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Isolation and Molecular Identification *of Candida Albicans* from Various Clinical Samples in Egypt

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Abstract

Candida albicans is responsible for candidiasis in Egyptian women, so a fast and accurate identification is needed for perfect prescription. A total of 15 samples, obtained from vaginal microenvironment of symptomatic women, were cultivated on a Sabouraud Dextrose Agar (SDA) and identified by API 20C AUX kit, germ tube test, chromogenic medium and molecular technique. Six strains of *Candida albicans* was isolated from the pregnant and nonpregnant women in Meniat El-Naser, Dakhelia, Egypt. Eighty percentage of patients are in the age ranged of 25-40 years and the remaining 20% are in age between 18 -25 and the highest level of infection (60%) in the elder one. Pregnant women are more infected (80%) than non-pregnant (20%). Non-educated ladies are more infected (80%) than educated one (20%). The fungal strains were identified as *Candida albicans* through growth at 45°C on CHROMagarTM *Candida* Medium and forming green colonies. Germ tube test and biochemical system using API 20C Aux were used in confirmation. The genomic DNA was extracted from the six samples and rDNA-ITS region was amplified and sequenced. A phylogenetic tree of the fungus was constructed. Both SEM and TEM Electron microscopy analyses were performed for showing *Candida albicans* structure.

Keywords: Candida albicans, Morphological, biochemical and molecular identification, SEM and TEM microscopy.

Introduction

Candida albicans caused a vaginal infection disease known as candidiasis that is a problem to a wide range of women and cause high healthcare costs all over the world. It is also isolated from healthy person and could induce candidiasis, as a result of its overgrowth in suitable environment (**Erdogan and Rao, 2015**). Although it is an opportunistic pathogen, a member of the normal gut flora, it can cause life-threatening infections in immunosuppressed patients.

C. albicans is the second, after bacteria (*Lactobacillus* spp.), in causing of human candidiasis (**Dadar et al., 2018; Lopez, 2022**). The fungus is a common African (78.3-96.1%) vaginitis causal organism (**Konate et al., 2014; Shaaban et al., 2015; Amer et al., 2015;**

Hussein et al., 2019). It is a common yeast of the human microbiota persists in mucosal surfaces and skin. It is detected in the gastrointestinal tract and mouth in 40–60% of healthy adults (**Gow and Yadav, 2017**).

Candida albicans exists various forms. Their cells (blastospores) are normally divide asexually by budding and a new bud grows from the previous new one, distally from the site of the scar caused by formed new bud, after which the phase of growth begins. Bud my occurs in short or long chains forming pseudohyphae and hyphae. After the end of growth phase, the cells divide, whereby the daughter separates from the original cell by creating a compartment (**Walker and White, 2017**).

The aim of this work is to perfectly be identifying the pathogenic organism of women vaginitis in some Egyptian symptomatic samples.

Materials and Methods

Patients and samples collection

A total of 15 pregnant and non-pregnant women with suffering from vaginitis participated in this study. Swabs from their vagina were collected at various obstetrics and gynecology clinics of Alaziz Bellah center, Meniat EL-Naser, Dakhelia, Egypt. The patient was in the bladder lithotomy position.

Isolation and identification

A combination of morphological and biochemical criteria in addition to molecular techniques were used in identification of isolated *Candida* sp.

Isolation using cotton Swab.

A saline lubricated speculum was inserted into the vagina, swabbed of vaginal secretion by a bit of dry cotton. The cotton swaps were either coated a slide from left to right evenly or putted into a clean tube with little saline solution for microscopic study. The cotton swab obtained from vaginal sample in sterilized distilled water, poured in a sterile plate followed by addition of 20ml of Sabouraud Dextrose Agar (SDA: 10 g peptone, 40 g dextrose and 15 g agar dissolved in 1 L of distil. water, sterilized at

121ºC and 1.5 bar for 15 minutes), and incubated at 37ºC for 2-3 days (**Ax´ell et al., 1985**).

Morphological Identification.

a. Microscopic Identification

The collected swaps were examined microscopically at 40x and 100x within 30 minutes for rapid identification.

b. Chromogenic test

The isolated yeast strains were grown on chromogenic medium (CHROMagarTM *Candida* medium), then they were incubated at 37 °C for 48 to 72 h (**Madhavan et al., 2011; Daef et al., 2014**). This medium incorporates substrates linked to chemical dyes to distinguish between *Candida* spp. by the developing colony color: where *C. albicans* colonies are ether in green or blue green colour.

c. Germ tube test

It is a rapid phenotypic testing to differentiate between *Candida* spp. It is an extending outgrowth from the yeast cells (**Ellis et al., 2007**) when growing at 45°C on the primary **SDA** medium for up to 10days. The germ-tube test involves inoculation of one or two colonies of target yeast to 0.5 ml horse serum which contains 0.5% of glucose in the Eppendorf tube and incubated at 37°C for 24-hrs. After that a smear taken from the growing *Candida* sp., fixed on to slides and stained (gram stain or periodic acid Schiff) to exam their outgrowth.

d. Biochemical Identification

This experiment is based on carbohydrate assimilation. It is the most suitable and current methods for *Candida* identification. Test carbohydrates are contained in plastic wells located on a test strip. Fresh cultures of the 15 isolates were made on SAD agar. A numerical system of identification was used commencing with inoculation of API20C Auxanogramme, according to the manufacturers' instructions and incubation at 37°C from 48 to 72 hrs. Where Developed colonies were picked up into suspension medium of API20C AUX Kit and agitated. Growth in each well is read by changes in turbidity or colour changes in the kit systems.

Then reads are converted into a numerical profile, which is read from a profile index that is interpreted using the manufacturer's reference manual (**De Louvois, et al., 1979; Ellepola and Morrison, 2005).** The fermented sugars by yeast isolates was determined and described by (**Van der Walt and Yarrow, 1984**). The API20C Aux kits (BioMerieux, France) consists of 19 fermentation and assimilation wells (cupules) each of them contains a single substrates for assimilation and a 0 well as a negative control for the comparison.

e. Molecular identification

Genomic DNA was extracted and purified from samples using ABT DNA mini extraction kit (Applied Biotechnology Co. Ltd, Egypt). PCR was carried out using the universal primer pair of ITS1/ITS4 (5'-TCCGTAGGTGAACCTGCGG-3'/ 5'-TCCTCCGCTTATTGATATGC-3'). PCR reactions occur in a volume of 50 µl (2x Red Master mix, 20 picomole of each primer and ca. 100 ng of genomic DNA) using a thermal cycler (MJ research, USA). PCR cycling conditions were as follows: one cycle of initial denaturation stage at 96 ℃ for 3 min, 35 amplification cycles [Denaturation at 95℃ for 30 s, annealing at 50 ℃ for 30 s, extension at 72 ℃ for 90 s], then final extension at 72 ℃ for 5 min. PCR-DNA products were visualized on 1.5% agarose gel by gel electrophoresis system and sent to Solgent Co Ltd (South korea) for gel purification and sequencing. The resulted sequences were trimmed and assembled in Geneious software ((http://www.geneious.com).

Consequently, the trimmed sequences were identified by search in basic local alignment Nucleotide Blast tool (BLAST) in GenBank. Obtained Nucleotide sequences were aligned using MAFFT alignment (**Katoh and Standley, 2013**). Phylogenetic trees were constructed using the Neighbor joining method (**Saitou and Nei, 1987**), employing the Tamura-Nei Model (**Tamura and Nei, 1993**). The trees were assessed using 1000 bootstrap replicates.

To confirm the targeted PCR amplification five ul of the PCR product was electrophoresed along with 100 bp DNA molecular weight 1 % agarose gel containing ethidium bromide (at the rate of 0.5 ug|ml) at contrast 80V for 30min in 1xTAE buffer. the amplified product was visualized as single compact band of expected size under UV light and documented by Samsung note4 smart phone

Electron microscopy

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) analyses were carried out at the Electron Microscopy Unit of Mansoura University Egypt. TEM and SEM electron microscopy analyses were performed on an overnight culture of *Candida albicans* ATCC 14053 cells. For SEM, fixed samples on nitrocellulose membrane were mounted on a stub and coated with gold-palladium, then examined using a SEM system (JEOL 200, Jaban) at 15 KV of acceleration.

For TEM, centrifuged (2500 g, 10 min) Cells washed with $0{\cdot}1$ mol l^{-1} $MgSO_4$ buffer and double fixed in 1 ml of $2{\cdot}5\%$ glutaraldehyde and 1% paraformaldehyde over night at 4°C. Pellets were resuspended in 200 µl of 2% low melting point agarose and kept at 4°C for 1 h. The agarized samples were cut in small cubes and added to 2% potassium permanganate solution and incubated for 3 h under gentle shaking at 4°C. Samples was then rinsed with distil. Water dehydrated in graded ethanol series (30–100%) and embedded in Epon–Araldite resin. Ultrathin sections were cut with a diamond knife using Ultramicrotome (Leica Ultracut UCT, Milan, Italy), double-stained with uranyl acetate and lead citrate, observed through a Philips CM 10 Joel, Japan, and TEM Microscope. Image analysis was performed using image j software.

Results

General demographic information

Table 1. illustrated that 15 married patients with symptoms of vulvovaginitis candidiasis, prior informed consent freely signed, participated in the study conducted at December 2019 to December 2021. Continuous variables were summarized using the mean as descriptive statistics as presented in Samples are randomly selected from married women (60% pregnant and 40% not pregnant). The results revealed that 40% of the symptomatic women were infected with *Candida albicans* who were diagnosed by clinical examination causal agent

of candidiasis infection. I t is also noticed that pregnant women are more infected (80%) than non-pregnant (20%) one. The majority of the tested patients (73%) are non-educated whom are more infected (80%) than educated one (20%). Eighty percentage of patients are the in the age ranged of 25-40 years and the remaining 20% are in age between 18 -25 and the highest level of infection (60%) the elder one. The final laboratory examination was confirmed that 40% of the patients were infected with *Candida albicans* (Table 1).

Table (1): General Socio-demographic information of participated women (N = 15)

Parameter		Percentage of occurrence	Percentage of infection
Age (in years)	18-25Y	20 %	40%
	25-40Y	80 %	60%
Educational Status	educated	73 %	20%
	None	27 %	80%
Marital Status	pregnant	60 %	80%
	Not pregnant	40 %	40%
Total Infection	Infected	40 %	
	Not Infected	60 %	

Identification of Candida albicans

Morphological identification

Six *Candida albicans* strains were isolated from total 15 vaginal sample swab on primary SDA medium. The cells are Spherical or oval, 3–6μm in Size, with single nucleus (Figure 1a, b). Buds are either solitary or in chains, with narrow attachment to mother cell, and thin walls. Characteristic pseudohyphae and or hyphae are pronounced (Figure 2).
Chromogenic medium (CHROMagar™ *Candida* medium), that combine substrate linked with chemical dyes to distinguish between *Candida* spp. by the color of the growing colonies. The chromatic of the colonies of *Candida* strains appear either in green or blue green colors which is characteristic to *C. albicans*. Figure 3 illustrated the result of germ-tube test. The induction of *Candida* germ tubes occurs when sub-cultured on the Chromogenic medium at 37°C for 2–4hrs. *Candida albicans* outgrowth appears in most of observed cells (nearly over 90%) in forms of blastospores, pseudohyphae, and hyphae.
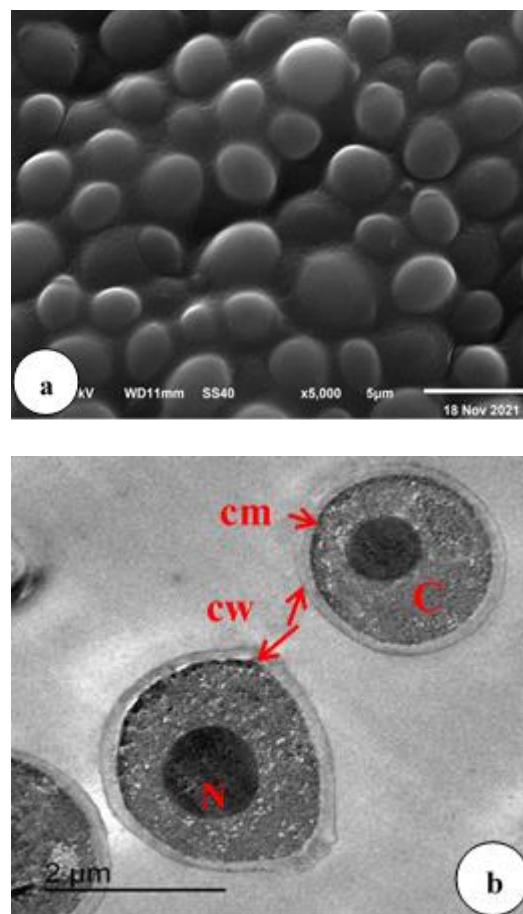

Figure 1: SEM and TEM micrographs of *Candida albicans* cells (a) and (b). a: show normal oval and round cell shapes, normal distribution of cells, cells are mostly of same size. Bar equal5μm. b: Oval and globose shaped cells, Normal intact Cell wall (cw), regular cell membrane (cm), normal intact nucleus (N). Also, cells in which the cytoplasm (C) is full of small electron-dense particles presumed to be ribosomes. Bar equal 2μm.
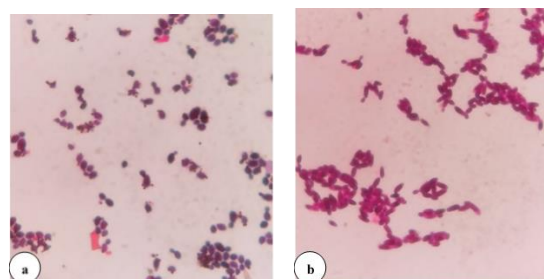

Figure 3: Germ tube test with several morphological forms. a: budding (blastospores) of *Candida albicans* cells; b: blastospores, pseudohyphae, and hyphae .mag. 10 X.

Biochemical Identification

API 20C AUX kit system was used in identification of *Candida* species. It is largely based on carbohydrate assimilation when

placed within wells or Eppendorf tube seeded yeast spp. Turbidity concentration and colour changes is an indicator for the presence of *C. albicans* (Figure 4). The degree of turbidity increases in each well produce a biochemical profile that translated into a code that is interpreted by the manufacturer's reference manual. As a result, of the 15 isolates tested, only six were identified as *C. albicans* according to API20C Aux.

Figure (4): Reaction of candida strains with carbohydrates showing degree of turbidity and colour change in a and b characteristic to *Candida albicans*.

Molecular identification:

The sequences of the six *C. albicans* strains generated in this work have been submitted to GenBank (Table 2). In order to construct a phylogenetic position of the *C. albicans*, MEGA program version 4 was used (Figure 5). The values were only considered and displayed next to the phylogenetic tree branches, with confidence levels estimated using 1000 bootstrap replicates. As a result of morphological examination and DNA sequencing consistent with the first matching identification of BLAST sequence database in NCBI using distinct sequences proved the identity of the 6 pathogenic strains of *C. albicans*. Their sequences of matched with that in the GenBank databases at 98–100% global similarity. The 6 *C. albicans* strains deposited in the GenBank nucleotide databases under accession numbers Mz674383, Mz674384, Mz674385, Mz674386, Mz674387 and Mz674388 (Table 1, Figure 5).

Table 2: Gene Bank codes of *C. albicans*

Patients	Strain Code	GenBank code
1	DAK100	*C. albicans* Mz674383
2	DAK101	*C. albicans* Mz674384
3	DAK102	*C. albicans* Mz674385
4	DAK103	*C. albicans* Mz674386
5	DAK104	*C. albicans* Mz674387
6	DAK105	*C. albicans* Mz674388

In order to reconstruct the ITS trees, multiple alignment of segment of ITS gene for several various Candida strains, ITS sequences of several Candida spp. were used as the out-group species to build their phylogenetic tree. The trees were reconstructed using neighbor joining analysis. The phylogenetic trees displayed that all strains of C. albicans were clustered with a few references in a single cluster (Figure 5).
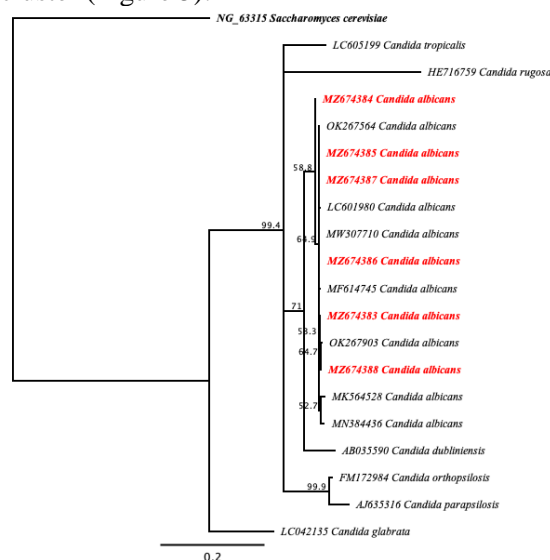

Figure 5. Phylogenetic tree of *Candida albicans* strains based on Geneious alignment of ITS nrDNA sequences and other related *Candida* species after exclusion of the non-alienable flanking regions (480 bp alignment; 20 sequences). The neighbour-joining was per-formed using the maximum composite likelihood method. The values on the nodes are confidence levels estimated by 1000 bootstrap replicates. Scale bar indicates number of substitutions per site.

Discussion

Vaginal microenvironment is important in maternal and fetal health. *Lactobacillus* spp. and various other microbes occupies 73% of microbial communities inhabiting vagina (**Stapleton, 2016**). Generally, *Candida albicans* is a commensal yeast fungus of the human body and was found to be is the second most common causal agent of vaginitis after bacterial vaginosis (**Gonçalves et al., 2016; Seyoum et al. 2022**). Our epidemiological study reveals that *C. albicans* is the causal of candiasis in Egyptian symptomatic women (40 %) especially the pregnant non-educated one. It is found that the percentage of infection in pregnant (80%) women is more than non-

pregnant one (20%), the non-educated ladies are more infected (80%) than educated one (20%) and the elder (25-40) married women are more infected (60%) than young 18-25 married one. Correspondingly, it is stated that *C. albicans* was the predominant cause of vaginal candidiasis in symptomatic women in Qena and Tanta of Egypt (**Amer et al., 2015; Hussein et al., 2019**) and in Multan of Pakistan (**Manzoor et al., 2018**). Conversely, it is found that women in 16 - 25 years of age were more infected (30%) than (24%) that in the ages of 25 - 35 years (**Amer et al., 2015**). It is also isolated from healthy, asymptomatic pregnant women in 10–55% (**Neville et al., 2015**). Furthermore, *C. albicans* hazard to non-pregnant women is approximately 20%, that increases to 30% throughout pregnancy and reached their maxima (50%) in the last trimester (**Gonçalves et al., 2016; Sangaré et al., 2018; Konadu et al., 2019; Waikhom et al., 2020).** The fungus cell wall often displays an external, fibrillary layer that consists of glycoproteins that linked covalently to an inner polysaccharidic layer (66 KDa glycoproteins) responsible for the bind with epithelial cells of the host tissue (**Gonzalez et al., 2010**), and playing a decisive role in the pathophysiology of candidiasis (**Senet 1998**). It is generally a mild in healthy individuals, but its overgrowth can lead to illnesses of the host from limited insignificant infections to systemic spread candidiasis (**Ponde et al., 2021**). Finally, it is reported that the majority (75%) of females will experience vulvovaginal candidiasis at least in a part of their lifespans, and 40–50% of initially infected one will suffer a retrogression (**Jaeger et al., 2013; Gonçalves et al., 2016; Seyoum et al. 2022**).

Macroscopic, microscopic, and molecular analysis using genomic DNA sequences were used to identify *C. albicans*. Six strains of *Candida* were isolated from vaginal samples were grown on SDA and identified as *C. albicans* on CHROMagar *Candida* medium on the bases of colony colour. SDA is the infamous primary *Candida* isolation medium (**Odds, 1991**). It is facilitating the growth of *Candida* and represses the bacterial growth due to its acidic pH. Their colonies on SDA are cream, smooth and pasty convex. The *C. albicans* cells are globose or oval, 3–6μm in Size, with single nucleus. **Klis et al. (2014)** reported that *C. albicans* cell is normally white have a globose to oval shape and about 5 – 6μm in size.

Various chromogenic media are used for quick and easy identification of yeast on colony colour bases. It includes CHROMAgar *Candida* medium (**Bouchara et al. 1996; Madhavan et al., 2011**). This medium provides advantage in reducing time of identification over traditional methods in differentiation between multiple *Candida* spp., in a single infection that is significant clinically in selecting treatment process (**Odds and Bernaerts, 1994; Marsh and Martin, 2009**). The specificity of this method for discrimination between *Candida* spp. is about 95% (**Pfaller et al., 1996**). This medium help in identifying all infected samples that turned to positive green colour that characteristic to *Candida albicans*.

Germ-tube outgrowth was also used for identifying *C. albicans*. *Candida albicans* outgrowth appears in most examined cells in forms of blastospores, pseudohyphae, and hyphae. Buds are either solitary or in chains, with narrow attachment to mother cell, and thin walls. Characteristic pseudohyphae and or hyphae are pronounced. Likely, **Williams and Lewis (2000),** stated that almost 95% of *C. albicans* isolates produce germ tubes. Where *C. albicans* strains can spontaneous changeover between different phenotypes of unicellular (yeast) and multicellular (hyphae, pseudohyphae) forms. This phenotypic exchange is attributed to change in expression of the regulatory gene. This evolutionary advantage allows the fungus to adapt greatly to environmental changes (**Santos et ai., 1999; Holland et al., 2014).** However, germ tubes production by *C. albicans* is not enough for identification, a Validation is required by biochemical reactions to establish or to confirm identity using API 20C (Aux) numerical profile. API 20C (Aux) is a valuable system, accurate, reliable, reproducible, unambiguous, perfect and more rapid in identification than the traditional methods (**De Louvois, et al., 1979; Ellepola and C. J. Morrison, 2005**). Where it can give us accurate result in two days comparable to other traditional method that need 14 days at least (**Hermansyah et al., 2017**). This test based on the capability of *C. albicans* to utilize various types of sugars (monosaccharides, disaccharides, and polysaccharides) as energy source. it has been observed that Glucose was directly utilized by *C. albicans* to perform metabolism in the cells via glycolysis and production of energy. The fungus can also hydrolyze disaccharides into its

monomers. It showed a positive reaction to maltose and sucrose and conversely on lactose due to lack of enzyme system. Ultimately, API 20C AUX is more practical in rapid identification of *C. albicans* because it requires less time without addition of any chemicals, **(Land et al., 1979).**

Evolutionary analysis of the *Candida albicans* DNA sequences from several valid and verified one deposited on GenBank together with the sequences of current study executed using MEGA software program **(Kumar et al. 2018)** were performed**.** The length of the obtained DNA fragment of the ITS region had the ideal conditions for amplification (**White et al., 1990)**. Analysis of BLASTn establish similarities of 98–100% global of the ITS sequences of *C. albicans* with previously identified on GenBank. The phylogenetic trees of the isolates reconstructed based on ITS sequences, using neighbor joining analysis, showed that all isolates belonging to the *C. albicans* are clustered with several reference in a single cluster.

Conclusions

Six strains of *Candida albicans* is responsible for 40% of symptomatic women candidiasis. Chromogenic media (CHROMagar *Candida* media), germ tube test, biochemical assimilation test (API 20C Aux) and Molecular analyses are valuable in accurate identification of the isolated strains to *Candida albicans.* Future work needed to find the best biocontrol method.

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الملخص العربي

عنوان البحث: التوزيع والتعرف الجزيئي لفطرة الكانديدا البيكنس المعزولة من عينات سريرية مختلفة في مصر

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فطرة الكانديدا البيكنس هي المسؤولة عن داء الكانديداياسس لدى النساء المصريات ، لذلك يلزم تعريف سريع ودقيق للحصول على علاج طبي مثالي. تمت زراعة ما مجموعة ١٥ عينة ، تم الحصول عليها من بيئة مكروبية مهبلية للنساء المصابات بالأعراض السابقة ، على الوسط الغذائي Sabouraud Dextrose Agar (SDA) وتم تعريفها بواسطة بروتكول API 20C AUX ، واختبار أنبوبة الإنبات، والوسط الغذائي الملون chromogenic medium والتقنية الجزيئية. تم عزل ستة سلالات من فطرة الكانديدا البيكنس من النساء الحوامل وغير الحوامل في منية النصر، محافظة الدقهلية، مصر. و قد وضح ان ٨٠% من المرضى تتراوح أعمارهم بين ٢٥-٤٠ سنة والباقي (٢٠٪) تتراوح أعمارهم بين ١٨-٢٥ وانت أعلى نسبة إصابة (٦٠٪) في الشريحة الأكبر سنا. النساء الحوامل أكثر إصابة (٨٠٪) من غير الحوامل (٢٠٪). النساء غير المتعلمات أكثر إصابة (٨٠٪) من المتعلمات (٢٠٪). و تم اثبات ان السلالات الفطرية على أنها الكانديدا البيكنس من خلال النمو عند ٤٥ درجة مئوية على CHROMagar TM Candida Medium وتكون مستعمرات خضراء عليها. كما تم استخدام اختبار أنبوب الإنبات والنظام الكيميائي الحيوي API 20C Aux في تأكيد ذلك. و تم استخلاص الحمض النووي من العينات الست وتم تكبير وتعدد منطقة rDNA-ITS وتسلسلها. كما تم بناء الشجرة الوراثية للفطر. بالإضافة الي إجراء تحليلات باستخدام المجهر الإلكتروني SEM و TEM لإظهار البيئية التركيبية لفطرة الكانديدا البيكنس.