Determinant 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. parapsilosis, 11 (4.4%) C. glabrata and 10 (4%) C. tropicalis. A 18S rRNA gene of C. parapsilosis 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 18S 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. However, vaginitis is usually occurred by disbalance of the vaginal flora, this means some pathogens become dominant such as Candida, Trichomonas or Mycoplasma. Normally, Candida species are found in the genital, mucosal alimentary and upper respiratory tracts of human and other animals. Candida parapsilosis is an opportunistic fungal pathogen and considered one of the most common Candida species found in the clinical specimens. Candida parapsilosis complex species show differences in the sensitivity to antifungals, geographical distribution, virulence and formation of biofilm. 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. Modrzewska and Kurnatowski 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. Pharkjaksu *et al.* confirmed that the clinical isolates of *C. parapsilosis* have many virulence factors like secretion phospholipase and protease enzymes and pseudohyphae 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 microscopic and traditional tests (Germ tube formation, Sugar fermentation, Chlamydoospore formation and Differential medium CHROM agar Candida) were used to identify *C. parapsilosis*.

**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.* 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 Macrogen 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

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'→3')</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S rRNA gene Candida parapsilosis</td>
<td>GCTACACTGACGGAGCCAG</td>
<td>507bp</td>
</tr>
<tr>
<td>C. parapsilosis HWPI</td>
<td>ATATGTTCCGCGGACGTG</td>
<td>351bp</td>
</tr>
<tr>
<td>Adhesion gene</td>
<td>CTTTGTGACCAACGGAATG</td>
<td></td>
</tr>
<tr>
<td>C. parapsilosis</td>
<td>GCCCTGGACCTTGTGCT</td>
<td>637bp</td>
</tr>
<tr>
<td>SAP1 proteinase gene</td>
<td>CTGCTCGCAACCACTTG</td>
<td></td>
</tr>
<tr>
<td>C. parapsilosis phospholipase gene</td>
<td>TGAGGATGGGCAAAATGACCT</td>
<td>407bp</td>
</tr>
<tr>
<td>C. parapsilosis hyphal formation CPH1 gene</td>
<td>GCGCTGCATTTTGCTCTTT</td>
<td></td>
</tr>
</tbody>
</table>

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. parapsilosis, 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 18S 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).
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 18S 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 18S 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-Blat strains, especially 4100050L39-1 (Identity: 99.34%).

Phylogenetic analysis of cph1 gene in C. parapsilosis isolate (MW960357) showed genetic closely related to NCBI-Blat 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-Blat 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/ Italy/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: 18S rRNA gene sequencing of local \textit{C. parapsilosis} (MW899046) isolate at total genetic changes (0.003-0.0005\%), B: \textit{cphl} gene partial sequence in local \textit{C. parapsilosis} (MW960357) isolate at total genetic changes (0.8-0.2\%), C: \textit{hwp1} gene partial sequence in local \textit{C. parapsilosis} (MW960355) isolate at total genetic changes (0.8-0.2\%), D: \textit{sap1} gene partial sequence in local \textit{C. parapsilosis} (MW960356) isolate at total genetic changes (0.8-0.2\%), E: \textit{plb1} gene partial sequence in local \textit{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. In the current study, *T. vaginalis* recorded 4.8% of 250 samples. The finding is nearly consistent with Kadir and Fattah, 2010. The determination of non-*C. albicans* species in vaginal candidiasis is essential where some of these species have resistance to theazole compounds used to treat this infection. 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. Bitew and Abebaw have recorded 2.3% for *C. parapsilosis* in their study of Candida isolated from 210 infected women with vulvovaginal candidiasis.

In the current study, 18S rRNA gene was used to identify *C. parapsilosis.* This gene was demonstrated to detect different *Candida* species regardless of morphological form of growth. 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. 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. Ramos et al. 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.

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. *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. There are many studies refer fungal phospholipases may promote virulence by damage membranes of the host cell.

29. Neji et al. 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.

In the present study, the *hwp1* gene has been detected in all *C. parapsilosis* isolates, this is consistent with Abastabar et al. and Nikmanesh et al. who observed that *hwp1* gene was an excellent marker to detect non- *Candida albicans* species. Modrzewska and Kurnatowski 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. have mentioned that expression of *hwp1* gene is high among strains isolated from patients with candidiasis.

The results showed that *cph1* 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.

**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 18S 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.

**Acknowledgments**

The authors would say thanks to the staff of Bint Al-Huda Hospital of Maternity and Children in Thi-Qar province for their helping to collect the samples. The study is not supported financially.

**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).

References:
تعداد الإصابة الطفيلية والفيطريّة المسببة لالتهاب المهبل في النساء في مدينة الناصرية/ العراق

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الخلاصة:

أهداف الدراسة الحالية للعثور على الأنواع المختلفة من Candida spp. وذلك لتعرف على السمات الطفيلية والفيطريّة و Trichomonas vaginalis في شريان النازحات من مدينة الناصرية/عراق.

كانت هذه الدراسة اكتشافية، حيث تم جمع عينات من شريان النازحات من الناصرية، بغداد، العراق. قام عيالي قسم العلوم الطبيعية، كلية الصيدلة، جامعة العين، محافظة ذي قار بتشخيص وتحديد الأنواع المختلفة من Candida spp. و Trichomonas vaginalis في نازحات الناصرية.

النتائج:

تم جمع 250 عينة من شريان النازحات من الناصرية، بغداد، العراق. تم تشخيص الأنواع المختلفة من Candida spp. و Trichomonas vaginalis في نازحات الناصرية. تشكلت الأنواع المختلفة من Candida spp. و Trichomonas vaginalis في نازحات الناصرية، بجانب الأنواع الأخرى مثل Trichomonas vaginalis. تم تعريف الأنواع المختلفة من Candida spp. و Trichomonas vaginalis في نازحات الناصرية، باستعمال الاختبارات الكيميائية المختلفة، بما في ذلك القياسات الفيزيولوجية والبيولوجية.

الاستنتاج:

تم تشخيص الأنواع المختلفة من Candida spp. و Trichomonas vaginalis في نازحات الناصرية، بأكثر من 100% من العينات. تم تشخيص الأنواع المختلفة من Candida spp. و Trichomonas vaginalis في نازحات الناصرية، باستعمال الاختبارات الكيميائية المختلفة، بما في ذلك القياسات الفيزيولوجية والبيولوجية.

Candida parapsilosis

Candida parapsilosis، في الميكوس، هو من الفصيلة Candida، وتعد هذه البكتيريا من أكثر البكتيريا شيوعًا في الجهاز المناعي البشري. يتميز بمتعدد البكتيريا الفطرية، مثل Candida albicans، حيث يتميز بوجود الفطر في هيئة تحولية. C. parapsilosis هو من الفصيلة Candida، ويتميز بوجود الفطر في هيئة تحولية، مما يجعله أكثر شيوعًا في الجهاز المناعي البشري.

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