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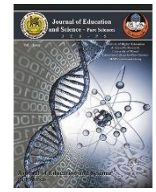

Detection of Enzymatic Virulence Factors in *Candida albicans* and *Candida glabrata* Isolated from Diabetic Patients

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Abstract

Candiduria is among the most common infections in diabetic patients, and its incidence has increased markedly in recent decades. *Candida* spp. are frequently isolated from patients, with non-*albicans* species being increasingly recognized as important etiological agents. The current study aimed to diagnose *Candida* spp. isolated from the urine of diabetic women aged 16-72 years and evaluated their ability to produce phospholipase, hemolysin, and esterase enzymes. Statistical analysis of enzymatic activity was performed ($P<0.01$). Midstream urine samples were collected from 178 diabetic women, including 86 type 1 and 92 type 2 diabetes. Urine samples were cultured on Sabouraud's Dextrose Agar (SDA) and subsequently identified using conventional methods, including cultural characteristis Gram stainning germ tube formationchlamydospore production, growth at 45°C, and growth on HiCrome Candida Differential Agar M1297A. The present study showed that three different species of *Candida* were identified: 30 (50%) *C. glabrata* isolates, followed by 18 (36%) *C. albicans* isolates and 2 (4%) C. *krusei* isolates. Also, the results showed that type 1 diabetic patients are more susceptible to *Candida* infection. The virulence test revealed that all *C. albicans* isolates (100%) were positive for phospholipase, hemolysin, and esterase with different production rates. In terms of *C. glabrata* isolates, one isolate only (3.3%) produced phospholipase, while all isolates (100%) produced hemolysin, and four isolates (13.3%) produced esterase.

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1. Introduction

In healthy humans, *Candida* species is considered a normal flora of the skin, mucous membranes, and gastrointestinal tract. However, these commensal yeasts can transform into opportunistic pathogens when predisposing factors are present. Among the most significant of these factors are immunosuppressive diseases, uncontrolled diabetes, high-carbohydrate diets, pregnancy, use of contraceptives, prolonged use of broad-spectrum antibiotics and corticosteroids, as well as the incomplete or extended use of antifungal agents; poor personal hygiene also plays a contributory role [1]. In recent decades, there has been a significant increase in the prevalence of invasive fungal infections globally, with pathogenic fungi posing an increasingly serious threat to human health [2].

Candiduria refers to the presence of *Candida* species in the urine [3] and can be detected either by microscopic examination or by fungal growth on culture media [4]. Urine culture is considered the gold standard method for diagnosing candiduria [5]. Some evidences indicate a decrease in the proportion of urinary tract infections caused by *Escherichia coli*, *Proteus* spp., and *Pseudomonas* spp., on the other hand, an increase in the proportion of infections caused by fungi, particularly *Candida* species [6].

Several studies have shown an increase in the prevalence of *Candida* species in urine samples from hospitalized patients, and the most common species are *C. albicans* (35-68%), *C. glabrata* (8-53%), and *C. tropicalis* (3-36%). It has also been noted a progressive increase in non-*albicans Candida* species in some countries [7]. Although *C. albicans* is the main etiologic agent in patients with candiduria, over the past few decades, there has been an increase in non-*albicans Candida* (NAC) species [8].

It has been noted that approximately 75% of females experience *Candida* infections at least once in their lifetime, *C. albicans* being the most common, accounting for 90% of isolates compared to non-*albicans* species. Despite that, a few years ago, studies indicated an increase in infections among women caused by non-*albicans* species such as *C. glabrata* and *C. krusei,* which have become more prevalent within developed countries, compared to other less common species like *C. tropicalis* and *C. parapsilosis* [9,10].

C. glabrata is the second most common pathogenic yeast after *C. albicans*, which is non-dimorphic and was previously thought not to be pathogenic, but in patients with a weakened immune system, *C. glabrata* may represent a highly opportunistic pathogen affecting the urinary, reproductive, and blood system [11].

Fungi can induce disease and disrupt the host immune system through many genes and proteins associated with pathogenicity, known as virulence factors, such as extracellular enzymes. The most important hydrolytic enzymes produced by *Candida* spp. are phospholipase, which play a significant role in their pathogenicity, acting by degrading phospholipids to facilitate tissue penetration [12]. Also, hemolysins are a group of enzymes that play a role in the pathogenicity of *Candida* by breaking down erythrocytes to use haemoglobin as an iron source [13]. In addition, esterase plays a significant role in the breakdown of triacylglycerol [14].

However, due to the significant role of virulence factors of *Candida* species in enhancing the severity of fungal infections and the health complications in women with diabetes, the current study aims to diagnose *Candida* species in urine samples from diabetic women and evaluate their ability to produce phospholipase, hemolysin, and esterase enzymes.

2. Methods and Materials

2.1 Samples Collection

Midstream urine samples were, in the morning, collected from women with both types of diabetes who attended the Al-Wafa Specialized Center for Diabetes and Endocrine Diseases in Mosul, Iraq. Women's ages were from 16 to 72 years old, and all exhibited symptoms of urinary tract infections. None of them was taking any antibiotics. A total of 178 samples were collected, including 86 samples from patients with type 1 diabetes and 92 samples from type 2. The samples were collected using sterile plastic containers with a capacity of 50 ml and a tight-fitting lid and transported to the laboratory within 2 hours for subsequent tests.

2.2 Samples Culturing

Urine samples from each patient were inoculated onto Sabouraud's dextrose agar (SDA) containing streptomycin (100µg/mL) by streaking. The plates were incubated at 37°C for 48 h. The plates that did not show any growth were retained in the incubator for 7 days before being discarded.

2.3 Identification of Isolates

The identification was carried out based on the appearance, color, shape, and texture of the growing colonies [15]. Further techniques were processed for identification using Gram stain and germ tube test using human serum [16], chlamydospores production on corn meal agar supplemented with Tween 80 [17], growth at 45°C [18], and growth on differential medium HiCrome Candida Differential Agar M1297A.

2.4 Determination of Virulence Factors

2.4.1 Determination of Phospholipase Activity

The test was conducted using the plate method described by Price *et al*. [19]. The suspension was prepared for each isolate of *C. albicans* and *C. glabrata* growing for 18-24 h on SDA at a concentration of 1×10^8 cells/mL, then 10 microliters of the suspension of each isolate were inoculated as a spot onto the surface of egg yolk agar, left to dry at room temperature, and then incubated at 37°C for 5 days [20]. Each isolate was tested in triplicate. The precipitation zone (Pz), which appears as a dense white area around the colonies, indicates a positive result for the production of phospholipase. The ratio obtained by dividing the colony diameter by the total diameter of the colony and the precipitation zone (Pz) represents a unit of measurement for phospholipase activity.

The activity of phospholipase production is classified into four categories in addition to the control: Pz value = 1.00 as negative (─), Pz value = 0.99-0.90 as weak activity (1+), Pz value = 0.89-0.80 as moderate activity (2+), Pz value = 0.79-0.70 as relatively strong activity (3+), if Pz $\leq$ 0.69, the activity is very strong (+4). Accordingly, a low Pz value indicates strong enzyme activity [21].

2.4.2 Determination of Esterase Activity

Esterase activity was assessed according to the method described by Noori et al. [22]. A yeast suspension was prepared at a concentration of 1×10^6 cells/mL for each isolate of *C. albicans* and *C. glabrata*, then 10 microliters of the suspension was inoculated for each isolate onto a Tween 80 opacity surface as spots. The plates were incubated at 30°C for 10 days, and checked daily; each isolate was tested in triplicate.

The appearance of a halo as a transparent white zone around the colony indicates the precipitation zone, which is a positive for esterase production. The ratio of the diameter of the colony to the total diameter of the colony and precipitation zone is measured as the value of esterase activity (Ez). Enzyme activity was measured as described for phospholipase activity.

2.4.3 Determination of Hemolysin Activity

The activity of hemolysin production by *C. albicans* and *C. glabrata* isolates was assessed using the modified plate assay described by Luo et al. [23], which was modified from the method described by Manns et al. [24]. *Candida* isolates were cultured on SDA for 18-24 h. Thereafter, a suspension was prepared for each isolate at a concentration of 1×10^8 cells/mL. Then 10 microliters of the suspension were inoculated as spots on sugar-enriched sheep blood agar, and incubated at 37°C in 5% CO_2 for 48 h. A positive hemolytic activity is indicated by the appearance of a translucent halo and/or a greenish-black halo around the colony, which can be observed using transmitted light.

The ratio obtained by dividing the total of the colony diameter and the halo diameter by the colony diameter indicates the hemolytic index (Hi), and this ratio is equal to or larger than 1 [23].

2.5 Statistical Analysis

The data obtained in our study were statistically analyzed using Duncan's multiple range test; P values less than 0.01 were considered statistically significant [25].

3. Results and Discussion

The present study showed that a total of 178 urine samples were tested for yeast growth on SDA; 60 (33.7%) were culture-positive. Out of 60 isolates, 50 isolates (83.3%) were positive to the Gram stain test. Additionally, the results revealed that 18 isolates (30%) were positive for germ tube formation, chlamydospore production, and growth at 45°C. The colors of yeast colonies on HiCrome Candida Differential Agar are shown in Figure 1, where 30 isolates (50%) exhibited cream to white color, which were identified as *Candida glabrata*, 18 isolates (30%) exhibited light green and were identified as *C. albicans*, and 2 isolates (3.33%) exhibited purple fuzzy were identified as *C. krusei*. The remaining 10 isolates (16.66%) had a different color appearance on HiCrome agar; therefore, it was difficult to identify them, so they were disregarded.

Many studies indicate that non-*albicans Candida* species have been increasing in recent years and become more prevalent as pathogens compared to *C. albicans*, as a result of non-*albicans Candida* species, including *C. glabrata*, are more adapted to the urinary tract environment and in some cases display resistance patterns to traditional antifungal agents, therefore, they are sometimes difficult to treat, they should be considered a health problem and a potential cause of treatment failure [26, 27,28].

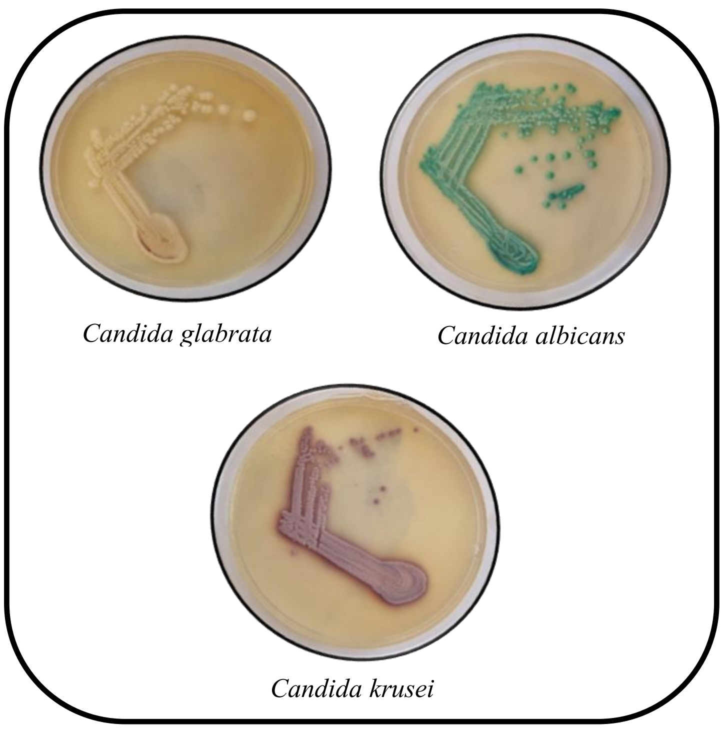

Figure 1. Three different *Candida* species colonies on HiCrome Candida Differential Agar medium

The current study also showed that the incidence of *Candida* sp. in type 1 diabetes was higher than in type 2, at 60% in type 1 and 40% in type 2 (Table 1). Rodrigues *et al.* [29] and Saud et al. [30] reported that uncontrolled hyperglycemia leads to an increased risk of fungal infections due to the impairment of the immune system, resulting in a deficiency of T lymphocytes, reduced neutrophil activity, and decreased cytokine secretion, leading to immunosuppression in type 1 diabetes. High blood glucose levels, urine acidity, and female gender have been associated with a high incidence of *Candida* species in the urine of diabetics.[31,32].

Table 1. Distribution of different *Candida* species among Type 1 and Type 2 diabetes

Candida spp.	Type of diabetes		Isolates No. (%)
	Type 1	Type 2	
C. albicans	11	7	18 (%36)
C. glabrata	17	13	30 (%60)
C. krusei	2	0	2 (%4)
Total	30 (%60)	20 (%40)	50 (%100)

3.1 Virulence Factors

In the present work, *C. albicans* and *C. glabrata* were chosen for the enzyme production assay due to their frequent appearance compared to other species. For differentiation purposes, the isolates of *C. albicans* were assigned codes ranging from CA1 to CA18, whereas those of *C. glabrata* were assigned codes ranging from CG1 to CG30.

3.1.1 Phospholipase Enzyme Activity

The results showed that all *C. albicans* isolates were positive for phospholipase production, as indicated by the appearance of a dense white area around the colonies, which represents the precipitation zone (Pz) of the calcium complex (Figure 2).

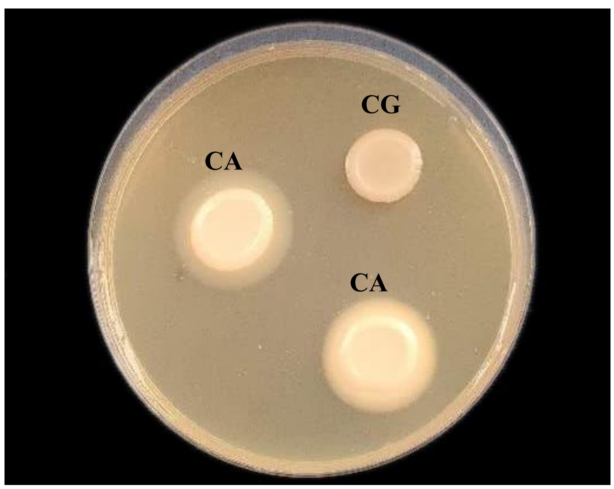

Figure 2. Phospholipase activity of *Candida* spp. on egg yolk agar; *C. albicans* (CA) positive; *C. glabrata* (CG) negative

In addition, significant differences ($P < 0.01$) were shown among *C. albicans* isolates in their ability to produce phospholipase (Table 2). The isolates CA11 and CA5 had the highest enzyme production, followed by the other isolates in decreasing productivity, while isolate CA6 showed the lowest productivity.

Table 2. The effectiveness of *C. albicans* isolates in producing virulence enzymes

Isolate code	Pz Values mean	Ez Values mean	Hi Values mean
CA1	*0.74^{e}	0.38	*2.66^{ab}
CA2	0.81^{cd}	0.37	2.50^{bcd}
CA3	0.85^{abc}	0.34	2.53^{a-d}
CA4	$0. 84^{abc}$	0.37	2.23^{de}
CA5	0.64^{f}	0.39	1.90^{fg}
CA6	0.87^{a}	0.40	2.83^{a}
CA7	0.72^{e}	0.41	2.60^{abc}
CA8	0.85^{abc}	0.38	2.30^{cde}
CA9	0.82^{bcd}	0.43	2.46^{bcd}
CA10	0.83^{bcd}	0.37	2.46^{bcd}
CA11	0.66^{f}	0.41	2.13^{ef}
CA12	0.86^{ab}	0.38	1.76^{g}
CA13	0.84^{abc}	0.40	2.00^{efg}
CA14	0.85^{ab}	0.43	2.50^{bcd}
CA15	0.80^{d}	0.37	2.00^{efg}
CA16	0.83^{bcd}	0.40	2.60^{abc}
CA17	0.85^{abc}	0.40	2.03^{efg}
CA18	0.80^{d}	0.39	2.60^{abc}

*Mean values within the same column followed by different superscript letters are significantly different ($P < 0.01$) according to Duncan's multiple range test

Moreover, the results showed that only one isolate of *C. glabrata* among 30 (3.3%) was a phospholipase producer, specifically isolate CG24. It had strong activity with a Pz value of 0.71 (Table 3). All 29 remaining isolates (96.7%) were non-enzyme producers, with no significant differences among them.

Table 3. The effectiveness of *C. glabrata* isolates in producing virulence enzymes

Isolate code	Pz Values mean	Ez Values mean	Hi Values mean
CG1	*1^{a}	*1^{a}	*3.06^{a}
CG2	1^{a}	1^{a}	1.73^{ij}
CG3, CG 13, CG 19	1^{a}	1^{a}	2.43^{bcd}
CG 4, CG 23, CG 26	1^{a}	1^{a}	2.33^{cde}
CG 5, CG 11, CG 20	1^{a}	1^{a}	1.80^{hij}
CG 6	1^{a}	1^{a}	2.66^{b}
CG 7, CG 25, CG 27	1^{a}	1^{a}	2.00^{f-i}
CG 8	1^{a}	0.57^{d}	2.10^{e-h}
CG 9	1^{a}	1^{a}	2.16^{d-g}

Isolate code	Pz Values mean	Ez Values mean	Hi Values mean
CG 10	1[a]	1[a]	2.23[d-g]
CG 12	1[a]	0.84[b]	2.10[e-h]
CG 14	1[a]	1[a]	2.23[d-g]
CG 15	1[a]	0.64[c]	1.96[ghi]
CG 16	1[a]	1[a]	2.63[bc]
CG 17	1[a]	1[a]	2.30[def]
CG 18	1[a]	1[a]	2.00[f-h]
CG 21	1[a]	0.57[d]	2.26[d-g]
CG 22	1[a]	1[a]	2.16[d-g]
CG 24	0.71[b]	1[a]	2.36[b-e]
CG 28	1[a]	1[a]	2.63[bc]
CG 29	1[a]	1[a]	1.63[j]
CG 30	1[a]	1[a]	2.20[d-g]

*Mean values within the same column followed by different superscript letters are significantly different ($P < 0.01$) according to Duncan's multiple range test

It has been demonstrated that the relationship between the activity of phospholipase secretion and the increased virulence of *Candida* species [33]. Additionally, different studies have shown that phospholipase activity in *Candida* isolates varies between 30 and 100% across different patient groups and infection sites. Thus, the differences in results among studies are due to several factors, such as the site of infection, which varies by species, and the gene encoding the phospholipase, which may be affected by growth conditions [34,35].

The significant difference observed in the present study between *C. albicans* and *C. glabrata* isolates in enzyme production efficiency may be attributed to the differences in virulence mechanisms [36].

3.1.2 Esterase Enzyme Activity

All *C. albicans* isolates showed positive for esterase production, and the Ez values ranged between 0.34 and 0.43, but no significant differences were seen among them (Table 2). The CA3 isolate produced the highest in enzyme production. Halos appeared around the colonies producing the esterase enzyme, which are deposits of the calcium complex (Figure 3). This is due to the binding of fatty acids, released by the action of the esterase, with calcium ions.

Moreover, only 4 isolates (13.3%) of *C. glabrata* produced esterase and differed significantly, as shown in Table 3. The Ez values for the three of these isolates, namely CG8, CG15, and CG21, ranged from 0.57 to 0.64, indicating very strong activity, while CG12 had mild activity. The remaining 26 isolates (86.7%) did not produce the esterase enzyme; their Ez value was equal to 1.

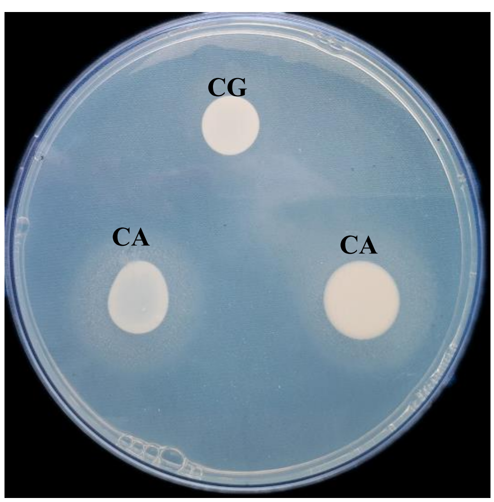

Figure 3. Esterase activity of *Candida* spp. on Tween 80 opacity medium; *C. albicans* (CA) positive; *C. glabrata* (CG) negative

3.1.3 Hemolysin Enzyme Activity

The results of this study showed that all *C. albicans* isolates produced hemolysin, as indicated by the formation of a lysis zone around the colonies. The lysis was alpha type (Figure 3), due to the appearance of a greenish-black halo around the colonies, which indicates incomplete hemolysis.

There were significant differences ($P < 0.01$) among *C. albicans* isolates in their ability to produce hemolysin, with Hemolytic Index (Hi) values ranging from 1.76 to 2.83. The CA12 isolate showed the lowest activity, while the CA6 isolate exhibited the highest level of activity, as shown in Table 2. *C. glabrata* isolates were also positive for hemolysin production. The isolates varied in their analysis indicators, which ranged from 1.63 to 3.06 with significant differences. The isolate CG29 showed the lowest efficacy, while the isolate CG1 had the highest efficacy in hemolysin production, as indicated in Table 3. Hemolysin production quantity and strength vary by species, isolation source, and fungal isolates differ in their secretion patterns [34]. Certain genetic traits may influence the efficacy of blood analysis in *Candida* isolates [37].

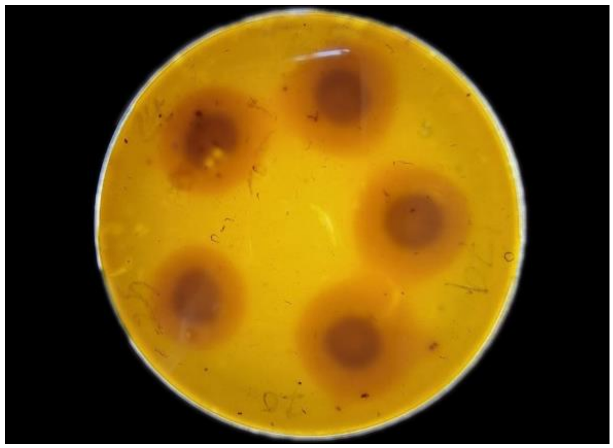

Figure 4. Positive hemolytic activity of *Candida albicans* isolates on sugar-enriched sheep blood agar

4. Conclusion

This work emphasizes how critical it is to identify Candida species in urine samples from diabetic patients and assess their capacity to secrete extracellular enzymes, which are essential for increasing *Candida* pathogenicity in diabetic patients. Therefore, further work in this approach is required with a focus on these enzymes as prospective therapeutic targets. This is a promising approach that may result in the creation of more potent therapies to lower infection rates and enhance diabetic patients' quality of life.

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6. Declarations

6.1 **Ethics approval and consent to participate**

Not applicable.

6.2 **Consent for publication**

Not applicable.

6.3 **Availability of Data and Materials**

Data will be provided upon receiving a valid request.

6.4 **Conflicts of interest**

The authors declare that there is no conflict of interest.

6.5 **Funding**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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الكشف عن عوامل الضراوة الانزيمية في *Candida albicans* و *Candida glabrata* المعزولة من مرضى السكري

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المستخلص:

يُعد داء المبيضات البولي Candiduria من اكثر انواع العدوى شيوعا لدى مرضى السكري، وقد ازداد معدل الاصابة بها بشكل ملحوظ خلال العقود الاخيرة. تُعزل انواع المبيضات .*Candida* spp بشكل متكرر من المرضى، مع تزايد ملحوظ للانواع غير البيضاء *Candida* Non- albicans بوصفها عوامل مسببة رئيسية للمرض. هدفت الدراسة الحالية الى تشخيص انواع المبيضات المعزولة من بول نساء مصابات بالسكري وتقييم قدرتها على انتاج انزيمات Phospholipase وEsterase و Hemolysin، كما اجري التحليل الاحصائي عند مستوى معنوية ($p < 0.01$). تم جمع عينات البول الوسطي Midstream من 178 امراءة مصابة بداء السكري، شملت 86 مصابة بالسكري من النوع الاول و 92 مصابة بالنوع الثاني. زرعت عينات البول على وسط Sabouraud's Dextrose Agar (SDA)، شخّصت العزلات باستخدام الطرائق التقليدية شملت الصفات الزرعية وصبغة غرام وإختبار تكوين أنبوب الأنبات وإنتاج الأبواغ الكلاميدية فضلاً عن إختبار النمو عند درجة حرارة 45مْ والتشخيص بقدرة العزلات على النمو بالوان مختلفة على الوسط التفريقي HiCrome *Candida* Differential Agar M1297A. اظهرت نتائج الدراسة تشخيص ثلاثة انواع مختلفة من المبيضات ، وكان النوع *Candida glabrata* الاكثر شيوعا بعدد 30 عزلة (50%) ثم تلاه النوع *Candida albicans* بعدد 18 عزلة (36%) ثم النوع *Candida krusei* بعدد عزلتين (4%)، كما بينت النتائج ان مريضات السكري بالنوع الاول اكثر عرضة للاصابة بعدوى المبيضات. كما اظهرت نتائج اختبارات عوامل الضراوة ان جميع عزلات (100%) *Candida albicans* كانت موجبة لانتاج انزيمات Phospholipase وEsterase و Hemolysin مع اختلاف في معدلات الانتاج، بينما عزلات النوع *Candida glabrata* فقد انتجت عزلة واحدة فقط (3.3%) انزيم Phospholipase واربع عزلات فقط (13.3%) انزيم Esterase في حين اظهرت جميع العزلات قدرة على انتاج Hemolysin.