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 healthcare 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
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 re-considered 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.
MATERIALS AND METHODS

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:

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

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

Table III. Three Readymade Culture media used in present study

<table>
<thead>
<tr>
<th>No.</th>
<th>Media</th>
<th>Company name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CHROM agar Candida</td>
<td>Himedia-India</td>
</tr>
<tr>
<td>2</td>
<td>Potato dextrose agar</td>
<td>Himedia-India</td>
</tr>
<tr>
<td>3</td>
<td>Sabouraud dextrose agar</td>
<td>Himedia-India</td>
</tr>
<tr>
<td>4</td>
<td>Brain heart infusion broth</td>
<td>Himedia-India</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Primer</th>
<th>DNA sequences (5'→3')</th>
<th>Target region</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITS3 F</td>
<td>5'-GCA TCG ATG AAG AAC GCA GC-3'</td>
<td>ITS2</td>
</tr>
<tr>
<td>ITS4 R</td>
<td>5'-TCC TCC GCT TAT TGA TAT GC-3'</td>
<td>ITS1</td>
</tr>
<tr>
<td>ITS1 F</td>
<td>5'-TCCGATGGTGAACCTGCGG-3'</td>
<td>ITS1</td>
</tr>
<tr>
<td>ITS4 R</td>
<td>5'-TCC TCC GCT TAT TGA TAT GC-3'</td>
<td>ITS2</td>
</tr>
</tbody>
</table>

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)
Table V. PCR mixture component used in the reaction

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA template</td>
<td>5 μl</td>
</tr>
<tr>
<td>Forward primer (10 pmol)</td>
<td>1.5 μl</td>
</tr>
<tr>
<td>Reverse primer (10 pmol)</td>
<td>1.5 μl</td>
</tr>
<tr>
<td>PCR water</td>
<td>12 μl</td>
</tr>
<tr>
<td>Total volume</td>
<td>20 μl</td>
</tr>
</tbody>
</table>

Table VI. PCR program that apply in the thermocycler

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Initial Denaturation</th>
<th>Temperature (ºC) / Time</th>
<th>Cycling Conditions</th>
<th>Final Extension</th>
<th>Cycles Number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Denaturation</td>
<td>Annealing</td>
<td>Extension</td>
<td></td>
</tr>
<tr>
<td>ITS2</td>
<td>95/5 Min</td>
<td>94/30 sec</td>
<td>56/1min</td>
<td>72/1min</td>
<td>72/5min</td>
</tr>
<tr>
<td>ITS1</td>
<td>95/5 Min</td>
<td>94/30 sec</td>
<td>56/1min</td>
<td>72/1min</td>
<td>72/5min</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of samples</th>
<th>No. of isolates</th>
<th>C. parapsilosis</th>
<th>C. krusei</th>
<th>C. albicans</th>
<th>C. tropicals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cancer</td>
<td>269</td>
<td>35</td>
<td>15</td>
<td>9</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Cardiac catheterization</td>
<td>140</td>
<td>3</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Renal failure</td>
<td>124</td>
<td>6</td>
<td>4</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>533</td>
<td>44</td>
<td>19</td>
<td>11</td>
<td>8</td>
<td>6</td>
</tr>
</tbody>
</table>
PREPARATION OF CULTURE MEDIA
Four Media used in this study listed in Table (II, III). All media were prepared according to the manufacturers 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 template 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, 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 *Candida spp.* 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 performed 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, 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, OK0902585, 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


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Conflict of interest:
The Authors declare no conflict of interest.

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