

## Isolation and genotyping of *Candida albicans* involved in vaginal candidiasis among pregnant women in Sulaymaniyah and Erbil cities

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### Abstract

**Background and objective:** Vaginal candidiasis is a common infection among pregnant women. The current study aimed to investigate the etiologic species of vaginal candidiasis and the genotypes of *C. albicans* isolated from vaginal samples among pregnant women in the Kurdistan region of Iraq.

**Methods:** Vaginal swabs were collected from pregnant women admitted to the Maternity hospital in Sulaymaniyah and Erbil cities from March 2016 to December 2018. *Candida* isolates were identified on CHROMagar medium, then confirmed with PCR depending on ITS region. All *C. albicans* isolates were confirmed using chitin synthase gene (CHS1) and subjected to genotypic analysis based on the transposable intron in 25S rDNA with using CA25S and CA-INT primers. DNA sequencing of 25S rDNA region was done by using CA and CA-INT primers.

**Results:** Among 340 women tested, 114 (33.53%) were positive for vaginal candidiasis. Five *Candida* species were identified, where they are *C. albicans* (56.14%), *C. glabrata* (24.56%), *C. kefyr* (11.40%), *C. tropicalis* (5.27%) and *C. krusei* (2.63%). It has been found that *C. albicans* significantly ( $P < 0.01$ ) higher than non-*albicans* species. The genotypes A (450 bp), B (840 bp), and C (450 and 840 bp) of *C. albicans* were detected. The Genotype A (54.69%) was the most prevalent, followed by Genotype B (34.38%) and Genotype C (10.94%). In regards to genetic variation, genotypes A and B were more similar compared to genotype C.

**Conclusion:** The current study revealed a high prevalence of vaginal candidiasis with different genotypes of *C. albicans* among pregnant women. Therefore, it is worth considering a vaginal swab culture with clinical symptoms during the diagnosis of vaginal candidiasis.

**Keywords:** *Candida*; Genotypes; Vulvovaginitis; Candidiasis; Prevalence.

### Introduction

*Candida* species are the most common cause of fungal infections, leading to a range of life-threatening invasive diseases such as bloodstream candidiasis to non-life-threatening mucocutaneous candidiasis such as genitourinary candidiasis, vulvovaginal candidiasis, and oropharyngeal candidiasis.<sup>1</sup> Genital involvement in women is one of the most common presentations due to *Candida*.<sup>2</sup> Vulvovaginal candidiasis (VVC) is caused by the overgrowth of *Candida* species in the vagina and is characterized by curd-like

vaginal discharge, itching, and erythema<sup>3</sup> and has increased dramatically in recent years as a worldwide health problem and affects millions of women annually.<sup>2</sup> It is estimated that 70%–75% of women of childbearing age worldwide experience at least one episode during their lifetime, and 5%–10% of women with a first episode of VVC experience frustrating recurrent infections. VVC is rare before puberty. Its incidence increases dramatically in the second decade of life, coinciding with the onset of sexual activity.<sup>4</sup> Some of the factors that predispose women to vaginal

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candidiasis are changing in pH, use of oral contraceptives, tight clothing, and personal hygiene.<sup>5</sup> However, it is reported that vaginal candidiasis is common during pregnancy due to the altered pH and sugar content of vaginal secretions. Also, increasing the estrogen level during pregnancy produces more glycogen in the vagina, which directly affects yeast cells, causing them to grow faster and stick more easily with the walls of the vagina.<sup>6</sup> It has been estimated that nearly up to 40% of pregnant women worldwide may have vaginal colonization by *Candida* species.<sup>5-7</sup> *Candida albicans* is the most common causative pathogen of candidiasis. Recently, other *Candida* species known as non-*albicans*, such as *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. krusei* and *C. dubliniensis* have emerged as important opportunistically infectious fungi.<sup>8-10</sup> Thus, it is well known that *C. albicans* is the leading agent of vaginal fungal infections and other gynaecological disorders. Almost 80-90% of VVC is caused by *C. albicans* except that only a minority of cases (10-20%) is caused by non-*C. albicans* species.<sup>11</sup> Genetic variability and key virulence factors such as adhesion capacity, biofilm development, cell surface hydrophobicity, morphological transition, and production of hydrolytic enzymes by *C. albicans* are recognized essential steps in the process of *Candida* colonization and subsequent infection.<sup>12</sup> Heterogeneity of these virulence properties in clinical *C. albicans* isolates is commonly observed; hence, molecular subtyping provides a useful tool to identify pathogenic subpopulations within this species. According to the presence and the size of transposable intron region in the large ribosomal subunit 25S rDNA, *C. albicans* can be divided into four subtypes based on different lengths of PCR products amplified namely, genotype A (450 bp), genotype B (840 bp), genotype C (450- and 840-bp), and genotype E (1400 bp).<sup>13</sup> Reports are indicating an association of certain *C. albicans* genotypes with fungal invasiveness and antifungal susceptibility.<sup>13</sup>

Thus, the current study aimed to determine the etiologic species of vaginal candidiasis and genotyping of *C. albicans* isolated from vaginal samples among pregnant women in the Kurdistan region of Iraq.

## Methods

### Sample Collection

Using sterile cotton tipped swabs, a total of 340 vaginal swabs were collected from symptomatic and asymptomatic pregnant women who were admitted to Maternity hospital in Sulaymaniyah and Erbil cities, Kurdistan Region, Iraq, from March 2016 to December 2018. The collection of vaginal swabs was done by a gynecologist. The swab stick was immediately replaced with its casing, labeled, and taken to the laboratory for culturing.<sup>14</sup> The participants filled out a consent form to participate in the research, which was approved by the Research Protocol Ethics Committee of the Kurdistan Board of Medical Specialties. Concomitant to each sample, a questionnaire was completed for each participant inquiring about their age, marital state, occupation, address, symptomatic state, pregnancy trimester, history of antibiotic use, taking birth control pills, associated medical conditions during pregnancy, and presence of stress factors.

### Isolation and Phenotypic Identification of *Candida* spp.

*Candida* spp. were isolated by direct plating of samples on Sabouraud dextrose agar (SDA) [Difco Laboratories Detroit, USA) supplemented with chloramphenicol (0.1 mg/mL, ASIA-Syria) and incubation at 37°C for 48 hours. As preliminary identification, *Candida* spp. were identified according to the morphology of the colonies, which were white to cream, round, curved, soft, and smooth to wrinkled, with a characteristic yeast odor, it was overgrowing. For species identification, all *Candida* isolates were cultured on CHROMagar *Candida* (Liofilchem, Italy), a loopful of an isolate was streaked on a CHROMagar plate and incubated at 37°C. The growth was

checked every 24 hours until 72 hours of incubation, and the species were identified by type and color of the colonies on CHROMagar media as per 'manufacturer's instructions'.<sup>15-17</sup>

#### Molecular Identification of *Candida spp.*

The molecular identification was performed for all the isolates using the primers shown in Table 1, also with a colony PCR to save material and time. Through an overnight culture, a single colony of *Candida* was picked then re-suspended in 40µl ddH<sub>2</sub>O. An incubation at 95°C for 20min done to release the DNA according to Lau *et al.*<sup>18</sup> and Alshahni *et al.*<sup>19</sup> The DNA was purified by centrifugation at 12000 rpm for 2min then 3µl of the supernatant used as a PCR template. ITS primers were used to identify different strains of the *Candida spp.*,<sup>20</sup> which amplified the intergenic spacer regions (ITS) (Figure 1) of ribosomal DNA (rDNA).<sup>21</sup> The PCR was performed using Prime Taq DNA Polymerase kit [Prime Taq Premix (2X)/ GeNet Bio) according to manual instructions. The program of the PCR was as follows: 1 cycle of initial denaturation at 95°C for 5min, followed by 35 amplification cycles of denaturation at

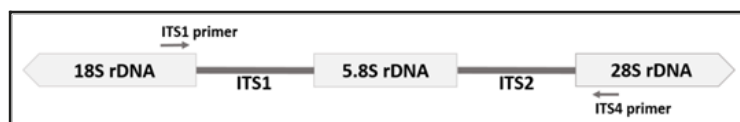
95°C for 30sec, annealing at 57°C for 30sec and extension at 72°C for 40sec, then finalized with the final extension at 72°C for 5min. The amplicon was separated by 1% gel electrophoresis then visualized by ethidium bromide.

#### Confirmation of the *Candida albicans* identification by PCR

For more confirmation, all the amplicon with the 532bp in size (previously amplified by ITS primers) were re-analyzed by another set of primers CHS1, according to Jordan.<sup>22</sup> The conventional PCR enables to amplify a portion of a chitin synthase gene (CHS1) from *C. albicans* with the size of 122bp. The colony PCR was also followed as previously mentioned with the same DNA polymerase kit. The PCR program was as follows: initial denaturation for 5min at 95°C, then in 40 cycles of denaturation at 95°C for 30sec, annealing at 52°C for 30sec, and extension at 72°C for 30sec were adapted. The PCR was terminated with one cycle of finale extension at 72°C for 7min. The products were also visualized with the 2% gel electrophoresis and ethidium bromides.

**Table 1:** List of the primers used in this study.

Name	Sequence (5' → 3')	Tm <sup>0</sup> C	References
ITS1-F	TCCGTAGGTGAACCTGCG	57 <sup>0</sup> C	Fujita <i>et al.</i> <sup>20</sup>
ITS4-R	TCCTCCGCTTATTGATATGC	57 <sup>0</sup> C	Fujita <i>et al.</i> <sup>20</sup>
CHS1-F	CGCCTCTGATGGTGATGAT	52 <sup>0</sup> C	Jordan <sup>22</sup>
CHS1-R	TCCGGTATCACCTGGCTC	52 <sup>0</sup> C	Jordan <sup>22</sup>
CA25S-F	CGATTCAGGGGAGGTAGTGAC	64 <sup>0</sup> C	Tantivitayakul <i>et al.</i> <sup>13</sup>
CA25S-R	GGTTCGCCATAAATGGCTACCAG	64 <sup>0</sup> C	Tantivitayakul <i>et al.</i> <sup>13</sup>
CA-INT-F	ATAAGGGAAGTCGGCAAATAGATCCGTAA	70 <sup>0</sup> C	Tantivitayakul <i>et al.</i> <sup>13</sup>
CA-INT-R	CCTTGGCTGTGGGTTTCGCTAGATAGTAGAT	70 <sup>0</sup> C	Tantivitayakul <i>et al.</i> <sup>13</sup>



**Figure 1:** Positions of the amplified region by both ITS1 and ITS4 primers on the intergenic spacer regions (ITS) of ribosomal DNA (rDNA).

**Nested PCR for Genotypes Detection**

Different genotypes of *C. albicans* were detected based on the transposable intron in 25S rDNA; for this purpose, the nested PCR method was followed using CA25S primers in the first round of the PCR. The method was started with one cycle of the initial denaturation at 95°C for 5min. The PCR was continued with the 35 cycles of denaturation at 95°C for 30 sec and annealing at 64°C for 30sec and extension at 72°C for 40 sec, then the final extension at 72°C for 5 min were finalized the PCR. The amplicon of the first round was used as a template of the second PCR with the same program with the exception of using CA-INT primers in the annealing temperature at 70°C.

**Sequencing**

The sequencing was done (Macrogen Company, Seoul, South Korea) for some of the samples amplified by CA25S and CA-INT forward and reversed primers (10pmol).

**Statistical Analysis**

Chi-square test was performed using the statistical package for the social sciences

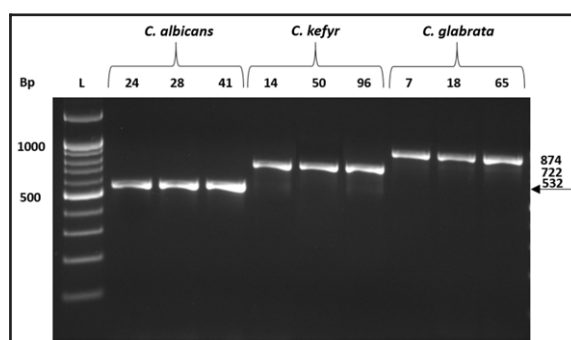
software (version 22.0), and differences were considered significant at  $P < 0.01$ .

**Results****Prevalence of *Candida* Species**

A total of 340 vaginal swabs were collected from pregnant women and reported in the present study. Of these, 114 (33.53%) women were positive for vaginal candidiasis, and 226 (66.47%) women were negative for vaginal candidiasis. All isolates were identified on CHROMagar *Candida* medium, then confirmed with PCR depending on ITS region, which is presented in Figure 2. Thus, as listed in Table 2, five *Candida* species were identified between all isolates, where they are *C. albicans*, *C. glabrata*, *C. kefyr*, *C. tropicalis* and *C. krusei*. It has been revealed that the highest frequency of vaginal candidiasis of pregnant women was caused by *C. albicans* (56.14%), which is significantly ( $P < 0.01$ ) higher than non-*albicans* species, where they are *C. glabrata* (24.56%), *C. kefyr* (11.40%), *C. tropicalis* (5.27%) and *C. krusei* (2.63%).

**Table 2:** Distribution and characteristics of isolated *Candida* species.

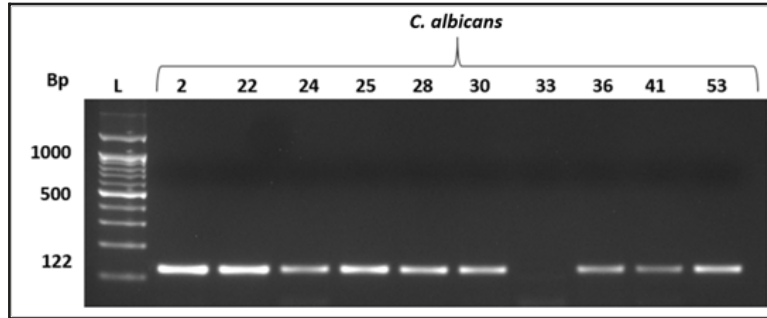
<b>Candida Species</b>	<b>Number of Isolates</b>	<b>Percentage (%)</b>	<b>Color on CHROMagar</b>	<b>Fragment size found by PCR with ITS1-ITS4 primers</b>
<i>C. albicans</i>	64	56.14	Light green	532 bp
<i>C. glabrata</i>	28	24.56	Pinkish purple	874 bp
<i>C. kefyr</i>	13	11.40	Dark pink	722 bp
<i>C. tropicalis</i>	6	5.27	Metallic blue	521 bp
<i>C. krusei</i>	3	2.63	Rose pink	500 bp
<b>Total</b>	<b>114</b>	<b>100</b>		

**Figure 2:** An example of *Candida* spp. Identification showing three species, which were *C. albicans* (532bp), *C. kefyr* (722bp) and *C. glabrata* (874bp). The marker was 100bp DNA [GeNet Bio].

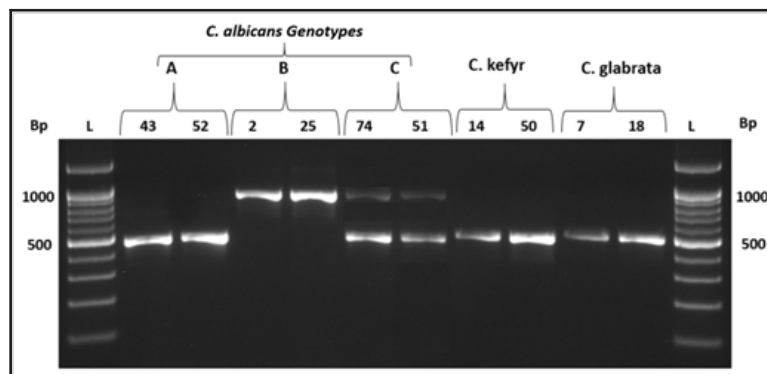
**Identification and Genotyping Analysis of *Candida albicans***

The identification of all *C. albicans* isolates were confirmed with PCR depending on the chitin synthase gene (CHS1), as shown in Figure 3, the fragment size was 122bp. Then all *C. albicans* isolates were subjected to genotypic analysis based on the transposable intron in 25S rDNA with using CA25S and CA-INT primers as

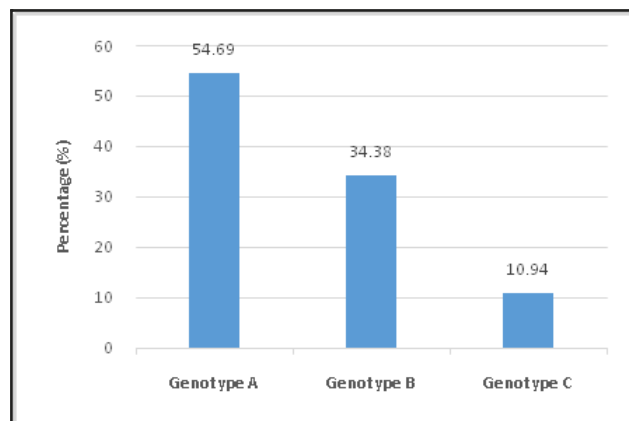
it is shown in Figure 4. It has been found that *C. albicans* isolates belong to three genotypes, where are genotype A (450 bp), genotype B (840 bp), and genotype C (450 and 840 bp). Results showed that out of 64 isolates, the Genotype A was the most prevalent with 35 isolates (54.69%), followed by Genotype B, with 22 isolates (34.38%), and Genotype C, with 7 isolates (10.94%) (Figure 5).



**Figure 3:** Confirmation of the *C. albicans* identification with different primers CHS1 (122bp). The marker was 100bp DNA [GeNet Bio].



**Figure 4:** Detection of three genotypes of *C. albicans*.

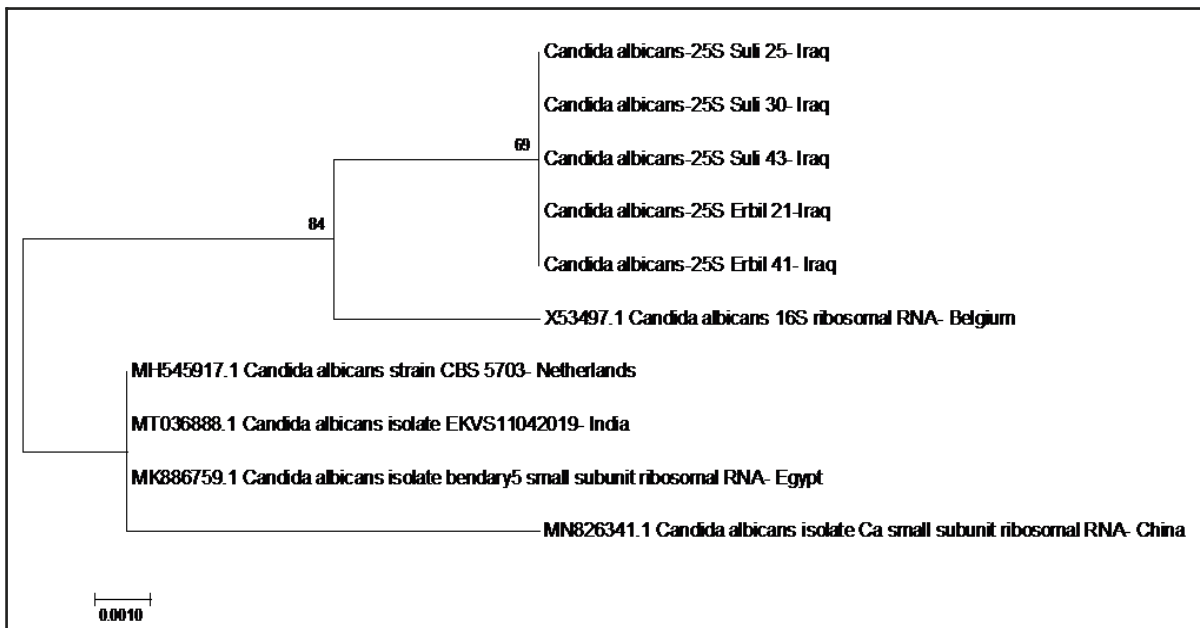


**Figure 5:** The percentages of *C. albicans* genotypes.

### Phylogenetic analysis

The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model. The tree with the highest log likelihood (-418.7355) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite

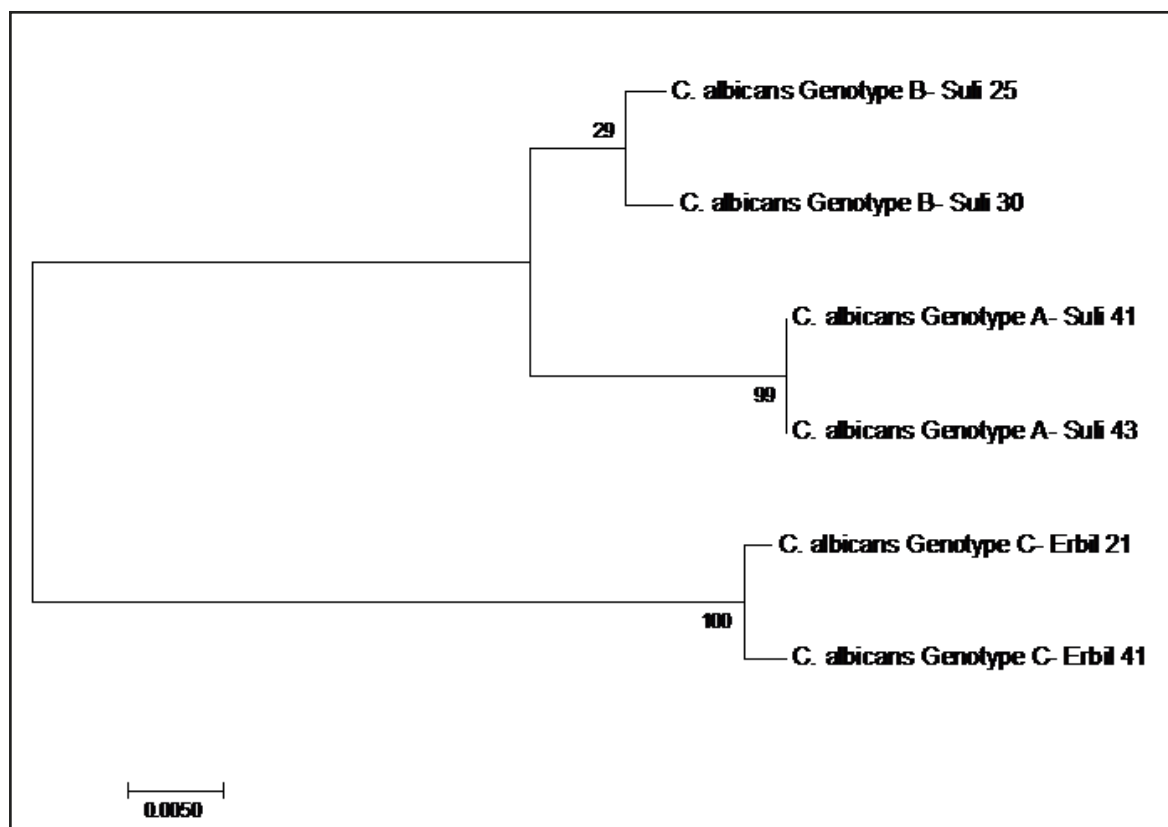
Likelihood (MCL) approach and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved ten nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 271 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.



**Figure 6:** Molecular Phylogenetic analysis by Maximum Likelihood method of the 25S region.

The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by

applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log likelihood value. The analysis involved six nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 439 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.



**Figure 7:** Molecular Phylogenetic analysis by Maximum Likelihood method of the three genotypes.

## Discussion

It is well known that *Candida* species is an opportunistic pathogen associated with vulvovaginal candidiasis in women, which varies from one population to another, and pregnancy is one of the risk factors. In the current study, the prevalence rate of vulvovaginal candidiasis was 33.53% between participated pregnant women. This result is lower than that reported in Lebanon (39%),<sup>7</sup> Pakistan (38%),<sup>6</sup> and India (42.37%).<sup>23</sup> However, another study reported a lower prevalence rate (29%) of VVC in Iraq.<sup>24</sup> This variation of VVC rate in pregnant women may be related to increased risk factors during pregnancy such as immunologic alterations, increased estrogen levels, and increased vaginal glycogen production mechanism.<sup>25</sup> Five *Candida* species were isolated in the present study, *C. albicans* was the predominant species (56.14%), followed by *C. glabrata* (24.56%), *C. kefyr* (11.40%), *C. tropicalis* (5.27%), and *C. krusei* (2.63%). These results are inconsistent with previous studies, where they stated that vaginal infection with *C. albicans* is predominated over other species. Nelson et al.<sup>26</sup> showed that *C. albicans* was the most common vaginal *Candida* species, followed by *C. glabrata* as a second species causing vaginal candidiasis among pregnant women, but other species such as *C. krusei*, *C. tropicalis* and *C. parapsilosis* are also encountered. Yadav and Prakash<sup>27</sup> reported high occurrence of *C. albicans* (64.04%) followed by *glabrata* (12.35%), *dubliniensis* (10.11%), *tropicalis* (5.61%) and *C. krusei* (3.37%). Another study by Sutaria et al.<sup>28</sup> reported *C. albicans* (35.55%) as the most common vaginal *Candida* species, followed by *C. glabrata* (9.63%), causing vaginal candidiasis among pregnant women. The high occurrence rate of *C. albicans* as a causative agent of vaginal candidiasis during childbearing age maybe due to its virulent factors, which include dimorphism and phenotypic switching. Also, *C. albicans* produces protease and phosphatase

enzymes, where they enhance its attachment to the human epithelium. It can also be found that the high occurrence rate of *C. albicans* could be due to increased physiological changes, estrogen, and rich glycogen content of the vaginal mucosa, thereby providing an adequate supply of utilizable sugar that favor its growth during pregnancy.<sup>14</sup> However, Ghaddar et al.<sup>7</sup> reported a low incidence of *C. albicans* (42%) comparing to non-*albicans Candida* species (58%), where include *C. glabrata* and *C. krusei*. Molecular subtyping of *C. albicans* provides a useful tool for studying the epidemiology of this commonly causative agent of candidiasis in humans for identification of pathogenic subpopulation within this species, thereby assist in developing appropriate therapeutic strategies to combat the disease.<sup>13,29</sup> In Iraq, there is insufficient data on vaginal candidiasis in pregnant women and the distribution of the vaginal *Candida* species; thus, this study considers as the first report of genotypic analysis of *C. albicans* isolated from vaginal candidiasis in pregnant women. In this study, 64 isolates of *C. albicans* were obtained from vaginal swabs in pregnant women, depending on the transposable intron in 25S rDNA all isolates were differentiated into three genotypes (A, B, and C), none of the isolates belonged to genotype (E). The data revealed that the most prevalent genotype among all isolates was Genotype A (54.69%), followed by Genotype B (34.38%) and Genotype C (10.94%). These results agree with those reported by previous studies,<sup>29,30</sup> whereas, disagree with the results of Ali Shtayeh et al.,<sup>31</sup> who reported that the dominant genotype is Genotype C (55%), followed by Genotype A (32.4%), and Genotype B (12.6%). The variation between three genotypes of *C. albicans*, may be due to the level of secretion and activity of exoenzymes such as phospholipase and proteinase since they are noteworthy virulence factors where contribute to the pathogenesis.<sup>32,33</sup>



Notably, there is limited information regarding phylogeny and genetic diversity of *C. albicans* strains isolated in vaginal candidiasis of pregnant women in Iraq. It is revealed that *C. albicans* population isolated from vaginal candidiasis in pregnant women despite the genetic variation depending on 25S rDNA, they are belonging to one clade (Figure 6). This clade of *C. albicans* was more similar to the *C. albicans* strain from Belgium (X53497.1), while genetically very different with strains from the Netherlands (MH545917.1), India (MT036888.1), Egypt (MK886759.1), and China (MN826341.1). The phylogeny results of three *C. albicans* genotypes are shown in Figure 7, and it is found that genotypes A and B are more similar to each other, comparing to the genotype C.

### Conclusion

The study concluded a high prevalence of vaginal candidiasis, and vaginal infection among pregnant women by *C. albicans* dominates other non-*albicans* species such *C. glabrata*, *C. kefyr*, *C. tropicalis*, and *C. krusei*. Molecular genotyping revealed that three genotypes (A, B, and C) of *C. albicans* causing vaginal candidiasis in pregnant women. In addition, this study also found a genetic variation between *C. albicans* genotypes. Therefore, future works should focus on the correlation of genotypes to the host predisposing factors, pathogen virulence factors, and their antifungal susceptibility.

### Competing interests

The author declares no competing interests.

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