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A type of yeast that causes illness was found in women with weak immune systems in the central Euphrates region of Iraq

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SUMMARY

AUTHORS' CONTRIBUTION: (A) Study Design · **(B)** Data Collection. **(C)** Statistical Analysis · **(D)** Data Interpretation · **(E)** Manuscript Preparation · **(F)** Literature Search · **(G)** Funds Collection

Background: A vaginal yeast infection, otherwise known as vaginal candidiasis, is a fungal infection that is characterized by irritation, discharge and itching of the vagina and vulva. It is estimated that most people assigned female at birth will experience at least one vaginal yeast infection in their lifetime, with many individuals typically experiencing multiple infections.

Aim: The study was designed to establish a catalogue of fungal species that cause infections in different patients, and to study their genes and compare them with standard types of fungus already known to science. Method: A total of 194 samples were collected from women who visited Al-Sadder Medical City in Al-Najaf province between January 2022 and March 2022.

Result: 17 samples gave positive growth of yeasts which were blood samples 2 (11.7%) and urine samples 15 (88.2%). Chromagar Candida medium revealed that five species of Candida depending on color of colonies which were C. glabrata, C. krusei, C. parapsilosis, C. albicans, and C. tropicalis and also unknown interesting yeast species with olive color. The results of alignment and distance phylogeny have been investigated for (A1) ITS2 region sequences, the strain (A1) strain found to be identical to Pseudozyma aphidis strain (HP1191), it is not detected with both PCR and chromagar identification. On the other hand, the results with ITS1 region revealed that (B1) strain found to be the nearest neighbor to P. aphidis strain (HP1191) with 86% identical. The present study, the first record of Basidiomycetious P. aphidis in Iraq using DNA sequencing of ITS1 and ITS2 regions.

Conclusion: The DNA sequencing is the best method for identification of fungi compared with chromagar Candida medium test and PCR analysis.

Keywords: Vaginal yeast infection; Fungal infection; Vaginal candidiasis; DNA sequencing

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INTRODUCTION

Candida is a normal flora of the mucous membrane of upper respiratory tract and also it becomes pathogen yeast which invades the mucous membrane and causes candidiasis (opportunistic infection) in immunocompromised individuals [1]. The invasive fungal infection in human being has risen greatly in past two superficial and serious systemic disease [2,3].

The classical approach to the identification of fungi involving morphological and cultural characters, microscopic and molecular identification, human fungal infections can be detected directly from patients samples using PCR assay but accurate identifications of the fungi are made by using DNA sequencing techniques on the isolated fungus [4]. The term DNA sequencing refers to methods for determining the order of the nucleotides bases of DNA, the knowledge of DNA sequences of genes and other parts of the genome of organisms has become indispensable for basic research studying biological processes, as well as in applied fields such as diagnostic or forensic research [5]. Based on available information in Iraq there are little data available associated with fungal species isolated from different clinical cases for this reason the present study is designed to fulfill these spaces, this study confirms the importance of fungi as causative agents to human and gives it a genetic and molecular identification profile to identify the exact identity of pathogen [6].

Therefore, the present study was aimed to find a database of fungal species causing infection of different clinical cases to give their genetic characterization and compare them with standard isolates documented in global sites NCBI. This goal was achieved *via* the following objectives:

1. Isolation and morphological identification of the yeast species from patients in Al-Najaf province.
2. Molecular identification of the yeast species.
3. Identifying some yeast isolates using DNA sequencing analysis and aligned with NCBI database using Blast software and multiple aligned to each other using BioEdit software.

MATERIAL AND METHOD

Collection of samples

A total of 194 samples were collected from women patients with different clinical cases were obtained from Al-Sadder Medical City in Al-Najaf province, from January 2022 to March 2022. There are two types of samples were collected as following: 137 blood samples and 57 urine samples, all the above samples were transferred to the laboratory of Microbiology/ Faculty of Medical techniques/ Islamic University Najaf for diagnosis and study.

Identification of fungal isolates

After 24-48 hours of incubation for yeast isolates, the shape, size, color, edge, and appearance of the fungal isolates were examined on SDA media. The chromagar test was utilized to aid in the diagnosis of the *Candida* species based on color; a single cell was selected from the yeast growth on SDA and culture planning using the loop method; the yeast isolates were incubated for 24-48 hours at 37 ˚C Molecular identification [7,8].

Identification by PCR technique

Preparing the primers

The lyophilized form of the primers used in this study was obtained from the manufacturer, who instructed preparation of the primers by adding deionized distill water to obtain a concentration of 100 pmol/µl for the stock solution; the primers' working solution was then prepared by adding 10 µl of stock solution to 90 µl of deionized distill water to obtain a final concentration of 10 pmol/µl.

Fungal DNA extraction

For the purpose of purifying the DNA of juvenile fungal colonies, a particular EZ-10 spin column fungal genomic DNA mini-preps kit (Favorgen Biotech Corp., Taiwan) was utilized.

1. Pouring a portion of the fungal culture into a 1.5 ml microcentrifuge container.
2. Pipetting 1 milliliter of FA buffer into the cells to resuscitate them.
3. Centrifuging the cells at 5,000 x g for 2 minutes to deplete them, then fully discarding the supernatant.
4. Adding 50 µl of lyticase solution and resuspending the cells in 550 µl of FB buffer, then thoroughly mixing by vortexing. The sample is incubated for 30 minutes at 37 ˚C.
5. Centrifuging the cells for 10 minutes at 5,000 x g to lower them. Removing all of the supernatant.
6. Pipette 350 µl of TG1 buffer and well mix. Moving the mixture of the sample to a bead tube.
7. Mixing thoroughly with a 5-minute Plus-vortex.
8. Including 20 µl of Proteinase K (10 mg/ml) and thoroughly blending with a vortex. Vortex for 30 seconds after every 5 minutes of incubation at 55 ˚C.
9. Centrifuge the cells at 5,000 x g for 1 minute to descend them, then transfer 200 µl of the supernatant to a fresh 1.5 ml microcentrifuge tube.
10. Pipetting in 200 µl of TG2 buffer and well mixing.
11. Adding 200 µl of ethanol (96–100%) and thoroughly blending for 10 seconds using a pulse-vortex method.
12. Inserting a small TG column into the collection tube. Carefully transferring the sample mixture to the TG micro column, making sure to include any precipitate. After a 30-second centrifugation at 11,000 x g, transfer the TG micro column to a fresh collecting tube.
13. Filling the TG micro column with 400 µl of W1 buffer. After 30 seconds of centrifugation at 11,000 x g, discard the flow-through. The TG micro column should be returned to the collection tube. ensuring that, upon initial usage, ethanol has been added to the W1 buffer.
14. Filling the TG micro column with 750 µl of wash buffer. After 30 seconds of centrifugation at 11,000 x g, discard the flow-through. The TG micro column should be returned to the collection tube. Upon initial use, confirm that ethanol has been introduced to the wash buffer.
15. To dry the column, centrifuge at maximum speed (around 18,000 x g) for an extra three minutes. Vital action.
16. Attaching an elution tube to the TG micro column.
17. Pouring 50–100 µl of H_2O or elution buffer into the TG mini column's membrane center. Use the TG tiny column for three minutes.
18. Centrifuge for one minute at maximum speed (around 18,000 x g) to extract all DNA.
19. Keep all DNA at -20 ˚C or 4 ˚C.

DNA quantification and quality determination

5 µl of DNA from each sample was used for the bio drop test to measure the concentration and purity of the DNA; the results showed that the concentration of DNA was 74.65 mg/ml and the purity of the DNA solution was within a range of 1.7 ± 0.2 [9].

a) Primer's selection: As indicated in **Tab. 1.**, Bioneer Company, Korea synthesized all of the primers used in this investigation.

b) PCR mixture: Optimization of PCR was performed after several attempts by using PCR mixture, thus, the following mixture is according to (Bioneer company, Korea), **Tab. 2**.

c) PCR Program: General program is listed in **Tab. 3.**

Electrophoresis is used to examine the PCR products and the ladder marker; the resolved band indicates the matching genes (ITS2, and ITS1). The resolved band's molecular weight identification is determined by how well it matches the ladder bands.

Tab. 1. Primers used for Candida spp. detecting of ITS1 and ITS2 region (10).

Primer	DNA sequence (5'-3')	Target region
ITS3- F	5 -CGACGCAAGAAGTAGCTACG-3'	ITS2
ITS4- R	5-TCCTCCGCTTATTGATATGC-3'	
ITS5- F	5 - GGAAGTAAAAGTCGTAACAAGG-3'	ITS1
ITS4- R	5 -TCCTCCGCTTATTGATATGC-3'	

Tab. 2. The mixture of PCR.

Mixture solution	Volume
Master mixture	5 µl
DNA template	5 µl
Forward primers	2.5 µl
Reverse primers	2.5 µl
Deionized water (dd water)	5 µl

Tab. 3. PCR program that apply in the thermocycler.

Gene Name	Temperature (°C) / Time					Cycles Number
	Initial Denaturation	Cycling Conditions			Final Extension	
		Denaturation	Annealing	Extension		
ITS2	95/5 Min	94/30 sec	56/1 min	72/1 min	72/5 min	30
ITS1	95/5 Min	94/30 sec	56/1 min	72/1 min	72/5 min	30

Detection of DNA content by agarose gel electrophoresis

DNA was detected using a UV transilluminator and gel electrophores [10,11].

Agarose preparation: In order to make 100 ml of agarose solution, 1.3 g of agarose and 100 ml (1X)TBE buffer were combined in a glass flask, heated to boiling in a microwave, cooled at 55 °C, and then mixed with 5 µl of ethidium bromide before being poured onto a prepared tray. After the agarose hardened, the comb was removed, leaving wells that were subsequently utilized to hold DNA samples [12].

Sample preparation: Each well of the agarose gel had around 5 µl of DNA material.

Agarose electrophoresis: The electrophoresis tank was filled with TBE (1X) buffer, and a tray containing agarose gel was submerged in it. A standard molecular weight DNA ladder (marker) and 5 µl of DNA sample were loaded into each well, with the first well being loaded first. The electrophoresis was run at 80 volts for one hour, during which time the gel was observed under a UV transilluminator and captured on camera using a digital camera [12].

DNA sequencing of PCR products

The PCR products of *Candida spp.* was sent to Macrogen Lab in USA and received the data of sequences for every Candida spp. and until sequencing reaction, purified PCR product with processor kit (Promega, Madison, USA) for PCR purification according to industrialization company, Subjected the sequencing results for multialignment setup on BioEdit software, Sanger's method is used for DNA sequencing [13].

RESULTS

Isolation and identification

Nineteen hundred and four samples (137 (70.6%) and 57 (29.3%) of urine were taken from randomly selected patients with various clinical conditions for the current investigation, **Tab. 4**.

The current study's outcome data included the following information: When compared to other species of *Candida*, *C. glabrata* shows the highest prevalence in both urine and blood (see **Tab. 5.**). These findings aligned with [14,15].

Tab. 4. Numbers and percentages of samples collected from patients in Al-najaf province.

Type of samples	No. of samples	Percentage (%)
Blood	137	70.6%
Urine	57	29.3%
Total	194	100%

Morphological identification

Identification on SDA medium

Sample that was gathered was cultivated on SDA. The colonies of *Candida spp.* ranged in color from cream to yellowish, matured quickly in 24-48 hours, and had a smooth, glossy, or dry texture. Figure (3.1A). There was agreement with these findings [16]. Except the interesting yeast spp. which grows on SDA medium with white, dry or wrinkled colonies depending on the species, Figure (3.1B). These results are in accordance with [17] who found the same results.

Identification of *Candida* spp. on chromagar medium

This study demonstrated that *C. glabrata* dark pink, *C. krusei* pink with white peripheral (fuzzy), and *C. parapsilosis* white pale pink colonies appear when using chromagar *Candida*, which is thought to be a differential

agar [18]. *C. albicans* is characterized by light green color smooth colonies and dull greenish blue of *C. tropicalis*, Figure (3.2). These findings are in line with those of [14,19,20], who discovered that the colonies of *Candida* spp. that surfaced on chromagar shared the same traits. Additionally, the intriguing yeast species grown on chromagar *Candida* displayed an olive hue, leading to a diagnosis of unknown yeast species, Figure (3.2).

Molecular identification

In order to ascertain the genotypes of yeast spp. isolates, amplified DNA products of the ITS1 and ITS2 regions from five *Candida spp.* and one unidentified yeast species were utilized as templates for PCR.

Sequences analysis

Sequencing for (A1 and B1) strains was received online and aligned to NCBI data base using blast software and multiple aligned to each other, using BioEdit software and submitted in fasta format to NCBI through sequin software. The primers used for yeasts were (ITS4- ITS5) and (ITS3-1TS4) amplified the ITS region for 2 samples, **Tab. 5.**, **Tab. 6.**, Figure (3.3) and Figure (3.4).

DISCUSSION

Out of 194 only 17 samples gave positive growth of yeasts which were blood samples 2(11.7%) and urine samples 15 (88.2%), **Tab. 5.**. The current study's outcome data included the following information: When compared to other species of *Candida*, *C. glabrata* shows the highest prevalence in both urine and blood (see **Tab. 6.)** Except the interesting yeast spp. which grow on SDA medium with white, dry or wrinkled colonies depending on the species, **Fig. 1.** The intriguing yeast species grown on chromagar Candida displayed an olive

Tab. 5. Numbers and percentages of positive growth samples collected from patients in Al-najaf province.

Type of samples	No. of samples	Percentage (%)
Blood	2	11.7%
Urine	15	88.2%
Total	17	100%

Tab. 6. Distribution of yeasts species isolated independent of samples from patients with different clinical cases.

Yeasts species	Samples	No.	Percentage (%)
C. albicans	Urine Blood	3 1	17.6% 5.8%
C. tropicalis	Urine	3	17.6%
C. glabrata	Urine	5	29.4%
C. krusei	Urine	2	11.7%
C. parapsilosis	Urine	2	11.7%
Yeast spp.	Blood	1	5.8%

Fig. 1. Macrograph showing A: Candida spp. colonies growing on SDA, 24-48 hr., 37 Cº B: unidentified yeast spp. colonies growing on SDA, 5 days, 37 Cº.

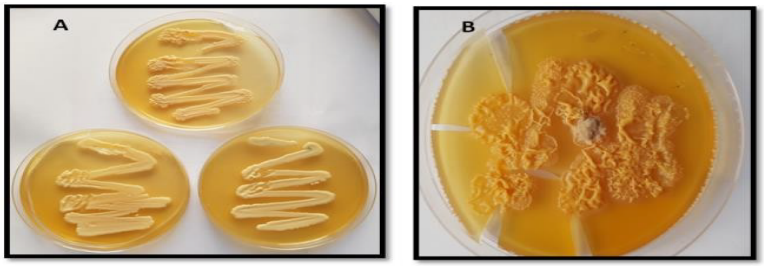

Fig. 2. Macrographs showing Candida spp. colonies growing on chromagar 24-48 hr. 37 Cº, A :*C. krusei*, B: *C. glabrata*, C: unknown yeast spp. , D: *C. tropicalis*, E: *C. parapsilosis* and F:*C. albicans.*

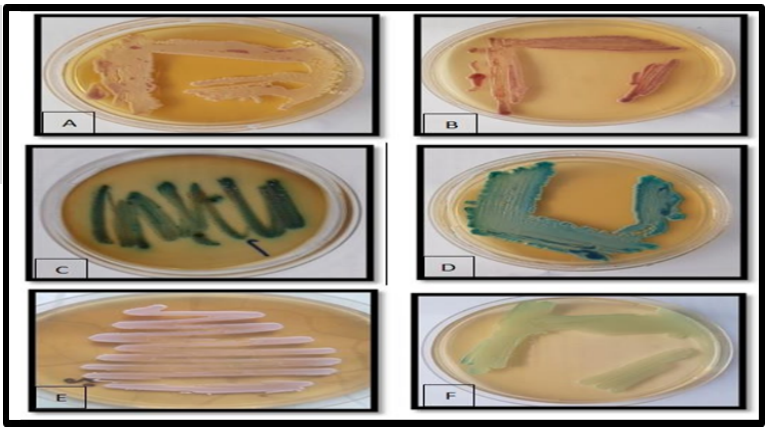

Fig. 3. DNA sequences alignments of isolated A1 (*P. aphidis*) in comparing with database obtained from NCBI website.

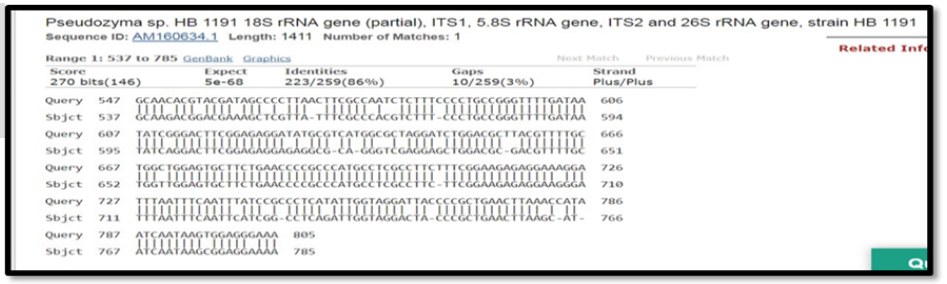

Fig. 4. DNA sequences alignments of isolated B1 (*P. aphidis*) in comparing with database obtained from NCBI website.

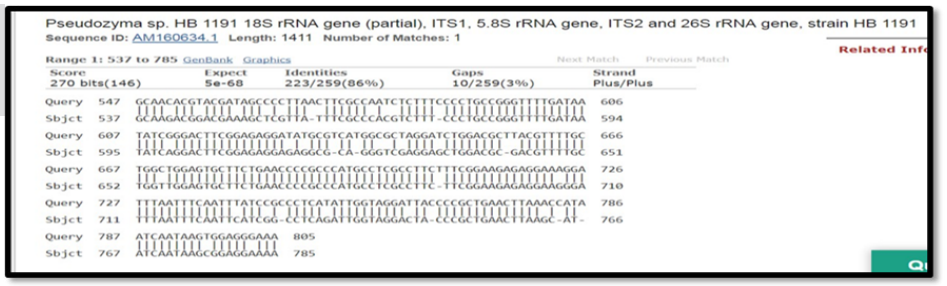

Fig. 5. Pairwise alignment of ITS1 region of B1(*P. aphidis*) strain HB 1191 using NCBI online.

hue, leading to a diagnosis of unknown yeast species, **Fig. 2.** The A1 was strain found to be the nearest neighbor to *P. aphidis*. Strain HP1191 with identity 99% **Fig. 3.** and **Fig. 4.**, this yeast was considered the first recorded in Iraq and was isolated from blood samples also we are not able to identify these isolates when using both PCR and chromagar identification. While the B1 strain found to be the nearest neighbor to *P. aphidis* strain HB1191 with identity 86% **Fig. 4.**, **Fig. 5.**, the researcher is not able to identify these isolates when using both PCR and chromagar identification.

Pseudozyma spp. was initially identified as a potential human pathogen associated with fungal pneumonia [21]. In 2008, a 7-year-old girl diagnosed with short gut syndrome became the first known human instance of *P. aphidis* infection [22]. Though

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