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Original Article

Isolation and Identification of *Candida tropicalis* as a Cause of Cutaneous Candidiasis in Kalar District, Iraq

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Abstract

Fungal infections are currently causing health issues all over the world, among which are *Candida* species that cause cutaneous infection. Numerous dermatological studies concentrated on a single species. However, the virulence factors and the spread of specific candidiasis in specific areas have remained poorly understood. Therefore, the current study was designed to shed light on *Candida tropicalis*, which has been identified as the most prevalent yeast among *Candida* non-*albicans* species. Forty specimens were collected from patients with cutaneous fungal infection (25 females and 15 males) and underwent examination. According to conventional identification based on macroscopic and microscopic examinations, eight isolates were identified as *C. tropicalis* from *Candida* non-*albicans*. Molecular diagnosis for internal transcribed spacers (ITS1 and ITS4) using conventional polymerase chain reaction (PCR) yielded an amplicon of 520 bp for all isolates. Further investigation of PCR-restriction fragment length using Mitochondrial sorting protein; Msp1 enzyme revealed two bands of 340 and 180 bp. The ITS gene sequence in one isolated species was found to be 98% identical to *C. tropicalis* strain MYA-3404 chromosome R ATCC CP047875.1. Another isolate shared 98.02% identity with *C. tropicalis* strain MA6 18S ribosomal RNA gene DQ666188.1, indicating *C. tropical* species identity, implying that non-*Candida* species should be considered when diagnosing candidiasis. This study demonstrated the significance of *Candida* non-*albicans*, particularly *C. tropicalis*, in terms of pathogenic potential, the ability to cause potentially fatal systemic infections and candidiasis, and acquired flucozonal resistance with a high mortality rate.

Keywords: Candidiasis, *Candida tropicalis*, ITS, PCR-RFLP

1. Introduction

Mycosis is an infection caused by fungi. The majority of pathogenic fungi are exogenous and they live in soil, water, and organic debris. The most common mycoses are candidiasis and dermatophytosis (1, 2). Candidiasis is a yeast infection caused by several species of *Candida*. These organisms are part of the normal flora of the skin, gastrointestinal tract, and mucous membranes (3). Candidiasis is the most common type of systemic mycosis, whose most prevalent species or agents are *Candida albicans*, *Candida tropicalis*, *Candida parapsilosis*, *Candida glabrata*, *Candida krusei*, and *Candida dubliniensis* (4). Cutaneous or mucosal candidiasis is characterized by damage to the skin or epithelium, allowing yeast and pseudohyphae to invade locally. Inflammatory reactions range from pyogenic abscesses to chronic granuloma in the cutaneous lesions. In tissue or culture, *Candida* species grow as oval yeast cells with a diameter of 3-6 mm and form pseudohyphae when the buds continue to grow and fail to release, resulting in chains of elongated cells. The morphological characteristics of *Candida*

spp. on agar plates after 24 h at 37°C are flat, soft, and cream-colored, with a yeasty odor (5, 6). *Candida albicans* has historically been the most common etiological agent of systemic and cutaneous candidiasis (7). The most clinically important species are *C. tropicalis, C. parapsilosis* (8). Recently, opportunistic infections caused by *Candida* species other than *C. albicans* have gradually increased (9). Antifungal overuse promotes the colonization of non-*albicans Candida* species (CAN) and increases resistance to antifungal medications (10, 11). Epidemiological databases from India show that CAN causes 69-90% of nosocomial candidaemia, with *C. tropicalis* being the most common species (12). Therefore, the current study was designed to shed light on *C. tropicalis*, which has been identified as the most prevalent yeast among CAN species.

2. Materials and Methods

2.1. Specimens Collection

Forty specimens were collected from patients with cutaneous fungal infection (25 females and 15 males) and examined under the supervision of specialized physicians at the Dermatology Unit of the General Hospital in Sulaimania Province, Iraq, from the middle of October 2019 to the end of December 2019. Specimens were gathered through skin scraping and nail clipping and then transferred to the microbiology laboratory of the Biology Department at the College of Education, University of Garmian, Kalar, Iraq, where they were immediately examined. All specimens were examined with 10% potassium hydroxide (KOH), cultured on Sabouraud's Dextrose Agar supplemented with both cycloheximide and chloramphenicol (SDACC), and incubated at 30°C for 2-3 days before being examined morphologically and microscopically by Gram stain as described in the Condalab kit (catNo.1089).

2.2. Carbohydrate Assimilation Test

This test aimed to investigate whether the yeast isolate could use the carbohydrate substrate as a carbon source in the medium plate. For 24 h, yeast was cultured on Yeast Extract Peptone Dextrose at 30°C. After centrifuging the cultures at 2800 g for 5 min, the pellet was washed three times and resuspended in normal saline. The suspension was placed in 2-ml suspended *Candida* spp., 300-L Yeast Nitrogen Base, and 25-ml sterile agar-agar medium in sterile petri dishes. Following solidification, 2-cm discs containing 2% carbohydrates (maltose, xylose, galactose, lactose, sucrose, and glucose) were placed. As a positive control, glucose was used. The plates were incubated at 30°C for 96 h. As previously stated, carbohydrate assimilation was observed by the appearance of a halo of growth around each carbohydrate (13).

2.3. Carbohydrate Fermentation Test

The principle of this test is to determine the ability of the yeast to acidify carbohydrate substrate in the medium detected by a pH indicator (phenol red). The purple broth was prepared by mixing peptone broth with a pH indicator. Each tube of purple broth was inoculated with specific carbohydrates (glucose, lactose, sucrose, and mannitol) and isolated colonies from a 24-hour pure culture. A Durham tube was provided in tubed broth media to collect the gas produced during fermentation. The pH indicator changed from purple to yellow when the acid produced from the fermentation was more than the alkaline as reviewed by Staudacher and Whelan (14).

2.4. Chlamydoconidia Production

This test was performed using rice agar medium, which was prepared by mixing 10 g of rice and 10 g of agar-agar with distilled water to a final volume of 1000 ml and supplemented with 10 ml of Tween 80. The samples were grown on SDA seeded as three parallel lines on rice agar between two slides and incubated at 30°C for 72 h. The formation of rounded spores or chlamydospores observed under 10X and 40X magnification by light microscope was indicative of *C. albicans* or *C. dubliniensis* (15).

2.5. Growth at 45°C

This test was used to identify *C. albicans* (appear growth) at 45°C and distinguish it from *C. dubliniensis*. This test was performed by inoculating the colony yeast

into SDA plates and incubating them at 45°C, and the growth was then examined daily for 10 days.

2.6. Germ Tube

Yeast colonies were incubated with 0.5 ml of human serum and incubated at 37°C for 2.5 h. Slides were prepared and examined to diagnose the germ tube, which appeared as a slender tube with a straight wall, without septa and construction at the junction between cells. Germ tube was indicative of *C. albicans* and *C. dubliniensis* (16).

2.7. Genomic DNA Extraction

The yeast colonies were sub-cultured on SDA and incubated at 30°C for 48 h. Genomic DNA was extracted from fungal colonies using OMEGA Fungal DNA Mini Kit (USA) based on the manufacturer's instructions.

2.8. Polymerase Chain Reaction

The Polymerase chain reaction (PCR) was performed following the manufacturer's instructions by mixing 12.5 µL of master mix (X2), 2 µL of DNA template, 9.5 µL of deionized water, 0.5 µL of forward primer ITS1 (5- TCC GTA GGT GAA CCT GCGC-3), and 0.5 µL of reverse primer ITS4 (5- TCC TCC GCT TAT TGA TAT GC-3). The PCR was conducted in a thermal cycle with the following settings: one cycle of initial denaturation at 95°C for 5 min followed by 35 cycles at 95°C for 30 sec, at 55°C for 1 min, and at 72°C for 2 min with a final extension at 72°C for 5 min and cooling at 4°C.

2.9. Restriction Digest

The PCR product was digested by the Msp1 enzyme (BioLab, UK). The digestion process was performed by mixing 42 µL of PCR product with 6 µL of 10 X NEB buffer and 2 µL of Msp1 enzyme to a final volume of 50 µL and incubated at 37°C for 1 h.

3. Results and Discussion

Out of 40 cutaneous specimens the recorded data showed that 10% of the samples were KOH positive and cultured on SDACC, only 10 (25%) specimens showed positive culture and were identified as *Candida* species. All isolates grew as smooth convex with entire margin colonies and creamy in color on SDA. Smooth and moist textures characterized the colonies with specific yeast odor (Figure 1). Eight isolates from the positive isolates were recognized as *Candida* non-*albicans.*

Microscopically, these isolates revealed gram-positive, oval-shaped budding yeast cells (Figure 2).

Figure 1. Colony of *Candida* species

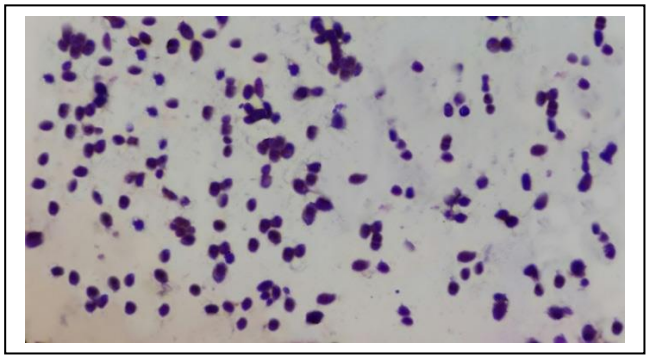

Figure 2. Microscopic examination with gram stain demonstrating globes to ovoid yeast cells (400x)

Biochemical tests, such as carbohydrate assimilation, revealed that all *Candida* non-*albicans* groups were positive for all carbohydrates (maltose, xylose, galactose, sucrose, and glucose) by converting the medium color from purple to yellow, except for lactose sugar; all isolates were negative to this sugar by keeping the primary color of the incubated plates.

Based on the carbohydrate fermentation test, eight isolates revealed positive reactions to glucose and sucrose, and negative reactions to lactose sugar were identified later molecularly as *C. tropicalis* (Figure 3), while two isolates showed positive results to glucose sugar, however, negative to sucrose and lactose by remaining the yellow color of the medium, which was later identified as *C. albicans*.

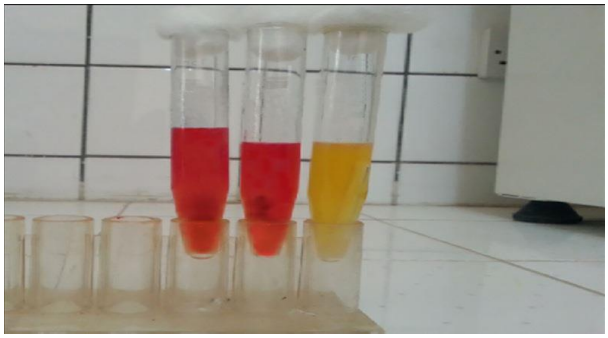

Figure 3. Carbohydrate fermentation by *Candida tropicalis*

Speciation of *Candida* non-*albicans* was carried by species-specific PCR (i.e., PCR-restriction fragment length polymorphism [RFLP]), and sequencing of the internal transcribed spacer (ITS) region using universal primers ITS and ITS4 were successfully amplified as the ITS1-5.8S rDNA-ITS2 region of their DNA producing DNA product 520 bp (Figure 4) when electrophoresed on 1.5% agarose gel. Digestion of ITS1-5.8S rDNA-ITS2 region in the PCR product by Msp1 enzyme exhibited two bands when electrophoresed on 3% agarose gel. These patterns of bands obtained from the digestion by Msp1-RFLP showed similarity with the findings of a study conducted by Mirhendi, Makimura (17). For species identification, sequencing of the ITS region *Candida* non-*albicans* group was aligned with the recorded references in the database by analysis of Basic Local Alignment Search Tool sequence ″http:www.ncbi.nlm.gov\BLAST″ from the National Center of Biotechnology. Depending on the sequencing of the ITS region, ten isolates of *Candida* spp. were identified as *C. tropicalis*, which had the pattern bands (340, 180 bp) (Figure 5). Sequencing of the ITS region in rDNA of eight isolates previously identified as *C. tropicalis* showed that one of these isolates had a similarity of about 98% with *C. tropicalis* strain MYA-3404 chromosome R *ATCC CP047875.1* (Appendix 1). Another isolate revealed 98.02% identity with *C. tropicalis* strain MA6 18S ribosomal RNA gene *DQ666188.1*.

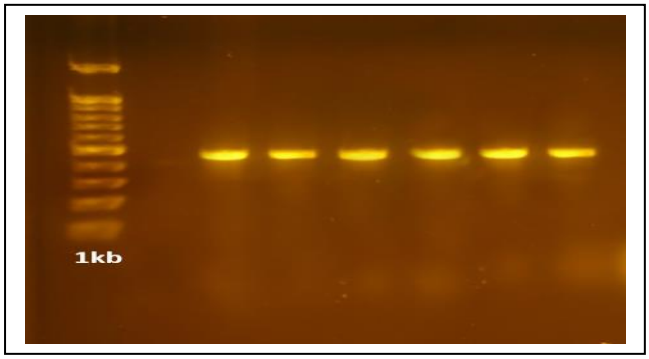

Figure 4. PCR for isolates of *C. tropicalis*
Samples were run on an agarose gel, and a 520-bp amplicon was detected for the ITS gene.

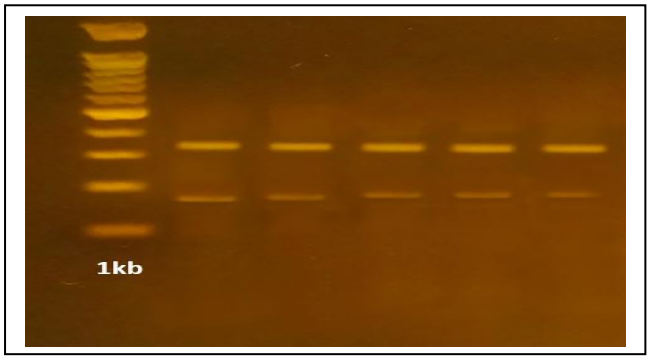

Figure 5. PCR-RFLP for PCR product of *C. tropicalis* isolates

The results of biochemical tests partially confirmed the molecular identification of yeast isolates. The germ tube test showed negative results for the isolates *C. tropicalis.*

The results of the chlamydospores test after incubation at 28°C for 2 days, then placed at 8°C for 1 week, showed the formation of terminal bodies similar to chlamydospores in *C. tropicalis* during the reduction of oxygen tension, leaving some growth uncovered (Figure 6).

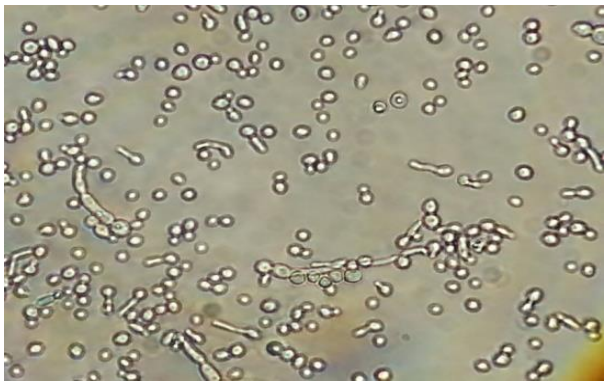

Figure 6. Chlamydospores in *Candida tropicalis* under a light microscope (400x)

The results of the thermotolerant test reported that only two isolates of *C. albicans* were grown at 45°C for 48 h, while the isolates of *C. tropicalis* did not grow under the same condition. The molecular results confirmed the other parameters conducted in the present study, which revealed that *C. tropicalis* was the most prevalent *Candida* non-*albicans* species that caused cutaneous infections and was identified in patients in Kalar city, Iraq. This finding highlights the urgent call for specialists to deal with the spread of *C. tropicalis* in the community. This result was in agreement with what was found in other research in other areas (18). The findings of recent studies have revealed that *C. tropicalis* increased virulence and pathogenicity, particularly in immunocompromised patients, such as coronavirus disease 2019 (COVID-19) patients. This may be due to tropiase protein released by the latest fungus and hemorrhagic activity and capillary permeability as reviewed by Arastehfar, Carvalho (19).

To conclude, *C. tropicalis* showed more infections than our expectations, which usually comes from *C. albicans*, suggesting that it has other virulence factors that need to be further investigated. Furthermore, with the current prevalence of COVID19 patients that currently have demonstrated a simultaneous infection with black fungi, there may be a close relationship with the case of *C. tropicalis*.

Authors' Contribution

Study concept and design: H. K. M.
Acquisition of data: S. J.
Analysis and interpretation of data: H. A.
Drafting of the manuscript: H. A.
Critical revision of the manuscript for important intellectual content: S. J.
Statistical analysis: H. K. M.
Administrative, technical, and material support: S. J.

Ethics

The study were approved by the Human Research Ethics Committee of the University of Garmian, Sulaymaniyah, Iraq.

Conflict of Interest

The authors declare that they have no conflict of interest.

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