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# Investigation of *Candida* species in the oral cavities of Iraqi patients with diabetes mellitus using molecular and phenotyping methods

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## ABSTRACT

**Aims:** This study aimed to identify *Candida spp*. isolates from the mouths of Anbar (Iraq) patients with diabetes mellitus using conventional and molecular markers. And to evaluate which redundant species.

**Methodology and results:** A total of 100 clinical samples were collected from the oral cavities of patients who were suffering from diabetic mellitus for four months (from December 2022 to March 2023). in two prominent hospitals located in Anbar province. We performed the identification of all samples phenotyping and by molecular identification. The 18s rRNA gene was detected using gradient PCR. All samples were applied to the KOH test and then cultured on SDA and chrome agar. There were 89 positive cultures on SDA, with 89 isolates (57.30%) having germ tube . The culture on chrome agar revealed four species of *C.albicans* was the most frequent species in 51(57.30%) followed by *C.* tropicalis in 16 (17.97%), *C.* glabrata in 13 (14.6%), and *C.krusei* 9(10.11%). Based on PCR technique to confirm isolates, 81 bacterial isolates carry the 18SrRNA. From 89 fungal isolates, there are 84 isolates were having the ITS gene sequence.

**Conclusion, significance, and impact of study**: Four fungal isolates were detected in this study by using conventional and modern methods to define the profile of oral microbial infection. This was done to give public health, general medical, and oral health practices a scientific basis that would guide and advise them, with a focus on how important it is for individuals with diabetes to get regular treatment.

*Keywords*: Diabetic mellitus, oral cavities, fungal infection

## Introduction

The term "microbiome" refers to the complex ecology of bacteria, fungus, protozoa, and viruses that lives in the human digestive system's upper and lower sections. It starts to form in the uterus even before birth, and it continues to grow for another two to three years after birth until the physiological changes brought on by senescence cause significant changes in its composition once again[1]. . The oral

cavity provides a window into overall health and is where dentistry and medicine converge [2]. Saliva and gingival crevicular fluid proteins either directly or indirectly control the establishment and growth of microorganisms in the oral cavity by acting as nutrients for the growth of microorganisms, supplying molecules for microbial attachment, or having an antimicrobial effect [3]. Because of its heterogeneity and the interactions between the various anatomic structures, the mouth harbors one of the most diverse microbial communities when compared to other body sites, like the skin and vagina [4]. A variety of factors, including phenotypic switching, dimorphism, adhesive characteristics, extracellular enzyme production, and the creation of biofilm can lead to Candida species taking advantage of an opportunity to colonize, grow, and hinder other microorganisms, resulting in recurrent infections in the oral mucosa when the host immune defenses weaken or the normal oral microbiota balance is upset [5]. Globally, diabetes mellitus (DM) has become more prevalent, accounting for the greatest number of cases. These chronic illnesses are characterized by cellular resistance to insulin action, insufficient insulin, or both, leading to hyperglycemia and associated metabolic disorders [6]. Members of the genus Candida have been reported to be the most prevalent commensals among the different organisms that can cause accidental infections in the oral cavity. Candida overgrowth occurs in the oral microenvironment due to preexisting conditions such as low salivary function, altered salivary pH, high salivary glucose, and poor nutrition [7]. Invasive Candida infections have become more common in the last ten years due to the increase in immunodeficient population [8]. Diabetes patients are more likely to develop oral candidiasis for a number of reasons, including high salivary glucose levels, low saliva release, weak chemotaxis, and a polymorphonuclear leukocyte deficiency-related phagocytosis defect.

A widespread oral opportunistic infection termed oral candidiasis is caused by an overabundance of candida species, mainly *C. albicans*. A group of infectious illnesses known as mycoses are brought on by the pathogenic activity of fungi. While the majority of fungal species are found in plants and the environment as saprophytes, where they are essential to the organic matter's natural recycling process, some of them can infect humans. Of the general population, 53% have common commensal organisms like candida in their oral cavities [9]. While *C. albicans* is the most common isolate from the oral cavity and is thought to be the primary cause of candidiasis, other non- *C. albicans* species, including *C. glabrata*, *C. tropicalis*, *C. krusei*, *C.parapsilosis, and C. dubliniensis*, have significantly increased over the past 20 years due to a variety of factors, including immune-suppressants and long-term use of anti-fungal and wide-spectrum antibiotics [10]. It is estimated that more than 80% of oral yeast isolates belong to the species *C. albicans* [11].

## MATERIALS and METHODS

### Sample Collection

This study was carried out in the labs of the Biology Dept / Faculty of Science at the University Of Anbar. A total of 100 clinical isolates were collected from the oral cavities of patients suffering from diabetic mellitus for four months (from December 2022 to March 2023). Isolates were obtained from Fallujah Teaching Hospital (76) and Karmah Emergency Hospital (24). Swabs of sterilized transport media were used to gather the samples, including the patients' oral cavities , who were diagnosed clinically by a specialist doctor as suffering from Diabetes. None of them had another systemic disease or were an alcoholic drinkers. Also, the blood glucose was measured by the Hemoglobin A1c test (HbA1c).

### Samples Culturing

All samples were cultured on ( Sabouraud dextrose agar ) SDA in aerobic conditions at 28°C for 2-3 days.

### Sample Identification

A sample collection for a total of 100 patients was collected from Ramadi Teaching Hospital for women and children in Anbar governorate. Samples were obtained from mother with mastitis and her baby. Using sterilized cotton swabs, samples were taken. The swabs were inoculated on SDA culture media for the isolation of pathogenic fungi. The fungi were examined under a microscope with KOH 10% and lactophenol cotton blue, and then candida was examined with a germ tube test, and also cultured on chrom agar to identify species of candida, All samples were preserved in Brain Heart Infusion Broth, and placed in the freezer for possible use until the end of the study, and finally, they were diagnosed with PCR.

## Microscopic Examination

### Potassium Hydroxide (10% KOH) test

The KOH test is a quick, easy, painless, accurate, and non-invasive method for detecting fungal infections [12]. To determine whether fungal infections were present, specimens were taken from the oral cavities and put on a slide with a KOH solution and then examined under a light microscope.

### Germ Tube:

The germ tube is used to differentiate fungi species, as this test used to detect *C.albicanes*. Three fresh drops of human serum were pipetted into test tubes that had been labeled by a Pasteur pipette. A portion of the yeast colony was added to the serum using a sterile inoculating loop, mixed well with serum , and then incubated for roughly three hours at 37° C. After incubation, a drop of the suspension was taken out from the test tube using a Pasteur pipette and placed on a clean sterile, dry slide, coated with a clean cover glass, and examined under a microscope with 10 X and 40 X objective lenses to observe the development of germ tubes. A germ positive tube

yeast is identified by a cylindrical filament that emerges from the blastoconidium and does not constrict at the point of origin or exhibit visible swelling along its length [13].

**Chrome Agar**

The samples that contain candida were cultured on chrome agar medium (Vavantor, Boland) which is used to differentiate between candida species based on the specific color colonies. The use of chrome agar for the identification of *Candida spp*. is a useful tool in the clinical microbiology laboratory, as it allows for accurate and rapid identification of *Candida spp*.

**Maintenance of fungal Isolates**

Brain Heart Infusion (BHI) broth (Himedia India) was used to store the fungal isolates. The process involved combining 20 mL of sterilized glycerol with 80 mL of brain heart infusion. The resulting mixture was divided into full-ring screw bottles, with 5 mL of the solution added to each bottle. After allowing the bottles to stand at room temperature for 30 minutes, they were transferred to a storage temperature of -20°C for future use [14].

**DNA Extraction**

The DNA was extracted using an easy Geneaid purification kit, and the concentration and purity of the DNA were determined using spectrophotometry .

**PCR application**

To find the 18S rRNA to separate the fungi, we used gradient PCR (Bio-Rad UK). The original PCR reagents and final concentrations of the protocol (Table 1), specific primers (Table 2) and certain conditions (Table 3):

Table 1: The original PCR reagents and final concentrations of the protocol.

| Component | Volume | Concentration |
|---|---|---|
| Green Master Mix, 2X | 12.5 µl | 1X |
| upstream primer | 0.75 µl | 10µM |
| downstream primer | 0.75 µl | 10µM |
| DNA template | 1 µl | <250ng |
| Nuclease-Free Water | Completed to 25 µl | N.A |

Table 2: Primer used throughout the current study .

| Primer Target | Oligo Sequence 5′→3′ | Product (bp) | Reference |
|---|---|---|---|

| | | | |
|---|---|---|---|
| ITS 18S ribosomal RNA | F TCCGTAGGTGAACCTGCGG<br>R TCCTCCGCTTATTGATATGC | 550 bp | [15] |

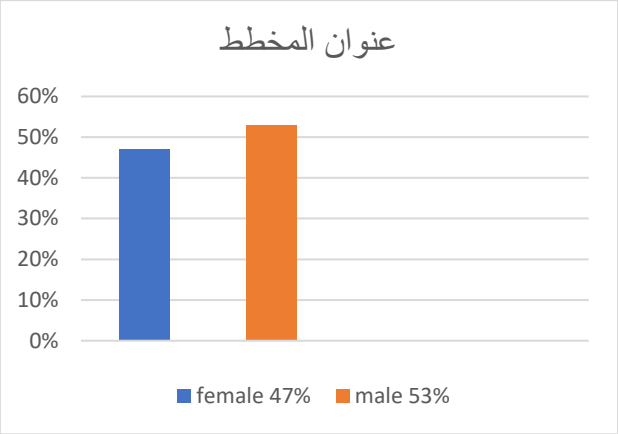
Table 3: program of PCR to detect for 18 srRNA

| Step | Temperature | Duration | Cycles |
|---|---|---|---|
| Enzyme activation | 95° C | 3 min | Hold |
| Denaturation | 95° C | 30 s | |
| Annealing | 63℃ | 35s | 30 |
| Extension | 72° C | 30s | |
| Final Extension | 72° C | 10 min | Hold |
| Hold | 4° C | ∞ | Hold |

Then agarose gel electrophoresis (Bio-Rad UK) was used to check for the presence of amplified genes following PCR amplification by using 1.5% gel agarose. Then the gel was applied to the gel document system (Bio-Rad UK) to observe the 18S rRNA bands.

## Results

In this study, 100 samples were collected from the oral cavity of patients who were suffering from diabetic mellitus. The samples were collected from oral cavity sources from December 2022 to March 2023. The majority frequent category age include 61–85 years old in 64 (64%) cases, followed by 20–39 years in 19 (19%) cases, and 40–60 years old in 17 (17%) instances (Table 4). Of the one hundred patients under investigation, 53 % males and (47%) were females (Figure 1). KOH test showed that 89% of the 100 samples from the mouths of diabetic patients who went to the above-mentioned hospitals had fungi in them. This was based on a random blood sugar test that was above 180 mg/dl and an HbA1c test that was above 6.5%. All samples cultivated on an (SDA) medium which allow the isolates to grow. There were 89 positive cultures.




Figure(1): The number and percentage of female and male

Table 4 : The distribution of age categories between male and female

| | | Gender | | | | Total | |
|---|---|---|---|---|---|---|---|
| | | Male | | Female | | | |
| | | N | % | N | % | N | % |
| Age Categories | 20-39yrs | 8 | 8.0% | 11 | 11.0% | 19 | 19.0% |
| | 40-60yrs | 9 | 9.0% | 8 | 8.0% | 17 | 17.0% |
| | 61-85yrs | 36 | 36.0% | 28 | 28.0% | 64 | 64.0% |
| Total | | 53 | 53.0% | 47 | 47.0% | 100 | 100.0% |

**Morphological examination of Different examined microorganisms**

***Candida* spp.**

The most significant opportunistic fungus is Candida; once barriers are removed, infection and spread may happen with potentially lethal results. The clinical manifestations were nonspecific, and the standard laboratory procedures were vague.

A popular selective and differential medium for isolating and identifying pathogenic fungi, such as various species of Candida, is Sabouraud Dextrose Agar (SDA). Additionally, it contains antibiotics such as chloramphenicol and cycloheximide that inhibit the growth of bacteria, thereby allowing for the selective growth of fungi [16]. With 100 samples taken from oral cavities, the presence of growth was 89 and no growth on the SDA medium was 11, as shown in (Figures 2,3) .

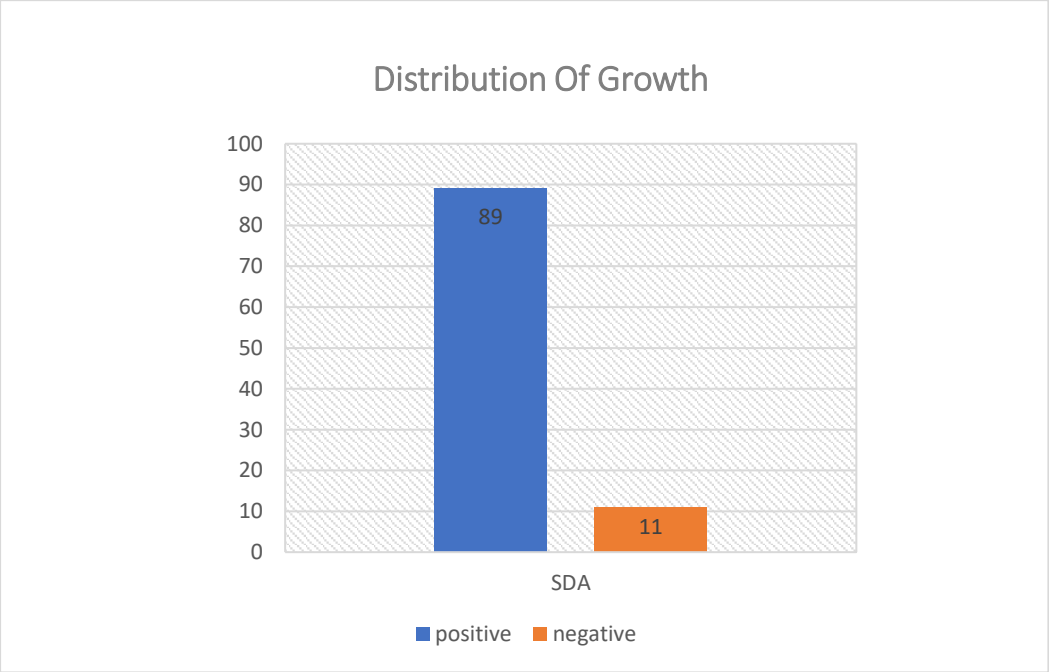



Figure (2) : percentage of samples growth on SDA medium

Figure (3): *Candida spp* isolate on SDA medium.

The separation of *Candida albicans* from other species of Candia is done using the germ tube test. After being incubated in serum, 95–97% *of Candida albicans* form germ tubes [17] . To identify *Candida albicans*, the germ tube test is utilized [9] from 89 fungal isolates which grow on the SDA medium, with 57.30% having germ tube. The germ tube test verified the isolates of *Candida albicans* (Figure 4,5) . All of the 89 isolates were tested for their growth ability on CHROM agar Candida medium (CHROMagar, Vavantor, Boland) at 28°C for 2-3 days. The use of Chrome agar for the identification of *Candida spp*. is a useful tool in the clinical microbiology laboratory, as it allows for accurate and rapid identification of *Candida spp*. [18].

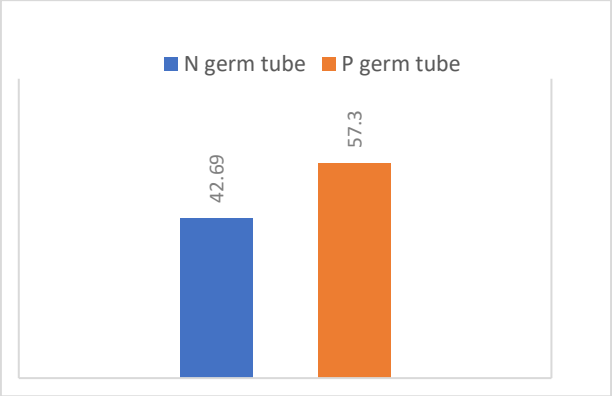



Figure (4):Germ tube percentage of 89 isolates . N:negative , P:positive

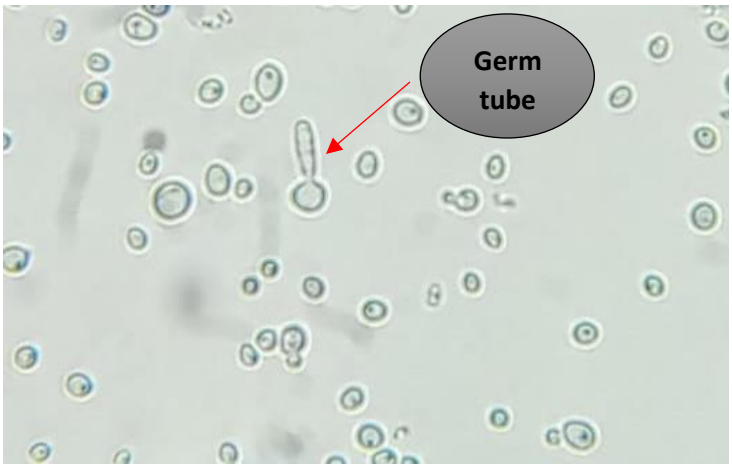



Figure (5): According to the germ tube there are 57.30% percent of isolates were *C.albicanes.*

The color of the colonies of *Candida albicans* is light to medium green, *Candida tropicalis* is dark blue to metallic blue, and *Candida krusei* is pink with a whitish border. Other yeasts may develop either light-to-dark mauve or cream colors(e.g., *C. glabrata*) on isolation media [19], [20] as shown in ( Figure 6).

Figure (6): Differentiation between *Candida spp*., on the basis of the specific color of colonies on chrome agar.

*C.albicans* was the most common species in 89 isolates 51(57.30%) and these results were closely related to a previous study done by [21] who found that the percentage of *C. albicans* was (57.1%) from 104 isolates, followed by *C. tropicalis* in 16 (17.97%), *C. glabrata* in 13 (14.6%), *C.krusei* 9 (10.11%).The 89 yeast isolates were distributed according to colony color on chrome agar after 72 hours.

**Molecular study of fungi**

The (Figure 7) shows the results of gel electrophoresis performed on genomic DNA extracted from 89 samples. A popular method in molecular biology for separating DNA molecules according to size and charge is gel electrophoresis. The presence of DNA bands on the gel indicates the presence of genomic DNA in the sample, 84 of the 89 fungal isolates tested positive for the ITS gen .

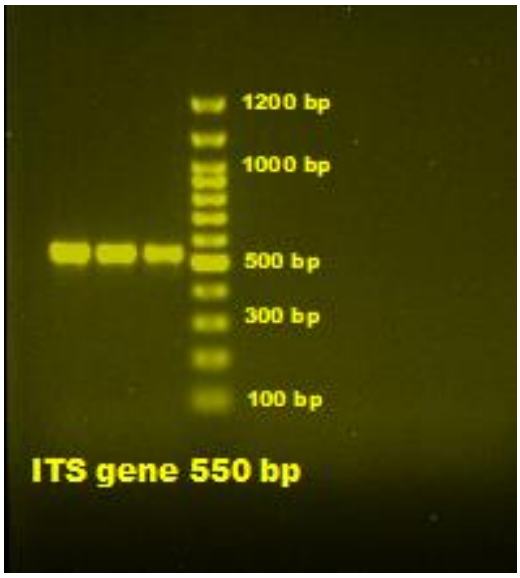



Figure (7): Gel electrophoresis of PCR product(ITS gene), 1.5% agarose gel, 90v for 10 min then 70v for 1:30 hours. Annealing temperature 58°C. DNA ladder (100 - 1200bp).

PCR was used for the detection of the ITS gene of *candida spp*. After PCR amplification, gel electrophoresis of the PCR product was done on 1.5% agarose. The target gene was found and its band size is 550 bp.

**Sequencing and Alignment of Amplicons**

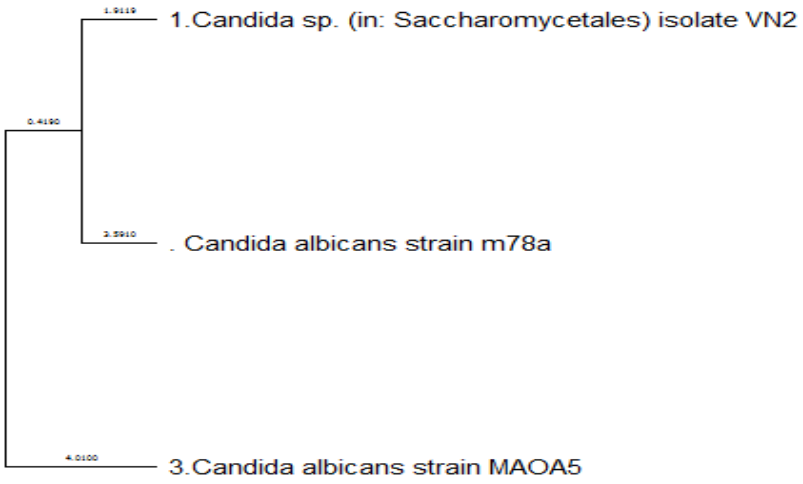
In the present analysis, the DNA extraction kit is simple and dependable, requiring no special equipment and producing high-quality DNA that is appropriate for researching fungal genes. As anticipated, the PCR specifically targeted the sequences between the 18SrRNA and 28SrRNA gene sequences. The amplicon amplified using ITS is 550 bp, according to the sequencing of the amplicons. (Figure 8)

When BLASTN queries were run against the GenBank database using the isolates' amplicon sequences. In (Table 5) multiple alignments between the two

sequences of three isolates were found in the results, indicating that ITS1/ITS4 amplicon of I1, I2, I3 were detected with its analog three isolates, whereas the ITS1/ITS4, amplicon of $I_1$ showed higher identity (81.5%) with the partial rRNA sequence of (acc.No.MT315066), $I_2$ showed identify (84.5%) with the partial sequence of (acc.No.MT159703.1), $I_3$ showed higher identify (94%) with the partial sequence of (acc.No.KP675208.1). The phylogenetic tree of the (ITS1/ITS4) amplicons RNA gene sequence of isolates revealed closely neighbor joining .

Table(5): The ITS of the rDNA fragment sequences of fungal clones was amplified using the BLASTN method and compared to an analogous sequence found in the Genbank database.

| Isolate | Amplicon of ITS | Species | Accession number | ID |
|---|---|---|---|---|
| $I_1$ | + | *Candida albicans strain MAOA54* | MT315066 | 81.5% |
| $I_2$ | + | *Candida tropicalis isolate 2Y196* | MT159703.1 | 84.5% |
| $I_3$ | + | *Candida albicans strain M37b* | KP675208.1 | 94% |




Figure(8):ITS and 18s rRNA gene regions were used to identify Taxa and other sequences in the phylogenetic tree of Candida isolates.

**DISCUSSION:**

When compared to healthy people, diabetic patients who practice poor oral hygiene may have higher levels of *Candida spp.* in their oral flora, which could lead

to both superficial and systemic fungal infections [22]. Antifungal agent resistance in *Candida species* has been documented recently, particularly in strains derived from immunocompromised individuals [23],[24] Our goal was to identify any *Candida species* that were present and colonized in the saliva of diabetic patients. We discovered that patients with diabetes had a higher incidence of Candida infection. More candida could be found in diabetic patients because the disease makes it easier for Candida to stick to epithelial cells and weakens tissues' ability to fight off infections. Similarly, a higher carriage rate of Candida in individuals with diabetes is associated with salivary glucose and pH levels [25],[26]. Based on the KOH test, there are 89 positive results. This test is important for fungal identification because KOH destroys every non-fungal cell. Therefore, by looking at the liquid under a microscope, it is possible to determine whether or not fungus is present. This test only detects the existence of fungi; it cannot identify the type of fungus present. According to our data, 89 (89%) of the patients with diabetes had fungal isolates . The SDA is a low pH (5.6) medium containing 4% dextrose, casein and animal peptic digest that is used to isolate both pathogenic and non-pathogenic fungal microorganisms (molds and yeasts). Because of its natural acidity, this agar prevents many bacteria from growing when they are grown on SDA medium [17]. The presence of germ tubes after 3 hours indicates that the *Candida spp*. isolates are likely *C. albicans* [13]. Germ tube formation by *Candida spp*. may be delayed, incomplete, or absent so the isolate-formed germ tube after 6 hours may be *Candida topicals* [27].

One of the most popular media in the mycology lab is CHROM agar. Depending on the characteristics of the colonies which include the color for identification of four species *C. albicans, C. glabrata, C. krusei and C. tropicalis* using CHROM agar. This study's findings were in agreement with previously published reports[28],[29]. Two chromogenic substrates are included in the CHROM agar medium to detect the activities of phosphatase and β-hexosaminidase [30]. With the help of the medium, it is possible to distinguish between *Candida tropicalis*, which forms blue colonies because it produces both enzymes, and *Candida albicans*, which forms green colonies because it produces β-hexosaminidase. Certain yeast species like *C. krusi* form pink colonies mainly because of their phosphatase activity, while other yeast species produce neither of these enzymes and develop as white colonies [31]. The main benefit of using chromogenic agars is their capacity to identify mixed cultures of yeasts, as distinct species often produce colonies with varying hues. These species mixtures could be identical and go unnoticed when combined on standard agars like Sabouraud agar plus chloramphenicol [21],[32] . The most common species (57.30%) found in the oral cavities of the diabetic patients in the current study based on germ tube and chrome agar was *C. albicans*, which was consistent with the results of earlier investigations [7],[33].
In this investigation *Candida albicans* exhibited the highest frequency in the oral cavit y among the species examined which is in agreement with investigation done by [11], which showed *C. albicans* to be the predominant yeast isolate (80%) from oral cavity swabs. The prevalence of oral candidiasis with other species, like *Candida glabrata*

and *Candida krusei,* has increased during the past 20 years[34]. The findings of the present study are more or less similar to those of the previous study. This could be due to variations in the geographical distribution of various *Candida species* [35]. The detection of the ITS gene in Candida using PCR followed by agarose gel electrophoresis is a common technique used in molecular biology research .The target gene was amplified and the PCR amplification was successful when there was only one band, measuring 550 bp, visible on the agarose gel. The ITS region (Internal Transcribed Spacer) is a highly conserved region found in the DNA genes of fungi, including *Candida species*. This region has been widely used as a target for molecular identification of fungal species using PCR-based techniques, including PCR amplification and agarose gel electrophoresis [36]. According to the results we obtained in this current study through the DNA sequence test demonstrated the results we obtained in the phenotypic and molecular tests , and confirmed as new types of fungi were discovered found in the oral cavity of people with diabetes in Al-Abar Governorate.

## Conclusion :

Presence of four fungal species ( *C.albicans ,C. tropicalis ,C. glabrata and C. krusei* ) was detected using a combination of phenotypic and genetic tests, and these results were confirmed using deoxyribonucleic acid sequencing. Conducting research in this field is very important in the coming period in order to control the fungal species that cause infections in the oral cavity in diabetic patients who are more susceptible to such diseases .

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