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RESEARCH ARTICLE

ANALYSIS OF FUNGAL SPECIES RECOVERED FROM THE OROPHARYNX OF THALASSEMIA PATIENTS

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ABSTRACT

This study characterized oropharyngeal fungal colonization in Iraqi transfusion-dependent thalassemia patients. Iron overload poses a risk of infection, but little is known about the local fungal epidemiology. We identified species biochemically and assessed amphotericin B and nystatin susceptibility from clinical isolates. Microbial growth was observed in fourteen of these samples. Following initial culture on Sabouraud dextrose agar, all isolates were sub-cultured on chromogenic agar (CHROMagar™ Candida) to differentiate Candida species based on colony color. Yeasts are recognized as prevailing etiological agents of life-threatening invasive fungal infections in severely immunocompromised thalassemia patients, often necessitating extended intensive care. C. albicans (11 colonies; 72.5%), C. parapsilosis (4 colonies; 22.4%), and C. tropicalis (2 colonies; 5.1%) made up the 17 speciated isolates. Critical virulence determinants, including biofilm formation, germ tube induction, and extracellular phospholipase activity, were profiled in order to assess the pathogenic potential of clinical Candida isolates. In order to perform phenotypic identification, samples were first cultured on Sabouraud dextrose agar (SDA) and then subcultured on chromogenic medium (CHROMagarTM). The results demonstrate that C. albicans isolates were proficient producers of biofilms, phospholipase, and germ tubes, expressing the full complement of virulence factors assayed. This study evaluated the in vitro antifungal efficacy of Nystatin and Amphotericin B against clinical isolates of Candida spp. using the disk diffusion method. Amphotericin B demonstrated superior antifungal activity compared to Nystatin. The speciated isolates (n=17), identified on CHROMagar™ medium, comprised C. albicans (69.9%, n=11), C. parapsilosis (20.5%, n=4), and C. tropicalis (9.5%, n=2). Furthermore, the virulence potential of the predominant C. albicans isolates was assessed, confirming their ability to produce key pathogenic factors germ tubes, biofilms, and extracellular phospholipase enzymes when cultured on specific indicator media.

KEYWORDS

Thalassemia; Oropharyngeal colonization; Candida albicans; Virulence factors; Antifungal susceptibility; Amphotericin B.

1. INTRODUCTION

Transfusion-dependent thalassemia (TDT) is a hereditary hemoglobinopathy necessitating lifelong blood transfusions, which inevitably lead to iron overload and subsequent immunologic dysfunction. This pathophysiological state significantly heightens patient susceptibility to opportunistic infections. Fungal pathogens, particularly commensal yeasts of the Candida genus, represent a grave clinical threat in this immunocompromised population, where oropharyngeal colonization can precede life-threatening systemic invasion (Basu et al., 2023; Farmakis et al., 2017).

Despite this recognized risk, the specific epidemiology and mycological profile of oropharyngeal fungal colonization in thalassemia patients, particularly within the Iraqi population, remain inadequately investigated. Furthermore, the appearance of critical virulence determinants, such as biofilm formation and hydrolytic enzyme secretion, which are pivotal for pathogenicity, is poorly characterized in clinical isolates from this cohort (Ballén et al.,2022). Compounding this issue is the emergent challenge of antifungal resistance, underscoring the necessity for local susceptibility data to guide effective prophylaxis and treatment. Therefore, this study was designed to comprehensively evaluate the oropharyngeal fungal colonization in Iraqi TDT patients by determining the prevalence and distribution of Candida species, profiling their key virulence factors, and assessing their in vitro susceptibility to conventional antifungal agents.

Since fungal pathogens are now a major etiological factor in infections linked to the community and healthcare, their epidemiological significance has increased significantly. The most clinically significant group of pathogenic fungi is represented by the genus Candida (Sardi et al., 2013). Only a small percentage of the more than 200 species in this genus are commonly isolated from human infections. Candida albicans is the most prevalent and dangerous of these, posing a significant threat to world health. Nonetheless, a number of Candida species that are not Albicans have a well-established pathogenic potential as supported by (Parslow and Thornton, 2022).

The following goals guided the design of this study:

- To perform laboratory mycological analysis on oropharyngeal swabs obtained from a group of thalassemia patients.
- To isolate fungal colonies following primary culture on Sabouraud Dextrose Agar (SDA).
- To cultivate Candida on chromogenic agar in order to identify the species.

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- To evaluate the isolates' ability to form biofilms, induce germ tubes, and produce the enzyme N-acetylglucosaminidase in order to describe their pathogenic profile.
- To ascertain the acquired Candida species' vulnerability to the antifungal drugs amphotericin B and nystatin.

2. Materials And Methods

This section is divided into four subsections: Sample Collection and Processing, Fungal Identification, Virulence Factors, and Antifungal Susceptibility. all of the subsections are highlighted in the paragraphs that follow.

2.1 Sample Collection and Processing

Oropharyngeal swab specimens were collected from fifty thalassemia patients at Karbala Medical Center, Iraq, between January 2022 and July 2025. All specimens were immediately transported to the microbiology laboratory for analysis. Primary culture was performed by inoculating each swab onto Sabouraud Dextrose Agar (SDA) plates. Following incubation, fungal growth was observed in fourteen samples.

2.2 Fungal Identification

Isolates from positive cultures were subcultured onto CHROMagar™ Candida medium to facilitate the presumptive identification of Candida species based on the distinctive colony morphology and color developed after incubation.

2.3 Virulence Factor Experiment

As explained below, three different types of experiments were conducted in this section.

2.3.1 Germ Tube Induction Experiment

To assess germ tube formation, a yeast inoculum derived from a single colony was suspended in 0.5 mL of human serum and incubated at 37°C for 2–3 hours. Prolonged incubation beyond this period was avoided to prevent nonspecific germ tube formation by non-albicans species. After incubation, a aliquot of the suspension was placed on a glass slide, covered with a coverslip, and examined under light microscopy for the presence of germ tubes (Makwana et al., 2012; Matare et al., 2017).

2.3.2 Biofilm Formation Experiment

Biofilm formation was evaluated using a modified tube adherence method (Goeres et al., 2019; Saxena et al., 2014). Isolates were inoculated into 10 mL of Sabouraud dextrose broth supplemented with 8% glucose and incubated for 24 hours at 37°C. After incubation, the broth was decanted, and the tubes were gently washed with phosphate-buffered saline to remove non-adherent cells. Adherent biofilms were stained with 0.1% safranin for 10 minutes. Excess dye was removed by rinsing under running tap water, and tubes were air-dried inverted. Biofilm formation was quantified based on the intensity of safranin staining as supported by (Heersema, 2019).

2.3.3 Phospholipase Activity Experiment

Extracellular phospholipase activity was determined using an egg yolk agar plate assay (Gubash, 1991; Jain et al., 2017). The medium consisted of 13.0 g/L Sabouraud dextrose agar (SDA), 11.7 g/L NaCl, 0.11 g/L $CaCl_2$, and 10% (v/v) sterile egg yolk emulsion in 184 mL distilled water. The base medium was autoclaved and supplemented with sterile egg yolk supernatant obtained by centrifugation at 500 × g for 10 minutes. Plates were inoculated with 10 µL of standardized fungal suspensions (10^8 cells/mL in saline) and allowed to dry. An overlay of 5 mL sterile 0.9% saline was applied to enhance zone visibility. After incubation at 37°C for 48 hours, phospholipase activity was quantified by measuring the precipitation zone (Pz) ratio, calculated as the colony diameter divided by the total diameter of the colony plus the precipitation zone as supported by (Alp and Arikan, 2008).

3. Results And Discussion

3.1 Candida Species Identification and Isolation

A total of fifty patients with transfusion-dependent thalassemia had their oropharyngeal swabs taken. Fungal growth was confirmed in 17 samples (28%), after they were cultured on Sabouraud Dextrose Agar (SDA) and then subcultured on chromogenic media. All isolates were presumptively identified as belonging to the Candida genus based on colony morphology and chromogenic characteristics.

3.2 Distribution of Candida Species

The distribution of the isolated Candida species is presented in Figure 1. Candida albicans was the predominant species, constituting 11 isolates (69.3% of the culture-positive samples). This was followed by Candida parapsilosis (4 isolates; 22.4%) and Candida tropicalis (2 isolates; 8.3%). The high prevalence of C. albicans is consistent with its well-established role as the most common commensal and opportunistic fungal pathogen in humans, particularly in immunocompromised hosts such as thalassemia patients . The presence of non-albicans Candida species, notably C. parapsilosis and C. tropicalis, is clinically significant due to their potential for reduced susceptibility to certain antifungal agents and their association with invasive infections .

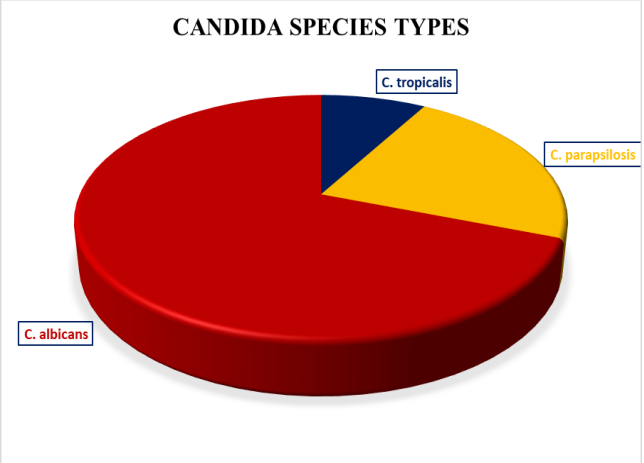


Figure 1: The Proportion (%) of Candida spp. Diagnosed from thalassemic subjects

3.3 Morphological Identification

3.3.1 Colonial Morphology on Sabouraud Dextrose Agar (SDA)

On Sabouraud Dextrose Agar (SDA), all fungal isolates showed distinctive growth after being incubated for 24 to 48 hours at 37°C (Reddy et al., 2013). The colonies had a dry, whole margin, a smooth to glistening texture, and a cream to yellowish pigmentation. Well-developed colonies usually formed in 48 hours, indicating rapid maturation. As seen in Figure 2, this morphological profile aligns with accepted descriptions of Candida species as supported by (Seneviratne, Fong, Wong, and Lee, 2015).

Figure 2: Colonial morphology of Candida spp. isolates cultivated on Sabouraud Dextrose Agar (SDA) after 48 hours of incubation at 37°C.

3.3.2 Diagnosis of Candida Species by Chromagar Medium

Species-level identification of isolates was performed using CHROMagar™ Candida, a chromogenic medium that facilitates the differentiation of Candida species based on colony color and morphology following incubation at 37°C for 48 hours (Charles, Kali, & Joseph, 2015). The observed colorimetric profiles were as follows: C. albicans developed light green colonies, C. tropicalis exhibited dark blue colonies, and C. parapsilosis presented as white to pale pink colonies Figure 3 demonstrates the characteristic colony colors used for differentiation: (A) C. albicans, (B) C. parapsilosis, and (C) C. tropicalis. These findings are consistent with previous reports and align with the manufacturer's specifications (Bloch et al., 2025).

Chromogenic media offer a rapid and reliable method for the presumptive identification of clinically relevant Candida species (Ruiz-Gaitán et al., 2023). This system functions through the cleavage of chromogenic substrates by species-specific enzymes, resulting in distinct colony colors that allow for differentiation even within polymicrobial samples. In this study, all yeast isolates grew successfully on the medium and produced characteristic pigmentation, enabling clear phenotypic discrimination.

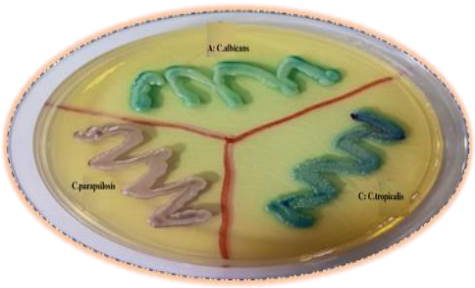


Figure 3: Speciation of Candida isolates on CHROMagar™ Candida. Distinctive colony morphologies and chromogenic properties are shown: (A) C. albicans (light green), (B) C. parapsilosis (pale pink/white), and (C) C. tropicalis (dark blue with a metallic sheen). Incubation was performed at 37°C for 48 hours.

3.4 Germ Tube Formation Assay

One important virulence factor that indicates hyphal growth, the ability to form germ tubes, was evaluated. To promote germ tube development, isolates were incubated in human serum for two to three hours at 37°C. In contrast to pseudohyphae, which show constrictions at the septal junctions, germ tubes were found to be lateral, non-constricted extensions from blastospores as visualize in Figure 4. For Candida albicans, which has continuously shown strong germ tube production in line with published research, this assay acts as a primary phenotypic marker (Du et al., 2012; Haghdoost et al., 2016; Zhang et al., 2023). Variability in this pathogenic trait is highlighted by the different results obtained from the tested species of Candida.

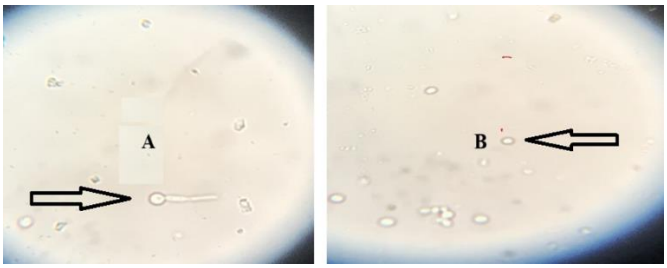


Figure 4: Germ tube formation assay. (A) Candida albicans exhibiting characteristic germ tubes (non-constricted extensions), indicative of a positive result. (B) A non-albicans Candida species (blastospores only), demonstrating a negative result.

3.5 Evaluation of Biofilm Formation

The ability of the Candida isolates to form biofilms, a key virulence characteristic, was assessed. Particularly for Candida albicans, the assay produced a positive result (Knobloch et al., 2002). Because these organized microbial communities have inherent resistance to antimicrobial agents and host immune defenses, biofilms provide a substantial survival advantage and significantly increase pathogenicity (Salari et al., 2018). One well-established factor contributing to Candida albicans' virulence is its capacity to form biofilms on both biotic and abiotic surfaces (Mathur et al., 2006). Figure 1 demonstrates robust biofilm architecture, as evidenced by the adherent cellular conglomerate and extracellular matrix stained with safranin.The results of this study see Figure 4, support the recent evidence that biofilm formation is a common mechanism in the pathogenesis of infections caused by diverse species of Candida (Corbu et al., 2024)

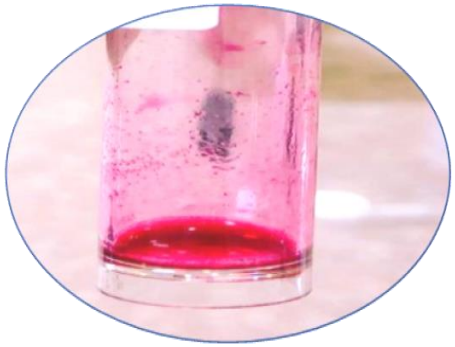


Figure 5: Biofilm formation by Candida albicans. The image demonstrates robust biofilm architecture, as evidenced by the adherent cellular conglomerate and extracellular matrix stained with safranin.

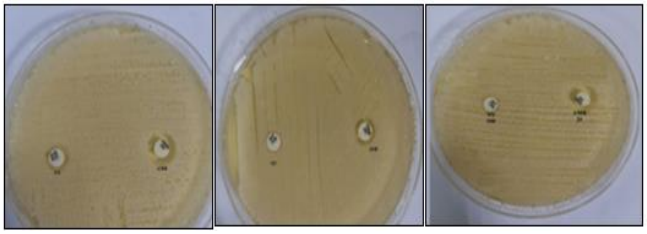
3.6 Phospholipase Activity Assay

Extracellular phospholipase activity, a key virulence trait, was assessed using an egg yolk agar plate assay (Alp and Arikan, 2008; Pasrija and Kumari, 2025). A positive result, indicative of enzyme production, was confirmed by the presence of a distinct precipitation zone surrounding the colony (Nyakeri et al., 2018). All clinical isolates of Candida albicans in this study demonstrated phospholipase activity, a finding consistent with previous reports that underscore its role in pathogenicity as supported by (Dabiri et al., 2018; Gácser et al., 2007; Padmavathi et al., 2025).

Figure 6: Extracellular phospholipase activity of Candida albicans on egg yolk agar. The formation of an opaque precipitation zone (Pz) around the colony indicates a positive result, demonstrating the hydrolysis of phospholipids.

3.7 Antifungal Susceptibility Profiling

The susceptibility of Candida isolates to polyene antifungals was evaluated. This class of antifungals, which includes nystatin and amphotericin B, exerts its effect by binding to membrane sterols, resulting in altered membrane permeability and cell death. The disk diffusion assay revealed that amphotericin B demonstrated superior in vitro efficacy against the tested isolates compared to nystatin, as indicated by larger zones of growth inhibition (see Figures. 7 a,b, and c).



(a) (b) (c)

Figure 7: The Sensitivity to NY and AMP of C.Albicans (a), C.parapsilosis (b) and C. tropicalis (c),

4. Theoretical And Practical Contributions Of The Study

4.1 Theoretical Contributions

- Elucidation of Local Mycological Epidemiology: This study provides the first comprehensive characterization of oropharyngeal fungal colonization patterns specifically within a cohort of Iraqi transfusion-dependent thalassemia (TDT) patients. It fills a significant gap in the literature regarding the prevalence and species distribution of Candida in this immunocompromised population in a Middle Eastern context, contributing valuable epidemiological data to the global understanding of fungal ecology in thalassemia.
- Virulence Profiling of Clinical Isolates: The research offers a detailed phenotypic analysis of key virulence factors—germ tube formation, biofilm production, and phospholipase activity—in clinically isolated Candida strains. The confirmation that C. albicans isolates consistently express this full complement of virulence determinants strengthens the theoretical framework linking specific pathogenic traits to the organism's success as an opportunistic pathogen in immunodeficient hosts.
- Validation of Phenotypic Identification Methods: The study reinforces the theoretical utility of chromogenic media (CHROMagar™ Candida) as a reliable and rapid tool for the presumptive identification of Candida species. By demonstrating a clear correlation between colony color and species identity, it supports the biochemical principles underlying these diagnostic methods, which rely on species-specific enzyme activities.

4.2 Practical Contributions

- Informing Clinical Surveillance and Prophylaxis: The high prevalence of Candida colonization (28% of patients) and the dominance of the

highly virulent C. albicans highlight the critical need for routine mycological surveillance in TDT patients. This finding provides a practical rationale for implementing regular oral screening protocols in thalassemia treatment centers to enable early detection and preemptive management of fungal infections.

- Guiding Empirical Antifungal Therapy: The antifungal susceptibility results provide immediate, actionable data for clinicians. The demonstrated superiority of amphotericin B over nystatin against the local isolates offers evidence-based guidance for selecting effective prophylactic or therapeutic regimens in this patient population, potentially improving treatment outcomes and slowing the development of resistance.
- Standardization of Laboratory Protocols: The detailed, replicable methodologies described for virulence factor testing (germ tube, biofilm, and phospholipase assays) serve as a practical laboratory guide. Other researchers and diagnostic labs can adopt these standardized protocols to assess the pathogenic potential of Candida isolates, facilitating comparative studies and consistent diagnostics.
- Public Health and Antimicrobial Stewardship: By identifying the specific Candida species and their antifungal susceptibility profiles circulating in this niche population, the study contributes crucial data to regional and national antimicrobial stewardship programs. This information is vital for developing targeted treatment guidelines and managing the growing challenge of antifungal resistance, ultimately aiding in public health policy decision-making.

5. Conclusion

This study offers a thorough examination of oropharyngeal fungal colonization in Iraqi patients with transfusion-dependent thalassemia, a group that is more vulnerable to opportunistic infections because of immunologic compromise and iron overload. Candida albicans (72.5%) dominated the mycological profile, with the remaining isolates being C. tropicalis (5.1%) and C. parapsilosis (22.4%). Given that every tested C. albicans isolate showed a strong virulence phenotype and demonstrated competence in germ tube formation, biofilm production, and phospholipase secretion—essential mechanisms that promote tissue invasion and immune evasion—this distribution is clinically significant.

The data on antifungal susceptibility shed more light on the therapeutic environment. The disk diffusion assay's conclusion that amphotericin B is more effective than nystatin offers crucial empirical support for treatment strategy guidance. Considering the patterns of acquired and intrinsic resistance seen in invasive candidiasis, this is especially pertinent.

All things considered, these results point to a triple risk in this patient group: a high frequency of pathogenic yeast colonization, the expression of several virulence factors that make invasive disease more likely, and a variable profile of antifungal response. In this context, Candida albicans reaffirms its position as the leading fungal pathogen. In order to lessen the burden of potentially fatal fungal infections in this susceptible group, these findings support the use of routine mycological surveillance in thalassemia patients and emphasize the need for continuous antifungal susceptibility testing to guide preventative and treatment choices.

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