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Conventional and Molecular Identification of *Candida* spp. And Antifungals Susceptibility Test in Pregnant Women

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Abstract*:** ***Candida **species are medically significant because they are the most frequent opportunistic mycosis in the worldwide. Because of hormonal and biochemical fluctuations, Fungal infections in the vagina are tenfold during pregnancy. Increased antibiotic usage and the presence of high levels of reproductive hormones during pregnancy both promote *Candida* species colonization. The study aimed to shed light on the relationship between Urinary tract infection UTI and candidiasis and determines the genetic patterns and their prevalence among women. There are 90 samples of urine and vaginal swabs collected from pregnant women with UTI at ages ranging from (15-40) years. All samples cultured on SDA, MSA and MEA. Several tests were used to identify the types of isolated yeasts, including growth on Chromogenic agar medium, as well as biochemical and molecular tests using primers $ITS_{1,4}$. The Results found that a nine species of *Candida* were isolated on Chromogenic: *C. albicans, C. glabrata, C. tropicalis, C. dubliniensis, C. parapsilosis, C. krusei, C. kefyr, C. membranifaciens* and *C. utilis*, Also, showed the number of yeasts that were genetically diagnosed by molecular diagnosis was eight species of yeasts as follows 3 isolates *C. albicans* from 33% and one isolate for each of the following types *C. orthopsilosis, C. glabrata, C. tropicalis, C. dubliniensis* and *C. parapsilosis* with 11% of all isolates where *C. albicans* showed the highest percentage. The results of the statistical analysis of the isolates showed that the most affected age group of pregnant women was (26-30), (21-25 year) in the rate of 33%, 30% respectively. Antifungal susceptibility was studied, as the results indicated that all *Candida* isolates were sensitive to Miconazole and itraconazole, except for *Candida albicans*, which was resistant to itraconazole. Furthermore, most types of *Candida* Were resistant to Nystatin and Amphotericin B, except for three type's *C. krusei, C. tropicalis*, and *C. utilis.* The most common pathogen in Urinary tract system is *Candida* spp. in pregnant women, the results indicated that the best treatment to eliminate *Candida* Miconazole, Itraconazol, Clotrimazol, Co-Trimoxazol, Ketoconazole, Fluconazole, Amphotricine B , and Nystatin respectively.**

Keywords: Urinary tract infection; Pregnancy; Candida spp.; PCR, ITS-rDNA; Antifungal Susceptibility.

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i. INTRODUCTION

Candida species are normally present in the vulvovaginal flora can cause opportunistic infections when the host's immunity is weakened. Because *Candida* spp. and the vaginal microbiota have a symbiotic relationship, asymptomatic colonization is common and can persist for years. *Candida* colonization in the vaginal tract can range from 20% in asymptomatic young women to 30% in pregnant women (Ghaddar *et al*., 2020) Presence of *Candida* species in urine (candiduria) are a common clinical finding that can indicate colonization or contamination of specimens, but they can also be etiological agents in urinary tract infections (UTIs), or signs of underlying genitourinary system disease or widespread candidaemia (Gajdács *et al*., 2019).

Candida species may be divided into two groups: *albicans* and non-*albicans*, both of which can cause infection, even in patients with somatic and immune-compromised conditions (Diekema *et al*., 2002). Among the *Candida* yeast species, *Candida albicans* is the most common human pathogen. It is one of the yeasts that may coexist in healthy individuals and is found in most environments, along with other *Candida* species such as *C. krusei*, *C. glabrata*, *C. paraphsilosis, C. dubliniensis*, and *C. tropicalis* (Kuhn *et al*., 2002) This yeast is also recognized for its ability to form a germ tube, the mechanical strength of which is attributed to the resistance immune cells or phagocytes in a host. In addition to secreting enzymes that destroy the host's cells and tissues, allowing the yeast to spread throughout the body host or extend between pus or chronic granulomas, or cause local infections such as inflammation of the skin, scalp, nails, vagina, vulvitis, and oral mucous membrane (Wood, 2001), Vulvovaginal candidiasis is the second most frequent infection of the female genital tract (Shatursky *et al*., 2014) advanced molecular methods, particularly PCR and sequencing analysis, are increasingly being used. The most common targets of PCR amplification are rDNA genes, which included typing of fungi's ITS1 and ITS2 regions, which were useful for quickly identifying clinically significant fungi (Turenne *et al*., 1999). Antifungal agents are a major part of the treatment for UTI, they are limited but an increasing number of antibiotics can be used to treat mycotic infections (Alsaggaf *et al*., 2016; Shatursky *et al*., 2014). The current study aimed to; molecular identification of *Candida* isolates by use Real-time PCR high-resolution melting analysis and investigation of the genetic variation of *Candida* species in the urinary system from pregnant women of attending for maternity hospitals in Thi –Qar Governorate.

ii. Patients and Methods

Sample Collection

Ninety samples were collected from pregnant of different ages and period of pregnancy and (30) samples from non-pregnant women that attending the Al Haboubi Hospital, Bint Al Huda Hospital and clinics for pregnant women in Thi Qar Governorate between December 2021 and March 2022. Urine samples were collected using a sterile urine container and vaginal were collected using a sterile cotton swabs with transport media**.** The urine and vaginal swab samples were transported to the Microbiology Laboratory, and cultured within the 2h of the collection.

Isolation *Candida* spp.

All samples were cultured on three mediums: sabouraud dextrose agar **(SDA),** it which was made according to the manufacture instructions, and mannitol salt agar **(MSA)** the medium was made by dissolving 111.02 g of Mannitol salt agar in 1 liter of D.W. and malt extract agar **(MEA)** The medium was made by suspend 50.0 grams in 1000 ml distilled water and soak for 15 minutes, and added chloramphenicol (250mg/ liter) to prevent bacterial contamination. Then-sterilize by autoclaving at 121 for 15 minutes, cooling until 50^{o}C and then mixed well before pouring into the plates (Aljaza, 2020). All plates were cultured incubated at 37˚C for 5 days , then the culture has been examined for pasty, creamy , and smooth white colonies, The colony morphology was examined and rehabilitated after cultivation and incubation on SDA, MEA , MSA.

Identification

Macroscopically and Microscopically Identification *Candida* spp.

The colonies were studied for their morphological characteristics such as size, color, and morphology of the colonies and production of the germ tube. This test was used to determine if whether yeast has germinated, using an a Pasteur pipette, 0.5 mL of serum was put to a tiny micro centrifuge tube and a colony of yeast was carried via a sterile wire loop and emulsified in the serum. After incubation at 37°C for 2-3 hours but no longer after mixing, a drop of the serum was placed on a slide for observation under a microscope with a 40X objective to see the Candida germ tube. Germ tubes are half the width and three to four times the length of the yeast cell they come from. Between the yeast cell and the germination tube, there was no shrinkage (Bhavan *et al*., 2010)

CHROM Agar *Candida*

Chromogenic media contain a combination of selective ingredients and chromogenic substrates that when incubated with the target yeast, produce a distinct color. This medium was made according to the manufacturer's instructions, which included dissolving 47 g of powdered medium in 100 ml distilled water, letting it boil in a water bath, putting it into Petri dishes, and keeping it at 4^{o}C until use (Khan *et al*., 2004).

All samples that diagnosis with *Candida* based on morphology and color of colonies were cultured on the chromogenic agar media. Where *Candida* was reactivated by streaking a loop full of culture from Sabouraud Dextrose Agar or Mannitol Salt Agar into CHROM agar media and incubating at 37°C for 72 hours, The *Candida* colonies were first recognized by colonial color when compared to standard color images provided by the manufacturer and also exhibited after 72 hours of incubation (Williams & Lewis, 2000).

KB006 HiCandida™ Identification Kit

Prepare the inoculum by picking 2-4 well isolated colonies and making a homogenous suspension in 2-3 ml sterile saline. The density of the suspension should be adjusted to 0.5 OD at 620 nm. Then inoculated, each well with 50 Ml of the above inoculum by surface inoculation method, incubated, the inoculum at 22.5°C for 24-48 hours interpret the results as per the standards given in the identification index.

Molecular Identification

Extraction of Yeast DNA

The kits has been used in this research by Geneaid Company.

The extraction was made according to the manufacturer's steps

Primers Preparation

The oligonucleotide of primers that were dissolved in the deionized water that to give to a final concentration of (100 picomols/µl) (as a stock solution) to that prepare 10 the picomols/µl concentrations and as work is primer, 10 µl the of stock that solution was is resuspended of in 90 µl of deionized to water and reach a final the volume at 100 µl

The PCR was performed in a total volume of 20 µL in each tube containing 5 µL master mix (BioNeer):Taq DNA polymerase, dNTPs, MgCl2, and reaction buffer, 5 µL of the template DNA, 1 µL of each primer (10 pmol final concentration of each primer), and 8 µL distilled water. The PCR condition was as follows after pre-incubation at 94˚C for 5 min, amplification for a total of 32 cycles was carried out that included initial denaturation at 94˚C, denaturation at 94˚C for 45s, annealing at 55˚C for 45s, extension at 72˚C for 60s, and a final extension step of 10 min at 72˚C. The amplified PCR amplicons were confirmed through gel electrophoresis using 0.8% agarose gel.

Antibiotics Susceptibility Test for *candida* spp.

Antibiotic discs were selected according to guidelines recommended by the Clinical and Laboratory Standard (CLSI, 2019). Nine pure isolated colonies from fresh culture *Candida* were suspended in 5 ml of sterile normal saline. Then inoculated mannitol salt agar plates by dipping a sterile swab into the inoculum, the swab stretched several times over the surface of the medium with the plate being rotated at an angle of 60˚ to finally ensure diffusion after each application. The swab was

also pressed around the edge. The plates were incubated for 5 days at 37˚C, after incubation, the diameters of the inhibition zone were observed and measured in millimeters.

C. Statistical Aanalysis

The statistical analysis of this case-control study performed with the statistical package for social sciences (SPSS) 25 program. It was determined (significant) $P \leq 0.05$ or (non-significant) $P \geq 0.05$, One and Two – way ANOVA were used determine the relationship.

II. Results and Discussion

This study showed the effect of pregnancy on infection with *Candida*, where the infection rate in pregnant women was great compared to non-pregnant women, These results agree with (Altayyar *et al*., 2016) and agree with (Ghaddar *et al*., 2019). The study also showed the percentage of *Candida* isolation affected by the type of samples, the isolation from vaginal swabs was more than isolations from urine, which agrees with (Sahal & Bilkay, 2018). That found that most of *Candida* isolates were isolated the vaginal swabs, figure 1, table 1, The data of the age distribution in the study showed that the most affected age groups with *Candida* were (26-30) with 33%, The increase in estrogen during pregnancy provides a suitable environment for the growth of *Candida*, However, there is no age group is that is completely free of infection This is in agreement with (Okonkwo & Umeanaeto, 2020) , (Nnadi *et al*., 2017) that found a high prevalence of *Candida* infection occurs in the age group (26-30) and disagree with (Manzoor *et al*., 2018) that found age group (30-35) the group most susceptible to infection with *Candida* Table 2.

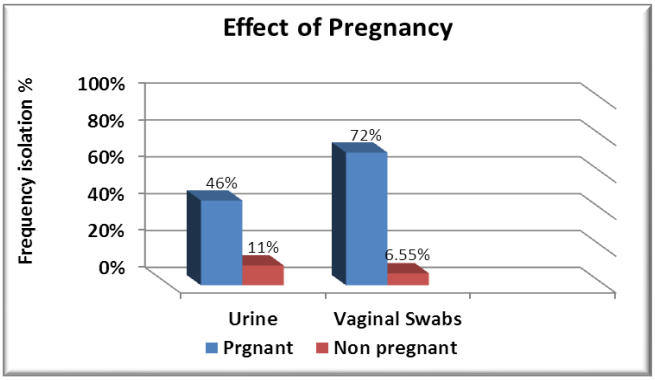

Figure 1: Effective of pregnancy on percentage% of Candida isolates in urine and vaginal swabs from UTI patients.

Table 1: Statically analysis of the type of pregnancy and sample on *Candida* isolates

Samples	Case		Mean	LSD	df	F
	Pregnant	Non-pregnant				
Urine	72.00 ± 1.50	11.00 ± 0.30	41.50	1.63	3	3471.59
Swabs	46.00 ± 0.50	6.73 ± 0.84	26.36			
Mean	59.00	8.86				

Sig = 0.000

Table2: Effect of age on Frequency of Candida spp

Age (Year)	No.of isolation	LSD	df	F	*P* Value
	Mean ± S.D.				
16-20	15.00 ± 1.04	2.27	4	228.24	0.000
21-25	30.06 ± 1.16				
26-30	33.00 ± 1.80				
31-35	10.00 ± 1.32				
36-40	11.00 ± 0.62				

After culturing samples on Chromogenic agar *Candida* which is selective media for *Candida* spp , and according to the color of colonies identifying the species of *Candida*, *C. albicans* green color, *Candida glabrata* cream to white, *C. parapsilosis* Cream to pale pink colonies, *C. krusei* Purple color, *C. dubliensis* pale green, *C. tropicalis* Blue with purple, *C. utilis* Pale pink to pinkish purple, *C. kefyr* Cream to white with slight purple center, A chromogenic agar medium is used for the differentiation of *Candida* spp. The resulting color is based on the interaction between the isolates and the medium (Musa *et al*., 2020) In general, this study's percentage of *Candida albicans* isolates in this study was more than the other non-*C. albicans* this agree with (Ahmad & Khan, 2009) and disagree with (Sahal & Bilkay, 2018) table 3, Figure-2.

Table 3: Statistical analysis of chrome agar test using Two ways Anova.

Candida spp.	Samples		Mean	*P* Value	F
	Urine	Swab			
C. albicans	**15.00 ± 1.00**	**16.00 ± 1.32**	**15.50**	**0.000**	**17**
C. dubliensis	**0.00 ± 0.00**	**1.00 ± 0.00**	**0.50**		
C. glabrata	**3.00 ± 0.50**	**3.00 ± 0.00**	**3.00**		
C. kefyr	**0.00 ± 0.00**	**4.00 ± 0.50**	**2.00**		
C. krusei	**18.00 ± 1.00**	**15.00 ± 0.50**	**16.50**		
C. membranifaciens	**1.00 ± 0.00**	**0.00 ± 0.00**	**0.50**		
C. parasilosis	**7.00 ± 2.00**	**8.00 ± 0.00**	**7.50**		
C. tropicalis	**8.00 ± 0.00**	**6.00 ± 1.00**	**7.00**		
C. utilis	**10.00 ± 1.00**	**8.33 ± 0.75**	**9.16**		
Mean	**6.88**	**6.81**			

L.S.D. = 1.29 df = 17

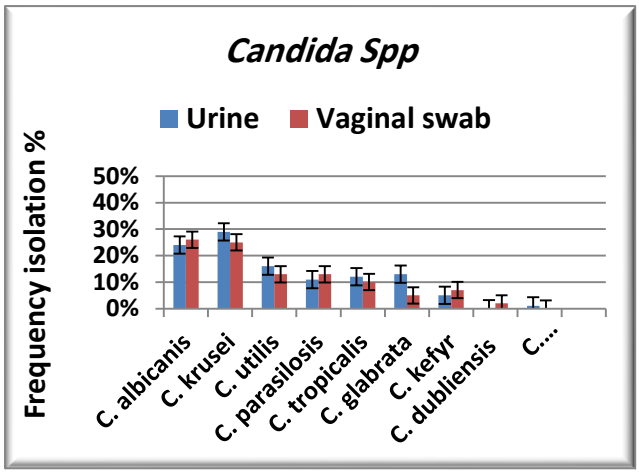

Figure 2: Variable percentage of *Candida* spp. isolated from urine and vaginal swabs using the chrome agar technique incubated at 37°C for 5 days. Bars indicated Stander error.

The results of current study documented that there is no similarity between chrome and KB006 Hi*Candida*™ Identification Kit techniques when are used for diagnosis of *Candida* Spp isolated from urine and vaginal swabs. All *Candida* spp diagnosed by chrome agar technique not corresponding with the same species when diagnosed using KB006 Hi Candida technique except *C. albicans* was found in the both techniques.

The sequencing results found that all isolated species using ITS_1 and ITS_4 primer pairs were confirmed to be *C. albicans* with a similarity percentage 97-99%, *C. glabrata* with a similarity of 97%, *C. tropicalis* 95%, *C. dubliniensis* with 90%, *C. orthopsilosis* 98% and *C. parapsilosis* 99% based on the NCB1 database The identification of yeast based on ITS sequence differed from the identification based on morphological characteristics (such as CHROMagar). This implies that identification using the ITS region sequence was more reliable because non-coding and is a conserved nucleotide sequence among fungal species (Nagla *et al*., 2019) Most studies found that sequencing the ITS regions is an accurate method for determining pathogenic yeast (Farahyar *et al*., 2020), Therefore, in this study, all yeast species isolated were identified using ITS primers (ITS1 and ITS4).

The results showed that the predominant percentage was *Candida albicans* prevalence of 33% followed by *C. glabrata*, C. *tropicalis, C. dubliniensis*, C. *orthopsilosis and C. parapsilosis* prevalence of 11%. This agrees with (Oufi *et al*., 2022) in his study, it was found that *Candida albicans* are dominant over other species. Figure 3

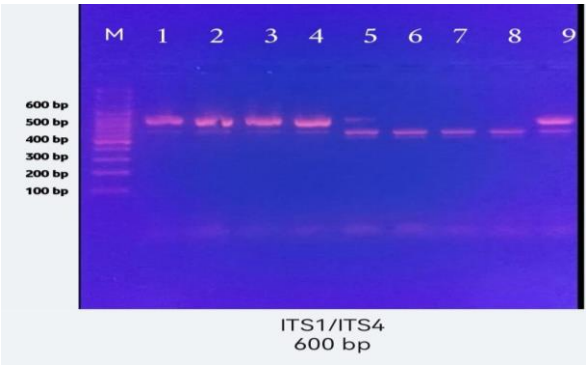

Figure 3: DNA bands of fifty strains isolated from urine and vaginal swabs in pregnant women with UTI patients using Gel electrophoresis for PCR product for ITS gene (600 bp) on 0.8% agarose gel using UV light after staining with ethidium bromide (80-120 V(1 volt/cm^2) / 30 minute. (M: 100 bp DNA Ladder; 1-9: Fungal spp).

Eight antifungals were tested against 9 *Candida* spp. isolated from urine and vaginal swabs samples. The results appearance that whole *Candida* Spp. was sensitive to Miconazole agrees with (Alikhani *et al*., 2022) and (Lomeli-Martinez *et al*., 2022) while all species except *C. tropical* , and *C. dubliensis* are resistant to Nystatin, this agrees with (Arastehfar *et al*., 2019) and disagree with (Mohamadi *et al*., 2015). Statistically, all antifungals were significantly affected ($P \leq 0.05$) the frequency of whole *candida* spp , except three species (*C. utilis, C. parasilosis* , and *C. albicans*) were unaffected ($P \geq 0.05$) by antifungal Table 4.

Table4: Summarise effective of different antifungals required for inhibiting fungal growth by nine *Candida* spp on Mannitol salt agar medium at 37°C.

Candida spp.	Resistance/ Sensitivity (mm)							
	Fluconazole	Itraconazol	Amphotericin	Nystatin	Clotrimazol	Miconazole	Ketoconazole	Co-Trimoxazol
Candida krusei	R	+++	++	R	R	+	R	+
Candida albicans	+	R	R	R	+	+	R	R
C. tropicalis	+	+++	R	+++	R	++	R	+
C. glabrata	R	+++	R	R	++	+	R	R
C. kefyr	R	+	++	R	+	+	+	+
C. membranifaciens	R	+	R	R	+	+	+	R
C. dubliensis	+	+++	R	+	+++	+	+	+
C. parapsilosis	+	+++	R	R	++	++	+++	+
C. utilis	+	+++	++	R	++	+	++	+

Key: R = Resistance; +++ = Sensitive (10 mm); ++ = (5 mm); + = (2 mm)

iii. Conclusion

Candida spp. Were one of main causes in increasing the incidence of urinary tract infection in pregnant women, and molecular assay and sequencing have been important tools in the identification and profiling of Candida species and the evolutionary relationship between isolates.

iv. Acknowledgment

We would present a special thanks to members of laboratories in Nasiriyah city's laboratories for helping in collection the samples and organize of data.

ETHICAL CONSIDERATION

To conduct the ethical research, permission was obtained from the hospital, and all participants in this work, pregnant and non-pregnant women, The patient selection was an accomplishment

with assistance of gynecologists in the hospital.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest

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