptive identification of Candida species and other yeasts**.** Finally molecular technique may provide accurate alternative than traditional methods.

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