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ORIGINAL STUDY

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Molecular Characterization and Genotyping of *Candida* Species Isolated from the Nasal Cavity of Patients with Nasal Infections in Iraq

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Abstract

Background: Nasal infections are commonly associated with opportunistic microorganisms, particularly *Staphylococcus aureus* and *Candida* species.

Objectives: This study aimed to molecularly characterize *Candida* species isolated from the nasal cavity of patients with nasal infections and to determine their phylogenetic relationships using the internal transcribed spacer (ITS) region.

Methods: A total of 88 nasal swab samples were collected from patients aged 1–80 years in Thi-Qar Province, Iraq, between December 2023 and March 2024. Samples were cultured on Sabouraud Dextrose Agar and Mannitol Salt Agar. Yeast isolates were identified phenotypically using CHROMagar Candida and germ tube tests. Molecular identification and genotyping were performed using conventional PCR with ITS1 and ITS4 primers, followed by sequence analysis using NCBI BLAST tools.

Results: Out of 88 samples, 20 (22.73%) showed positive yeast growth. Molecular analysis demonstrated that *Candida parapsilosis* and *Candida zeylanoides* were the predominant species, each representing 25% of isolates, followed by *Candida orthopsilosis* (20%) and *Candida albicans* (10%). *Candida glabrata*, *Clavispora lusitaniae*, *Debaryomyces hansenii*, and *Filobasidium oeirense* were detected at lower frequencies (5% each). Sequence analysis revealed several nucleotide mutations among different genotypes, particularly within *C. zeylanoides* isolates. Phylogenetic analysis using the UPGMA method clustered the isolates into distinct species-specific clades according to ITS sequence variations.

Conclusion: The findings indicate that ITS region sequencing is a reliable molecular approach for accurate identification and genotyping of *Candida* species isolated from the nasal cavity and provides important information regarding their genetic diversity and evolutionary relationships.

Keywords: *Candida* species, ITS region, PCR, Nasal cavity, Phylogenetic analysis

1. Introduction

The respiratory mucosa, which lines the lungs, includes the nasal mucosa. Nasal mucosa is intimately linked to the nasal conchae periosteum or perichondrium. It connects to the nasal mucous membrane via the choanae and the epidermis via the nostrils. The thickest and most vascular is the nasal conchae mucosa. Above the nasal septum, goblet cells produce more nasal mucus, making it thicker. It is one of the most diseased tissues in children and adults. Irritated tissue may cause stuffy noses, headaches, mouth breathing, and other symptoms that disrupt daily activities [1].

The nasal cavity, from the nostrils, the exterior apertures of the respiratory system, to the throat, the upper-most part of the larynx, is entirely lined with a mucosa, also called respiratory mucosa [2].

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Fungal infection is one of the four major microbiological subgroups. There are two types of fungi. Yeasts are single-celled creatures, while moulds are branching filamentous organisms and therefore are the simplest of the fungi to recognize. Fungi may switch between these two states to some degree [3, 4]. Candida and Aspergillus species [6] are the most often encountered fungal species in medical practice. These fungi are most often associated with opportunistic fungal illness in immunocompromised patients, like Mucormycosis [5].

Fungal spores are everywhere and often meet ENT doctors' anatomical targets. However, some fungi only become harmful under certain environments. Normal sinonasal flora includes inhaled fungi. Healthy immune cascades destroy these fungi. However, inadequate ventilation, dark and wet conditions, and immunocompromised individuals after extended use of antibiotics disturb these immune mechanisms, and fungal infections become more probable [6].

2. Materials and methods

2.1. Patient

Flu, sinusitis, and immunocompromised cancer patients' specimens have been collected. The samples were obtained from patients of varying ages and genders at hospitals in Thi-Qar Province between December 2023 and March 2024.

Patient samples were taken with sterile swabs. Swabs were placed in sterile tubes with transport medium and submitted to the Public Health Laboratory within 1 hour. They were subcultured on blood, MacConkey, Mannitol, and SDA agar at 37 °C for 24 to 48 hours.

2.2. Sample collection

The present study included 88 clinical samples from the nasal cavity. These samples were collected from Al- Habboby Hospital. The patients' ages ranged from one to eighty years of both genders.

2.3. Identification of Fungi

The culture was checked for white colonies that were buttery, pasty, and silky. The colony morphology was examined, and the colonies were recovered after incubation and culture on SDA.

2.3.1. Chromogenic Candida Agar (CAC)

Media was prepared aseptically by streaking a loop of culture from Sabouraud Dextrose Agar to Chromogenic Candida Agar medium and incubated at 37 °C for 72 hours. After 72 hours of incubation, the *Candida* colonies were phenotypically identified by comparing their color with the colony and with the standard color photos provided by the manufacturer, and also represented [7].

2.3.2. Germ tube test

It has been used as a yeast-germination test and to identify Candida albicans. A rapid test based on nutritional biomarkers is used to distinguish C. albicans from non-albicans species. 0.5 ml of serum sample was transferred to a small microcentrifuge tube with the help of Pasteur pipette. The yeast colony was passed onto a sterile wire loop and made into a serum emulsion. The serum was mixed and incubated at 37° for 2–3 h. The serum was then placed on a slide and checked for the Candida germ tube at 40X magnification. Germ tubes are extruded around one-half the breadth and three to four times the length of the yeast cell from which they arose. In this case, neither the yeast cell nor the germination tube shrivelled [8].

2.4. Extraction of yeast DNA

Using an inoculating loop, transfer 50–200 mg of yeast/fungus colonies (up to 2×108) from an agar plate to a 1.5 ml microcentrifuge tube containing 600 μl of Sorbitol Buffer.

Transfer the yeast or fungal cells from the broth into a 1.5 ml microcentrifuge tube. Centrifuge for 10 minutes at 5,000 x g and discard the supernatant. For DNA extraction, weigh 50–200 mg of moist material (up to 2×10^8). If necessary, centrifuge the yeast/fungal cells once more and gather them in the same microcentrifuge tube. Re-suspend the cells in 600 μL of Sorbitol Buffer.

To ensure the transparency of the sample lysate, add 200 μl of GB Buffer, vortex for 10 seconds, and incubate for at least 10 minutes at 70 °C. Invert the tube every 3 minutes throughout the incubation procedure. Meanwhile, warm the required amount of Elution Buffer (200 μl per sample) to 70 °C for use in step [7] of DNA elution.

2.4.1. Estimation of DNA concentration

The Nano-drop spectrophotometer is employed to verify the integrity of the genomic DNA extracted by measuring the DNA concentration (ng/μl) and observing the absorbance at (260/280 nm).

2.5. PCR master mix

AccuPower®PCRPreMix is a premixed, ready-to-use solution that includes all the necessary reagents

Table 1. PCR mixture components for each gene.

Component	Volume
Master Mix	5 μl
Forward primer	2 μl
Reverse primer	2 μl
DNA template	2 μl
Nuclease free dH2O	14 μl
Final volume	25 μl

Table 2. ITS gene program in PCR prossece in the thermocycler.

Gene	Cycle	Phase	Temp	Time
ITS	30	Initial denaturation	95 °C	5min
		Denaturation	95 °C	30 s
		Annealing	58 °C	30 s
		Extension	72 °C	1 min
		Final extension	72 °C	5 min

for PCR, with the exception of water, primers, and template. A compound required for direct loading onto an agarose gel, as well as two tracing dyes (blue and yellow), are included in the Ready to Load format. These dyes enable the monitoring of electrophoresis progress.is a pre-mixed, ready-to-use solution that is designed for the amplification of DNA templates using conventional PCR.

For each gene, the PCR mix was prepared individually in a total volume of 25μl as shown in the table. Then, use the micropipette to thoroughly combine the components and place the tubes in the microcentrifuge for a few seconds to ensure that the components are installed at the bottom of the tube.

2.6. Genetic analysis

All nucleotide sequences were aligned using BioEdit software, which includes a graphic view tool to show the genetic variations (such as mutations) among nucleotide sequences. The Mega X software was also used to construct the phylogenetic tree, which was categorized into groups. The phylogenetic tree was constructed using the unweighted pair group method with arithmetic mean (UPGMA).

2.7. Statistical analysis

The data were exported to Excel for statistical analysis. *P*-value was calculated for male and female distribution using unpaired samples *t*-tests (two-tailed distribution and two samples with equal variance).

3. Results and discusion

3.1. Distribution of patients according to age group

The samples have been divided according to age into groups, each group of ten years. The age group of females (11–20), (21–30), (41–50), (51–60) 4.55% are the largest groups of the total samples. (71–80) 1.14% it's the lowest age group of the total sample.

In this study, the frequency of nasal infections in males was higher than in females. This might be due to the nature of men's work, which requires them to communicate with different environments in Iraqi society. This result comes in accordance with a study in

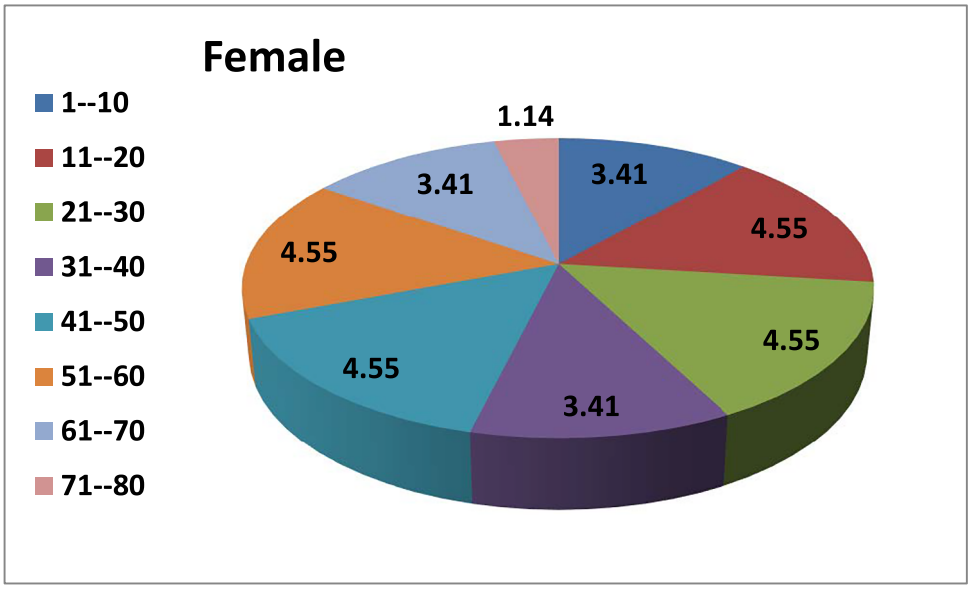

Fig. 1. Details of male patients depending on age groups.

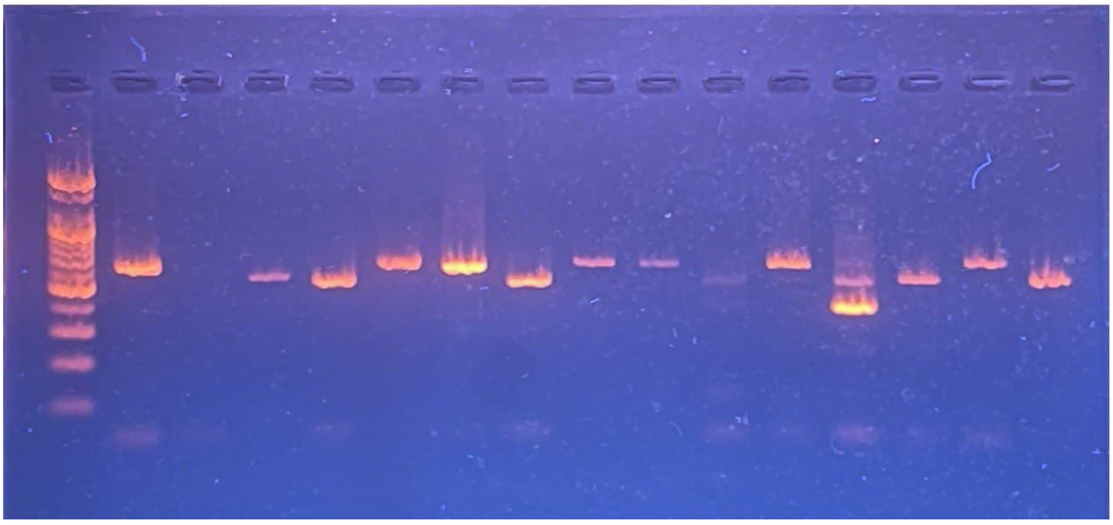

Fig. 2. Agarose gel electrophoresis at 80 v. for 30 min. for ITS gene. PCR products visualized under UV light at 280 nm after staining with ethidium bromide. M : 2000 bp ladder; the positive sample with a product size of 643, 996 bp.

Erbil;were 62.5% in males, and were 37.5% in females [9]. Depending on the study sample,

Age groups of (21–30), (31–40) and (41–50) had the highest ratios of nasal infection frequency in both males and females. These age groups include workclass individuals, which needs to communicate with different environments in Iraqi society. Therefore, the researcher believes that these results may indicate a relationship between the frequency of nasal infections and the age groups of (21–30), (31–40) and (41–50).

3.2. Culturing on Sabouraud Dextrose Agar

Among the 88 samples cultivated on Sabouraud Dextrose Agar (SDA), twenty (22.73%) were positive for at least one yeast species. *C. albicans* is ubiquitously present, and nasal colonization is relatively common [12]. *C. albicans* is recognized as the primary etiologic agent of candidiasis, an opportunistic infection associated with both local and systemic predisposing factors.

All isolated yeast species were subcultured on CHROMagar petridishes, and identification was done depending on colonies color, which indicates that all the yeast was identified under the Candida genus. In addition, the results of the germ tube test indicated that there were only two isolates of *C. albicans*. These results were well interpreted in the genetic section of this chapter.

The total percentage of yeast isolates in this study was (22.73%), which was more than the percentage of *Candida* species (13.8%) isolated from immunocompromised patients with nasal infections in Baghdad [13].

3.3. Culturing on CHROMagar

The colonies of the different yeast isolates showed various colors. The green color of the colonies indicates *C. albicans*, after incubation for 48 hours. Creamy color indicates *Candida parapsilosis*, the yeast colony in purple were seen in isolates of *Candida glabrata.*

3.3.1. Result of PCR technique

All *Candida* isolates (n = 20) have been identified, and their genotypes have been determined using the conventional PCR technique and through the amplification of (ITS1 (F), ITS4 (R)) genes. The results that had been obtained from BLAST tools in NCBI (GenBank) showed that 5 isolates (25%) of *C. parapsilosis*, 5 isolates (25%) of *C. zeylanoides*, 4 isolates (20%) of *C. orthopsilosis*, 2 isolates (10%) of *C. albicans* and the lowest percentage for *C. glabrata*, *Clavispora lusitaniae*, *Debaryomyces hansenii* and *Filobasidium oeirense* with 1 isolate (5%)

The genetic variations have also been observed between the *Candida zeylanoides* (genotype ZHIQN13) isolates in this study in comparison with the reference isolate in genbank (accession number: KR089867.1) at the locus no = 27(A-C). The genotype ZHIQN15 exhibited multiple genetic variations as follows: 506, 507 (A-T), 525 (G-A), 528 (T-A), 548 (G-T), 550 (C-G). Another isolate with genotype ZHIQN21 showed mutations at the following sites: 580 (A-C), 587 (T-C), 600 (T-A). There were two genotypes of *C. zeylanoides* that did not show genetic mutations.

The rapid detection and identification of fungi is highly beneficial in all disciplines, including medical, scientific, and commercial applications, due to the

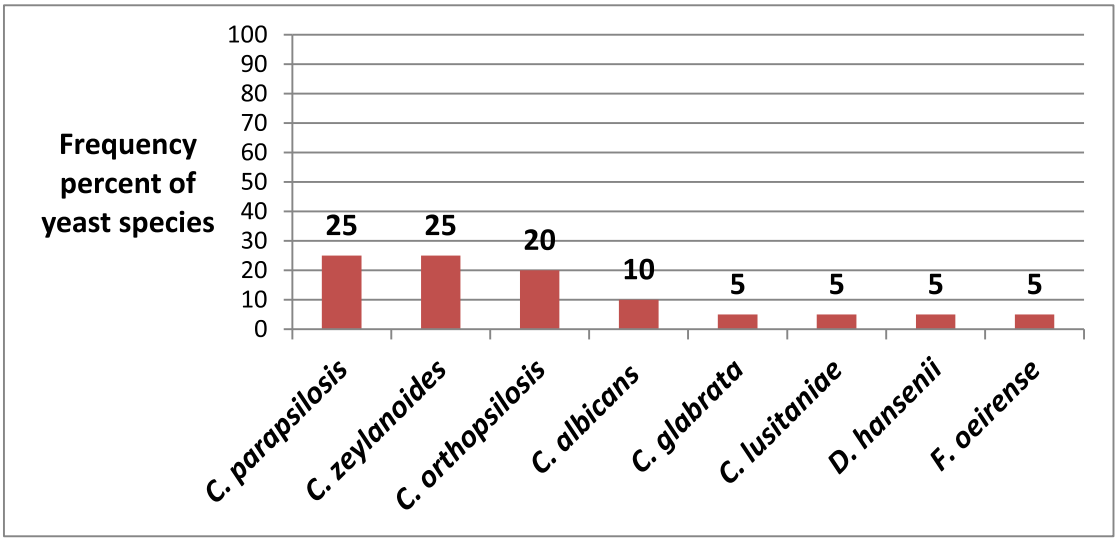

Fig. 3. Frequency percent of yeast species from total positive samples. Yeast identification by DNA sequence.

development of molecular technologies. Several targets within the fungal genome were assessed, with a particular emphasis on the internal transcribed spacer (ITS) region, a nucleotide sequence that is relatively conserved among fungi [14]. Consequently, the ITS primers (ITS1 and ITS4) were employed to identify and genotype all yeast species that were isolated in this study.

The process of identifying yeast using the ITS sequence was significantly distinct from the process of identifying yeast using morphological features (e.g., CHROMagar). The ITS region sequence was more reliable for identification, as it is a conserved nucleotide sequence in fungal species and is non-coding [15–17].

ITS sequence allows separating *Candida* isolates into clades depending on the species level. Each clade included specific genotypes of Candida species according to mutations in nucleotide sequences. The result obtained from this method (ITS sequence) was similar to genotyping methods (such as the Multilocus sequence method) [18].

Ethical approval

This study was approved by Ethics Committee, Mazaya University College (Approval NO.BU- MED 2020–33).

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Conflict of interest

There was no conflict of interest during the preparation of this article.

Author Contributions

Amran M. AL-Erjan conceptualized and designed the study. he was responsible for patient recruitment, sample collection, and laboratory analysis. he performed the microbology assays, conducted the statistical analysis, interpreted the data, and drafted the manuscript. The author critically revised the manuscript and approved the final version for publication.

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