Pro Index Journal of Science in Medicine and Life
Volume: 2 Issue: 2 Year: 2024
http://journals.proindex.uz

Molecular Diagnosis of Yeasts Isolated From Women with Vaginal Candidiasis

Budoor Riyadh Ibrahim Al–Ameri, Hawazin Ahmed Abid, Noor Maath Ahmed
Department of Life Sciences, College of Science, Tikrit University

Abstract: This study aimed to isolate and diagnose the fungi causing genital tract infections from married women and extract DNA from the isolated and diagnosed samples on chrome agar medium. Studying the extent of genetic variation of Candida isolates using Specific-PCR Determine the sequences of the nitrogenous bases of the products of the Specific-PCR reaction and compare them with the sequences of the standard strains present in Gen Bank to reveal the degree of similarity and to find a relationship between them and the standard strains from Gen Bank.

Keywords: Candida, ITS1,ITS4, DNA, Real-time PCR.

المستخلص

هدفت هذه الدراسة الى عزل و تشخيص الفطريات المسببة لالتهاب الجهاز التناسلي من نساء متزوجات واستخلاص ال DNA للعينات المعزولة والمشخصة على وسط كروم اكار.

دراسة مدى التباين الوراثي لعزلات نوع المبيضات وذلك بأستخدام التفاعل التضاعفي المتسلسلSpecific-PCR .

تحديد تسلسل القواعد النتيروجينية لنواتج تفاعل البلمرة Specific-PCR ومقارنتها بتسلسلات السلالات القياسية الموجودة في بنك الجينات للكشف عن مدى التشابه ولايجاد صلة القرابة بينها وبين السلالات القياسية من بنك الجينات.

الكلمات المفتاحية : المبيضات, ITS1,ITS4, DNA,,Real-time PCR

Introduction

Candidiasis is a primary or secondary fungal infection caused by species of the genus Candida spp. This infection may be either acute, moderate, or chronic. The pathogen causes local infection in the mouth, pharynx, skin, vagina, fingers, nails, and trachea. It is possible to spread within the body and infect the lungs, gastrointestinal tract, or other infections. Systemic, as the presence of fungi in the blood is a pathological condition known as Fungemia, or it may be endocarditis or meningitis, and the clinical appearance of the disease varies depending on the location of the infection (Richardson,2019).

In recent years, the importance of research on fungal infections and their recurrence has increased, due to the increasing number of people suffering from diseases that weaken the immune system, such as diabetes, tuberculosis, leukemia, and HIV/AIDS (2020, Chelsea). Although *Candida* is part of the normal flora in the mouth and digestive system, as it is present in small quantities and does not cause any infection when it is present with Lactobacillus bacteria, which inhibit their growth and adhesion, in the absence of beneficial bacteria, these yeasts turn into pathogenic organisms that have the ability to cause infection in areas Different as it is an opportunistic fungus (Naglik,2023).

The molecular study of the *Candida* genome began in 2004 as Genomic, Genetic and Molecular Biology Information about *Candida albicans*. At that time, due to the rapid and significant advances

in cell biology, a series of new diagnostic techniques aimed at identifying the unique molecular fingerprint of each pathogenic microorganism came into use in Diagnostics of infectious diseases quickly became widely available and highly effective for diagnosing some diseases, Such as vaginal candidiasis (Ahmad,2022), and in fact, molecular methods, the most important of which is PCR, are used every day in routine clinical practice and have replaced traditional diagnostic procedures for a variety of human infections (Tirodker,2013), their simplicity and ease of use The short response time is among its most important advantages compared to traditional techniques (Maaroufi, 2014). PCR has revolutionized scientific research since it was first introduced to the outside world in the 1980s.

PCR technology has also found applications in mycology and parasitology, by enabling early identification of microorganisms, thus aiding in the diagnosis and effective treatment of fungal and parasitic infections.

The vital function of nitrogenous bases is to bind nucleic acids to each other. In the nitrogenous base there is a nitrogen atom that has basic properties, and the basic properties of the nitrogenous base are attributed to a pair of electrons in the nitrogen atom (Wellinghausen,2019).

DNA sequencing technology is a method of determining the arrangement of nucleotides in complex DNA. DNA sequencing has revolutionized genomics. Since 1995, DNA sequencing has made it possible to analyze the genomes of more than 50,000 different organisms (2022, Schell).

The raw data is sequenced in order to obtain biologically relevant information, so that no sequence information remains without scientific value (Kasai, 2022).

Materials and working methods

Sample collection

150 samples were collected from women with vaginal candidiasis, aged between 17 years and 62 years, by taking a vaginal swab and diagnosed clinically by a female gynecologist in the women's consultation at the Heet General Hospital, Ramadi General Hospital, and outpatient clinics for a period of from December 2022 until March 2023.

Isolation of species on chrome agar medium

This detection was carried out by taking a portion of a yeast colony growing on SDA medium at the age of 24 hours and grown on chromium agar and incubated for 24-48 hours at a temperature of 37 °C, until *C. albicans* appeared green in color and *C. glabrata* light pink to cream, while *C. krusei* is purple, and *C. tropicalis* appears blue or bluish-green (Hassan, 2023).

DNA Extraction

DNA was isolated from the yeast species *Candida* sp. The kit prepared by GENEAID was used for the purpose of extracting genetic DNA.

Gel electrophoresis

Genomic DNA samples were electrophoresed on a 1% agarose gel to ensure the presence and integrity of the DNA. This technology was discovered in the seventies, as this technology replaced a density gradient device. It is a quick way to separate, diagnose, and purify DNA fragments as they pass through the gel. It is used to separate a mixture of DNA fragments and to calculate the molecular weight of the molecules separated from the gel, comparing it with the Marker indicator according to the molecular weight (Sambrook et al, . 1989).

Polymerase Chain Reaction

1-Initiator

The primers shown in(Table1) and their sequences shown in the table, prepared by the Korean company BioNEER, were used.

(Table 1) Primers used in the study

No.	Primer Name	Sequence 5´→→→ 3´	Size (bp)	Reference	Annealing
1	ITS1 (forward)	5' – TCCGTAGGTGAACCTGCGG - 3'	600		58
	ITS1 (R)	5' – TCCTCCGCTTATTGATATGC - 3'	600		58

2 - Components of the PCR Pre Mix kit used in the polymerase chain reaction

The volume of the PCR reaction in this study was 20 microliters, and the components required to perform this reaction were supplied by BioNEER in 0.2 ml Eppendorff tubes, and each tube contained within it the components listed in Table (2 and 3).

Table (2): Components of the AccuPower PCR PreMix Kit

The concentration of the PCR reaction is 20 microliters	components
(U) 1	(DNA) polymeraseTaq polymerase
Micromolar per 250 nitrogenous base	Nitrogenous base dCTP, dATP, dTTP) dGTP
Milli Mueller 10	(Tris-HCl buffer (pH 9
Milli Mueller 30	KCl
Milli Mueller 1.5	MgCl2
Stabilizer and tracking dye	

Table (3) Components of the gene diagnostic reaction mixture

components	size	ug / µl التركيز
Pre Mix	2 µl	
Primer (F + R)	2 µl	10 pmol
DNA	4 µl	
Free Water	12 µl	
Final volume	20 µl	

Table(4): Ideal conditions for the polymerase chain reaction

No	Phase	Tm (℃)	Time	NO. of cycle
1	Initial Denaturation	95℃	5 min	1 Cycle
2	Denaturation	95℃	45 Sec	40 Cycle
3	Annealing	58℃	1 min	
4	Extension – 1	72℃	1 min	
5	Extension – 2	72℃	7 min	1 Cycle

To determine the nucleotide sequence, the Sanger method was applied, based on the source (Ramsden ,2015). The results of the nucleotide sequence are (-15-16-17-18). Relying on both the Molecular Evolutionary Genetics Analysis MEGAX program sites and the National Center Biotechnology and Informatics NCBI site, the nucleotide sequencing results of the selected isolates were analyzed. For the purpose of comparing isolates, the MEGAX program was used, while NCBI was used for the purpose of finding percentages of identity with international standard strains.

DNA Sequencing Analysis

The nucleotide sequence of the amplified gene of the ITS1-5.8- ITS4 gene, which is confined between ITS4 and ITS1, was determined in the direction of ITS1 (Forward Primer) immediately after obtaining the gene duplication product, by sending 25 µl of the PCR product and 100 µl at a concentration of 17.5 pmol of each primer to the Korean company. Macrogen, Then the results were compared using an online computer program, BLAST, with a database on a global site for genetic information, the National Center for Biotechnology Information (NCBI), which in turn enters the nucleotide sequences into the BLAST field to perform alignment, sequence comparison, and matching of the genetic sequence of the fungi that are to be diagnosed to determine their species. And their type with the sequences in the previously identified and known database, and the sequences are converted into a statistical application that expresses the presence of a positive moral correlation whenever the expected value E - Value decreases. The sequences received from the company were processed using the MEGAX program to remove and trim the anomalies in the sequences.

Genetic tree analysis

The sequence data were analyzed using the Dendogram based on the genetic dimension, and the genetic relationship diagram based on the genetic distance table was drawn using the UPGMA method (The unweighted pair group method for the arithmetic average) using the MEGA X program.

Ethical consideration

The study was conducted in accordance with ethical principles. It was performed after obtaining patients' consent. The study protocol and subject information were reviewed and approved by the institutional ethics committee (date set as reference: 55 dated 10/30/0202).

Results

The test results showed that all isolates were able to grow on TMHiCrome Candida Differential Agar, as shown in Figure (1). Four species were isolated. The colonies appeared in different colors and matched the instructions of the company that prepared the TMHiCrome Candida Differential Agar. The yeast colonies appeared in light green, which belong to the species. *C. albicans*, while *C. krusei* yeast colonies appeared in purple, *C. tropicalis* appeared in blue, and *Rhodotorula* yeast colonies appeared in pink color Figure (2)

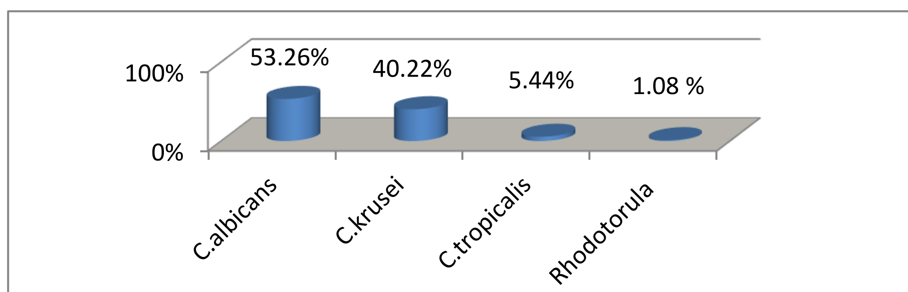
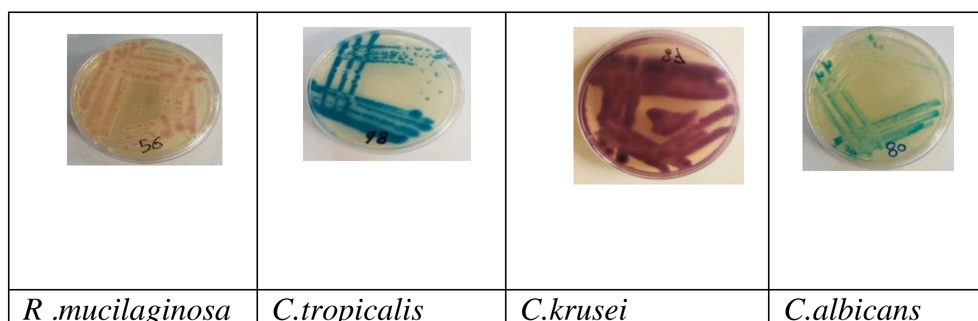


Figure (1): Types of yeasts isolated *Candida* spp on CHROM agar medium



R .mucilaginosa	*C.tropicalis*	*C.krusei*	*C.albicans*

Figure (2): Types of yeasts isolated *Candida* spp on CHROM agar medium

After diagnosing the isolates using traditional methods on SAD medium and Hi Crome Candida Differential Agar. DNA was isolated from (4) isolates of the yeasts studied, Figure (3), and the genomic DNA isolation method was used using the extraction kit prepared by (GENEAID). The amount of extracted DNA and its purity were estimated based on the absorbance of the ultraviolet spectrum at wavelengths (260 and 280) nanometers. From Figure (3), we notice that the DNA bands are equal in size because they traveled at equal distances and dimensions, due to the small distance traveled by the agarose gel for all samples of genomic DNA purified from yeast isolates

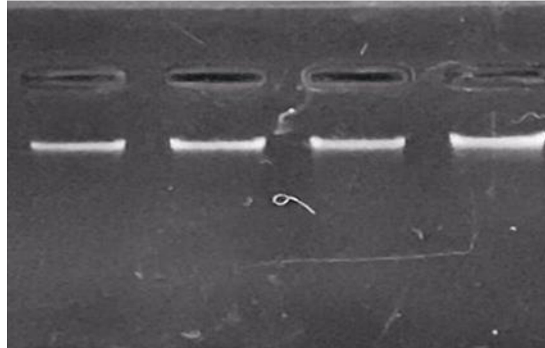


Figure (3) represents the results of isolating genomic DNA from *Candida* sp. Phase on agarose gel at 1% concentration

Amplification results of the target region of the primer pair ITS4 - ITS1 appeared when the electrophoresis product was analyzed using agarose at a concentration of 2%, with molecular sizes ranging from bp 541 to 600 for Candida spp. The amplification volume was determined based on the beam's location in relation to the Ladder volume guide (Figure 4).

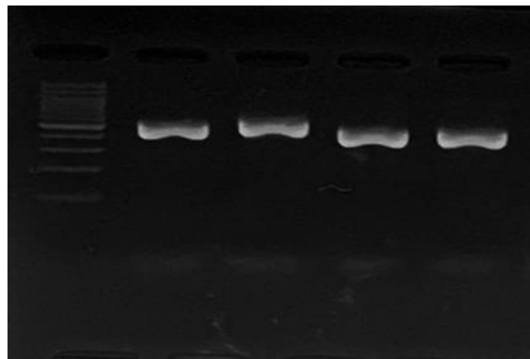


Figure (4) represents the products of the replication of the initiator ITS1, ITS4 and the phase on a 2% agarose gel.

According to the NCBI website, it was found that the samples completely matched the standard strains of fungal samples in the ITS region (Sample 1, 2, 3, 4), where the match rates were 100% with the reference sample. The matching results of the sequence via the BLAST application on the NCBI website also showed that there was a 100% match.

Table (5) Sequence analysis of the ITS fragment within segment 5.8 of RNA compared with standard fungal isolates (GENE BANK).

Sample .NO	Source	Sequence ID	Score	Expect	Identities
Sample 1	*Candida albicans*	OR526605.1	880 bits(476)	0.0	476/476(100%)
Sample 2	*Rhodotorula sp.*	MK646042.1	1005 bits(544)	0.0	544/544(100%)
Sample	*Pichia*	MT071789.1	830	0.0	449/449(100%)

3	*kudriavzevii Candida kruseii*		bits(449)		
Sample 4	*Candida tropicalis*	KX664620.1	808 bits(437)	0.0	437/437(100%)

By comparing them with NCBI isolates using its Forword Primer, the ITS sequence was chosen for this analysis because it has been shown to be more informative with different sections of the genus Candida (Muhammad, 2021).

The tree diagram of the genotype, the Phyogenetic tree, was shown in Figure (5). It was noted that the local isolate (C1) was close to the standard strain *Candida* sp., close to the *Candida albicans* strain, and close to the isolate (C4). As for the isolate C4, it was close to the *Candida tropicalis* strain and close to the *Candida dubliniensis* strain and the strain. *Cryptococcus neoformans*. As for the isolate (C3), it was close to the *Picha Kudriavzevi* strain and close to the *Candida*.

Figure (5) Phylogenetic tree of the ribosomal ITS1 -5.8S - ITS4 gene segment of an isolate of *Candida* spp. With isolates from the World Gene Bank through the use of reverse primers

Discussion

The incidence of various causes of fungal infections has increased widely over the past decades. *Candida* species are widely spread among segments of society(Capoor,2005).Vaginitis and vulvovaginitis are one of the most common symptoms of *Candida* infection. In this study, four types of yeast were detected after purifying and growing them on cultural and diagnostic media. The growth test on chromium agar medium is one of the effective and rapid tests in diagnosing *Candida* species according to color after culture and incubation at 37°C for 24-48 hours. This medium contains a substance called chromogenic hexosaminidase, which in turn interacts with the enzymes secreted by *Candida*, leading to the appearance of colored colonies. Thus, the types of *Candida* can be diagnosed according to the color of the colony (Peng, 2007).

The appearance of these bands of DNA with an approximate size of 541 bp-600 bp was the result of the presence of similar and common sequences in the sequence of nitrogenous bases in the genomic DNA of these isolates, which were able to complement those present in the two primers and to cause a specialized replication reaction and generate DNA bands of similar sizes.(Nadâs et al. 2014) We note that the DNA bands are equal in size because they traveled in equal distances and

dimensions, due to the small distance traveled by the agarose gel for all samples of genomic DNA purified from yeast isolates. (Abdulrahman et al., 2020).

Polymerase chain reaction (PCR) was performed for specific diagnostic sites in the fungal genome. The region (5.8s of nuclear ribosomal DNA) containing (ITS1, ITS4) internal transcribed spacer was amplified using the primers ITS1 and ITS4 (5' - TCCGTAGGTGAACCTGCGG -3') ITS1 Primer (5'-TCCTCCGCTTATTGATATGC-3′) ITS4 Primer(White et al,. 1990) at the ITS region as an approved taxonomic genetic indicator for diagnosing and studying genetic variation among isolates of different yeasts in this region. In fact, molecular methods are used, the most important of which is the polymerase reaction. serial, every day in routine clinical practice and has replaced traditional diagnostic procedures for a variety of human infections (Tirodker, 2013). Its simplicity, ease of use and short response time are among its most important advantages compared to traditional techniques (Maaroufi, 2014).

The diagnosis of most isolates using the polymerase chain reaction technique showed agreement with the diagnosis using the DNA sequencing technique. The current study showed the high sensitivity of the polymerase reaction technique based on ITS in diagnosing the types of isolated yeasts compared to other traditional methods, and DNA sequencing is considered one of the best rapid standard methods. To diagnose fungi (Alkhwailidy, 2022).

These results prove that the isolates belong to *Candida* spp. One of them belongs to the yeast *Rhodotorula* sp. This confirms the results obtained from the Specific PCR technology and proves the validity of the results of the diagnostic and molecular tests.

To use DNA sequencing technology to assemble the genome sequence. This genome serves as a reference for genetic and experimental studies. Among the use of the API system, the Vitec system, PCR, and DNA sequencing technology, diagnosis with the DNA sequencing system is the most accurate (Huang et al, 2021).

Conclusion

The four isolated samples are completely identical to the standard strains of fungal samples in the ITS region, which are (Sample 1, 2, 3, 4, which are each of *C. albicans*, *C. krusei* ,*C. tropicalis*, and a yeast of the *Rhodotorula* sp type. The match rates were 100% with the reference sample.

Author's contributions statement

All authors on this manuscript have made significant contributions: Hawazin A. A developed and carried out the scientific plan for the current research; Budoor R. I collected the sample and analyzed all parameters Noor M. A, also wrote part of the manuscript and statistical analysis of the data. All authors read the manuscript carefully and approve the fina version of their MS.

Acknowledgments

The author is grateful to the University of Tikrit / College of Science for providing all facilities to achieve this study.

Financial support and sponsorship

Nil

Conflicts of interest

There are no conflicts of interest.

References

1. Abdulrahman, Z. Q., Al-Attraqchi, A. A., Al-Taee, H. N., and Ghazi, H. F. (2020). Detection of pulmonary blastomycosis in patients with different respiratory diseases using conventional and molecular methods. Annals of Tropical Medicine and Public Health, 23: 232-110
2. Ahmad S, Khan Z, Mustafa AS, Khan ZU. 2022. Seminested PCR for diagnosis of candidemia: comparison with culture, antigen detection, and biochemical methods for species identification. J. Clin. Microbiol. 40:2483–2489. 10.1128/JCM.40.7.2483-2489.2002
3. Alkhuwailidy ,Hawraa A. M. Alkhuwailidy, Muhammad M. Alrufae DNA SEQUENCING OF NOVEL YEAST ISOLATED FROM BLOODSTREAM INFECTIONS IN AL-NAJAF PROVINCE AL-KUFA UNIVERSITY, KUFA, IRAQ.
4. Capoor, M. R., D. Nair, M. Deb, P. K. Verm, L. Srivastava and P. Aggarwal. 2005. Jpn. J. Infect. Dis. 58: 344-348.
5. Chelsea Marie and Theodore C.white.,2020. Genetic Basic of Antifungal Drug Resistance.Curr Fungal Infect Rep.1;3(3):163-169.
6. Hassan, Alaa Saad.2023. Role of fungal infections in pathogenicity of some inflammatory bowel disease in lraqi patients. College of Education for Pure Science -Ibn Al-Haitham/ University of Baghdad.
7. Huang , C. J. ; Lu , M. Y. ; Chang , Y. W. and Li , W. H. (2018) Experiment Evolution of yeast for High – Temperature Tolerance. Mol. Biol. Evol.35(8):1823-1839.
8. Kasai M, Francesconi A, Petraitiene R, Petraitis V, Kelaher AM, Kim HS, Meletiadis J, Sein T, Bacher J, Walsh TJ. 2022. Use of quantitative real-time PCR to study the kinetics of extracellular DNA released from Candida albicans, with implications for diagnosis of invasive candidiasis. J. Clin. Microbiol. 44:143–150. 10.1128/JCM.44.1.143-150.2006
9. Maaroufi Y, Ahariz N, Husson M, Crokaert F. 2014. Comparison of different methods of isolation of DNA of commonly encountered Candida species and its quantitation by using a real-time PCR-based assay. J. Clin. Microbiol. 42:3159–3163. 10.1128/JCM.42.7.3159-3163.2004
10. Muhammad, Sahar Basil, (2020) A descriptive diagnostic study of different local isolates of Candida spp Khmer College of Education for Pure Sciences - University of Mosul.
11. Nadă, G. C., Kalmár, K., Taulescu, M. A., Chirilă, F., Bouari, C. M., Răpuntean, S., ... and Fiţ, N. I. (2014). Comparative identification of Candida species isolated from animals using phenotypic and PCR-RFLP methods. Bull. Vet. Inst. Pulawy.58(1): 219-222
12. Naglik JR , Challacombe SJ, Hube B. Candida albicans secreted aspartyl proteinases in virulence and pathogenesis. Microbiol Mol Biol R2023;67:400–28
13. Peng, C.F., Lee, K.M., and Lee, S. H., 2007. Characterization of twochromogenic media of Candida ID 2 and CHROMagar Candida for preliminary identification of yeasts. J. Biomed Lab. Sci., 19(5):63-8.
14. Ramsden,Sophie, Abiola Abidogun, Emma Stringer, Sara Mahgoub, Artemis Kastrissianakis, Paul Baker.A peer-led teaching initiative for foundation doctors.2015.p. 236-240
15. Richardson JP, Willems HME, Moyes DL et al. Candidalysin drives epithelial signaling, neutrophil recruitment, and immunopathology at the vaginal mucosa. Infect. Immun, 2018; 86(2), e00645–17. doi: 10.1128/IAI.00645-17. Print2018 Feb.
16. Sambrook J., Russell D.W. Molecular cloning: a laboratory manual. Cold spring harbor laboratory press cold spring harbor NY. 2001; (2)

17. Schaller M , Bein M, Korting HCet al. . The secreted aspartyl proteinases Sap1 and Sap2 cause tissue damage in an in vitro model of vaginal candidiasis based on reconstituted human vaginal epithelium. Infect Immun2023;71: 3227–34.

18. Tirodker UH, Nataro JP, Smith S, LasCasas L, Fairchild KD. 2013. Detection of fungemia by polymerase chain reaction in critically ill neonates and children. J. Perinatol. 23:117–122. 10.1038/sj.jp.7210868

19. Wellinghausen N, Siegel D, Winter J, Gebert S. 2019. Rapid diagnosis of candidaemia by real-time PCR detection of Candida DNA in blood samples. J. Med. Microbiol. 58:1106–1111. 10.1099/jmm.0.007906-0.

20. White, T. J., T. D. Bruns, S. B. Lee, and J. W. Taylor. Amplification and direct sequencing of fungal ribosomal RNA Genes for phylogenetics.1990. pp.315-322