RESEARCH ARTICLE

Identification of *Candida* Species Using Phenotypic and Molecular Methods Isolated from Women Infected with Vulvovaginal Candidiasis in Maysan, Iraq

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ABSTRACT

Vulvovaginal candidiasis (VVC) caused by *Candida* spp. is a serious health that primarily affects women of childbearing age, accounting for 15–25 % of vaginitis patients. There have been few studies on the VVC in Maysan province/Iraq. Thus, the aim of this study was the phylogenetic and molecular identification of *Candida* spp. Isolated from women with VVC in Maysan/Iraq. A total of 100 samples were taken from women of reproductive age attending the Maysan Maternity Hospital. The cultures were positive for 45 (45%) and six *Candida* species, including; *Candida albicans* (44.4%), *Candida glabrata* (17.8%), *Candida dubliniensis* (15.6%), *Candida parapsilosis* (11.1), *Candida krusei* (6.7%) and *Candida Kefyr* (4.4%). The colors of colonies of tested *Candida* on CHROMagar *Candida* confirmed our isolates. *C. albicans* and *Candida dubliniensis* showed capability to form chlamydospores and germ tubes and grow in the presence of cycloheximide. *C. dubliniensis* and *C. parapsilosis* showed no growth at 45°C. The majority of tested *Candida* species revealed 9%-100% identity with many reference strains in GenBank.

Keywords: Candida species, Candida albicans, Vulvovaginal candidiasis, CHROMaga, Polymerase chain reaction (PCR), Phylogenetic tree.

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INTRODUCTION

Yeasts are eukaryotic microorganisms that have a multipurpose group and are found in a broad range of environments.[1] Moreover, yeasts are heterogeneous in their nutritional capabilities and can adapt even in extreme conditions.[2,3] The unicellular yeasts have the ability to live in the depths of liquids and moist and irregular surfaces.[4] Some yeasts are pathogenic fungi and responsible for many diseases, such as candidiasis.[5] *Candida* is a source of fungal contamination in the body as it is a class of yeast. Moreover, candidiasis is a yeast which considered as an infecting agent and belongs to the genus *Candida*.[6]

About 200 different species belong to the genus *Candida*, which are different in shapes and effectiveness,[7] nonetheless, only around 20 *Candida* species are harmful and responsible for pathological manifestations in humans that cause a variety of clinical syndromes, which can be superficial infections or invasive diseases.[8] *Candida* species form part of the human flora and spread in many parts of the human body, such as the gastrointestinal tract, vulva, groin, anus, vaginal canal, and oral cavity of healthy people.[9] *Candida* species become pathogenic agents under different conditions. Therefore, it is classified under opportunistic fungi,[7,10] and turned to pathogens in persons with an immune-suppressed system, especially newborns, patients on antibiotic therapies, human immunodeficiency virus (HIV) acquired immunodeficiency syndrome (AIDS), and cancer therapy patients.[11-13] Moreover, the number of systemic infections has increased over the past 15 years, and are an important infection agent due to the appearance of large numbers of invasive *Candida*, decreased natural human immunity, the wide use of antibiotics, and other factors.[11,14]

Vulvovaginal candidiasis (VVC) is a very common and widespread mucosal vaginal or vulva infection,[15] and represents a primary opportunistic infection and secondary with internal or external features.[16] Moreover, almost 75% of women become infected with *Candida* spp., which leads to VVC. and is one or two times infected in a year or at least one time in their life.[17-19] Meanwhile, Trisnadewi[14] indicated that VVC infections are considered the most common disease and

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represent the second infection kind after bacterial vaginosis. Candidiasis affects women of reproductive age, especially married and pregnant women, with an immune-suppressed system that includes about 15–25% of vaginitis cases.[20,16]

C. albicans is the main cause of VVC in women and represents about 80–95%, while non-*albicans Candida* species are responsible for (5–20%) of VVC infections. Non-*albicans Candida species, including C. glabrata, C. arapsilosis, C. tropicalis, C. krusei*, and others are stated as causative agents of VVC. However, *C. glabrata* are responsible for (10–20%) of VVC infections.[21,22] The present study aims to isolate and identify *Candida* species isolated from women infected with VVC in Maysan province, Iraq.

MATERIALS AND METHODS

Samples Collection

A total of 100 samples were collected from women of reproductive age attending the Maysan Maternity Hospital (Maysan, Iraq) from December 2021 to April 2022 using sterile vaginal swabs. Authentic approvals were obtained from the College of Science to conduct the current study. Vaginal swabs were taken using sterile cotton swabs, placed in test tubes containing normal saline, and brought to the laboratory. Samples were cultured onto sabouraud dextrose agar (SDA) and incubated for 24–48 hours at 30°C. For yeast isolation. The following identification tests were used to identify the *Candida* spp.

CHROMagar Culture

Purified single colony was streaked on CHROMagar *Candida* (Hi-Media, India) and incubated for 24 to 48 hours at 37°C. The colors of colonies on CHROMagar are considered a taxonomic characteristic used to distinguish between *Candida* species.[23]

Germ Tube Test

Cells suspension of *Candida* species was cultured into a test tube containing 0.5 mL of fresh human serum using a loopful, incubated at 37°C for 2 to 4 hours. The drop was put on a slide and examined under a light microscope (40X) to observe the germ tube, which is visible as short, slender tubes without constriction at their point of origin and arising from the mother yeast cell.[24]

Chlamydospores Formation Test

Casein agar medium was used for producing chlamydospores and incubated at 37°C for 24 to 48 hours. Chlamydospores were examined under light microscope using lactophenol cotton blue.[25]

SDA Plus Cycloheximide Test

used to estimate the growth of *Candida* species in the presence of cycloheximide.[26]

Growth of Candida Species at 45°C

Candida cells suspension was inoculated on SDA, and incubated for 24–48 hours at 45°C, growth at 45°C id considered a taxonomic characteristic of some species.[27]

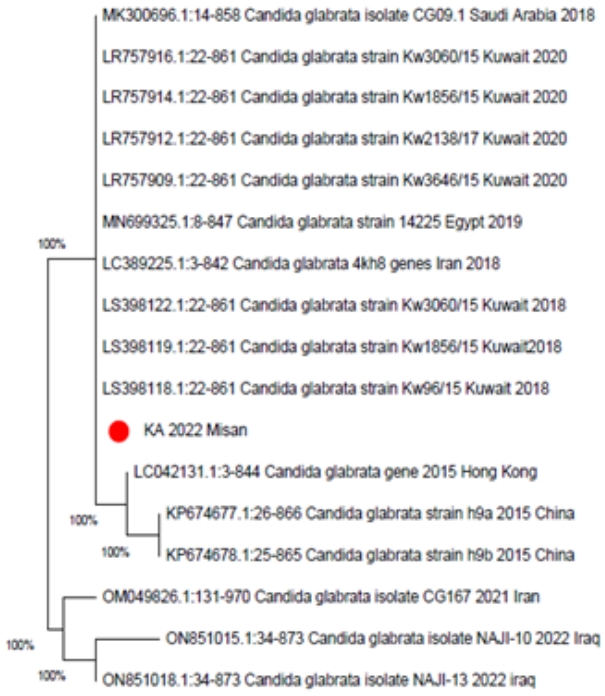

Figure 1: Phylogenetic tree based on sequencing results of *C.glabrata*1.

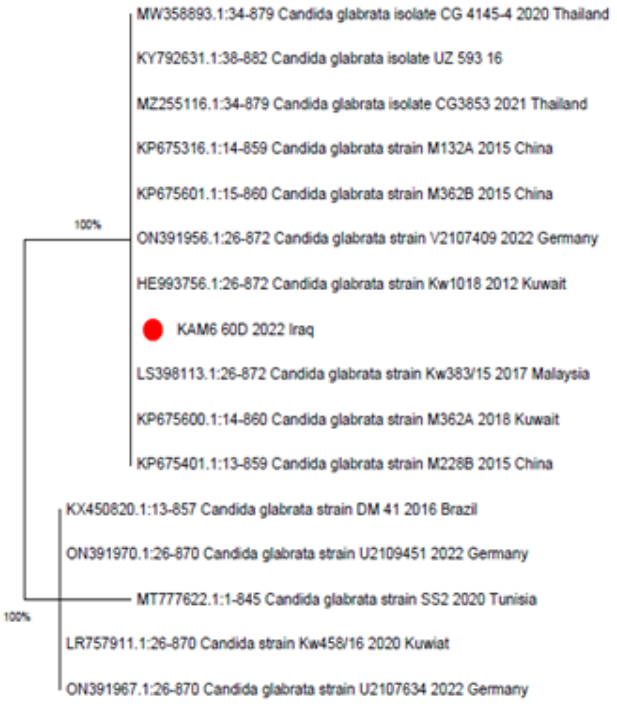

Figure 2: Phylogenetic tree based on sequencing results of *C.glabrata*2.

Molecular Methods

Eight isolates were selected according to the results of identification tests as representative of all Candida species (Two isolates form both *C. albicans* and *C. glabrata*) in this study for DNA extraction and PCR assays.

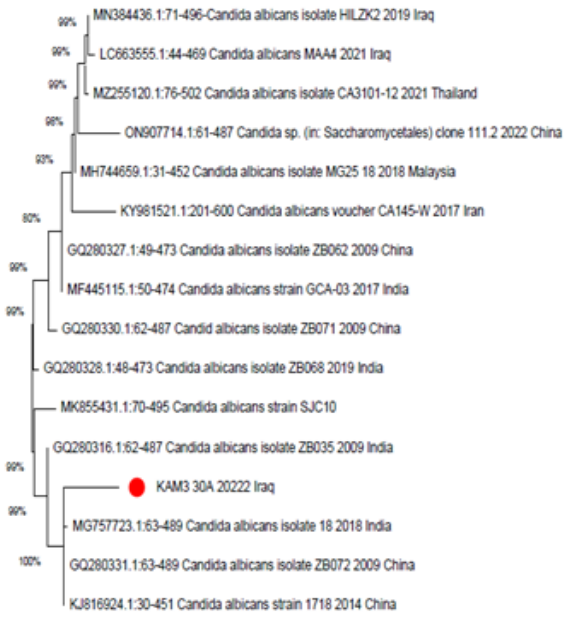

Figure 3: Phylogenetic tree based on sequencing results of *C.albicans*1.

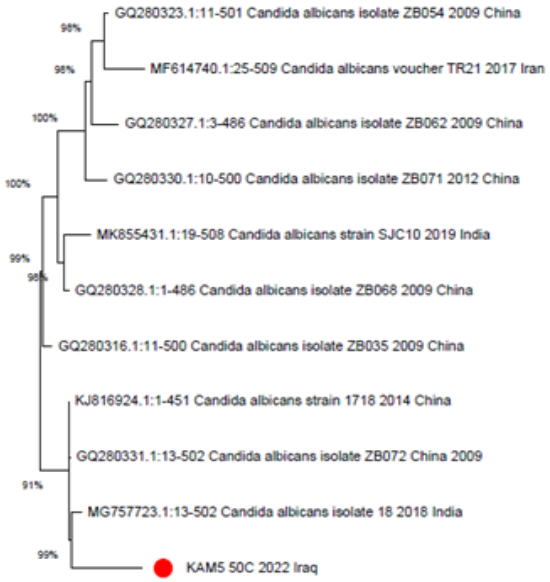

Figure 4: Phylogenetic tree based on sequencing results of *C.albicans*1.

Table 1: The PCR process program used in this study

PCR Cycle	*Temp.(˚C)*	*PCR product size*		
		100-500 bp	*500-1000 bp*	*1-5 kb*
Initial denaturation	94	2 min	2 min	2 min
Denaturation	94	20 sec	20 sec	20 sec
Annealing	50-65	10 sec	10 sec	20 sec
Extension	65-72	20-30 sec	40-50 sec	1 min/kb
Final extension	72	Optional, Normally, 2-5 min		

Extraction of DNA

DNA of tested *Candida* isolates was extracted using the method described by[28] and following the protocol instructions provided by the kit (Presto™ Mini gDNA Yeast Kit, Genaid, USA); the steps in brief as below:

PCR using Candida Species Universal Primers

Universal primers ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) were used to amplify ribosomal internal transcribed ITS region.

Table 2: Identification of vaginal *Candida* species using some identification tests

Species	*No. of isolates and occurrence %*	*Colony color on CHROMagar*	*Germ tube*	*Chlamydospore*	*SDA plus cycloheximide*	*Growth at 45°C*
C.albicans	20, 44	light green	+ 95%	+	+	+
C. glabrata	8, 17.8	white-cream	-	-	-	+
C. dubliniensis	7, 15.6	dark green	+ 100%	+	+	-
C. parapsilosis	5, 11	cream-purple	-	-	-	-
C. krusei	3, 6.7	red pink	-	-	-	+
C. kefyr	2, 4.4	dark pink	-	-	-	+

Table 3: The Phenotypic, Molecular identification and number of reference to *Candida* strains in GenBank.

Phenotypic identification	*Molecular identification*	*Number of references*	*GenBank sequence accession numbers*
*C.glabrata*1	*C.glabrata*1	OP042382	SUB11829667 KAM1_10
*C.glabrata*2	*C.glabrata*2	OP042386	SUB11829667 KAM6_60D
*C.albicans*1	*C.albicans*1	OP042383	SUB11829667 KAM3_30A
*C.albicans*2	*C.albicans*2	OP042385	SUB11829667 KAM5_50C
C. kefyr	*Kluyveromyces marxianus*	OP042384	SUB11829667 KAM4_40B
C. krusei	*Pichia kudriavzevii*	OP042387	SUB11829667 KAM7_70E
C.parapsilosis	*C.parapsilosis*	OP042388	SUB11829667 KAM8_80F
C. dubliniensis	*C.dubliniensis*	OP042389	SUB11829667 KAM9_90G

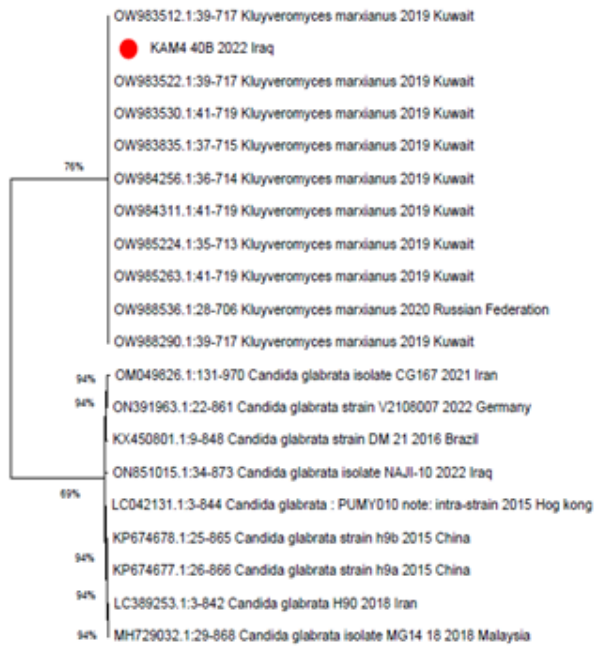

Figure 5: Phylogenetic tree based on sequencing results of *C. kefyr*.

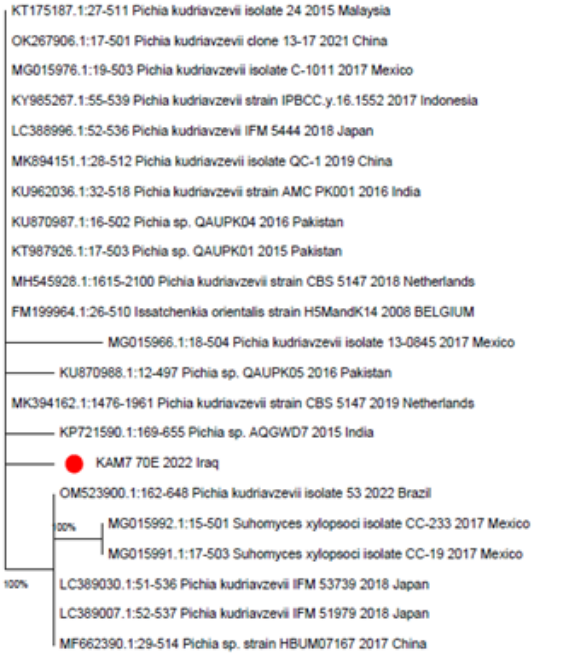

Figure 6: Phylogenetic tree based on sequencing results of *C. krusei.*

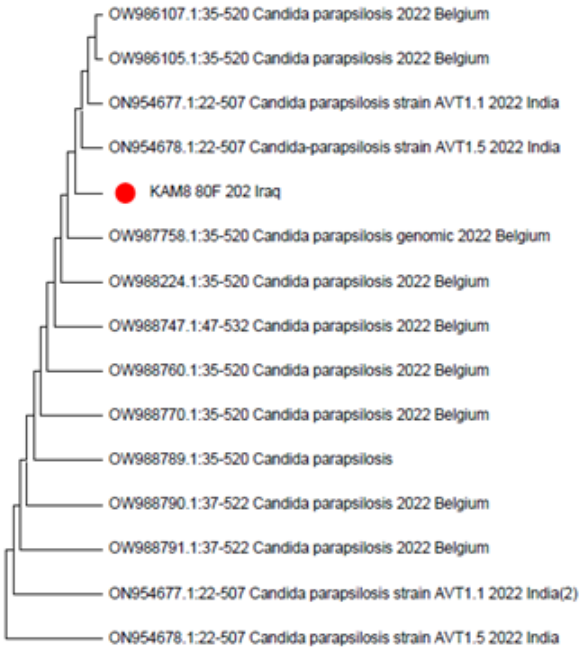

Figure7: Phylogenetic tree based on sequencing results of *C. parapsilosis.*

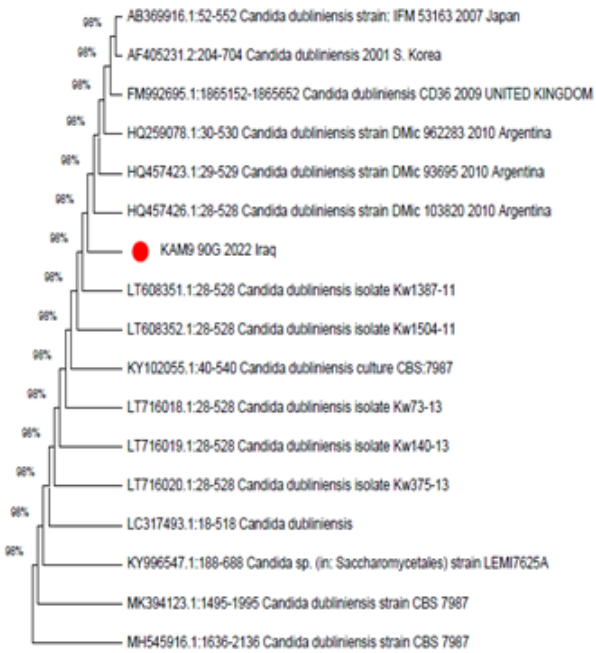

Figure 8: Phylogenetic tree based on sequencing results of *C. dubliniensis.*

Polymer Chain Reaction

The PCR reactions were carried out with conditions according to below (Table 1), using Maxime PCR Premix Kit. The samples send to Psomagen Center/USA (https://www.psomagen.com/) to determine DNA sequences.

NCBI BLAST

The results obtained from DNA sequences were used to found closest matched sequences in the GenBank database. Moreover, the MEGA 11.0 software package used to obtain the phylogenetic tree of our isolates with reference strains available in GenBank.

RESULTS AND DISCUSSION

The majority of women infected with vaginal candidiasis which represents the second kind of vaginal infection.[20] VVC is a disturbing infection that infects more than 75% of women at least once during their lifetime.[18] Identification experiments

showed that out of 100 vaginal samples, forty-five (45%) revealed positive results on SDA,[20] (44%) isolates showed as light green were diagnosed as *C. albicans*, other isolates belonged to five species of *Candida* which are *C. glabrata* (8 isolates), *C. dubliniensis* (7 isolates), *C. parapsilosis* (5 isolates), *C. krusei* (3 isolates) and *C. kefyr* (2 isolates) showed different colors (Table 2), this result is in agreement with most studies, except for some exceptions.[29] The results indicated that both *C. albicans* and *C. dubliniensis* have the ability to form germ tubes and chlamydospores and grow in SDA-containing cycloheximide in contrast to other species. All species are able to could grow at 45°C except for *C. dubliniensis* and *C. parapsilosis*. Gomez-Gaviria and Mora-Montes[30] showed that *C. albicans* is the extremely common etiological agent of candidiasis and found in abundance in various parts of the body such as mouth, ear, vagina and gastrointestinal tract; outermost, many studies reported that the mots infection factor of VVC patients were *C. albicans* and *C. glabrata*.[31] Other species collectively, these species are the causative agents of non-albicans candidiasis. *C. albicans* can shift from its filamentous to hyphal morphisms, increasing the pathogenicity which help *C. albicans* adhere to surfaces of tissue and cause infection.

Furthermore, *C. albicans* infection may be lead to death in women with low immunity.[32] Hasanvand *et al.*[33] showed that C. albicans, *C. tropicalis, C. glabrata,* and *C. parapsilosis* isolated from vaginitis patients.[33]

The colors of colonies of tested *Candida* on CHROM agar *Candida* confirmed our findings and agreed with several studies (Table 2).[34,20,22]

C. albicans and *C. dubliniensis* showed capability to form germ tubes and chlamydospores and grow in the presence of cycloheximide, this result agrees with Alam *et al.*[35] and Farahyar *et al.,*[36]. Four species grow well at 45°C, except *C. dubliniensis* and *C. parapsilosis*. Alam *et al.*[35] reported that *C. albicans* grows at high temperatures, reached to 45°C. Moreover, *C. dubliniensis* grow at 30 and 37°C but does not grow at 42°C.[25]

Many physiological and biochemical procedures have given useful data for the taxonomy, the nucleotide sequences of DNA likely to provide an accurate differentiation of organisms and also refer to evolutionary and phylogenetic relationships.[37]

To estimate genetic differences and relationships between *Candida* species, PCR products of eight clinical *Candida* isolates, therefore, the PCR-RFLP used for identification of the tested *Candida* spp. Using the ITS1 and ITS4 primers to amplify the ITS region, all tested *Candida* isolates yielded fragments of 500–700 bp. Table 3 showed that the sequences of all tested *Candida* species were 100% identical to the sequences of reference strains, except *C. albicans*2 (99%), *C.parapsilosis*, and *C. dubliniensis* (98% for each). *C. albicans*1 and *C. albicans*[2] are identical to the same reference strains (MG757723 reference strains), but with 100 and 99%, respectively.

All *C. glabrata*1 strains showed 100% identity with many reference strains such as LC042131, KP674677, KP674678, LS398118, and LS398119. On the other hand, it is genetically far OM049826, ON851018, and ON851015 reference strains (Figure 1). *C. glabrata*2 indicated 100% similarity to ON391956, KP675601, HE993756, etc. reference strains (Figure 2). Figure 3 showed that *C. albicans*1 showed similarity (100%) to MG757723, GQ280331, KJ816924, and GQ280316 reference strains. Figure 4 shows the closest reference strains to *C.albicans*.2 While, *C. kefy* (*Kluyveromyces marxianus*) showed 100% identity with 10 reference strains (OW983512, OW983522, OW983530 and OW983835((Figure 5). Figures 6-8 showed that *C. krusei* (Pichia kudriavzevii), *C. parapsilosis*, and *C. dubliniensis* displayed 100, 100 and 98%, respectively, identity with reference strains.

Several studies indicated the use of PCR method and restriction enzymes for the accurate identification of organisms that have also used many procedures with universal primers for the identification of different fungi. The ITS1 and ITS2 regions are enclosed by the 5.8SrDNA gene,[38] which are suitable for the identification of fungi and medically significant. Universal primers are often used as a useful method for identification of clinical microbiology.[28]

CONCLUSION

The results of this study showed that *Candida albicans* is the most common *Candida* species in VVC among women with a prevalence of 4.44% followed by (17.8%). Chromogenic Candida agar and a multiplex PCR allowed the presumptive identification of all *candida* species.

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