ORIGINAL ARTICLE

DNA SEQUENCING OF NOVEL YEAST ISOLATED FROM BLOODSTREAM INFECTIONS IN AL-NAJAF PROVINCE

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ABSTRACT

The aim: To identify of fungal isolates using PCR techniques with universal primer (ITS1-ITS4 and ITS3-ITS4). A total of 533 blood samples from cancer patients, renal failure patients and patients who underwent cardiac catheterization have been included in this study.

Materials and methods: Devices and tools were used to preceding the study as shown in table (I), while biological and chemical materials are given in table (II).

Results: Three groups, 44 isolates of *Candida spp* were isolated: 35(79.6%) isolates from cancer patients, 6(13.6%) isolates from patients with renal failure, and 3 (6.8%) isolates from patients with cardiac catheterization. These yeasts were diagnosed by conventional methods and by CHROMagar Candida medium, as well as by molecular methods to detect the regions of ITS2, ITS1, and the isolates were diagnosed as belonging to the yeast *Candida spp*. These isolates were also diagnosed using DNA sequencing detection technology and 12 new strains were recorded for the first time In the name of the researcher by the Japan Gene Bank.

Conclusions: It was concluded that there was high susceptibility of the polymerase chain reaction technique based on ITS 1, ITS2 primers in diagnosing the types of yeasts isolated from the bloodstream with high accuracy and speed compared to other traditional methods. Therefore, the DNA sequencing method is considered one of the best rapid standard methods for the diagnosis of fungi.

KEY WORDS: ITS regions, *Candida spp.*, DNA sequencing, bloodstream infection

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INTRODUCTION

Diseases caused by fungi and yeasts are known as fungal infections, and they are usually chronic diseases because the fungi grow slowly, as well as the difficulty of treating them because they are eukaryotes, which made them of great medical importance [1]. In normal conditions, fungi are less pathogenic and have a low ability to invade the body, but when the host's immune mechanism is weakened due to diseases or the use of immunosuppressive drugs, they will find the appropriate opportunity to invade the body and cause diseases. Although these fungi are of less importance than bacteria and viruses, they are opportunistic and have become It is of increasing importance, especially for patients with cancer, immunodeficiency syndrome, renal failure, and those taking immunosuppressive drugs [2]. Pathogenic fungi have the ability to cause severe damage to humans due to the secondary metabolic compounds they produce such as enzymes and toxins. In most cases, it is difficult to control fungal infections because they need specialized antifungal, in addition to the lack of vaccines against fungi, and the indiscriminate use of antifungal has led to the emergence of pathogenic strains. Resistance to antifungal due to fungi possessing genetic mechanisms of resistance, or a change in the permeability barrier or target site, and all of these methods are encoded by genetic factors that fungi possess [3]. The incidence of fungal bloodstream infections caused by pathogens such as Candida spp. has increased in recent years, especially in intensive care units (ICUs). Candida bloodstream infections have been reported to be the fourth highest in-hospital infection [4]. Studies has shown that the incidence of fungal bloodstream infections in ICUs is 0.22–4.1% in developing countries and 0.024–0.687% in developed countries [5]. These rates appear to coincide with more widespread use of broad-spectrum antifungal drugs, Glucocorticoids, and immunosuppressive agents, as well as the low immunity of ICU patients and use of central venous catheter technology and other invasive procedures; however, early symptoms of fungal bloodstream infection lack specificity and low culture-positive rates can lead to a misdiagnosis [6]. Bloodstream fungal infections constitute a serious health problem because of the excessive hospital stay, added health care costs, and high morbidity and mortality attributed to the diseases. *Candida albicans*, *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, *C. krusei*, and *Cryptococcus neoformans* are the most common yeasts causing bloodstream infections. These six species may account for 95 to 98% of all blood yeasts. *C. guilliermondii* and other, minor species maybe isolated occasionally [7]. Candidiasis is a parasitic fungal infection in or on any part of the body, such as fungi, caused by any of the species of Candida species, among which Candida albicans is the most common causative species. The infection is technically referred to as candidosis. These infections are broad-spectrum, ranging from

Table I. Devices and Tools used to preceding this study

No.	Devices and Tools	Company name
1	Agarose gel tank	Company/China
2	Autoclave	Harayma (Japan)
3	Bunsen burner	Iraqi
4	Centrifuge	Heraeus (England)
5	Compound light microscope	Olympus/Japan
6	Disposable Petri dishes	BBL- USA
7	Electrophoresis apparatus	Mupid-one (Japan)
8	Eppendorf centrifuge	Hettiich EBA.20/Germany
9	Eppendorf tube	Sigma/England
10	Gel electrophoresis unit	LKB/Swden
11	Incubator	Memmert , Germany
12	Micro pipettes	Gillson instruments/France
13	Nano drop	Labnet/ Germany
14	Slides and cover slides	BBL-USA
15	Standard loop 0.01	Himedia(India)
16	Sterile cotton swabs	Himedia(India)
17	Vortex mixture	Buchi (Germany)

superficial oral thrush and vaginitis to the deeply cited, often life-threatening systemic infection. Systemic infections are commonly referred to as candidemia and are one of the prominent co-infections in immunocompromised patients such as those suffering from cancer, HIV, renal replacement therapy, central-venous catheter (CVC) use (prolonged catheterization, type of catheter material, and anatomical site of catheter insertion), and healthcare practice (weak barrier methods during catheter insertion as well as patients in emergency surgery) with extensive use of powerful antibiotics and immune-suppressive therapies during organ transplant or anti-leukemia therapies [8]. Molecular identification has become an important approach, with sequences in rRNA genes and spacers being used in DNA sequence analysis, microarrays or peptide nucleic acid probe based fluorescent hybridization assays. Non-culture based approaches to the diagnosis of yeast infections have been suggested as ways to improve the sensitivity and speed of a diagnosis, though currently few have been proved as sufficient and reliable alternatives to culture. [9]The term DNA sequencing refers to methods for determining order DNA nucleotide bases, knowledge of DNA sequences of genes and other parts of organisms' genomes have become indispensable to basics research in biological processes, as well as in areas such as diagnostics or forensic research [10]. Based on the information available in Iraq, there is little data available associated with fungal species isolated from bloodstream infections, and due to the increase in the number of immunosuppressed patients made many opportunistic yeasts more virulent and caused many cases of severe bloodstream infections, which are considered life-threatening for these groups of patients. The genetics of these yeasts may therefore be reconsidered in the strategies used in the treatment of these patients for these reasons , this study was designed to meet these spaces, this study confirms the importance of fungi as human causative agents and gives them Genetic and molecular profile for accurate identification of pathogens. Therefore, the current study aimed to find a database of fungi species that cause bloodstream infections to give their genetic characteristics and compared with standard isolates documented in the NCBI global sites. And to monitor and measure the occurrence of bloodstream infections in hospitals for immunocompromised patients and the detection of the most common pathogen among the cases of infection, this was achieved through the following objectives:

- Isolation and identification of fungal species from the blood of immunocompetent patients by microscopic examination and the formation of the germ tube and growth on CHROMagar medium etc.
- Identification of fungal isolates using PCR techniques with universal primer (ITS1-ITS4 and ITS3-ITS4).
- DNA sequencing analysis and aligned with NCBI database using Blast software and multiple aligned to each other using BioEdit software for identifying some fungal isolates.
- Registration of new strains and giving them an accession number by gene bank through the NCBI website.

THE AIM

To identify of fungal isolates using PCR techniques with universal primer (ITS1-ITS4 and ITS3-ITS4). A total of 533 blood samples from cancer patients, renal failure patients and patients who underwent cardiac catheterization have been included in this study.

Table II. Biological and chemical materials were used in this study

No.	biological and chemical materials	Company name
1	Agarose	Sigma (USA)
2	De-ionized water	Bioneer /Korea
3	DNA Ladder	Promega/USA
4	DNA Loading Dye	Promega
5	EDTA	Promega/USA
6	Ethidium bromide	Sigma (USA)
7	Glycerol	Sigma (USA)
8	Gram stain kit	Himedia-India
9	Human serum	Iraqi
10	Lacto phenol cotton blue	Himedia-India
11	Lacto phenol cotton blue stain	Fluka / Switzerland
12	DNA Ladder	Promega/ USA
13	Master Mix	Promega / USA
14	Normal saline	Haidylena (Egypt)
15	Primers	Bioneer/ China
16	TBE buffer	Promega/USA
17	TE buffer	Promega/USA

Table III. Three Readymade Culture media used in present study

No.	Media	Company name
1	CHROM agar Candida	Himedia-India
2	Potato dextrose agar	Himedia-India
3	Sabouraud dextrose agar	Himedia-India
4	Brain heart infusion broth	Himedia-India

Table IV. Specific primers used in this study and their Oligonucleotide sequences for Candida species detecting

Primer	DNA sequences (5 `---3`)	Target region
ITS3 F	5 ´-GCA TCG ATG AAG AAC GCA GC-3′	ITS2
ITS4 R	5´-TCC TCC GCT TAT TGA TAT GC-3′	
ITS1 F	5′-TCCGTAGGTGAACCTGCGG-3′	ITS1
ITS4 R	5´-TCC TCC GCT TAT TGA TAT GC-3′	

MATERIALS AND METHODS

MATERIALS

DEVICES AND TOOLS

The following devices and tools were used to preceding the study table (I)

BIOLOGICAL AND CHEMICAL MATERIALS

The biological and chemical materials in table (II) were used for proceeding of experiments and tests in this study such as following:

READYMADE CULTURE MEDIA

Three Culture media illustrated in table (III) were used as readymade for proceeding of experiments and tests in this study.

PCR MATERIALS

KITS

A specific EZ-10 spin column fungal genomic DNA mini-preps kit (Favorgen Biotech Corp, Taiwan), was used for purification of young yeast colonies.

REACTION MIXTURE

Accu power PCR Pre Mix. Favorgen Biotech Corp, Taiwan, (0.1ml) thin-wall 8-strip tubes with attached cup /96 tubes were used.

MOLECULAR WEIGHT MARKER

DNA marker Favorgen Biotech Corp, Taiwan, gene ruler, 100-1500bp, Kit size 50µl (0.1 µg/µl) were used.

PRIMER

The following primers were used in the study for genome amplification [11], table (IV).

PCR REACTION MIXTURE

The following components were mixing in the Thermal cycler to produce PCR products table (V)

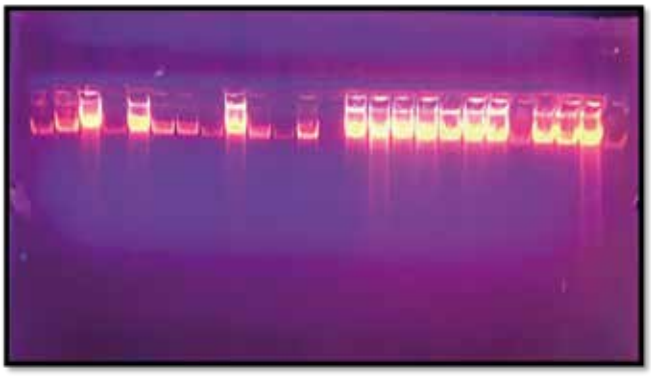

Fig. 1. Quantitative DNA concentration was measured using a nanophotometer

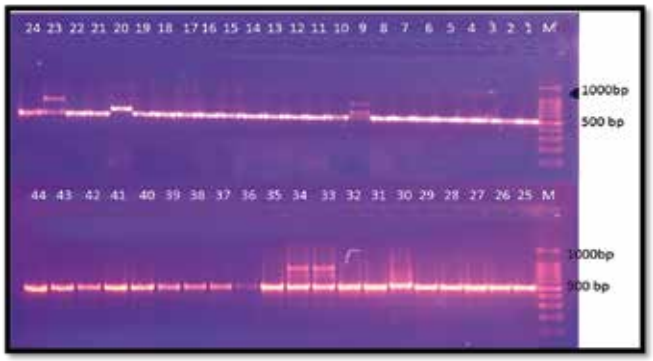

Fig. 2. Agarose gel electrophoresis of ITS1 regions PCR product by pair primers (ITS1-ITS4) of different yeast spp. (1.3g agarose gel, 80 volts for 1 hour) (M: DNA ladder; lane 1-44: yeast spp)

PCR THERMO CYCLER CONDITIONS

PCR PROGRAM

General program is listed in table (VI), using to amplification at target gene .

The PCR products and the ladder marker are analyzed by electrophoresis on 1.2 % agarose gel. The resolved band is indicative of the corresponding of (ITS2 and ITS1) genes. The molecular weight identification of resolved band is based on their correspondence to the ladder bands.

Table V. PCR mixture component used in the reaction

PCR Master mix	Volume
DNA template	5µl
Forward primer (10 pmol)	1.5µl
Reveres primer (10 pmol)	1.5µl
PCR water	12µl
Total volume	20µl

METHODS

533 blood samples were collected from hospitals in An-Najaf Governorate from patients undergoing catheterization 140 samples (26.27%), cancer patients 269 (50.47%) and patients with renal failure 124 samples (23.26%) during the period from 15 August 2020 to 21 March 2021. Data was retrieved from patient data register, individual patient records bloodstream infection was defined as the isolation of a micro-organism from bloodstream of patients with or without systemic symptoms was drawn under sterile conditions by venipuncture from a site not previously pricked. Two ml of blood was transported to the mycology laboratory in faculty of sciences in university of Kufa, and this blood specimens was immediately added to bottles containing 20ml of brain heart infusion broth and The bottles were incubated at 37ºC for up to five days. Subcultures were made on PDA and SDA agar plates after 5 days or in between if visible turbidity appeared, also CHROMagar Candida medium was performed and incubated at 37C° for 1-3 days for visible growth of Candida *spp* colonies and molds [12].

Table VI. PCR program that apply in the themocycler

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

Table VII. Number of samples and isolates from the three categories of patients

Species / Patients	No. of samples	No. of isolates	*C. parapsilosis*	*C. krusie*	*C. albicans*	*C. tropicals*
Cancer	269	35	15	9	5	6
Cardiac catheterization	140	3	0	2	1	0
Renal failure	124	6	4	0	2	0
Total	533	44	19	11	8	6

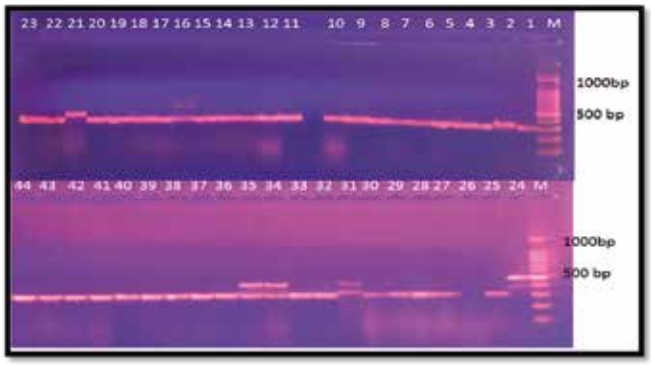

Fig. 3. Agarose gel electrophoresis of ITS2 regions PCR product by pair primers (ITS3-ITS4) of different yeast spp. (1.3g agarose gel, 80 volts for 1 hour) (M: DNA ladder; lane 1-44: yeast spp).

PREPARATION OF CULTURE MEDIA

Four Media used in this study listed in Table (II, III). All media were prepared according to the manufactures fixed on their containers.

YEAST ISOLATES IDENTIFICATION

All yeast isolates were diagnosed depending on the culture characteristics, microscopic features, biochemical test and molecular characteristics.

PCR ASSAY

The PCR assay was performed to amplify 16S rDNA sequence for identification of *Candida spp*. using universal primers table (IV) for species identification.

GENE AMPLIFICATION

Amplification of genes was carried out according to the experimental protocol of Accu power TLA PCR Premix tub under conditions of cycling as mentioned in cycling parameters of table (VI). The PCR reaction mixture was prepared as 5µl of Favorgen Biotech Corp, Taiwan, which is supplied in a ready to use in 100 µl PCR tube, followed by 5 µl temple DNA extract, 2µl of 10 pmol/µl upstream (reveres) primer specific solution as mentioned in table (V), µl of 10 pmol/µl downstream (forward) primer specific solution and the volume was completed to 20 µl with deionized distilled water, the tube were mixed with vortex to dissolved the lyophilized blue pellet, and briefly spin down.

DETECTION OF DNA CONTENT BY AGAROSE GEL ELECTROPHORUS

Gel electrophorus was used for detection of DNA by UV transuliminator.

AGAROSE PREPARATION

To prepare 100 ml of agarose solution, 1.8g of agarose was added to 100 ml(1X) TBE buffer into a glass flask,

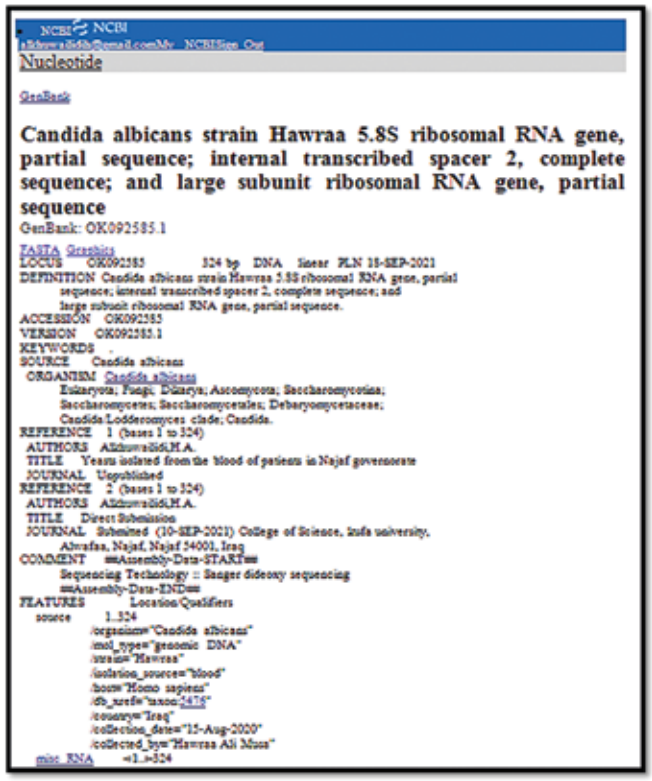

Fig. 4. Report of Candida albicans strain

then leave to boiling in a microwave, followed by cooling at 55°C,then 5µl of ethidium bromide is added to agarose and poured on preparing tray. Comb was removed after hardening of agarose leaving wells which are used later to put DNA samples [13].

SAMPLE PREPARATION

About 5 µl of DNA sample was placed in each well in agarose gel.

AGAROSE ELECTROPHORESIS

TBE (1X) buffer was poured to the electrophoresis tank; tray with agarose gel was immersed in electrophoresis tank. Each well is loaded with 5µl of DNA sample and standard molecular weight of DNA ladder (marker) is loaded in a first well. Electrophoresis run at 80 volts for 1hr, gel was visualized under UV transuliminator and photographed by using digital camera [14].

DNA SEQUENCING OF PCR PRODUCTS

The PCR products of *Candidaspp* were sent to Macrogen Lab in USA and received the data of sequences for every *Candida* spp which sent until sequencing reaction, purified PCR product with processor kit (Promega, Madison, USA) for PCR purification according to industrialization company. Subjected the sequencing results for multi-alignment setup on BioEdit software at finely phylogeny tree is commands and the multigene are perform via Mega 6

software. The sequencing results of the PCR products of several SSCP patterns were edited, aligned, and analyzed as long as with the respective sequences in the reference database using BioEdit Sequence Alignment Editor Software Version 7.1 (DNASTAR, Madison, WI, USA). The primary protein structure building of each SSCP genotype was begin by mutating the available reference NCBI DNA sequences of each coding genetic fragment, by substituting each observed SNP from the BioEdit/Laser gene software into its accurate position in the whole reference DNA sequence to represent each altered variant.

RESULTS AND DISCUSSION

Depending on the table (I) the current study included the collection of 533 specimens from three groups of patients: cancer patients, patients with renal failure and patients with cardiac catheterization, only 44 samples gave positive growth results, the results were distributed as follows: 35 positive samples from cancer patients, 3 samples from patients undergoing cardiac catheterization, and 6 samples from patients with renal failure, in addition to the 39 control samples that were taken from healthy people, all gave negative results. The blood samples were grown on BHI broth from two to seven days at a temperature of 37 C^{o}**,** and by observing the turbidity of the culture medium, Gram stain test was carried out for all positive isolates, and all of them gave a positive result of this test. Non-albicans Candida (NAC) constituted the majority of isolates, and *C. parapsilosis* was the most frequent in terms of NAC isolates. these results agreed with [15] Where they found that the group Candida nonalbicans is the main reason for the appearance of invasive Candida was heading upwards overtime while *Candida albicans* is decreasing, while disagreed with [16]were the most frequent from *Candida albicans,* and the study showed a remarkable high rate of fungal infection in a group of cancer patients relative to the other groups under study, where the number of fungal infections in this group was 35 out of a total of 269 patients due to the use of chemotherapy and radiation for these patients, which weakens their immune system significantly, which it makes them more susceptible to infection with various pathogens, especially fungi, which constitute a large proportion of the normal flora on the human body and its digestive canal, which is opportunistic in those cases (table VII).

Molecular diagnosis of isolated yeasts was carried out using total chromosomal DNA with PCR technology to identify the isolates, chromosomal DNA was extracted from Isolates using extraction kit (Favorgen Biotech Corp, Taiwan) according to Manufacturer's instructions. DNA concentration was measured Quantification using a nanophotometer and DNA purity was determined in the range of 1.7-2.0 figure (1).

The results showed that our different molecular sizes of the ITS region are *Candida spp.* Primers was used for the same sample, is (ITS1-ITS2) ITS regions have been targeted It was followed according to Key [18], PCR products of these isolates in figures (2) and (3).

PCR products were sent to Macrogen Lab in USA for sequencing analysis, (forward and reverse strand and our sequences were compared with reference global sequences in national center biotechnology information (NCBI) Gene Bank and strains was received online and aligned to NCBI database using blast software and multiple aligned to each other, using Bio Edit software and submitted in fast a format to NCBI through sequin software. Online NCBI blast software was used to compare resulting sequences to the NCBI .The diagnosis of most of the isolates using PCR technique showed agreement with the diagnosis by DNA sequencing technique, and 12 new strains were first named after the researcher Hawraa by the Japanese Gene Bank according to the reports of the NCBI website, Each isolate has been given its own accession number as follows:

OK090878,OK090877,OK090876,OK090875,OK090874,OK090873,OK090872,OK090871,OK090870,OK090869,OK092585,OK083693, as in the report below (fig 4).

Through the current study, it was found that most of the yeast isolates were isolated from cancer patients after undergoing chemotherapy, as their weak immunity and their long stay in the hospital for the purpose of receiving treatment made them highly susceptible to infections due to their exposure to nosocomial infections, also yeast infections can occur in women who take steroids or antibiotics to treat or prevent bacterial infections, and the patient may succeed in overcoming cancer. But he dies due to a fungal infection. This results agree with [19] When collecting patient data at the Oncology Center, it was noted that the most prevalent type of cancer is breast cancer in women, followed by leukemia, followed other types of cancer when the isolates were diagnosed by biochemical tests, and the results were confirmed by culturing them with CHROMagar Candida Medium, the current study showed the high susceptibility of the polymerase chain reaction technique based on ITS 1, ITS2 primers in diagnosing the types of yeasts isolated from the bloodstream with high accuracy and speed compared to other traditional methods and The DNA sequencing method is considered one of the best rapid standard methods for the diagnosis of fungi.

CONCLUSIONS

It was concluded that there was high susceptibility of the polymerase chain reaction technique based on ITS 1, ITS2 primers in diagnosing the types of yeasts isolated from the bloodstream with high accuracy and speed compared to other traditional methods. Therefore, the DNA sequencing method is considered one of the best rapid standard methods for the diagnosis of fungi.

REFERENCES

1. Benedict K., Richardson M., Vallabhaneni S. Emerging issues, challenges, and changing epidemiology of fungal disease outbreaks. Lancet Infect Dis. 2017; Lancet Infect Dis. 2017; 17(12): e403–e411. doi: 10.1016/S1473-3099(17)30443-7.

2. Calderone R.A., Fronzi W.A. Virulence of Candida albicans, Trends in Microbiology. 2021; 9(7): 327.
3. Sobel J.D. Recurrent Vulvovaginal candidiasis. Am. J. Obstet. Gynecol. 2016; 214(1):15-21.
4. Pfaller M.A., Yu W.L. Antifungal susceptibility testing: new technology and clinical applications. Infect. Dis. Clin. N. Am. 2001; 15:1227-126.
5. Xiao G., Liao W., Zhang Y. et al. Analysis of fungal bloodstream infection in intensive care units in the Meizhou region of China: species distribution and resistance and the risk factors for patient mortality. BMC Infect Dis. 2020; 20(599): 2-8.
6. Barchiesi F., Orsetti E., Gesuita R. et al. Epidemiology, clinical characteristics, and outcome of candidemia in a tertiary referral center in Italy from 2010 to 2014. Infection. 2016; 44:205.
7. Chen P.Y., Chuang Y.C., Wang J.T. et al. Comparison of epidemiology and treatment outcome of patients with candidemia at a teaching hospital in Northern Taiwan, in 2002 and 2010. J. Microbiol. Immunol. Infect. 2014; 47: 95–103.
8. Hani U., Hosakote G.S., Vaghela R. et al. Candidiasis: A Fungal Infection-Current Challenges and Progress in Prevention and Treatment. Journal of Infectious Disorders-Drug Targets. 2015; 15: 42-52.
9. Otasˇevic´ S., Momcˇilovic S., Stojanovic´ N.M. et al. Non-culture based assays for the detection of fungal pathogens. Journal de Mycologie Me´dicale. 2018; 28: 236–248.
10. Murphy K., Berg K., Eshleman J. Sequencing of genomic DNA by combined amplification and cycle sequencing reaction. Clinical chemistry. 2005; 51(1): 35–39.
11. White T.J., Burns T.D., Lee S.B., Taylor J.W. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, In PCR Protocols: A Guide to Methods and Applications. 1990, 322p.
12. Loveday H.P., Wilson J.A., Pratt R.J. et al. National evidence based guideline for preventing health care associated infections in NHS hospitals in England. Journal of hospital infection. 2014; 86(1): 51-70.
13. Sambrook J., Russell D.W. Molecular cloning: a laboratory manual. Cold spring harbor laboratory press, cold spring harbor NY. 2001; (2).
14. Mishera V., Nag V.L., Tandon R., Awsthi S. Response surface Methodology based optimization of agarose gel electrophoresis for screening and electropherotyping of rotavirus. Appl. Biochemi ND Biotech. 2010; 160(8): 2322-2331.
15. Smith A.C., Hussey M.A. Gram Stain Protocols. American Society for Microbiology, A website for publishing research. 2016. https://www.jove.com/v/10513/microscopy-and-staining-gram-capsule-and-endospore-staining [date access 11.12.2021]
16. Alkharashi N., Aljohani S., Layqah L. et al. Candida Bloodstream Infection: Changing Pattern of Occurrence and Antifungal Susceptibility over 10 Years in a Tertiary Care Saudi Hospital. Journal Canadian Journal of Infectious Diseases and Medical Microbiology. 2019 ;2015692. doi: 10.1155/2019/2015692.
17. Al-Akeel R.A., El-Kersh T.A., Al-Shaikh Y.A., AL-Ahmedy Z.Z. Prevalence and comparison for detection methods of Candida species in vaginal specimens from pregnant and non pregnant Saudi women. African journal of microbiology research. 2013; 7(1):56-65.
18. Bellemain E., Tor C., Christian B. et al. ITS as an environmental DNA barcode for fungi: an in silico approach reveals potential PCR biases. BMC Microbiology. 2010; 10: 189.
19. Teoh F., Pavelka N. How Chemotherapy Increases the Risk of Systemic Candidiasis in Cancer Patients: Current Paradigm and Future Directions. Journal pathogen 2016; 5(6): 2-16.

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The Authors declare no conflict of interest.

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A – Work concept and design, **B** – Data collection and analysis, **C** – Responsibility for statistical analysis, **D** – Writing the article, **E** – Critical review, **F** – Final approval of the article