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Original Article

Phenotypic and Genotypic Identification of Yeast Species Isolated from Diabetic Foot Patients in Al-Najaf Province, Iraq

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Abstract

Opportunistic yeasts, such as *Trichosporon* and *Candida* species (spp.), are reported to cause high rates of morbidity and mortality in immunocompromised and underlying patients. This study was conducted to investigate the phenotypic and genotypic identification of yeast spp. isolated from diabetic patients in Al-Najaf province, Iraq. Samples were collected from the depth of diabetic foot patients' wounds. They were then cultured on Sabouraud Dextrose Agar (SDA) and incubated at 30˚C to 35˚C for 5 to 7 days for the growth of yeast spp. The colonies were identified based on their microscopic features. Afterward, these yeast samples were cultured in CHROMagar for the isolation and identification of yeast spp. All collected samples were cultured on the SDA through the use of CHROMagar, which is considered a differential agar since the colonies obtained from *Candida intermedia* and *Trichosporon asahii* appear in different colors on this media. The Polymerase Chain Reaction assay was performed to amplify the internal transcribed spacer 1 (ITS1) and Internal transcribed spacer 4 (ITS4) sequences for the identification of the yeast spp. Furthermore, the products were sequenced by the Sanger method and compared to the reference global sequences in the national center for biotechnology information Gene Bank. The results showed different molecular sizes of the ITS regions of yeast spp. The primer pair was used for the same sample (i.e., ITS1-ITS4) and targeted the ITS regions. Yeast spp. can be considered the most common fungal agent of life-threatening invasive infections in patients with severe immunodeficiency or underlying diseases, and the treatment of these infections requires long stays in the intensive care units.

Keywords: *Candida intermedia*, Diabetic, ITS1-ITS4, *Trichosporon asahii*

1. Introduction

Fungal infections cause serious diseases that greatly impact human and animal health since they are by microorganisms (fungi) similar to the metabolism and cellular activity of host cells. Therefore, it is difficult to diagnose and treat them. It is also challenging to identify fungal species (spp.) (1). *Candida* is a normal flora of the mucous membrane of the upper respiratory tract. It can also become a pathogen yeast, which then invades the mucous membrane and causes candidiasis (opportunistic infection) in the immunosuppressed system (2). Invasive fungal infections have noticeably increased in humans in the form of superficial and systemic diseases (3). *Candida intermedia* (*C. intermedia*) is a *Candida* that is rarely found as a microflora on the surface of the cheese and in humans' oropharyngeal cavity; therefore, it has seldom been associated with human diseases (4). This organism has been reported in a case series of fungemia; however, there is still a lack of detailed clinical and microbiological information (5). *Trichosporon* spp. are naturally found in water and soil; they may also be a part of normal human flora and colonize the skin, respiratory tract, gastrointestinal tracts, vagina, as well

as urine (6). *Trichosporon asahii* (*T. asahii*) is a systemic fungal infection found in hosts with immunosuppressed, immunocompromised, and immunodeficient systems. It has been identified as a cause of fatal invasive fungal infection in patients with severe anthropogenic cancer, as well as hematopoietic cell transplant recipients. There are a few reports of *T. asahii* infection in patients with single organ transplants, human immunodeficiency virus infection, burns, catheter-related infections, peritoneal dialysis, and prosthetic heart valves (7). Based on the immune efficiency of the host, clinical signs vary from localized skin lesions in the immune-competent hosts to disseminated systemic infections in immunocompromised patients (8). Culture characteristics of *T. asahii* resemble the *Candida* spp.; however, it does not form the germ tube as *Candida albicans* do (9). Due to the importance of *T. asahii* in patients with immunodeficiency and underlying diseases, this study was performed to isolate and identify *T. asahii* in diabetic patients in Iraq.

2. Materials and Methods

2.1. Sampling

Samples were collected from the depth of diabetic foot patients' wounds (taking aseptic precautions) after the debridement. These samples were collected from Al-Sadder Medical City in Al-Najaf province, Iraq, from November 2020 to January 2021. All of the above samples were transferred to the Laboratory of Advanced Mycology in the Faculty of Sciences, University of Kufa, Kufa, Iraq.

2.2. Isolation in Culture Medium

Samples were inoculated into Sabouraud Dextrose Agar (SDA) and incubated at 30˚C to 35˚C for 5 to 7 days for the growth of fungal microorganisms. The colonies were identified based on their macroscopic and microscopic features. Yeast samples were cultured in the CHROMagar for the isolation and identification of *Candida* spp.

2.3. Molecular Identification and Sequencing

Total DNA was extracted from the culture broth, and 1.5 ml of culture broth was pipetted into Eppendorf tubes. Afterward, it was centrifuged at 4.300×g for 5 min, and the supernatant was discarded. Subsequently, 200 ul of TE buffer was added, vortexes well were boiled for 10 min, and then put on ice immediately for 1 min. This was centrifuged again at 6.700×g for 10 min, and the supernatant was collected, which was used as the DNA template.

The Polymerase Chain Reaction (PCR) assay was performed to amplify the internal transcribed spacer 1 (ITS1) and the Internal transcribed spacer 4 (ITS4) sequences for the identification of yeasts. In this study, the primer pair was ITS1-F (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4-R (5'-TCCTCCGCTTATTGATATGC-3') (10). The PCR mixture was prepared as 5 µl of master mix, which was supplied to be followed by 5 µl template DNA extract, 2.5 µl of 10 pmol/µl reveres primer, and 2.5 µl of 10 pmol/µl forward primer. The volume was then completed to 20 µl with deionized distilled water, and the tube was mixed with the vortex. The PCR amplification system was utilized as follows: initial denaturation for 4 min at 94˚C, 30 sec at 94˚C, 30 sec at 56˚C, 30 sec 72˚C, 35 cycles, and 7 min at 72˚C. The PCR products were then separated by electrophoresis on 1.3% (w/v) agarose gel and stained in ethidium bromide. Afterward, the DNA sequencing of the PCR products of fungal spp. was sent to the Macrogen Lab in the USA to receive the data of sequences for every fungal spp. Until the sequencing reaction, the PCR product was purified with the Promega kit (Madison, USA), according to the instructions given by the company. Afterward, it was subjected to the sequencing results for a multi-alignment setup on Bio Edit software. The PCR product was then sequenced by the Sanger method.

3. Results and Discussion

Microscopic examination is a preliminary test to diagnose candidiasis. In the present study, the microscopic examination was performed at the Laboratory of Advanced Mycology in the Faculty of Sciences, University of Kufa, Kufa, Iraq. Each sample

was stained with Lacto-phenol cotton blue, and a direct microscopic examination was conducted for the identification of the chlamydospores and pseudohyphae *C. intermedia* (Figure 1).

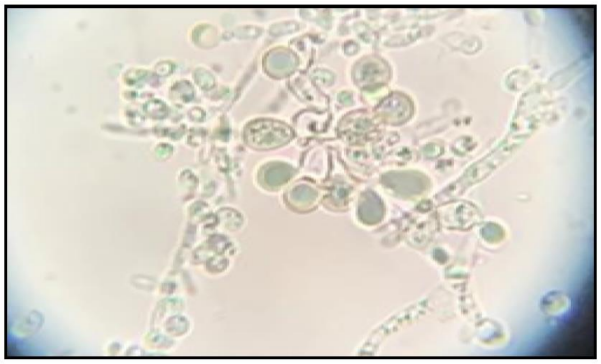

Figure 1. Micrographs showing *Candida intermedia* (chlamydospore and pseudohyphae) (40×)

The genus *T. asahii* is characterized by the ability to form arthroconidia, blastoconidia, hyphae, and pseudohyphae (11).

All collected samples were cultured on the SDA. Colonies of *C. intermedia* were smooth, butyrous, and wrinkled margin, while the isolated *T. asahii* was characterized by smooth, flat, and creamy colonies; however, both grew at 30˚C to 35˚C for 5 to 7 days (12) (Figure 2).

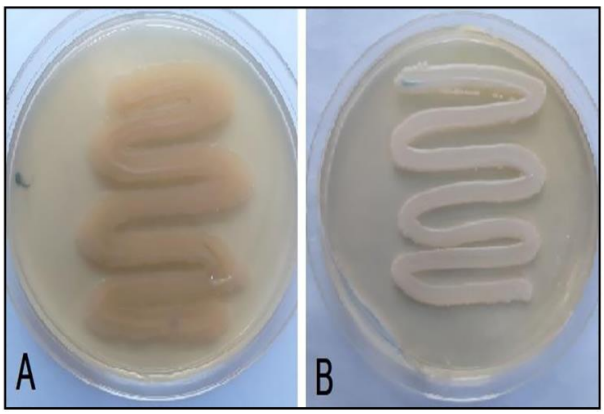

Figure 2. A) *Candida intermedia* and **B)** *Trichosporon asahi* colonies growing on the Sabouraud Dextrose Agar for 5 to 7 days at 30˚C to 35˚C

This study showed that using CHROMagar, as a differential agar, was suitable for the diagnosis of *Candida* spp., especially *C. intermedia*. The colonies of *C. intermedia* appeared onion color with wrinkled peripheral. This finding is in disagreement with the results of a study conducted by Agrawal, Bhagwat (12), which detected the colonies of *C. intermedia* as dark purple and those of *T. asahii* as light blue on the CHROMagar. However, the findings are consistent with the results obtained in another study by Lu, Lo (13), in which the same characteristics were found with regards to *T. asahii* colonies that appeared on CHROMagar; thereby disagreeing with the results of Agrawal, Bhagwat (12), in which it appeared pale lavender with a white rim of *T. asahii* on CHROMagar (Figure 3).

Figure 3. Yeast spp. colonies growing on CHROMagar for 5 to 7 days at 30˚C to 35˚C, **A)** *Candida intermedia*, **B)** *Trichosporon asahi*

Chromogenic media are effective and rapid ways of testing for the diagnosis of *Candida* spp., especially *C. intermedia* at the spp. level regarding the resulting color after inoculation. Compared to other culture traditional methods, the medium greatly facilitates the detection of specimens containing mixtures of yeast spp. due to changes in color produced by the reactions of species-specific enzymes with a proprietary chromogenic substrate. All of the tested yeast isolates grew on CHROMagar *Candida* after 5 to 7 days of incubation at 30˚C to 35˚C, the majority of the tested yeasts had grown well, as specified in the manufacturer's instructions (14).

The results showed that the molecular sizes in the ITS region were different in the detected yeast spp. The PCR products of isolates are shown in figure 4 and table 1. The primer pair was used for the samples (i.e., ITS1-ITS4) and targeted the ITS regions. The identification of *C. intermedia* was performed according to the methodology proposed by Bellemain, Carlsen (15).

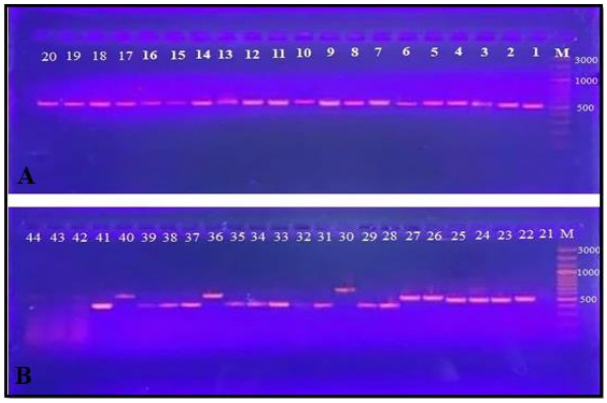

Figure 4. Agarose gel electrophoresis of the ITS regions of PCR product by pair primers (ITS1-ITS4) (M: DNA ladder, **A)** *Trichosporon asahii*; **B)** *Candida intermedia*)

Table 1. Molecular weight of bands produced by the amplification of ITS1 and ITS4 regions from yeast spp. using UVIB software

Yeast identifies	Molecular weight (bp)
Trichosporon asahii	535–537
Candida intermedia	384-388

The six samples of the amplified PCR products (forward and reverse strands) were sent to the Macrogen Lab in the USA and were compared with reference global sequences in the national center for biotechnology information (NCBI) Gene Bank.

Strain sequencing (L3, L29, L31, L33, L34, and L35) and alignment with the NCBI database were performed using BLAST software. Multi-sequence alignment was conducted by Bio Edit software and immediately submitted to the NCBI through sequin software.

The L3-LF strain was found to be the nearest neighbor to *T. asahii* strain KTSMBNL-12 with an identity of 99.21%, which was isolated from poultry feces. The L3-LR strain was identified as the nearest neighbor to *T. asahii* strain KY495727.1 with an identity of 98.97%. The L29-LF strain was found to be the nearest neighbor to *C. intermedia* strain KY495735.1 with an identity of 98.87%, and the L29-LR strain was identified as the nearest neighbor to *C. intermedia* strain KY102157.1, which was isolated from a human with an identity of 99.70%. The L31-LF strain was the closest neighbor to *C. intermedia* strain KM246246.1 with an identity of 99.44%. The L31-LR, as well as L33-LR strain, was found to be the nearest neighbors to *C. intermedia* strain DQ657830.1 with an identity of 99.58% and 98.87%, respectively. The L33-LF strain was the closest neighbor to *C. intermedia* strain MT38726.1 with an identity of 99.71%. The L34-LF strain was found to be the nearest neighbor to *C. intermedia* strain MT138722.1 with an identity of 98.87%. The L34-LR strain was found to be the nearest neighbor to *C. intermedia* strain DQ657830.1 with an identity of 98.88%. The L35-LF, as well as L35-LR strain, were identified as the nearest neighbors to *C. intermedia* strains DQ6828337.1 and DQ657830.1 with an identity of 99.15% and 98.60%, respectively.

The taxonomic predicted to discriminate yeast spp. at 98.41% for the ITS and 99.51% for the large subunit ribosomal (LSU) gene, respectively. It also showed that the strains of yeast spp. differ by less than 1% in the ITS and LSU regions (16). Accordingly, they are considered a suitable marker in the identification and diagnosis of yeasts. Due to the importance of the growth of these yeasts in patients with immunodeficiency and underlying diseases, such as diabetes, it is essential to identify and diagnose them to take appropriate treatment measures. In addition to traditional methods and culture media, the use of gene markers is also recommended in identifying wound contaminants.

Authors' Contribution

Study concept and design: A. Y. K.
Acquisition of data: M. M. A.
Analysis and interpretation of data: M. M. A.
Drafting of the manuscript: A. Y. K.

Critical revision of the manuscript for important intellectual content: M. M. A.
Statistical analysis: A. Y. K.
Administrative, technical, and material support: M. M. A.

Ethics

This study was approved by the Ethics Committee of Sciences at the University of Kufa, Kufa, Iraq, and all participants were requested to sign a consent form before participating in this study.

Conflict of Interest

The authors declare that they have no conflict of interest.

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