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Identification and Evaluation of Drug Resistance and Exoenzymes in *Candida albicans* Isolated from Nails

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ABSTRACT

Background and Objective: Candidiasis is the most common yeast-related infection worldwide, with infections caused by *Candida albicans* increasing over the past two decades. Limited information exists regarding its pathogenic characteristics. This study aimed to evaluate drug resistance, pathogenicity, and biological mechanisms of *C. albicans* to inform prevention and treatment strategies.

Materials and Methods: Antifungal susceptibility to fluconazole, voriconazole, amphotericin B, and caspofungin, phospholipase and proteinase activities, and biofilm formation were assessed in 26 *C. albicans* isolates obtained from patients' nails. Identification was confirmed on CHROMagar medium.

Results: Phospholipase activity was high in 6 isolates, moderate in 14, and low in 6. Proteinase activity was high in 3 isolates, moderate in 5, and low in 18. All isolates formed biofilms. MIC testing revealed fluconazole susceptibility in 89.5% of isolates; amphotericin B, 100%; voriconazole, 90%; and caspofungin, 80%. Intermediate or dose-dependent responses were observed in the remaining isolates, with 10.5% resistance to caspofungin.

Conclusion: *Candida albicans* employs multiple pathogenic mechanisms, posing a persistent risk for infections. Antifungal susceptibility assessment is essential for selecting effective treatments and designing preventive measures.

Keywords: *Candida albicans*, candidiasis, virulence factors, antifungal susceptibility, phospholipase, proteinase, biofilm, MIC.

Introduction

Fungi are eukaryotic organisms that lack motility (except for oomycetes) and do not perform photosynthesis. The optimal pH for their growth is approximately 6; however, fungi can tolerate pH values ranging from 2 to 10, and sometimes even beyond this range. They are aerobic organisms that generally grow best at temperatures between 20°C and 30°C. Fungi are broadly classified into two main categories: filamentous fungi and yeasts. Some pathogenic fungi are dimorphic, exhibiting a yeast form when growing on nutrient-rich media at 37°C and a filamentous form in nature or when cultured at 25°C on standard laboratory media. Certain yeasts, such as *Candida albicans*, can produce elongated chains of cells known as pseudohyphae within animal tissues or on specific culture media (1).

Dermatophytosis is a type of fungal skin infection that manifests with symptoms such as itching, inflammation, skin changes, and secondary infections. It is a superficial fungal infection affecting the skin, hair, and nails. More than 150 Candida species are known to inhabit various parts of the human body, including the oral cavity, skin, gastrointestinal tract, and vagina (2).

C. albicans is a diploid, dimorphic fungus that reproduces asexually and is found both in the environment and as part of the human normal flora on skin and mucosal surfaces. Identification of *C. albicans* from other Candida species relies on morphological and biochemical characteristics, germ tube testing, and molecular techniques. *C. albicans* is an endogenous commensal present in the gastrointestinal tract of nearly all mammals and birds. Although it is a unicellular organism, it forms true hyphae, thus being referred to as a yeast-like fungus. Because it can exist both as a yeast and as a hyphal form, it is considered reverse dimorphic and can colonize the oral cavity, lower gastrointestinal tract, and female genital tract(3).

The ability of Candida species to cause invasive infections is associated with several

virulence factors, including extracellular enzymes such as phospholipases, esterases, and proteinases; biofilm formation; and mutations or overexpression of genes such as *ERG11*, *HWP1*, and *SAP1–2*. Biofilm formation is one of the characteristic features of *C. albicans*, providing protection against antifungal agents and the host immune system. Moreover, *C. albicans* biofilms on mucosal surfaces and medical devices are responsible for nosocomial infections(4).

Among the major virulence factors, secreted aspartyl proteinases (*SAPs*) are known as extracellular hydrolytic enzymes produced by Candida species. The products of *SAP1–8* genes are secreted extracellularly, while those of *SAP9* and *SAP10* are membrane-bound. *SAP1* and *SAP3* play key roles in mucosal infections by promoting adhesion, tissue damage, and evasion of host immune responses. These genes are especially significant in oropharyngeal candidiasis and in tissue invasion during *C. albicans* infections. *SAP2* is highly expressed in oral candidiasis, and overall, *SAP* enzymes play critical roles in colonization and tissue invasion by Candida species (5).

Ergosterol is the main sterol component of fungal cell membranes. It was first isolated from ergot by Charles Tanret in 1889. Its physiological roles include maintaining membrane integrity and fluidity, both essential for fungal cell cycle completion. Ergosterol is also involved in mating processes, influencing cell morphology, which in turn affects cell fusion during mating. Most antifungal drugs used to treat Candida infections target either the ergosterol biosynthetic pathway or ergosterol itself. One of the key genes in the ergosterol synthesis pathway of Candida cell walls is *ERG11*. Mutations or altered expression of this gene result in resistance to antifungal agents. Any compound that inhibits ergosterol synthesis can cause fungal death, as Candida cannot survive without ergosterol(6).

Voriconazole is an antifungal drug belonging to the azole class. Resistance of Candida species to azoles is the most common form of antifungal resistance. Clinically, azole resistance is most frequently observed in two conditions: (1) candidemia in immunocompromised patients and (2) mucosal candidiasis in individuals with AIDS (7).

Phenotypic switching and biofilm formation in *C. albicans* facilitate evasion of host defense mechanisms. During the early stages of infection, yeast cells are typically eliminated by phagocytic mechanisms; however, the remaining cells can convert into hyphal forms, which are more invasive and resistant to phagocytosis due to their larger size. Phospholipases concentrated at hyphal tips further enhance tissue invasion(8).

The localized mucocutaneous form of candidiasis results from overgrowth of *C. albicans* in the oral cavity or gastrointestinal and urinary tracts. Predisposing factors include cellular immune deficiency, comorbid diseases, disruption of normal flora due to prolonged antimicrobial therapy, and mucosal injury from catheters. Damaged mucosa becomes thickened and hyperemic. Hematogenous dissemination may occur following vascular invasion by hyphae or pseudohyphae, leading to systemic lesions(9).

Onychomycosis is a fungal infection of fingernails or toenails caused by various dermatophytes, yeasts, and saprophytic molds, accounting for approximately 50% of all nail diseases. In some studies, onychomycosis represents up to 30% of superficial fungal infections. Its prevalence varies widely across regions; recent reports estimate an incidence of about 20% among individuals aged 40–60 years. The most common dermatophyte associated with onychomycosis is Trichophyton rubrum, while the predominant yeast pathogen is *C. albicans*. Generally, onychomycosis caused by molds and dermatophytes affects toenails, whereas yeast-induced cases more often involve fingernails(10).

Several predisposing factors have been identified, including impaired local circulation, peripheral neuropathy, diabetes mellitus, repetitive microtrauma, mild immunodeficiencies, and AIDS. Despite the availability of effective antifungal agents, onychomycosis remains a persistent health concern. Accurate diagnosis relies on microscopic examination, fungal culture, and histopathological tests, while species identification is commonly based on phenotypic characteristics such as colony morphology, biochemical properties, and sugar assimilation/fermentation profiles. Although these methods are generally reliable, they are time-consuming and sometimes limited in distinguishing less common species. Newer approaches focus on genotypic analysis, such as sequencing specific genomic DNA regions of

yeasts.

Studies conducted in Iran have identified *C. albicans* as the most prevalent species, along with several other common Candida species. However, less frequent isolates are often collectively referred to as "other species" (11).

Materials and Methods

1. Cultivation of Fungal Strains

Thirty fungal strains obtained from the Pasteur Institute Mycology Bank were cultured on Sabouraud Dextrose Agar (SDA) medium supplemented with chloramphenicol. The cultures were incubated at 37°C for 2–3 days to obtain fresh isolates for use in various parts of the study (13).

2. Evaluation of Fungal Growth on CHROMagar Medium

Yeast cells exhibit considerable diversity in appearance, including variations in size, shape, and color. Even pure cultures of a single species may display distinct morphological features depending on the culture medium. When cultured on CHROMagar medium, different Candida species produce colonies of varying colors, allowing for presumptive identification (14).

Clinical isolates were inoculated on CHROMagar Candida plates and incubated at 28°C for 24–48 hours, following local laboratory standard procedures. Although standard protocols recommend 35–37°C for optimal growth and color differentiation, the lower temperature was used to maintain consistency with previous experiments in our laboratory Species identification was performed based on colony color according to the manufacturer's instructions. Only isolates identified as *C. albicans* were included in subsequent analyses (15).

3. Antifungal Susceptibility Testing (Serial Dilution Method)

Antifungal susceptibility testing was performed according to the Clinical and Laboratory Standards Institute (CLSI) guideline M27-A3(16). Initially, serial dilutions of each antifungal agent were prepared in RPMI 1640 medium as indicated in the standard protocol.

A yeast cell suspension equivalent to 0.5 McFarland standard was prepared for each isolate by transferring a small portion of colony into sterile physiological saline. The optical density (OD) of the suspension was measured using a spectrophotometer. An OD reading between 0.75 and 0.77 corresponded to the 0.5 McFarland standard. The final working suspension was prepared by diluting the 0.5 McFarland suspension 1:1000 in RPMI 1640 medium.

The diluted suspensions were dispensed into 96-well microtiter plates. Serial dilutions of fluconazole, voriconazole, caspofungin, and amphotericin B were added to the wells containing the yeast suspensions. Plates were incubated at 35°C for 24 hours as recommended by CLSI M27-A3 guidelines. The minimum inhibitory concentration (MIC) — the lowest concentration of drug preventing visible growth — was determined. For amphotericin B, the MIC was defined as the lowest concentration showing complete growth inhibition; for caspofungin, the first concentration reducing growth by 50%; and for azoles, the first concentration showing a noticeable reduction in yeast growth. Interpretation of results was based on the CLSI M27-A3 standard Table 1 (17).

4. Determination of Phospholipase Activity

Extracellular phospholipase activity was determined by measuring the precipitation zone formed on solid medium containing egg yolk emulsion. A 5 µL aliquot of each yeast suspension was inoculated onto the egg yolk agar medium, and the assay was performed in duplicate for each isolate. Plates were incubated at 37°C for 5 days(18).

The presence of phospholipase activity was indicated by the formation of an opaque zone surrounding the yeast colony. Phospholipase activity (Pz value) was calculated as the ratio of the colony diameter to the total diameter of the colony plus the precipitation zone (colony diameter / [colony diameter + halo diameter]) (17,18).

Table 1. **Stock solution concentration and solvent used for the antifungal agents**

Drug name	Maximum drug concentration (mg/mL)	Drug solvent according to CLSI
Fluconazole	64	DMSO
Voriconazole	16	DMSO
Amphotericin B	16	DMSO
Caspofungin	16	DMSO

Table 2. Interpretation guide for phospholipase and proteinase enzymatic activity

Enzymatic Activity	Pz Value
High	< 0.6
Moderate	0.7–0.9
Low	0.9
Absent	1.0

5. Determination of Proteinase Activity

Among the major protease families, aspartyl proteases are particularly important. These enzymes are encoded by a ten-gene family (*SAP1–SAP10*) and facilitate colonization of yeast cells within host tissues by degrading host mucosal membranes and breaking down defense-related proteins and immunological components. Aspartyl proteinases are capable of degrading cell membrane proteins such as collagen, mucin, keratin, antibodies, and complement proteins (17). Exposure to subinhibitory concentrations of antifungal agents can promote the emergence of resistant strains exhibiting increased production of these enzymes. Typically, drug-resistant isolates express higher levels of proteinase activity compared to susceptible ones. Proteinase activity was assessed using bovine serum albumin (BSA) agar medium composed of 1.17% yeast carbon base, 1.01% yeast extract, and 0.2% BSA. The pH of the medium was adjusted to 5.0, filtered, and mixed with 2% sterilized agar. Plates were incubated at 37°C for 5 days. After incubation, opaque zones surrounding the colonies were measured, and proteinase activity was evaluated based on Pz values as presented in Table 2.

6. Biofilm Formation Assay

The initial stage of Candida infection involves adhesion to host cells or prosthetic surfaces, leading to colonization and subsequent biofilm development. This process plays a crucial role in Candida pathogenicity. The ability of Candida species to adhere is attributed to specific cell wall proteins. Following initial attachment, yeast cells proliferate and produce a biofilm — a structured community of microorganisms enclosed within an extracellular polymeric matrix. This matrix restricts antifungal penetration and protects fungal cells from host immune responses (19). Biofilm-forming ability was tested for each clinical isolate. Yeast cells were first cultured on Sabouraud Dextrose Agar plates at 37°C for 24 hours. After incubation, a cell suspension was prepared for each isolate. For biofilm induction, 180 µL of Sabouraud Dextrose Broth and 20 µL of the yeast suspension were added to each well of sterile, flat-bottom 96-well microtiter plates. The plates were incubated at 37°C for 90 minutes to allow initial adhesion. After the adhesion phase, the supernatant containing planktonic (free-floating) cells was removed, and each well was washed with sterile phosphate-buffered saline (PBS) to eliminate non-adherent cells. Fresh medium was then added, and plates were incubated again at 37°C for 48 hours.

Biofilm formation was quantified using the crystal violet staining method. Wells were washed twice with 200 µL PBS and air-dried. Then, 100 µL of 99% methanol was added to each well for 15 minutes to fix the biofilm. The methanol was removed, and the plate was air-dried for 20 minutes. Subsequently, 100 µL of 4% crystal violet solution was added to each well and incubated at room temperature for 45 minutes. After staining, the wells were washed four times with sterile water, and 150 µL of 33% acetic acid was added to each well to solubilize the dye. The optical density (OD) of the resulting solution was immediately measured at 590 nm using a microplate reader (19).

7. Statistical Analysis

All experiments were performed in duplicate or triplicate, and data are presented as frequencies or percentages. The association between categorical variables, such as enzyme activity levels (phospholipase, proteinase) and antifungal susceptibility patterns, was evaluated using the Chi-square (χ^2) test. This non-parametric test assesses whether observed frequencies differ significantly from expected frequencies under the null hypothesis of no association between variables. A p-value < 0.05 was considered statistically significant. All analyses were conducted using [specify software, e.g., SPSS version 26.0](20).

Results

Morphological Identification

On CHROMagar medium, *Candida albicans* colonies appeared green, *C. tropicalis* blue, *C. krusei* pink, and *C. glabrata* purple. Based on colony color, four non-*C. albicans* isolates were identified and excluded from further analysis, leaving 26 *C. albicans* isolates for subsequent testing.

Antifungal Susceptibility Testing (Serial Dilution Method)

Table 3. MIC values of the antifungal

Drug name	MIC Endpoint	Breakpoint CLSI
Fluconazole	50%<growth reduction	8> Susceptible 64< Resistant
Voriconazole	50%<growth reduction	0.125> Susceptible 1> Resistant
Amphotericin B	50%<growth reduction	0.25> Susceptible 1> Resistant
Caspofangin	100%-90 growth reduction	1-o,5 Susceptible 2< Resistant

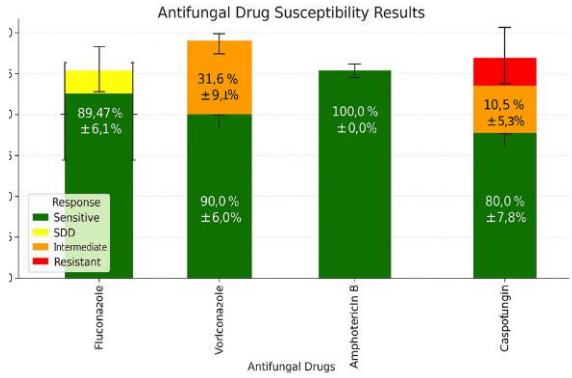

Figure 1. Antifungal susceptibility results

Antifungal susceptibility was determined for 26 *C. albicans* isolates using the standard CLSI M27-A3 microdilution method. The MIC values (µg/mL) for each antifungal agent are summarized in Table 3. Statistical analysis was performed to calculate the mean, standard deviation (SD), and range of MICs.

Reference: Clinical and Laboratory Standards Institute (CLSI). Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard—Second Edition. CLSI document M27-A3.

For Fluconazole: 23 isolates (88.5%) were fully susceptible, 3 isolates (11.5%) exhibited dose-dependent susceptibility. MIC range: 0.25–16 µg/mL, mean ± SD: 2.5 ± 4.2 µg/mL(21). For Voriconazole: 23 isolates (88.5%) were susceptible, 2 isolates (7.7%)

demonstrated intermediate response, 1 isolate (3.8%) resistant. MIC range: 0.03–2 µg/mL, mean ± SD: 0.4 ± 0.5 µg/mL(22). For Caspofungin: 21 isolates (80.8%) were susceptible, 4 isolates (15.4%) intermediate response, 1 isolate (3.8%) resistant. MIC range: 0.03–1 µg/mL, mean ± SD: 0.2 ± 0.3 µg/mL(23).

Phospholipase Activity

Extracellular phospholipase activity was determined by measuring the precipitation zone (Pz) around colonies grown on egg yolk agar medium. Plates were incubated at 37 °C for 5 days. The Pz values of the 26 C. albicans isolates ranged from 0.55 to 0.70, with a mean ± SD of 0.62 ± 0.05, indicating moderate phospholipase activity. Statistical analysis using one-way ANOVA showed no significant difference between isolates ($p > 0.05$)(24).

Proteinase Activity

Protease enzyme production was evaluated on bovine serum albumin (BSA) agar medium. Plates were incubated at 37 °C for 5 days. Pz values ranged from 0.85 to 0.95, with a mean ± SD of 0.90 ± 0.04, indicating low proteinase activity among most isolates.

Statistical comparison among isolates revealed no significant variation ($p > 0.05$)(24).

Biofilm Formation Ability

Biofilm formation was quantified in 96-well microtiter plates using crystal violet staining and measuring OD at 590 nm(25). The percentage of blocked light transmission (Tbloc%) was calculated as:

$$Tbloc\% = \%T_{control} - \%T_{\_test}$$

Biofilm Scoring:

Negative: <10%
Weak (1+): 10–20%
Moderate (2+): 20–35%
Strong (3+): 35–50%
Very strong (4+): ≥50%

Discussion and Conclusion

In recent decades, numerous studies have focused on *Candida albicans*, particularly its biofilm formation, secreted enzymes (such as proteinases and phospholipases), antifungal resistance, and epidemiology. Populations at risk—including preterm neonates, patients with malignancies or autoimmune diseases, and immunocompromised individuals—along with the widespread use of invasive devices (e.g., catheters, prostheses), have led to an increasing incidence of candidiasis. Our findings align with previous reports indicating that *C. albicans* remains a major nosocomial pathogen, highlighting the urgent need for effective treatment and preventive strategies(1,26).

The antifungal susceptibility patterns observed in this study revealed that fluconazole resistance was present in a subset of isolates, whereas caspofungin generally exhibited full susceptibility. These results are consistent with previous studies reporting high azole resistance and preserved echinocandin activity(1,26). Fluconazole resistance is frequently associated with *ERG11* mutations or overexpression of efflux pumps (*CDR* and *MDR*), providing a plausible biological explanation for the observed decreased sensitivity in some isolates(23).

Amphotericin B showed full susceptibility in all isolates, consistent with prior studies demonstrating its efficacy against planktonic *C. albicans* forms (18). Nevertheless, clinical efficacy may vary due to differences between MIC and MFC values, as reported by several authors. Caspofungin displayed strong activity against most isolates, in agreement with other reports; however, some studies have shown reduced efficacy against biofilms compared to planktonic cells, emphasizing the importance of considering biofilm-associated infections when selecting therapy.

Our phenotypic assessment of biofilm formation and hydrolytic enzyme activity revealed that isolates produced more biofilm at 30°C than at 37°C. This observation is in line with previous findings indicating that environmental conditions, including temperature and growth medium, strongly influence biofilm development and matrix maturation. Moreover, variability in biofilm formation has been reported between clinical and laboratory isolates, as well as between young and mature biofilms, highlighting the need for careful extrapolation of laboratory data to clinical settings (19,22).

The concurrent presence of biofilm formation and hydrolytic enzyme activity in our isolates may contribute to reduced azole susceptibility. Biofilm matrices can trap antifungal molecules, creating microenvironments with sub-inhibitory drug concentrations that promote survival of tolerant cells. These findings are consistent with reports showing correlations between enzyme secretion, biofilm formation, and azole resistance.

Phospholipase and proteinase activities were generally moderate and low, respectively, which aligns with prior studies showing variable enzyme activity among *C. albicans* isolates depending on strain, source, and culture conditions. Importantly, the simultaneous assessment of biofilm formation and enzyme activity provides a more comprehensive view of virulence factors contributing to *C. albicans* pathogenicity(17,26).

Overall, the present study reinforces previous observations that most *C. albicans* strains are capable of biofilm formation, and some can also produce phospholipase and proteinase enzymes. Environmental temperature significantly affects these phenotypes, and these factors together can influence antifungal susceptibility and clinical outcomes.

Conclusion

In conclusion, our study demonstrates that *C. albicans* clinical isolates exhibit strong biofilm formation, moderate phospholipase activity, and low proteinase activity, with variable azole susceptibility and consistent echinocandin sensitivity. These findings are consistent with previous reports and emphasize the clinical importance of assessing virulence factors alongside antifungal susceptibility. Effective management of *C. albicans* infections, particularly those associated with biofilms on prosthetic devices or catheters, requires careful selection of antifungal agents and consideration of the pathogen's enzymatic and biofilm-forming capabilities. Future studies should focus on elucidating the molecular mechanisms linking enzyme production, biofilm formation, and antifungal resistance to inform better therapeutic strategies.

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