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Determination of fungal and parasitic infections caused vaginitis: molecular identification of Candida parapsilosis in Al-Nasiriyah city, Iraq

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Abstract:

The current study aims to determine the prevalence of *Trichomonas vaginalis* and *Candida* spp., and also to identify *Candida parapsilosis* and some virulence genes. It was conducted in Bint Al-Hoda Hospital of Maternity and Children in Thi-Qar province, south of Iraq for the period from the beginning of January to the end of December 2020. Two hundred and fifty samples were collected from the female genital tract for women whose age ranged between 17-50 years. Microscopic, traditional and molecular tests were used in the sample examination. The results recorded 12 (4.8%) samples infected with *T. vaginalis* parasite, whereas 130 (52%) samples showed *Candida* yeast distributed as follows: 75 (30 %) *C. albicans*, 20 (8%) *C. krusei*, 14 (5.6%) *C. parapsilosisas*, 11 (4.4 %) *C. glabrata* and 10 (4%) *C. tropicalis*. A 18*S rRNA* gene of *C. parapsilosisas* appeared in all samples confirmed with biochemical tests and CHROM agar *Candida.* The *cph1* and *hwp1* genes were observed in all of *C. parapsilosis* isolates (100%), whereas *sap1* and *plb1* genes showed different proportions (64.3% and 57.1%, respectively). Depending on phylogenetic analysis, there was a slight genetic variation between local isolate sequences compared with global recorded strains. The current study confirmed that 18*S rRNA* gene is highly precise to identify *C. parapsilosis*. The appearance or absence of the genetic variation of some virulence genes may cause different clinical manifestations.

Keywords: 18S rRNA, C. parapsilosis, T. vaginalis, Trichomonal vaginitis, vulvovaginal candidiasis.

Introduction:

Vaginitis is considered a common disease among all women that it has a wide spectrum of clinical manifestations. This disease develops to become a chronic, if continued for more than a year[1]. However, vaginitis is usually occurred by disbalance of the vaginal flora, this means some pathogens become dominant such as *Candida*, *Trichomonas* or *Mycoplasma*[2]. Normally, *Candida* species are found in the genital, mucosal alimentary and upper respiratory tracts of human and other animals [3]. *Candida parapsilosis* is an opportunistic fungal pathogen and considered one of the most common *Candida* species found in the clinical specimens[4,5].

Candida parapsilosis complex species show differences in the sensitivity to antifungals, geographical distribution, virulence and formation of biofilm[6]. However, biofilms formation in *Candida* spp. is important for its pathogenicity and also considered as substantial virulence factors. In addition, the site of the infection, species and strain, and the micro-environment play a crucial role in the ability of *C. parapsilosis* to generate biofilms. So, the cell surface proteins, such as *Als1*, *Als2*, *Hwp1*, the cell wall-related protein and *Sun41* have an effective role in occurrence, the adhesion process and the virulence of the biofilm[7]. Modrzewska and Kurnatowski[8] have mentioned that *ALS*, *EPA*, *HWP1* are specific proteins existing on the cell wall at *Candida* spp. that have an important role in adhesion. The production of hydrolytic enzymes, such as phospholipases (PLs) and aspartyl proteinases

(SAPs) is mainly responsible for pathogenicity of *Candida* species, which play an essential role in *C. parapsilosis* adherence, tissue penetration, and host invasion[9]. Pharkjaksu *et al*. [10] confirmed that the clinical isolates of *C. parapsilosis* have many virulence factors like secretion phospholipase and protease enzymes and psedohyphae formation.

For the importance of *C. parapsilosis* as an essential causative agent causes vulvovaginal candidiasis. Moreover, molecular studies are considered necessary to determine and to identify *C. parapsilosis* virulence factors that can be targeted by antifungal agents to control the disease. Generally, this study aims to detect the prevalence of *T. vaginalis* and identify *C. parapsilosis* with PCR technique and to sequence some virulence genes that may increase pathogenicity in *C. parapsilosis*.

Materials and methods:

Sample collection:

Randomly, vaginal swabs and urine samples were collected from 250 women who attended the consulting clinic at Bint Al-Huda Hospital of Maternity and Children in Al-Nasiriyah city, Thi-Qar province/ Iraq. The current study was conducted for the period from the beginning of January to the end of December 2020. The age of the women ranged between 17- 50 years. Urine samples were directly collected then centrifuged and examined with microscopy to determine *T. vaginalis.* All vaginal swabs were cultivated on sabouraud dextrose agar to diagnose *Candida* spp. that may grow.

C. parapsilosis isolates

The phenotype of fungal colonies was checked and identified after the cultivation and incubation on SDA depending on[11] microscopic and traditional tests (Germ tube formation, Sugar fermentation, Chlamydospore formation and Differential medium CHROM agar Candida) were used to identify *C. parapsilosis*[12-14].

Genomic DNA Extraction

Using EZ-10 Spin Column Fungal Genomic DNA Mini-Preps Kit, genomic DNA of *Candida* spp. was extracted according to the produced company protocol and then DNA was checked with Nanodrop-spectrophotometer. Finally, the extracted DNA was stored at -20˚C until to used with PCR technique.

PCR amplification

In the study, 18S *rRNA* gene was used to identify *C. parapsilosis* isolates according to Mousavi *et al.*[15]. The conventional PCR technique was also used to determine some virulence genes (*hwp1, plb1, sap1, cph1,*) in all *C. parapsilosis* isolates. The primers were designed online with NCBI database and Primer 3. They were provided by Bioneer Company (Korea) (Table 1). The PCR master mix was prepared according to AccuPower®PCR PreMix kit (Bioneer, Korea). The PCR master mix reaction components consisted of 5 µl of DNA template, 10 pmol of each F and R primers and 12 µl of PCR water then placed in standard PCR tubes. PCR thermocycler conditions consisted of an initial denaturation set at 95 °C for 5 min, then 30 cycles at 95 °C for 20 s., 60 °C for 20 s. and 72 °C for 1 min. Final extension was at 72 °C for 5 min. PCR products of each gene were electrophoresed using 1% agarose gel with 3µL of ethidium bromide. In each comb well, 10 µl of PCR product was added and also 5 µl of ladder (100bp) was added in one well. Next, the gel tray was fixed and filled with a (1X) TBE buffer inside the chamber. After that, the current was set at 100 volts and 80 mA for 1 hr. Finally, PCR amplification products were imaged with an UV transilluminator.

phylogenetic analysis

Using a purification kit, the gene samples were purified, and one sample was sent to Macrogene Company (Korea) in order sequencing deposited into GenBank for an obtainment of accession numbers. The recorded nucleotide sequences of *C. parapsilosis* isolates were compared with *C. parapsilosis* strains in NCBI GenBank to detect mismatching between genes sequence using the NCBI-Blast, (http://blast.ncbi.nlm.nih.gov/Blast.cgi). phylogenetic tree analysis has based on molecular evolutionary genetic analysis using Mega V. 6.

Table 1. Oligonucleotide primers designed and used in PCR technique

Primer	Sequence (5'-3')		Product Size
18S rRNA* gene *Candida parapsilosis	F	GCTACACTGACGGAGCCAG	507bp
	R	TGCGAGAACCAAGAGATCCG	
***C. parapsilosis HWP1 Adhesion* gene**	F	ATATGTTCCGCGGACGTGTT	351bp
	R	CCTTGCTGACCAACGGAATG	
***C. parapsilosis SAP1 proteinase* gene**	F	GCCTTGGACTTTGATGTGCT	637bp
	R	CTTGCTCGGCAACCAACTTG	
***C. parapsilosis phospholipase* gene**	F	TGAGGATGGGCAAAATGTACCT	407bp
	R	TCAATCTGGTTGTCGCTGCA	
***C. parapsilosis, hyphal formation CPH1* gene**	F	CAGGTTCTTTGGGCAGCAAC	546bp
	R	GCTGCTGCATTTGCCTCTTT	

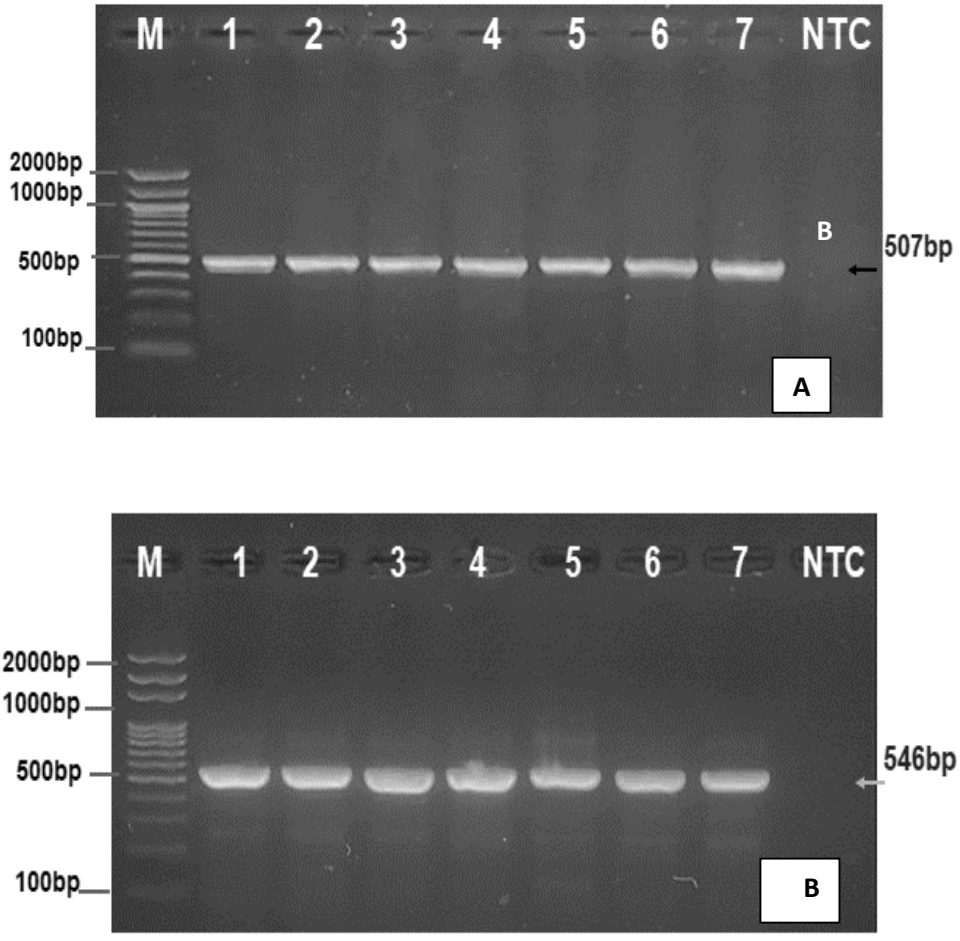
Results:

A total 250 women suspected with vaginitis, 12 (4.8%) samples were infected with T. *vaginalis* parasite (parasitic infection) with microscopy, whereas 130 (52%) samples showed *Candida* spp. yeast (fungal infection) with biochemical tests and CHROM agar *Candida* that distributed as following: 75 (30 %) *C. albicans*, 20 (8%) *C. krusei*, 14 (5.6%) *C. parapsilosisas*, 11 (4.4 %) *C. glabrata* and 10 (4%) *C. tropicalis*.

Molecular identification of C. parapsilosis

All isolates of *C. parapsilosis* were identified with biochemical tests and CHROM agar Candida have confirmed their diagnosis through the presence of 18*S rRNA* gene at 507bp (Fig 1A).

In the current study, PCR product of *cph1* and *hwp1* genes showed in all of *C. parapsilosis* isolates which were molecularly identified in this study at 546bp and 351 bp (Fig 1B, C), respectively. The *sap1* gene appeared in 9 (64.3%) *C. parapsilosis* at 637bp, whereas *plb1* gene detected in 8 (57.1%) of these isolates, which generated at 598bp (Fig 1D, E).



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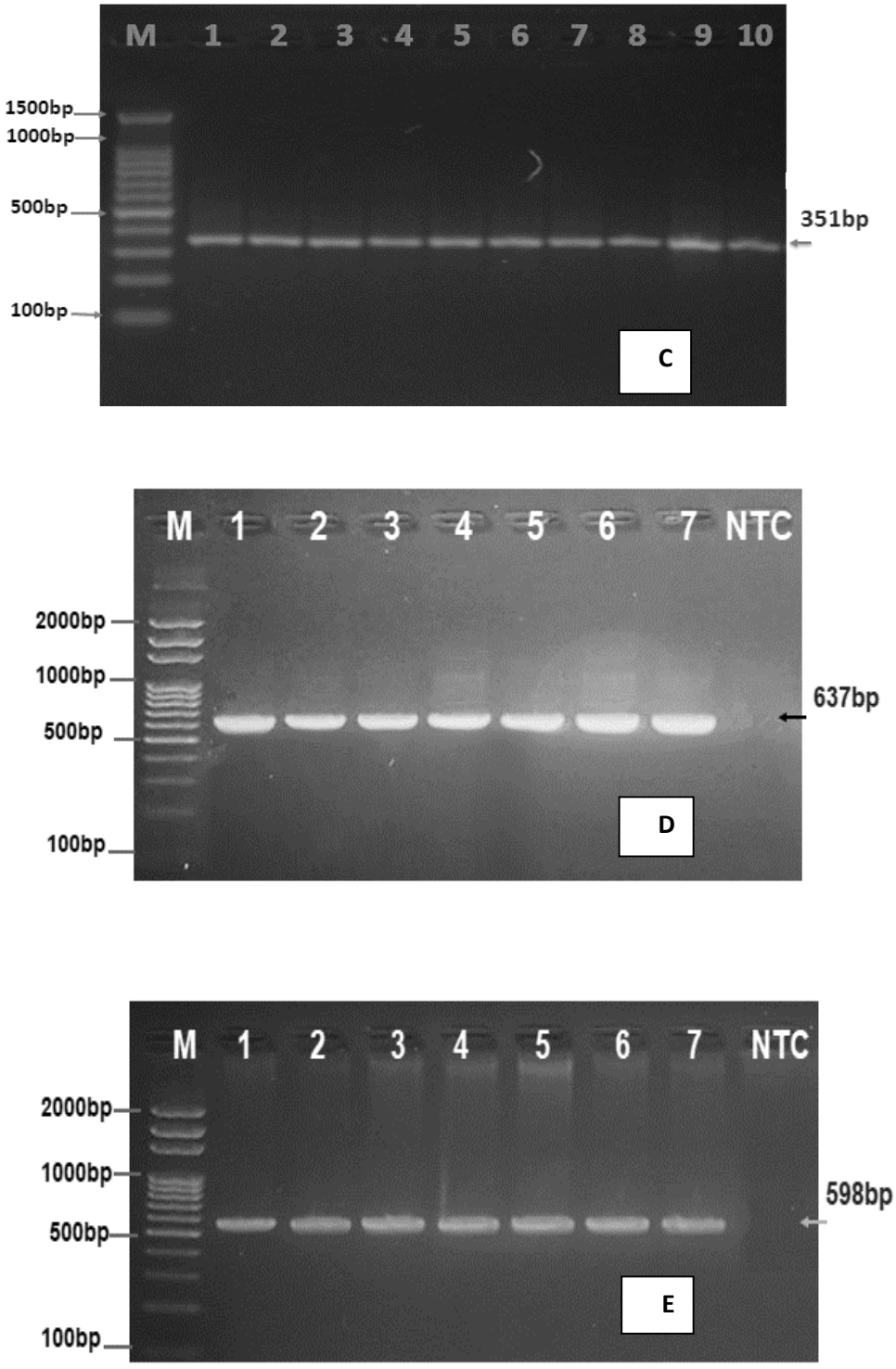
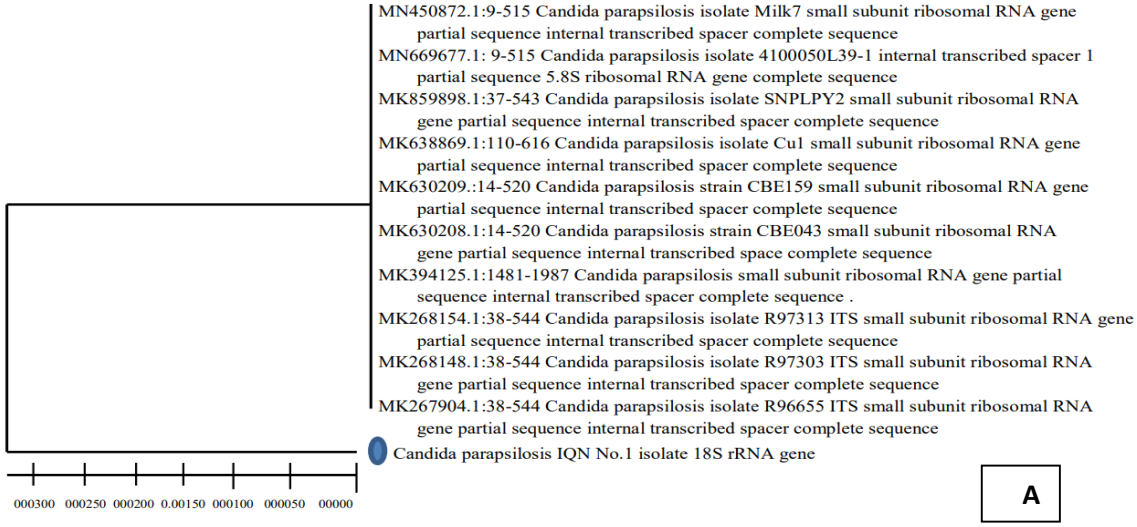


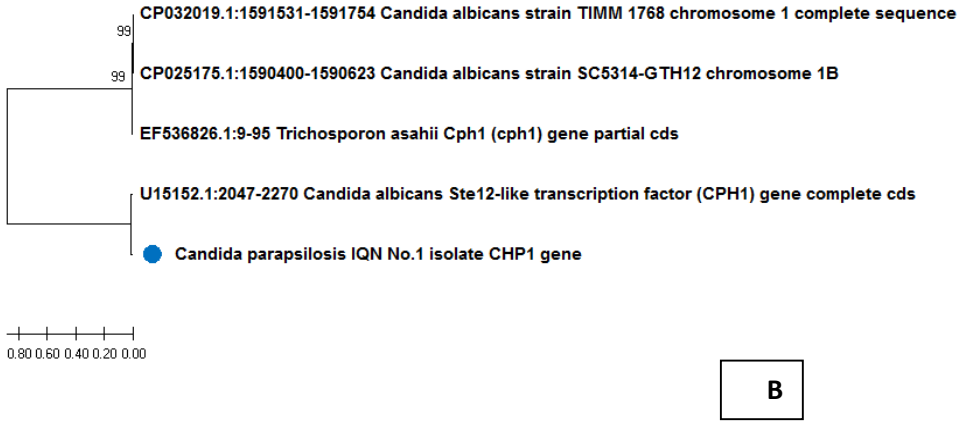
Figure 1. Agarose gel electrophoresis of *C. parapsilosis*. Where Marker ladder (100-2000bp), lane (NTC): Non template negative control. A: Lanes (1-7) positive for 18*S rRNA* gene (A) at 507bp, B: *cph1* gene at 546bp, C: *hwp1* adhesion gene at 351bp, D: *sap1* proteinase gene at 637bp and E: *plb1* gene at 598bp. (Thermal condition of the PCR reaction included an initial denaturation at 95 °C for 5 min, then 30 cycles at 95 °C for 20 s., 60 °C for 20 s. and 72 °C for 1 min. and finally, final extension was at 72 °C for 5 min.).

Phylogenetic analysis

Among all *C. parapsilosis* isolates containing virulence genes in their genome; one isolate was selected and sent for sequencing in order to conduct multiple alignment analysis. The nucleotide sequence of 18*S rRNA* gene showed upstream slight genetic variation. Phylogenetic analysis was observed in the genetic affinity between local isolates and the global strains, where *C. parapsilosis* isolate (MW899046) has showed relatively similarity and genetically related to NCBI-Blast strains, especially 4100050L39-1 (Identity: 99.34%).

Phylogenetic analysis of *cph1* gene in *C. parapsilosis* isolate (MW960357) showed genetic closely related to NCBI-Blast *C. parapsilosis* (U15152.1/USA/ 1995) (Identity: 97.31%) at total genetic change (0.8-0.2 %). AS for *hwp1* gene recorded simple genetic variation (MW960355). It was near NCBI- BLAST isolate (KX758629.1) isolated in Italy 2016, (Identity: 99.00%) at genetic change (0.08- 0.2%). Phylogenetic tree of *sap1* gene of local *C. parapsilosis* isolate (MW960356) showed a similarity related to NCBI-Blast *C. parapsilosis* isolate (FJ560879.1) (Identity: 99.32%) at total genetic change (0.4 -0.1 %), while local *C. parapsilosis* isolate of *plb1*gene (MZ020782) showed homology identity to NCBI-BLAST isolate (AY544775.1/ Japan/2018) (Identity: 98.91%) at total genetic change was (0.4 -0.1 %) (Fig. 2).





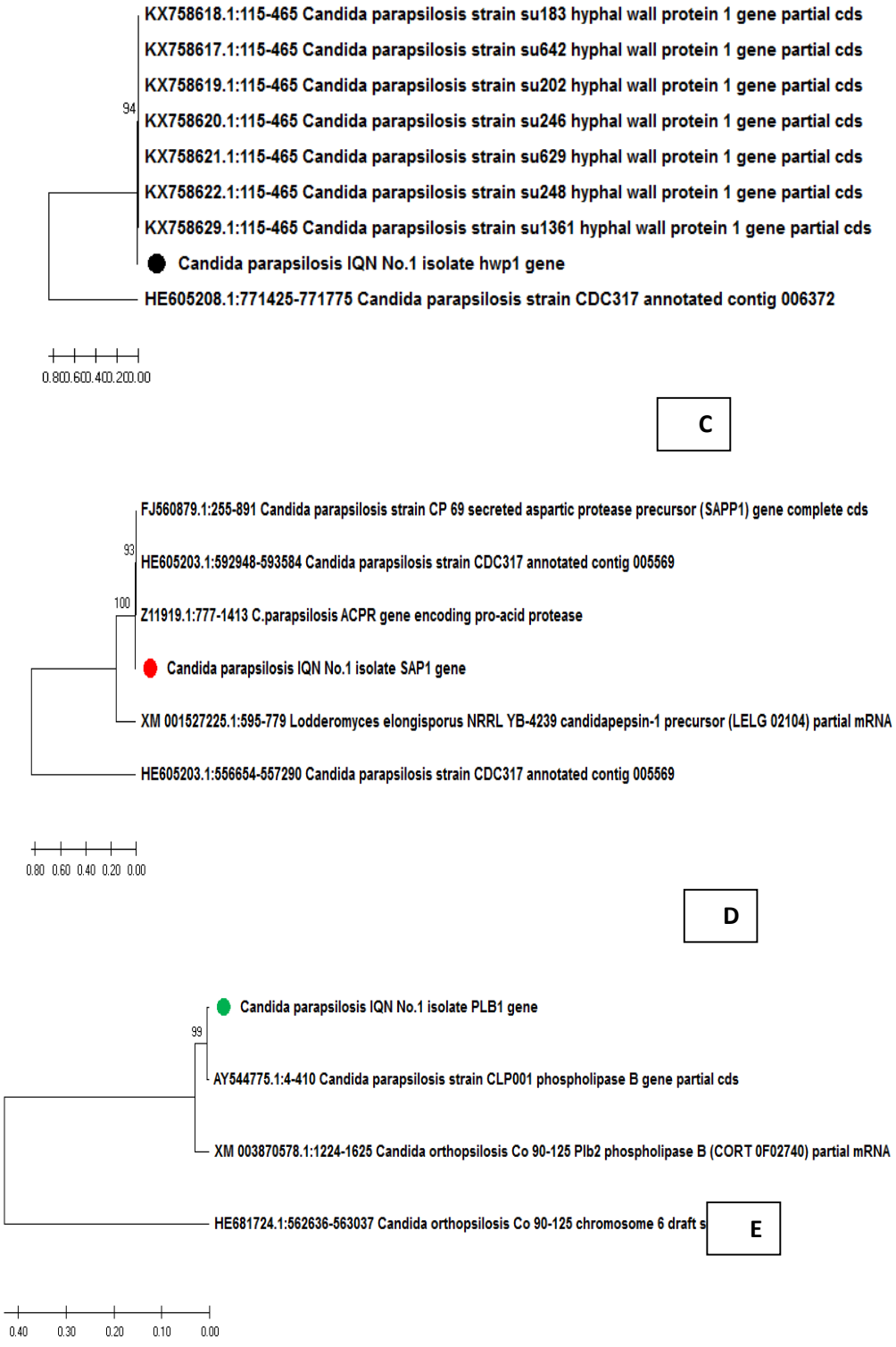


Figure 2. Phylogenetic analysis using Maximum Likelihood method (MEGA 6.0 version). A: 18*S rRNA* gene sequencing of local *C. parapsilosis* (MW899046) isolate at total genetic changes (0.003-0.0005%), B: *cph1* gene partial sequence in local *C. parapsilosis* (MW960357) isolate at total genetic changes (0.8-0.2%), C: *hwp1* gene partial sequence in local *C. parapsilosis* (MW960355) isolate at total genetic changes (0.8- 0.2%), D: *sap1* gene partial sequence in local *C. parapsilosis* (MW960356) isolate at total genetic changes (0.8- 0.2%), E: *plb1* gene partial sequence in local *C. parapsilosis* isolate at total genetic changes (0.4- 0.1%).

Discussion:

Overall, T. *vaginalis* parasite causes a common infection that is transmitted by sexual contact [16, 17]. In the current study, *T. vaginalis* recorded 4.8% of 250 samples. The finding is nearly consistent with Kadir and Fattah, 2010[18]. The determination of non-*C. albicans* species in vaginal candidiasis is essential where some of these species have resistance to the azole compounds used to treat this infection[19, 20]. The common species recorded in this study included *C. tropicalis*, *C. glabrata*, *C. krusei*, and *C. parapsilosis*. According to this study, the prevalence of *C. parapsilosis* was 10.7%. Other authors have reported the prevalence of 8.5% for *C. parapsilosis* in patients with VVC[21]. Bitew and Abebaw [22] have recorded 2.3 % for *C. parapsilosis* in their study of *Candida* isolated from 210 infected women with vulvovaginal candidiasis.

In the current study, 18*S rRNA* gene was used to identify *C. parapsilosis*. This gene was demonstrated to detect different *Candida* species regardless of morphological form of growth[23]. The results showed the presence of this gene in all isolates at 507bp and this is comparable with isolation methods with chemical tests as well as by CHROM agar *Candida*. This result is consistent with Kanwal *et al*. [24] whose study used specific regions (18S, 5.8S and 28S) of ribosomal RNA genes to diagnose *C. parapsilosis*.
In the study, virulence genes showed a variation in its presence inside *C. parapsilosis* genome. During infestation, pathogenic fungi excrete several hydrolytic enzymes so as to facilitate a penetration of the host. Generally, these secreted enzymes deactivate the membrane of the host cell and damage the extracellular matrix and host tissues. Hydrolytic enzymes in fungi may support cell adhesion, intracellular survival or biofilm formation [4]. Ramos *et al*. [25] have found that the clinical isolates of *C. parapsilosis* taken from patients of cutaneous candidiasis (15/16) actively produced protease (saps). Conventional-PCR was performed for determination of virulence factors [26].

There is a broad diversity of sap production among *C. parapsilosis* isolates derived from surfaces of the host like skin or vaginal mucosal layer, but these isolates secrete more than those obtained from the environment or systemic infections. This explains that surface isolates are more virulent[4]. *C. parapsilosis* secretes proteases that support an existence of the yeast in the host, raise the resistance of phagocytosis, and destroy the host cell and also facilitate intracellular survival. In addition, they inhibit host defense proteins such as antimicrobial, complement or antibodies[27, 28]. There are many studies refer fungal phospholipases may promote virulence by damage membranes of the host cell [27, 29]. Neji *et al*.[6] have found 111/ 172 (63.5%) clinical *C. parapsilosis* isolates were positive for phospholipase enzyme production. The *plb* may play an essential role through direct breakdown of host cell membranes. The lesion would allow elements of fungal hyphal to more effectively cross the vascular endothelium, this leads to increase the rapidity of dissemination to and invasion of target organs[30, 31].

In the present study, the *hwp1*gene has been detected in all *C. parapsilosis* isolates, this is consistent with Abastabar *et al*.[32] and Nikmanesh et al. [33] who observed that *hwp1* gene was an excellent marker to detect non- *Candida albicans* species. Modrzewska and Kurnatowski [8] have mentioned that *als, epa, hwp1* are considered the most important adhesins found on the cell wall of *Candida* spp. It is also considered responsible gene of the fungal hyphae formation. Naglik *et al*.[34] have mentioned that expression of *hwp1* gene is high among strains isolated from patients with candidiasis.

The results showed that *cph*1 gene was found in all isolates of *C. parapsilosis.* The transcription factor genes *cph1* regulate cell wall biosynthesis and involvement in virulence and hyphae are necessary to develop a biofilm. In addition are positive regulators of hyphal morphogenesis[11].

Conclusion:

There are few studies on identifying and detecting non-*C. albicans* species responsible for vaginitis in Thi-Qar province. The study has shown that 18*S rRNA* gene has high sensitivity to identify *C. parapsilosis*. The virulence genes show a variation in its presence within *C. parapsilosis* genome, which may explain a difference of the disease severity. In addition, the genetic variation of virulence gene sequences among isolate may result in different clinical manifestations.

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Authors' declaration:

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are ours. Besides, the Figures and images, which are not ours, have been given the permission for re-publication attached with the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee in Al-Ayen University.

Authors' contributions statement:

MSA collected the samples. ERA design of the work and wrote original manuscript. MHF analyzed data. MHF and KRH read and modified the manuscript carefully.

Ethical approval

The protocol of this study was obtained from college of pharmacy, Al-Ayen University (16 at 8/1/2020) and approved by management of training and human development department/ Thi-Qar Health Office (2022133).

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تحديد الإصابة الطفيلية والفطرية المسببة لالتهاب المهبل: التشخيص الجزيئي لـ *Candida parapsilosis* بين النساء في مدينة الناصرية/ العراق

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الخلاصة:

هدفت الدراسة الحالية لتحديد انتشار طفيلي *Trichomonas vaginalis* وفطريات *Candida* spp. وكذلك التعرف عن *Candida parapsilosis* وبعض جينات الضراوة. اجريت هذه الدراسة في مستشفى بنت الهدى للولادة والاطفال في محافظة ذي قار, جنوب العراق للفترة من بداية شهر كانون الثاني الى نهاية شهر كانون الاول 2020. تم جمع 250 عينة من المنطقة التناسلية الانثوية لنساء تتراوح اعمارهم من17-50 سنة. استخدم كل من التشخيص المجهري والتقليدي والجزيئي في فحص العينة. سجلت النتائج 12 (4.8%) عينة مصابة بطفيلي *T. vaginalis*, بينما 130 (52%) عينة اظهرت مصابة بـ *Candida* spp. والتي توزعت كالاتي: 75 (30%) *C. albicans*, 20 (8%) *C. krusei*, 14 (5.6%) *C. parapsilosisas*, 11 (4.4%) *C. glabrata* و 10 (4%) *C. tropicalis*. ظهر جين *18S rRNA* في كل عينات *C. parapsilosisas* التي اكدت مع الفحوصات الكيموحيوية ووسط CHROM. لوحظت الجينات *cph1* و*hwp1* في كل عزلات *C. parapsilosis* بنسبة (100%), بينما جينات *sap1* و*plb1* ظهرت مع نسب مختلفة (64.3%, 57.1%) على التوالي. اعتمادا على تحليل الشجرة التطورية, كان هناك تغاير جيني خفيف بين تتابعات العزلات المحلية مقارنة مع السلالات المسجله عالميا. اكدت الدراسة الحالية ان جين *18S rRNA* يمتلك حساسية عالية في تشخيص *C. parapsilosis*. ان ظهور او غياب و/او وجود التغاير الجيني في بعض جينات الضراوة قد يسبب اعراض سريرية مختلفة.

الكلمات المفتاحية: فطر*C. parapsilosis*, طفيلي *T. vaginalis*, *18S rRNA*, التهاب المهبل المشعري, داء المبيضات المهبلي