University of Thi-Qar Journal of Science (UTJsci)
E-ISSN: 2709-0256, P-ISSN: 1991-8690, Vol. 11, No. 2, Dec. 2024

Isolation, identification and antifungal susceptibility of *Candida species* in postmenopausal women

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Received: 2024-05-02, Revised: 2024-05-25, Accepted: 2024-06-04, Published: 2024-12-05

***Abstract*— The term "*vulvovaginal candidiasis*" refers to an inflammatory, non-erosive Vulvovaginitis characterized by symptoms such as itching, pain, dyspareunia, and discharge. The condition is treated with antifungal medication if a positive culture from a low vaginal swab reveals *Candida*. It should be mentioned that these people had ongoing medical conditions. VVC had previously been classified as acute or recurring. It appears that estrogen is a key permissive factor in VVC. Clinical findings have demonstrated that VVC is uncommon in premenopausal and postmenopausal women and that the post-menopausal vagina rarely contains isolated Candida. The study aimed to know the relationship between Vulvovaginitis and candida in postmenopausal women and effectiveness of some antifungals. A total of 100 postmenopausal women With Vulvovaginitis, aged 45 to 69, had vaginal swabs taken from them. Upon arrival at the laboratory, all samples were cultured on Sabouraud dextrose agar . The species of the isolated yeasts were identified by a variety of methods, including molecular analysis with primers ITS1–ITS4, growth on Chrom agar medium, and biochemical analyses. Result: candida in postmenopausal women is less than in per menopausal due to effectiveness of estrogen hormone in reproductive age . The outcomes On Chrom agar showed, six species of candida—C. albicans, C. glabrata, C. tropicalis, C. parapsilosis, C. krusei, and c. rugosa—were isolated. Among the other species, C. glabrata was discovered to be the most prevalent. The findings demonstrated that four species of yeast-like fungi were identified using molecular diagnosis and genetic testing as follows: Three isolated C. krusei (25%), four isolated C. albicans (33%) and four isolated C. glabrata (33%), and one isolated C. tropicalis (8%). The percentage that was highest in non-C. albicans. A test for antifungal susceptibility was also done; the findings showed that, with the exception of C. parapsilosis, which showed resistance to amphotericin B, all Candida isolates were susceptible to both Nystatin and Amphotericin B. The outcomes showed that nystatin, amphotericin B, Itraconazole, and clotrimazol were the most effective treatments for getting control of candida.**

Based on the results mentioned above, it can be said that postmenopausal women are less likely than women in reproductive age to have Candida spp.

Keywords*— *CHROM Agar; Candida Spp; Vulvovaginal Candidiasis ; ITS1–ITS4

I. INTRODUCTION

A common organism found in the gastrointestinal and reproductive mucosa, *Candida* is a yeast-like fungus that may be isolated from the oral cavity. As a result, as much as eighty percent of the healthy population is found to be susceptible to common fungal diseases like candidiasis. *Candida species* can cause a wide range of infections, from mucocutaneous disorders that are not life-threatening to invasive situations that can cause serious infections or even the death of essential organs [1]. *Candida colonization* has unknown benefits, however there is information about its negative effects. Ten percent of women get recurrent instances of vaginal candidiasis, which affects seventy-five of women at least once in their lives. In about ten percent of sexually active women, asymptomatic vaginal carriage is present; during pregnancy, this percentage rises to nearly thirty percent [2]. The prevalence of broad-spectrum antibiotic use and the rise in HIV-positive and immune compromised individuals have both contributed to an increase in the incidence of *Candida* infections in recent years. Complex factors such as intrapersonal and interpersonal factors, which vary based on the healthcare system and the sociocultural setting, influence biological elements in menopausal syndrome. Before puberty, the vaginal environment is sterile; during puberty, hormonal changes cause lactobacilli to colonize the vagina [3].

Vaginal tissues become thinner and less elastic in postmenopausal women due to decreased levels of progesterone and estrogen. Up to 47% of postmenopausal women experience these vaginal atrophic alterations. Vulvovaginal atrophy is one of the many alterations that take place following menopause as a result of the aging ovaries' reduced capacity to release estrogen [4]. Atrophic vaginitis, or vulvovaginal atrophy (VVA), is one of the numerous disorders associated with the menopausal transition that may impact disease activity [5]. Even so, *Candida albicans* is still the most prevalent species., non-albicans species like *Candida glabrata, Candida tropicalis, Candida guilliermondii, Candida dubliniensis,*

https://doi.org/10.32792/utq/utjsci/v11i2.1241

Candida parapsilosis, and Candida krusei are also becoming more common as pathogens and colonizers that can lead to both systemic and superficial infections. When *Candida species* are present, vulvovaginal candidiasis (VVC), also known as *Candida* vaginitis, is a frequent clinical illness that manifests as vaginal and vulval irritation [6]. The ITS1 region gene, which is based on the ribosomal RNA gene of *Candia species*, was molecularly detected using PCR . The goals of this research were to investigate the molecular identification of the main spp. of *Candida separate* from postmenopausal women in the Thi-Qar Governorate using high-resolution melting analysis with real-time PCR, and its susceptibility to some antifungal

II. Materials and Procedures

A. Samples Collection

One hundred samples in total were collected from women suffering from vaginal infections. Patients ranged in age from 45 and 69 years old. These patients received treatment at Al-Rifai General Hospital in Thi-Qar from different areas within the Thi-Qar Health Department. Vaginal swabs were collected using sterile cotton swabs and transport media.

B. Isolation Candida spp.

Every sample was cultivated Sabouraud dextrose agar (SDA), which was prepared in compliance with the guidelines provided by the manufacturer. (SDA) to make the medium, 47 grams were suspended in One thousand milliliters of purified water and allowed to soak for fifteen minutes. To prevent bacterial contamination, 250 milligrams of chloramphenicol were added per Liter **.** After autoclaving for 15 minutes at 121 ^{o} C, cool the mixture to 50 o C, and thoroughly mix it before putting it into the plates [7]. After five days of incubation at 37˚C, the culture was checked for pasty, creamy, and smooth white colonies on each plate. Following cultivation and incubation on SDA the colony morphology was analyzed and restored. The morphological traits of the colonies, including their size, color, and shape as well as the formation of the germ tube, were examined.

C. chrom Agar Candida

Selective components and chromogenic substrates are combined to create chromogenic media, which, when incubated with the chosen yeast, provide a distinctive color. The manufacturer's instructions were followed to make this medium, which called for mixing 47 grams of powdered medium with one hundred milliliters of purified water, heating it in a water bath until it boils, placing it in Petri plates, and maintaining it till usage, keep it at 4oC [8] **.** On chromogenic agar media, Every sample that was recognized as having *Candida* based on the color and colony was grown. When a loop rich in cultural references from Sabouraud Dextrose-Agar was streaked into CHROM agar media and incubated for 72 hours at 37°C, the Initially, *Candida colonies* were discovered. by their colonial color in contrast to the manufacturer's typical color pictures. They also became visible after the 72-hour incubation period [9].

D. KB006 HiCandidaTM Identification Kit

A standardized test system called KB006 can be used to distinguish and identify different species of *Candida*. A standardized colorimetric identification system using twelve common biochemical assays is included with every KB006 Kit. The test's foundation is the idea of PH shifts, which are represented by an a spontaneous change in the media's color [10]. Select two to four well-isolated colonies and create a homogeneous suspension in two to three milliliters of sterile saline to prepare the inoculum. At 620 nm, the suspension's density needs to be adjusted to 0.5 OD. Next, using the surface inoculation method, inoculate each well with 50 milliliters of the above inoculum. For 24 hours, incubate the colony at 22.5°C. to 48 hours. Interpret the outcomes using the guidelines provided in the identification index.

E. Molecular Detection

The universal primers target for the ribosomal RNA (ITS1) region gene were used to build the PCR primers for the identification of *Candida* species. (Fujita *et al*., 2001) **.** The extracted amount DNA was examined using the Thermo Scientific UV Visible Spectrophotometer, Nano Drop Lite (USA) to determine the content of DNA (ng/µL) and Analyze the RNA's purity at wavelength (260/280 nm). After opening the Nano Drop application, select the relevant application (DNA, nucleicacid). The pedestals for measurements were repeatedly cleaned with a dry handkerchief. Subsequently, 2µl of free nuclease water was carefully pipetted and deposited onto the lower measurement pedestals' surface to blank the device . A 1µl DNA sample was measured after the Nano drop sampling arm was lowered. The conditions for the PCR were as follows: following a 5-minute pre-incubation at 95˚C, amplification was conducted for 35 cycles total, comprising the first denaturation at 95˚C, subsequent denaturation at 95˚C for 5min, Heating to 55˚C for 30s, extension at 72˚C for 1min, and a final 5-minute phase of extension at 72˚C. gel electrophoresis using 1.5% Agarose gel to confirm the amplified PCR.

E. Antifungal Susceptibility Test for Candida spp.

The selection of antifungal discs was carried out in compliance with the Clinical and Laboratory Standard (CLSI, 2019). Five milliliters of sterile normal saline were used to suspend Six completely separated fresh-culture Candida colonies. Afterwards, Muller Hinton Agar plates were contaminated by touching the inoculum with a sterile swab. and applying it over the medium's surface more than once while rotating the plate at a 60˚ angle to finally guarantee diffusion following each use. Additionally, the swab was forced against the edge. Following three days of

incubation at 37˚C, the sizes of the inhibitory zone were measured in millimeters and viewed on the plates [11] .

F. Statistical Analysis

Version 25 of the statistical software for social sciences (SPSS) was used for all statistical analyses. To present the data, the frequency and percentage were utilized. The normally distributed data for all the chi-square test was utilized to assess the qualitative factors., with a less than 0.05 for the p-value being deemed significant.

III. Result and Discussion

The present study involved 100 patients with vaginal infections whose age ranged from 45 to 69 years, Out of the 100 people who had their fungal illnesses diagnosed; only 25 had *Candida* infections as in (Fig.1). The study also noted a statistically significant difference in the prevalence of *Candida* in postmenopausal women at p. value (0.05)

According to this result, [12]. As ovarian estrogen production decreases and eventually stops in menopausal women, the vulva and vagina see profound changes in

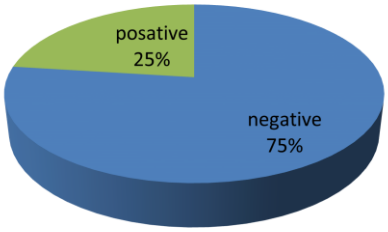

Fig. (1): Prevalence of *Candida* isolates in postmenopausal with vaginal infection.

appearance and structure. The vaginal epithelium thins and the vestibular glands secrete less fluid. The lower rate of vaginal colonization by *Candida* organisms in the post-menopausal vagina is most likely caused by the decline in estrogen receptors .That being said, these women may experience atrophic vaginitis more frequently. These concur with [13] which reported 29.1% infection with *Candida spp*. It would appear that inflammatory Vulvovaginitis is extremely unlikely to be caused by Candidiasis in postmenopausal patients who do not take hormone replacement therapy. These result agree with [14] VVC occurs at lower vaginal pH conditions (< 4.5) that are common in premenopausal women[15] .For this reason, VVC is expected to be less prevalent among postmenopausal vaginal conditions, with reduced glycogen and higher vaginal pH > 5.0 [16]. Due to colonization by anaerobic bacteria and fewer lactobacilli, conditions frequently observed in postmenopausal women. The low incidence of candidiasis in my study following menopause highlights the condition's hormonal reliance. this agree with [17].

After a 5-day incubation time at 37°C and sub culturing on Chrom agar media, *Candida* species were identified using the Chrom agar technique. Based on color changes and morphological diagnoses, the results indicated that there are about six species (Fig:2). The most common species found in vaginal samples were *C. glabrata*, *C. krusei*, *C. albicans*, *C. parapsilosis*, *C . tropicalis , and C. rugosa*, according to the frequency of isolation of these samples.

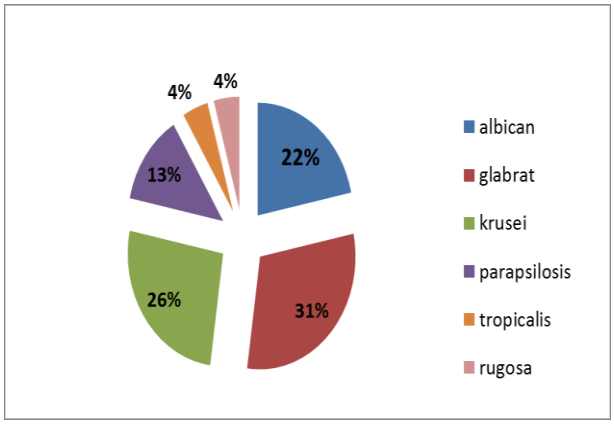

FiG. (2): Chrome identification of *Candida species* isolated in vaginal swabs from postmenopausal women with Vulvovaginitis.

Table (3): Check the isolation of Candida spp. using KB006 Hi Candida TM Identification Kit Chrom agar and molecular diagnosis

Diagnosis by CHROM agar	Diagnosis by KB006 Hi *Candida*	Diagnosis by (PCR)
C.rugosa	*Pintolopesii*	*C.krusei*
C. albicans	*Albicans*	*C. albicans*
C. parapsilosis	*Lambica*	*C.krusei*
C.trubicalis	*Tropicalis*	*Tropicalis*
C. glabrata	*Zeylanoides*	*C. glabrata*
C.krusei	*Pintolopesii*	*C.krusei*

The majority of *Candida species* may be isolated and distinguished using the simple, quick, and accurate Chrom agar medium method [18]. Using information from the chromogen diagnostic medium, which allows colonies to produce different colors depending on the type of *Candida*, the growth of *Candida* on chrome agar medium was examined. Type *C. albicans* showed up in a shade of pale green that sets it apart from the other species. Contrarily, It looks purple, *C. krusei*. due to the release of its own enzymes, which interact with the basic substance that is impacted by these enzymes to produce a purple appearance. According to [19] *C. glabrata* (39%), *C. albicans* (26%) and *C. tropicalis* (17%)and [20] *C. glabrata* was isolated in 68 (61.3%) and *C. albicans* in 32 (28.8%) of 111 subjects. the percentage of non-*C. albicans* isolates in this investigation was generally higher than the percentage of *C. albicans* isolates. findings

indicate that most often isolated species in diabetic women with VVC is *C. glabrata*. There is disagreement over the cause of the comparatively increased prevalence of *C. glabrata* infection in patients with diabetes mellitus [10]. The current study's findings showed that molecular diagnostic, KB006 Hi *Candida* TM Identification Kit, and Chrome techniques different from one to the other. When they were utilized to diagnose *Candida Spp* that was collected from vaginal swabs Table (3) shows all *Candida species* identified by the Chrom agar method do not match those identified by the KB006 method. Hi *Candida* method and PCR except for C.*albicans and C. tropicalis* were found in the three techniques as in Table: (3). *Candida* Species isolated by using this kit was *C. albicans, , C. Zeylanoides, C. tropicalis, C. Lambica, C. Pintolopesii* .

From Vulvovaginitis signs in vaginal swabs which were tested using two primers ITS1 and ITS4. The results found DNA of all *Candida* Spp. which were extracted, and the target region (ITS) of the DNA was amplified by using the universal primers, ITS1 and ITS4. The gel photo visualized six bands in the same molecular size between 500-900 bp.

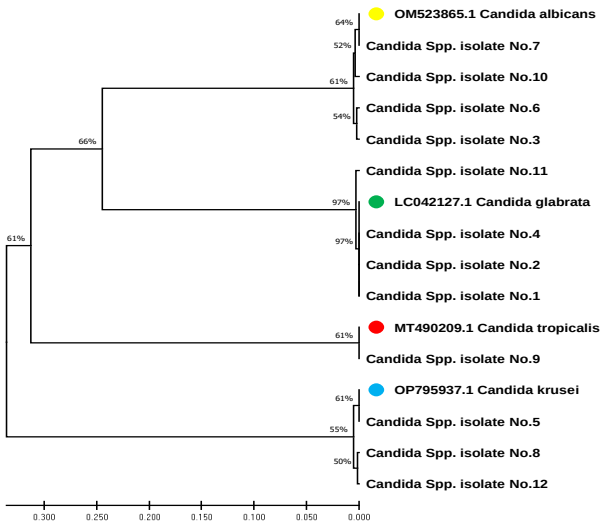

Fig. (3): Internal Transcribed Spacer Ribosomal RNA Gene Partial Sequence From Local Isolates Of *Candida Spp*.

It Was Utilized For Phylogenetic Tree Analysis To Determine Genetic Relationships. The Evolutionary Distances Were Calculated Using The Maximum Composite Likelihood Technique in (MEGA 6.0 Version), And The Phylogenetic Tree Was Built Using The Un Weighted Pair Group Method With Arithmetic Mean (UPGMA Tree Method). Closed Relationships Between The Local Isolates Of *Candida Spp*. No.1, No.2, No.4, And No. 11 and NCBI-BLAST *Candida glabrata* Were Found. The Isolates Of Local *Candida Species* No. 3, No. 6, No. 7, And No. 10 were shown to be closely associated with NCBI-BLAST *Candida Albicans*. The Isolates Of Local *Candida Species* No. 5, No. 8, And No. 12 Were Shown To Be Closedly Associated With NCBI-BLAST: *Candida Krusei*. The Isolate Of Local *Candida Spp*. No. 9 Was Shown To Be Closely Associated With NCBI-BLAST *Candida Tropicalis*. Overall Genomic Alterations (0.0300-0.050%).

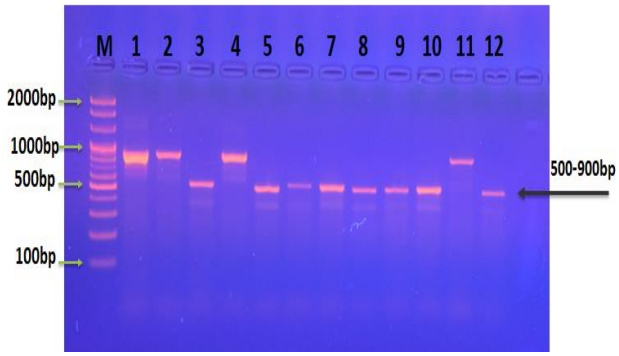

Fig. (4): Agarose gel electrophoresis1.5 % of PCR products for internal transcribed spacer ITS1-ITS2 regions (including 5.8S r DNA gene): Lane M:2000-100 bp . was showed positive *Candida species* ,Lane1:*C.glabrata* , Lane 2: *C. glabrata* lane3: *c. albicans* ,lane ,3:c.glabrata, lane 5:*c.krusei*, lane 6:*c:albicans*, lane 7:*c albicans* ,lane 8:*c.krusei*, lane 9:*c.tropicalis* ,lane 10:*c.albicans*, lane 11:*c.glabrata* lane 12:*c.krusei*. isolates at (500-900bp) PCR product.

Using ITS1 and ITS4 primer pairs, the sequencing findings showed that all isolated species were, in fact, *C. albicans*, with a similarity percentage of 99.64%. C. glabrata that is comparable 99.76% ,C. *tropicalis* 99.12%,*C. krusei* 99.60%, .*Candida* was identified differently depending on the ITS sequence than depending on morphological features (like Chrom agar). This suggests that since the ITS region sequence is non-coding and shared among fungal species' nucleotide sequences [21]), identification utilizing it was more accurate. Since most research has shown that sequencing the ITS regions is a reliable technique for identifying pathogenic yeast [22], all isolated yeast species in this investigation were identified and genotyped using ITS primers (ITS1 and ITS4).

The findings indicated that *C. albicans* constituted the majority percentage. *and c. glabrata of* 33% followed by *C. krusei* , *C. tropicalis*, .These agree with [23] According to the research, the most often isolated species were *C. glabrata and C. albicans*, with percentages of 40% and 30%, respectively,and disagree with [24]. According to his research, *C. albicans* predominates over other species. Six *Candida spp*. were isolated from vaginal swab samples, and antifungals were tested against them. The results of this study showed resistance of all types of *Candida* to fluconazole which agree with [25]. and disagree with [26] that showed sensitivity *of C. albicans, C. tropicalis, C. parapsilosis* to fluconazole . all types of candida are sensitive to Amphotericin-B (AP) except *C. parapsilosis* these results agree with [27] all type of *Candida* are sensitive to nystatin this result agrees with Mohamadi *et al.*, 2015 and disagre with[28] that show all type resistant to nystatin except the *C . tropicalis*. The following species *C. krusei, , C. tropicalis, C. glabrata* have been shown to be resistant to ketoconazole the results agree with [29] disagree with Bittencour *et al.* 2020 except, *C. parapsilosis*, which was sensitivity for ketoconazole All antifungals showed statistically significant effects ($P \leq 0.05$).

IV. CONCLUSION

Candida spp is, less common in postmenopausal women than women in reproductive age due to a decrease in estrogen hormone in these women Important. instruments for identifying and characterizing *Candida species* and determining the evolutionary relationships between isolates are molecular assay and sequencing . Chrom agar is a rabid and simple test for diagnosis of *candida* increased prevalence of *C. glabrata* infection in this age may be due to diabetes mellitus disease in this grope . The best antifungal for *candida* infection is nystatin.

ACKNOWLEDGMENT

We would like to thank the Al Rifai and Nasiriyah city's laboratories personnel for their help in gathering the samples and organizing the data.

CONFLICT OF INTEREST

The authors declare no conflicts of interest

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