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Conventional and Molecular Identification, Incidence and Species Distribution of *Candida* Associated with Vaginal Candidiasis among Women Attending Gynecological Clinic at Duhok Province, Kurdistan – Iraq

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Abstract

Vaginal Candidiasis is one of the most common sources of inflammation of the vagina in patient women attending gynecological clinics. The present study aimed to isolate and identify the *Candida* species in pregnant and non-pregnant women of different age groups attending maternity hospitals at Duhok, Iraq, using conventional and molecular methods. Vaginal swab samples were collected from 385 women aged 15-60 years with suspected vulvovaginal candidiasis. All swab samples were initially cultured on Sabouraud's dextrose agar (SDA) supplemented with 0.05% (Weight/Volume) chloramphenicol and incubated at 37ºC for 48 hours. For the presumptive identification of *Candida* species, all growing colonies were sub-cultured on Harlequin TM Candida Chromogenic agar (CHROM agar Company, Acumedia Neogen, UK) incubated at 35ºC for 48 hours. Molecular identification was achieved by sequencing the ITS1 and ITS4 regions of rDNA. Four *Candida* species were identified by both conventional and molecular methods. Based on PCR detection, *C. albicans* (47%) was the most common species, followed by *C. glabrata* (36.2%), then *C. krusei* (7.4%) while *C. tropicalis* (2.2%) showed the least incidence. Vaginal candidiasis is a common problem among women in Duhok city and was more common in age groups of 15-36 years of both pregnant and non-pregnant women. Sensitive Chromogenic agar for detection of pecies showed 91.7%, while the PCR-based technique was 97.49%.

Keywords: vaginal candidiasis, *Candida* spp., Candida chromogenic agar, ITS-rDNA, Duhok, Iraq.

Резюме

Вагиналната кандидоза е един от най-честите източници на възпаление на влагалището при пациентки, посещаващи гинекологични клиники. Настоящото проучване има за цел да изолира и идентифицира видовете *Candida* при бременни и небременни жени от различни възрастови групи, посещаващи родилни болници в Духок, Ирак чрез конвенционални и молекулярни методи. Взети са проби от вагинални тампони от 385 жени на възраст 15-60 години със съмнение за вулвовагинална кандидоза. Всички проби първоначално се култивират върху Сабуро-декстрозен агар на (СДА с добавен 0.05% (тегло/обем) хлорамфеникол и се инкубират при 37°C за 48 часа. За предполагаемо идентифициране на видовете *Candida*, всички растящи колонии са субкултивирани върху Harlequin TM Candida Chromogenic агар (CHROM agar Company, Acumedia Neogen, UK) при 35°C за 48 часа. Молекулната идентификация е постигната чрез секвениране на ITS1 и ITS4 регионите на рДНК. Четири вида *Candida* са идентифицирани както чрез конвенционални, така и чрез молекулярни методи. Въз основа на резултатите от PCR анализа, *C. albicans* (47%) е най-често срещаният вид, следван от *C. glabrata* (36.2%), *C. krusei* (7,4%), докато *C. tropicalis* (2.2%) показва най-ниска честота. Вагиналната кандидоза е често срещан проблем сред жените в град Духок и е по-честа във възрастовите групи от 15-36 години както на бременни, така и на небременни жени. Резултатите от проучването върху чувствителен хромогенен агар за идентифициране на видове *Candida* показва наличието им в 91.7% от пробите, а PCR-базираната техника установява видове *Candida* в 97.49% от тях.

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Introduction

Vaginal Candidiasis is the most common source of inflammation of the vagina in patient women attending maternity hospitals.*Candida* infection is likely to occur during pregnancy or reproductive age (Anderson *et al*., 2004). The probability of infection with *Candida* for the non-pregnant individual is less than 20%, while it may reach 30% during pregnancy (Aguin and Sobel, 2015). Vulvovaginal infection is invasion and colonization of *Candida* species in the vagina or vulva which as consequence showed signs of itching, vagina soreness, pruritus in the vulvae, burning sensation and irritation during urination, malodorous discharge, besides painful sexual intercourse (Fan *et al*., 2008; Willems *et al*., 2020). *Candida albicans* is considered to be the most frequently isolated species with a prevalence of around 90% (Ramirez-Lozada *et al*., 2019). Some other studies reported high occurrence of *Candida* spp., especially *C. glabrata, C. krusei*, *and C. parapsilosis* (Babić and Hukić, 2010; Mohammed *et al*., 2015). Certain factors may affect the incidence of infection in the reproductive system such as microbial diversity, incidence of some microflora such as *Lactobacillus* spp., which play major roles in equilibrium and their absence may result in the overgrowth of opportunistic pathogens leading to development of vulvovaginal candidiasis (Ma *et al*., 2012; Kamińska and Gajecka, 2017). Although *C. albicans* presents as vaginal mycobiota and cause no infection, in certain situations *Candida* changes into a pathogen, causing candidiasis and colonizing different sites of the body (Yang, 2003; Goncalves *et al*., 2016).

As stated in many research papers, almost 75% of maternity infections are candidiasis, with women having the infection at least once in their lives (Ilkit and Guzel, 2011; Gonçalves *et al*., 2016). The rate of infection increases due to some factors such as pregnancy, diabetes mellitus, steroid and contraceptives drugs and immuno-suppressive treatment, HIV patients (human immunodeficiency virus), patients with indwelling catheters and prosthetic implants, who have long broad-spectrum antibiotics treatment (Goncalves *et al*., 2016; Godoy *et al.*, 2018). The classical techniques for detection of the species of *Candida* include microscopic examination, production of germ tube and chlamydospore, and the morphological characteristics of colonies on chromogenic candida agar (Pfaller *et al.,* 1996; Çetinkaya *et al.*, 2003). Molecular analysis such as Polymerase Chain Reaction (PCR) based techniques is the best method for detection of *Candida* spp. with high precision and sensitivity (Neppelenbroek *et al.*, 2006).

In Iraq, most research studies on vaginal candidiasis focus on epidemiology, identification of causative yeasts and study of some predisposing and risk factors (Abdullah *et al.*, 2001; Khudor *et al*., 2002; Habib *et al*., 2007; Alsharifi, 2017; Abdullah, 2020). Few studies in Iraq have used molecular methods for the detection of *Candida* species involved in vaginal candidiasis (Imran and Al-Shukry, 2014; Mohammed *et al*., 2015; Habib *et al.,* 2016).

The present study aimed to isolate and diagnose *Candida* species from pregnant and non-pregnant women showing vaginal candidiasis, using both conventional and molecular methods.

Materials and Methods

Vaginal swab samples were prospectively collected from 385 patients with suspected vulvovaginal candidiasis in Zakho Maternity hospital. The study was carried out from 1st February to April 2019. High vaginal swabbing was cultured on (SDA) Sabouraud's dextrose agar with the addition of (0.05%) chloramphenicol to inhibit any bacterial contamination, and incubated at 37ºC for 48 hours (Bhavan *et al*., 2010). Growth colonies were sub-cultured on Harlequin™ Candida Chromogenic agar (CHROM agar Company, Academia, Neogen, UK) at 35ºC for 48 hours. Presumptive detection of *Candida* spp. was made depending on the changes of colony color (Pfaller *et al*.,1996).

All *Candida* colonies were sub-cultured on enrichment peptone Dextrose (YPD) Broth at 37ºC for 24 hours for further DNA analysis. The genomic DNA extract used genomic yeast DNA purification kits with spin column technique. All extracted genomic DNA was suspended in 50 µl of Elution buffer (EB), then frozen at 4°C. The concentration and purification of all isolated DNA were checked with the Nanodrop. For DNA sequencing two sets of primers ITS1 (5′- TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) were used for amplification (White *et al*., 1990).

The PCR reactions were performed in a 30 µl reaction tube consisting of approximately 15 µl of Crystal Hot Star Taq DNA Master Mix 2X, 2 µl of target DNA, 2 µl of forwarding primer ITS1 and 2 µl of reverse primers and 9 µl of PCR-grade water. The reaction mixture was kept in a single tube. The PCR cycling protocol was achieved by using Touchdown PCR protocols, as follows: one cycle of initial temperature of denaturation step was 95°C

for 5 min. Then ten cycles of denaturation at 94°C for 30 seconds, the annealing step was at a temperature of 60°C for 45 seconds, and extension step was at 72°C for 90 seconds, and 25 cycles followed by denaturation at 94°C for 30 seconds, annealing temperature at 55°C for 45 seconds, and extension at 72°C for 90 seconds. Then the final extension step at a temperature of 72°C for 10 minutes. The PCR products were run on agarose gel electrophoresis. All PCR products obtained from *Candida* species were sent to Macrogen Company (Korea) for sequencing of DNA and sequence analysis database such as Basic Local Alignment Search Tool (BLAST) was used to identify the obtained DNA sequences (http:// www.ncbinlm.nih.gov/BLAST). All sequences were purified and aligned using BioEdite.

Data analysis

SPSS software was used to analyze the data (Statistical Package for the Social Sciences, version). The relative proportions were calculated with a confidence interval of 95%. To determine the association between variables, the chi-squared (x^2) test was used; a p-value < 0.05 was considered significant, and more than that insignificant.

Results

High vaginal swab samples (n=385) were collected for detection of *Candida* species from women attending maternity hospital, 35% of samples (n=135) had growth of *Candida*. In general, the prevalence of *Candida* distribution related to age reveals that women within the age range 26 to 36 years have the highest percentage (53%) followed by women from 15 to 25 years (44%) (Table 1). The result among pregnant women shows that the highest ratio of infections is 31% (n=31) among women aged within 26-36 years, followed by 28% (n=28) among women aged 37-47 years, while 21% (n=21) was documented among women within the age range from 15 to 25 years. The age group 48-60 years shows the least percentage of 8.2% (n=7). As the table shows, on the one hand, there is a significant difference in age groups 15-25 and 48-60 among non-pregnant, (23% and 0%), respectively. The same can be said about the age group 37-47, which scored (3%) which is near to (0%). On the other hand, in the age groups 26-36 and 47-60 for pregnant, it is clear that the difference is significant, 31% and 8.2, respectively.

All positive species on SDA were again sub-cultured on Chromogenic Candida agar (Figure 1). The percentages were as follows; *C. albicans* was 44.4%, 33.3% for *C.glabrata* , *C.tropicalis* was 2.2% and *C. krusei* was 8.1%, while the percentage of subjects infected with both *Candida* species (*albicans* and *glabrata*) was 4.4%. Only 1% of the samples did not growth on the medium. The percentage of infection among pregnant women (71.1%) was higher than in non-pregnant women (28.8%) (Table 2). CHROMOgenic Candida Agar was used as it was considered as one of the primary identification mediums for the detection of four species of *Candida* based on colony colors (*C. albicans, C. glabrata, C. krusei* and *C. tropicalis*) (Fig. 1).

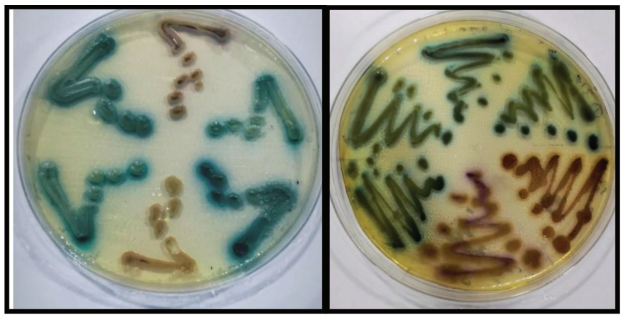

Fig. 1. Appearance of *Candida* colonies on chromogenic candida agar showing different colors for different species: *C. albicans* (green), *C. tropicalis* (blue), *C. glabrata* (white to purple), *C. krusei* (purple to pink)

Table 1. Prevalence of *Candida* spp. on SDA amongst different age group women patients related to pregnancy

Age Group (Years)	No. of Sample examined	Vaginal Swab		
		Non-Pregnant No. (%)	Pregnant No. (%)	Total Positive No. (%)
15 – 25	100	23/100 (23%)	21/100 (21%)	44/100 (44%)
26 – 36	100	22/100 (22%)	31/100 (31%)	53/100 (53%)
37 – 47	100	3/100 (3%)	28/100 (28%)	31/100 (31%)
48 – 60	85	0/85 (0%)	7/85 (8.2%)	7/85 (8.2%)
Total	385	48/385 (12.4%)	87/385 (22.5%)	135/385 (35%)

P value = 0.0002 analyzed using the SPSS software using the chi-squared (x2) test.

Table 2. The occurrence of *Candida* spp. on CHROM genic Candida agar among women

Species	Vaginal Swab		
	Non- Pregnant No (%)	Pregnant No (%)	Total Positive No (%)
C. albicans	(20/60) 33.3%	(40/60) 66.6%	(60/135) 44.4%
C. glabrata	(14/45) 31.1%	(31/45) 68.8	(45/135) 33.3%
C. tropicalis	0	(3/3) 100%	(3/135) 2.2%
C. krusei	(2/11) 18.1%	(9/11) 81.8%	(11/135) 8.1%
C. albicans & *C. glabrata*	(2/6) 33.3%	(4/6) 66.7%	(6/135) 4.4%
Not detected on Chromogenic agar	(1/10) 10%	(9/10) 90%	(10/135) 7.4%
Candida spp. identification	(38/39) 97.4%	(87/96) 90.6%	(125/135) 92.5%
Total	(39/135) 28.8%	(96/135) 71.1%	(135/135) 100%

P value: 0.66 analyzed using the SPSS software using the chi-squared (x2) test

Regarding the PCR results, two universal primers (ITS1 and ITS4) were used to amplify DNA fragments from DNA template isolated from clinical samples, all PCR products were run on agarose gel and showed sizes ranging from 500 to 1000 base pair as shown in Fig. 2.

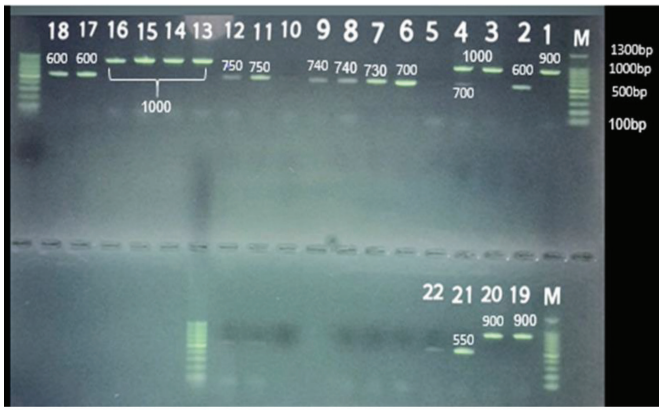

Fig. 2. PCR products showed specific bands on agarose gel electrophoresis *C. albicans* (Lane 2, 17, 18), *C. glabrata* (Lane 1, 3-4, 13-16, 19-20), *C. krusei* (Lane 6-9, 11, 12), *C. tropicalis* (Lane 21, 22).

In all PCR products BioEdite software program was used DNA sequencing alignment, then the resulting sequences were compared against Genebank database and linked to other strains of *Candida* (references) isolated from human hosts with a match ranging between 96% and 100% (Table 3).

Data of DNA sequencing of *Candida* species were analyzed by the Neighbor-Joining method using MEGA 7 (Tree- View software) to construct a phylogenetic tree and to compare with references strains (Fig. 3).

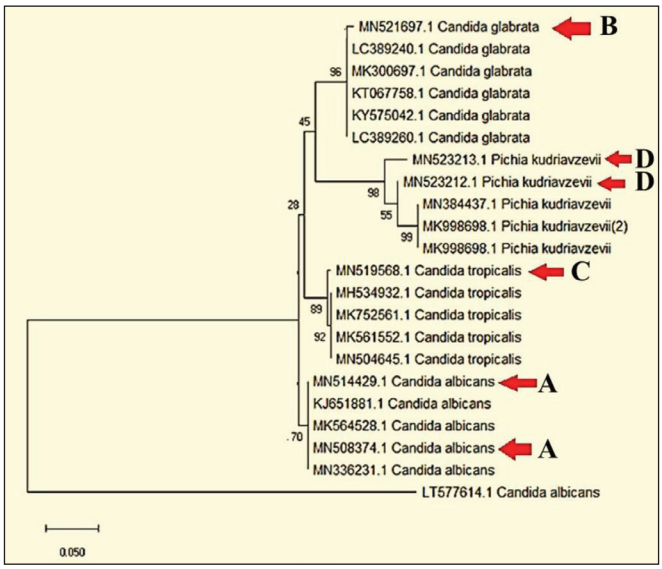

Fig. 3. Phylogenetic tree for *Candida* species using Neighbor-Joining method for *Candida* species isolated from vaginal samples.

Table 3. and Fig. 3. illustrate sequences of isolated species from vaginal swabs as follows: the two isolated strains of *C. albicans* (MN514429) isolated from pregnant women and (MN508374) isolated from non-pregnant women; both showed 99% similarity to *C. albicans* from Brazil (KJ651881), Saudi Arabia (MK564528), Iraq (LT577614, MN336231) and Iran (MN336231). The strain *C. glabrata* (MN521697) isolated from non-pregnant women shared 99% similarity to *C. glabrata* strain from Saudi Arabia (MK300697) and 98% similarity to other strains found in Iran (LC389260 and LC389260), Mexico (KY575042) and China (KT067758). The sequence of *C. krusei* (*Pichia kudriavzevii*) (MN523212) isolated from pregnant women and (MN523213) isolated from non-pregnant women shared 100% similarity to

Table 3. Accession numbers of isolated *Candida* spp. compared to references strains isolated from human from different locations

Species	Accession No.	Strain No.	Host (Human)	Region	Similarity %
C. albicans	MN514429	h55b	Vagina/Pregnant	Iraq	100 %
C. albicans	KJ651881	HC19C	Vagina	Brazil	99 %
C. albicans	LT577614	IQMunaff2 9	Oral	Iraq	99%
C. albicans	MK564528	CA10.2	Vaginal Swabs	Saudi Arabia	99%
C. albicans	MN508374		Vagina/Non-Pregnant	Iraq	100 %
C. albicans	MN336231		Vagina	Iran	99 %
C. krusei (Pichia kudriavzevii)	MN523212	CY902	Vagina/Pregnant	Iraq	100 %
C. krusei (P. kudriavzevii)	MN523213	m76b	Vagina/ Non- Pregnant	Iraq	100 %
C. krusei (P. kudriavzevii)	MN384437	ZAHZK	Human	Iraq	100 %
C. krusei (P. kudriavzevii)	MK998698	RCPF 1406	Vagina	Iran	100 %
C. krusei	MK998698	RCPF 1406	Vulvovaginitis	Russia	98%
C. glabrata	MN521697	CG-3PI	Vagina Non- Pregnant	Iraq	100 %
C. glabrata	LC389260	H155	Vagina	Iran	98%
C. glabrata	KY575042	cvv15m	Vaginal Swab	Mexico	98%
C. glabrata	KT067758	36(O) (1_0330)	Clinically	China	98%
C. glabarta	MK300697	CG09.2	Vagina	Saudi Arabia	99 %
C. glabrata	LC389240	A113b	Vagina	Iran	98%
C. tropicals	MN519568	19	Vagina Non- Pregnant	Iraq	100%
C. tropicals	MH534932	AUMC 13542	Vagina	Egypt	100 %
C.tropicalis	MK752561		Vaginal	Iraq	99%
C.tropicalis	MK561552	CT02.1	Vaginal Swabs	Saudi Arabia	99%
C. tropicals	MN504645		Urine DM	Iraq	100 %

other strains of *C. krusei* from Iraq (MN384437), Russia (MK998698) and Iran (MK998698). The phylogenetic tree analysis for the species *C. tropicalis* (MN519568) isolated from non-pregnant women showed 100% homology with the *C. tropicalis* strains from Iraq (MN504645) and Egypt (MH534932) and also showed 99% homology to strains from Saudi Arabia (MK561552) and Iraq (MK752561).

The method which was used for inferred evolutionary history was Neighbor-Joining. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is close to the branches. Evolutionary analyses were conducted in MEGA7. (A) Accession numbers of *C. albicans* isolated in this study (MN514429, MN508374) compared with reference strains, (B) accession numbers of *C. glabrata* isolated in this study (MN521697) compared with reference strain, (C) Accession number of *C. tropicalis* isolated in this study (MN519568) compared with reference strain, (D) accession numbers of *Pichia kudriavzevii* (*C. krusei*) isolated in this study (MN523212, MN523213) and reference strains.

The molecular study of collected specimens indicated that about 92.8% of the samples had single *Candida* spp., 47% of individual shave *C. albicans*, *C. glabrata* showed 36.2%, 7.4% for *C. krusei*, 2.2% for *C. tropicalis* and 3.7% *C. albicans* and *C. glabrata*. Sequencing of the PCR products produced bands for 131 samples (97 %) out of 135 samples (Table 4).

The percentage of specificities in the present research is as follows: Chromogenic agar showed 91.7%, while PCR-based technique was 97.49% (Fig. 4).

Table 4. PCR based detection of *Candida* spp.

Candida spp.	PCR-based identification
	Vaginal samples
C. albicans	(64/135) 47%
C. glabrata	(49/135) 36.2%
C. tropicalis	(3/135) 2.2%
C. krusei	(10/135) 7.4%
C. albicans + *C. krusei*	----------
C. glabrata + *C. tropicalis*	----------
C. albicans + *C. glabrata*	(5/135) 3.7%
Not identification	(4/135) 2.9%
PCR-based for identification *Candida* species	(131/135) 97%

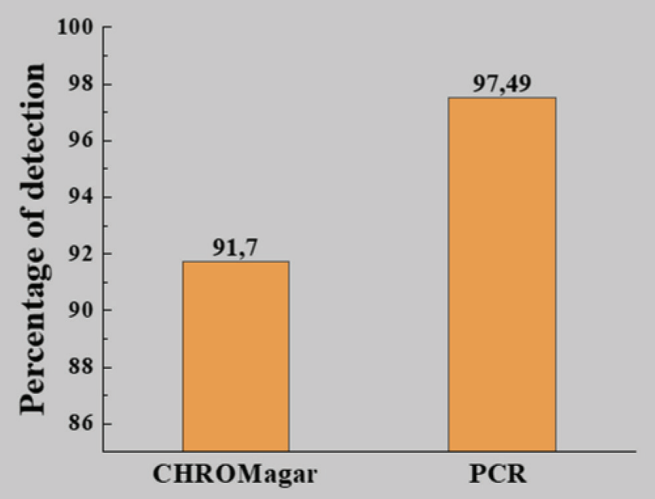

Fig. 4. Sensitivity of identifying methods for clinical isolates of *Candida* species.

Discussion

The highest proportion of candidiasis is among women with ages ranging from 26 to 36 years. This finding is in agreement with other research papers reporting women in the age range from 26 to 36 years as having a high ratio of candidiasis (Hedayati *et al*., 2015; Alsharifi, 2017; Kiasat *et al*., 2019; Waikhom *et al*., 2020). Likewise, women aged 26 to 36 years display changes in the level of hormones during pregnancy, therefore, vulvovaginal candidiasis is more common in women during pregnancy (Nohmi *et al*.,1995; Ahmad and Khan, 2009; Waikhom *et al*., 2020).

In the current study, isolated species of *Candida* were sequencing by using the two primers ITS-1 and ITS-4, and were likened with other isolated species (China, Iran, Saudi Arabia, Egypt, Russia and Mexico). The investigation shows that the similarity between the isolated strains and references strains of *Candida* is between 98% and 100%. The phylogenetic tree reveals high kinship (Fig. 3).

In general, our study concludes that *C. albicans* is the most commonly isolated strain with a prevalence of 47%, while *C. glabrata* is considered as the second most isolated species with a ratio of 36.2%. This finding is in line with other papers conducted by (Khudor *et al*., 2002; Güzel *et al*., 2013; Kiasat *et al*., 2019) but differs from other research studies (Trama *et al*., 2005; Mohammed *et al*., 2017; Waikhom *et al*., 2020), which shows a high prevalence of some non-albicans *Candida* strains particularly *C. glabrata*. This may be due to the development of antifungal resistance among *Candida* species besides the low immune system against the infection or due to alteration in hormones levels (Trama *et al*., 2005). Our results demonstrate that the ratio of mixed infection is highly related to other research studies conducted in China (Fan et al., 2008), the United States (Richter *et al*., 2005), and Iran (Mahmoudi Rad *et al*., 2012).

As shown in the phylogenetic tree (Fig. 3), the molecular weights of PCR products are diverse among *C. albicans, C. glabrata, C. krusei,* and *C. tropicalis*. The use of PCR methods with the ITS1 and ITS4 primer is rapid and accurate. Although the detection based on morphological characteristics of yeast colonies on Chromogenic Candida agar is considered as the possible identification for four species of *Candida*, it is impossible to distinguish other species of *Candida*, hence the combination of the phenotypic and genotypic method is the best method for detection of all species of *Candida*. Our finding is in agreement with other papers (Kirkpatrick et al., 1998; Sullivan and Coleman, 1998; Costa *et al*., 2010).

Conclusion

Vaginal candidiasis is a common infection among women attending maternity clinics in Duhok province, Iraq, and is more common in the age groups of 15-36 years for both pregnant and

non-pregnant women. Four *Candida* species were identified by both conventional and molecular methods with a prevalence showed by *C. albicans* followed by *C. glabrata*. Accurate identification of yeast species involved in vaginal candidiasis is essential for treatment, particularly the species showing resistance to antifungal drugs. The PCR-based technique is the first choice for accurate species diagnosis.

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