Genotyping, antifungal susceptibility, enzymatic activity, and phenotypic variation in *Candida albicans* from esophageal candidiasis

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Abstract

**Background:** Esophageal candidiasis is the most frequent form of esophagitis. The pathogenicity of *Candida* spp. is related to a combination of microbial factors, hydrolytic enzyme secretion and phenotypic switching. This study was designed to investigate esophageal candidiasis, antifungal activity, enzymatic activity patterns, phenotyping, and genotyping profiles of *Candida albicans* species.

**Methods:** Nine hundred thirty-three visited patients were evaluated, and esophageal biopsies from patients were included in this study during 2019–2020. Direct smear, Gram staining, and culture on CHROMagar were performed for each sample. Isolated species were identified with conventional procedures and PCR-RFLP. Susceptibility to antifungals was determined according to CLSI guidelines. ABC typing, phenotype switching, hemolysin, proteinase, phospholipase, and esterase activity were also determined with the appropriate protocols.

**Results:** Twenty-three (2.5%) patients (mean age 55.2 years) were diagnosed with esophageal candidiasis. The species isolated were 19 (82.6%) *C. albicans*, 3 (13.1%) *C. glabrata*, and 1 (4.3%) *C. tropicalis*. Genotype A (57.9%) was the predominant type in *C. albicans* isolates. 50% of *C. albicans* isolates exhibited a white phenotype. A high level of phospholipase (47.4%), hemolysin (68.4%), and proteinase activity (36.8%) was observed in the *C. albicans* isolates. Only three *C. glabrata* isolates displayed non-wild type susceptibility to voriconazole and itraconazole.

**Conclusion:** This study shows that *C. albicans* are still the most frequent isolates from patients with esophageal candidiasis. The predominance of genotype A, the white phenotype, and strong hemolysin activity may indicate a high prevalence of pathogenicity in these isolates. Sensitivity to antifungal drugs was greatest for amphotericin and fluconazole.

**Keywords**
antifungals, *Candida albicans*, enzymatic activity, esophageal candidiasis, genotyping, phenotypic variation
INTRODUCTION

Candidiasis is a yeast infection with worldwide distribution. Although superficial forms of the disease are benign, deep forms of candidiasis are life-threatening, especially in immunocompromised patients. Candida spp. are common opportunistic pathogens originating from the mycobiota of human mucosal sites such as the oropharynx, esophagus, and vagina.\textsuperscript{1,2} The yeast takes advantage of an impaired immune system, which allows proliferation in the esophageal mucosa in the form of adhesive plaques.\textsuperscript{3,4} Esophageal candidiasis is the most frequent form of infectious esophagitis and is usually common among patients infected with the human immunodeficiency virus.\textsuperscript{5,6} The prevalence of esophageal candidiasis ranges from up to 5.2% in the general population to 9.8% in HIV-positive patients.\textsuperscript{7,8} Associated risk factors reported to date include increasing age, HIV infection, use of antibiotics, herbal medication, heavy drinking, and high-dose corticosteroids.\textsuperscript{8,9}

The pathogenicity of Candida spp. is related to a combination of microbial factors such as their ability to grow at 37°C, polymorphism, biofilm formation, hydrolytic enzyme secretion, and phenotypic switching.\textsuperscript{10-12} Candida spp. can secrete lipolytic enzymes such as esterase and phospholipase. The secretion of phospholipases can disrupt phospholipids in the host mucosal epithelium, which is a major constituent of cell membranes.\textsuperscript{13} Esterase activity is also useful in differentiating some Candida spp.\textsuperscript{14} Clinical Candida isolates produce considerably more hemolysin than environmental isolates.\textsuperscript{15} About ten proteinase isoenzymes with various functions have been described in Candida albicans. Some of them are related to adherence, tissue damage, and changes in the immune response, and increased activity can lead to severe candidiasis.\textsuperscript{11}

Candida albicans as a major human fungal pathogen\textsuperscript{16-19} can switch between different morphological phenotypes. Histopathological examination in a murine model demonstrated the presence of pseudohyphae and C. albicans yeasts in kidney tissue.\textsuperscript{20} The best described fungal phenotype switch is the white-to-opaque transition.\textsuperscript{21} Different cell types vary in certain features including cell and colony appearance, mating, adherence, enzyme secretion, and their susceptibility to antifungals.\textsuperscript{22,23} A reproducible and discriminatory strain typing system for C. albicans is beneficial for clinical and epidemiological studies, because it can provide useful information especially on properties such as virulence and antimicrobial resistance.

ABC genotyping is a reproducible and straightforward method of strain typing for C. albicans. It is valuable for primary clinical and epidemiological studies because it provides information especially on the relations between strain types and properties such as virulence and antimicrobial resistance. According to this method, C. albicans is divided into several genotypes based on the electrophoresis banding pattern, for example, A, B, C, D, and E.\textsuperscript{24,25} In oral, vulvovaginal, and nail samples, the A genotype was the predominant type in previous studies.\textsuperscript{26-28}

According to the guidelines of the Infectious Diseases Society of America (IDSA), antifungal treatment for Candida infections is restricted to three major classes of antifungal drugs: azoles, polyenes, and echinocandins.\textsuperscript{29} Although antifungal resistance in C. albicans is less frequent than in other species, an increasing number of resistant strains are emerging.\textsuperscript{22,30} However, despite the profusion of data on antifungal susceptibility patterns of C. albicans, there is only limited information on the association between enzymatic activity, phenotypic variation, and antifungal susceptibility.\textsuperscript{30-32}

This study was designed to investigate etiologic agents of esophageal candidiasis, their antifungal susceptibility profiles, virulence patterns including enzymatic activity (phospholipase, protease, esterase, and hemolysin), phenotype variations, and genotyping methods for C. albicans isolates.

MATERIALS AND METHODS

2.1 Sampling and isolation

The present cross-sectional study was carried out in the department of internal medicine of Golestan Hospital affiliated to Ahvaz Jundishapur University of Medical Sciences, Iran. All patients who underwent endoscopy between 2019 and 2020 were included in the initial screening. Twenty-three esophageal biopsy samples from patients with candidiasis plaques who underwent gastrointestinal endoscopy were included in this study. Samples were transferred to normal saline and sent to the Medical Mycology Laboratory affiliated to Ahvaz Jundishapur University of Medical Sciences. Direct smears from each specimen were prepared and used for Gram staining; the presence of gram-positive budding yeast cells with pseudohyphae indicated candidiasis. The samples were cultured on CHROMagar Candida medium (CHROMagar Candida) and incubated at 35°C for 48–72 h.

Identification of isolates

Yeasts were initially identified by conventional methods, using CHROMagar Candida culture medium, germ tube formation in human serum, growth at 42°C, and the production of chlamydocnidia on cornmeal agar (HiMedia). For accurate identification, the genomic DNA of each strain was extracted with the DNA extraction protocol described by L"{o}"{o}ke et al\textsuperscript{33} Briefly, one yeast colony was picked up from an overnight culture of purified yeast with a sterile inoculation needle. Cells were suspended in 100 μl of 200 mM LiOAc (CDH) in 1% SDS (CinnaGen) solution. The suspension was incubated for 5 min at 70°C, and then, 300 μl absolute ethanol (Mojallali) was added. DNA and cell debris were spun down. The pellet was washed with 70% ethanol and dissolved in 100 μl deionized water.

Molecular identification of Candida species was performed with a PCR-RFLP method previously described by Mirhendi et al\textsuperscript{34} Briefly, the ITS1-5.8SrDNA-ITS2 region was amplified with a PCR mixture consisting of 12.5 μl Taq DNA Polymerase 2× Master Mix RED with 1.5 mM MgCl\textsubscript{2} (Ampliqon), 5 pmol of both ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCC
TCCGCTTATTGATATGC-3') primers, and 2 μl extracted DNA in a final volume of 25 μl. The PCR cycling conditions were as follows: initial denaturation at 94°C for 5 min, followed by 34 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 45 s, and extension at 72°C for 30 s, with a final extension at 72°C for 7 min. Ten microliters of each PCR product was separated by electrophoresis on 1.5% agarose gel. Subsequently, the PCR products were digested with MspI (Thermo Fisher Scientific) restriction enzyme according to the manufacturer’s protocol. Finally, 10 μl RFLP products was separated by gel electrophoresis on 2% agarose gel. Bands of 238, 297 bp; 320, 561 bp; 186, 340 bp; and 520 bp were identified, respectively, as C. albicans, C. glabrata, C. tropicalis, C. krusei, C. guilliermondii, and C. parapsilosis.

2.3 Genotype determination

ABC genotyping was carried out with 1 μl template DNA. 12.5 μl Taq DNA Polymerase 2× Master Mix RED with 1.5 mM MgCl₂ (Ampliqon), and 5 pmol of both CAIN-L (5′-ATAAGGGAAGTCGG CAAAATAGATCCGTAA-3′) and CAIN-R (5′-CTTGGCTGTTGCAGTAGATAGATAG-3′) primers. The PCR cycling conditions were as follows: initial denaturation at 94°C for 5 min, followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, with a final extension at 72°C for 7 min. Ten microliters of each PCR product was separated by electrophoresis on 1.5% agarose gel. Candida albicans isolates can be separated into three genotypes depending on the presence or absence of an intron in the internal transcribed spacer 1 (ITS1) region of 26 s rDNA. According to the PCR products, the genotypes of C. albicans can be categorized as group A (450 bp), group B (840 bp), and group C (450 and 840 bp).

2.4 Antifungal susceptibility testing

Antifungal susceptibility testing was done according to CLSI M27 4th edition. Briefly, all isolates were cultured on Sabouraud dextrose agar (SDA) (Pronadisa, Spain) before susceptibility testing to ensure purity and viability. Yeast inoculum was prepared by suspending colonies of each isolate from an overnight culture in sterile distilled water, and suspensions were adjusted to a turbidity of 0.5 McFarland standard to obtain 1 × 10⁶ to 5 × 10⁶ CFU/ml. Antifungal drugs were obtained from their respective manufacturers as standard powders. Stock solutions of amphotericin B (Sigma-Aldrich), nystatin (Sigma-Aldrich), itraconazole (Sigma-Aldrich), fluconazole (Serva), caspofungin (Sigma-Aldrich), econazole (Sigma-Aldrich), luloconazole (APIChem), and voriconazole (Sigma-Aldrich) were prepared in dimethyl sulfoxide (Merck). The final drug concentration ranged from 16 to 0.03 μg/ml for amphotericin B, nystatin and fluconazole; 0.5 to 0.0001 μg/ml for caspofungin, voriconazole, and luloconazole; and 8 to 0.01 μg/ml for itraconazole and econazole.

Serial twofold dilutions of the different drugs were prepared in RPMI 1640 medium (with l-glutamine, without bicarbonate) (Bio Basic) and buffered to pH 7.0 with a 0.165 M solution of MOPS (Bio Basic). Yeast suspensions were diluted in RPMI to a concentration of 1 × 10⁶ to 5 × 10³ CFU/ml. Minimum inhibitory concentrations (MICs) were determined in 96-well plates as follows: inoculated plates were incubated at 35°C and the plates were read visually after 24 h. The MIC was considered the concentration of a drug that elicited 100% inhibition of growth (amphotericin B and nystatin) or 50% inhibition of growth (fluconazole, itraconazole, caspofungin, voriconazole, econazole, and luloconazole). Susceptibility results were interpreted according to the CLSI breakpoints (M27-S4) and percent ECVs.

2.5 Evaluation of Candida albicans microcolonies with fluconazole E test strip

Isolates that exhibited trailing in response to fluconazole were evaluated to identify alterations in antifungal susceptibility profile and phenotype. With E test strips, trailing is apparent as the presence of microcolonies in the inhibition zone. Yeast suspensions were prepared as described in CLSI M27 4th edition. Minimum inhibitory concentrations were determined with fluconazole E test strips (TANA Giotech) after 24 and 48 h of incubation. For microcolonies within the elliptical inhibition zone, one colony situated nearest to the highest concentration was picked up with a sterile needle and cultured on SDA. After 24 h, fluconazole susceptibility was determined with the E test a second time. The MIC of all other antifungal drugs in the present study was also determined, and phenotype variations were assessed. This process was repeated three times.

2.6 Phenotype switching

Yeast extract peptone dextrose agar (YPD; Merck) supplemented with phloxine B (5 mg/L) (Merck) was used for phenotype switching assays. Phloxine B stains opaque colonies dark pink and gray colonies light pink, while white colonies remain white. Yeast suspensions were prepared from 24-h-old cultures in sterile distilled water and were adjusted to 500–1000 CFU/ml. Thirty microliters of each suspension was spread on YPD plus phloxine B plates and incubated at 37°C for 5 days.

2.7 Enzymatic activity

Proteinase production was evaluated on medium containing 1 g KH₂PO₄ (Merck), 0.5 g MgSO₄ (Merck), 0.1 g yeast extract (Merck, Germany), 10 g glucose (PanReac), and 2 g bovine serum albumin (Merck, Germany) in 100 ml distilled water at pH 5.0. The solution was sterilized by filtration and added to previously autoclaved agar solution (20 g agar powder in 900 ml distilled water). The inoculum
was placed on the agar medium and incubated for seven days at 28°C. The proteolysis of bovine serum albumin was visualized as a clear halo around the inoculated zone after staining with 1% amido black (Cellogel).

Phospholipase production was visualized in egg yolk agar plates as described by Samaranayake et al.\(^{41}\) The medium consisted of 65 g SDA, 4.58 g NaCl (Mojallali Chemical Laboratory), and 5.5 g CaCl\(_2\) (Merck in 920 ml distilled water. The medium was sterilized, and

| No. | Genotype | Amp | Flu | Vor | Itr | Cas | Nys | Eco | Lul | Hemo | Pro | Phos | Ester | Phe swi |
|-----|----------|-----|-----|-----|-----|-----|-----|-----|-----|------|-----|------|-------|---------|
| C. albicans 4 | A | WT | S | S | S | S | 4 | 1 | 0.00195 | VS | VS | M | VS | G |
| C. albicans 6 | A | WT | S | S | S | S | 4 | 1 | 0.0156 | VS | VS | VS | VS | Wh |
| C. albicans 8 | A | WT | S | S | S | S | 4 | 4 | 0.0156 | N | M | S | VS | Wh |
| C. albicans 9 | A | WT | S | S | S | S | 4 | 4 | 0.0156 | VS | M | S | VS | Wh |
| C. albicans 10 | A | WT | S | S | S | S | 4 | 0.25 | 0.0156 | VS | N | VS | VS | Wh & O |
| C. albicans 12 | A | WT | S | S | S | S | 2 | 1 | 0.125 | M | N | VS | VS | Wh |
| C. albicans 13 | A | WT | S | S | S | S | 4 | 0.016 | 0.000976 | VS | VS | W | VS | G |
| C. albicans 16 | A | WT | S | S | S | S | 4 | 0.25 | 0.0156 | VS | M | M | VS | Wh & G |
| C. albicans 17 | A | WT | S | S | S | S | 4 | 0.125 | 0.0078 | M | N | S | VS | G |
| C. albicans 18 | A | WT | S | S | S | S | 4 | 0.062 | 0.0039 | M | M | S | VS | Wh & G |
| C. albicans 19 | A | WT | S | S | S | S | 4 | 0.125 | 0.0078 | W | N | VS | VS | Wh & G |
| C. albicans 1 | B | WT | S | S | S | S | 4 | 0.5 | 0.0156 | VS | S | VS | VS | Wh |
| C. albicans 2 | B | WT | S | S | S | S | 4 | 4 | 0.0156 | VS | S | VS | VS | G |
| C. albicans 7 | B | WT | S | S | S | S | 4 | 1 | 0.031 | VS | VS | M | S | Wh |
| C. albicans 11 | B | WT | S | S | S | S | 4 | 0.5 | 0.031 | VS | N | M | VS | Wh |
| C. albicans 14 | B | WT | S | S | S | S | 2 | 0.25 | 0.000976 | VS | N | S | VS | Wh & G |
| C. albicans 15 | B | WT | S | S | S | S | 4 | 0.125 | 0.0039 | W | VS | VS | VS | Wh |
| C. albicans 3 | C | WT | S | S | S | S | 4 | 4 | 0.0156 | VS | VS | VS | VS | Wh |
| C. albicans 5 | C | WT | S | S | S | S | 2 | 4 | 0.0156 | VS | VS | VS | VS | G |
| C. glabrata 1 | – | WT | S | NW | NW | S | 8 | 4 | 0.000976 | VS | N | W | N | Wh |
| C. glabrata 2 | – | WT | S | NW | NW | S | 8 | 4 | 0.00976 | M | M | N | N | P |
| C. glabrata 3 | – | WT | S | NW | NW | S | 8 | 4 | 0.000976 | VS | M | M | VS | P |
| C. tropicalis | – | WT | S | S | WT | I | 4 | 0.5 | 0.031 | VS | N | VS | VS | Wh & P |

Note: Abbreviations: Amp, Amphotericin Bl; Cas, Caspofungin; Eco, Econazole; Ester, Esterase; Flu, Fluconazole; G, Gray; Hem, Hemolysin; I, Intermediate; Itr, Itraconazole; Lul, Luliconazole; M, Mild; N, Negative; Nys, Nystatin; O, Opaque; P, Pink; Phe swi, Phenotype switch; Pho, Phospholipase; Pro, Proteinase; S, Strong; SDD, Susceptible dose-dependent; Vor, Voriconazole; VS, Very Strong; W, Weak; Wh, White.
after cooling, it was supplemented with 80 ml egg yolk added to the medium. The plates were incubated at 37°C, and the diameters of the colonies with the zones of precipitation were measured after seven days of incubation.

The medium to evaluate esterase activity was prepared as described by Slifkin with 10.0 g Bacto Peptone, 5.0 g NaCl, 0.1 g CaCl₂, 15.0 g agar (Mirmedia), and 1000 ml distilled water. The medium was autoclaved and cooled to about 50°C, and then, 5 ml autoclaved Tween 80 was added. The plates were inoculated and then incubated at 37°C, and the diameters of the colonies with the zones of precipitation were measured after seven days of incubation.

Hemolysin activity was evaluated with blood agar plates prepared as described by Negri et al. Sabouraud dextrose agar was supplemented with 3% glucose and 7% sheep blood (Baharafshan). The plates were inoculated and incubated at 37°C for five days. Positive hemolysin activity was demonstrated as a ring of lysis around the colonies.

Enzymatic activity was evaluated by dividing the colony diameter by the clear or precipitation zone plus colony diameter. Values equal to 1 were considered negative, 0.9–0.99 as weak, 0.8–0.89 as mild, 0.7–0.79 as strong, and less than 0.69 as a very strong activity.

3 | RESULTS

Among 933 patients who underwent endoscopy, 23 (2.5%) had visible Candida plaques. The diagnosis was confirmed in 23 patients with Gram staining of tissue specimens obtained from these plaques. The mean age of patients with esophageal candidiasis was 55.2 years with a range of 17–91 years, and 58.3% of patients were female. Culture of the isolates with CHROMagar Candida medium yielded 19 green, three violet, and one blue colony. All isolates that produced green colonies grew at 42°C and were positive for chlamydoconidia and germ tube formation. A total of 23 Candida isolates including 19 (82.6%) C. albicans, three (13.1%) C. glabrata, and one (4.3%) C. tropicalis were detected. Among 19 C. albicans isolates, 11 (57.9%) were genotype A, six (31.6%) genotype B, and two (10.5%) genotype C (Table 1 and Figure 1).

3.1 | Antifungal susceptibility

The in vitro susceptibilities of isolated yeasts to amphotericin B, nystatin, caspofungin, voriconazole, fluconazole, itraconazole, econazole, and luliconazole are summarized in Tables 1 and 2. Among C. albicans isolates, 15.8% and 26.3% demonstrated intermediate susceptibility to caspofungin and itraconazole, respectively. All of the C. albicans isolates were wild type (isolates without mutational or acquired resistance mechanisms), and all of these C. albicans isolates were susceptible to fluconazole and voriconazole.

3.2 | Evaluation of Candida albicans microcolonies with fluconazole E test strip

Thirteen C. albicans isolates (68%) demonstrated a trailing effect to fluconazole. Because of biases inherent in the visual readings, the level of the trailing effect was not categorized with the E test method. The susceptibility rate across all three generations was almost identical. Some alterations in phenotype over generations were observed in five isolates as isolates: colonies from two isolates changed from gray to white, and colonies from three isolates changed from white to gray.

3.3 | Phenotype switching

Nine C. albicans isolates exhibited white colonies on YPD agar medium containing phloxine B. Five C. albicans isolates produced gray colonies. We observed a combination of white-gray colonies in four C. albicans isolates, and white-opaque colonies in one isolate. There were no significant differences between genotypes in the frequency of phenotype switching \((p \leq 0.232)\) (Table 1).

3.4 | Enzymatic activity

A high level of phospholipase activity was observed in nine (47.4%) of the C. albicans strains. Five (26.3%) C. albicans strains
### TABLE 2  The in vitro susceptibilities of isolated species

| Candida spp. | Antifungals | Minimum inhibitory concentration, MIC (μg/ml) | Break points | %ECV | W | Non-W |
|--------------|-------------|-----------------------------------------------|--------------|------|---|-------|
|              |             | MIC range | MIC<sub>50</sub> | MIC<sub>90</sub> | MIC<sub>GM</sub> | %S | %I/S | %D | %R |
| Candida albicans (19) | Amphotericin B | 0.031–0.5 | 0.125 | 0.25 | 0.144 | – | – | – | 100 | – |
|              | Nystatin | 2–4 | 4 | 4.5 | 3.585 | – | – | – | – | – |
|              | Caspofungin | 0.00097–0.5 | 0.031 | 0.5 | 0.028 | – | – | – | 84.2 | 15.8 | – | – |
|              | Voriconazole | 0.00097–0.0156 | 0.0039 | 0.0156 | 0.003 | – | – | – | 100 | – | – | – |
|              | Fluconazole | 0.125–2 | 0.5 | 1 | 0.432 | – | – | – | 100 | – | – | – |
|              | Itraconazole | 0.0156–0.5 | 0.125 | 0.25 | 0.093 | – | – | – | 73.7 | 26.3 | – | – |
|              | Econazole | 0.0156–4 | 0.5 | 4 | 0.537 | – | – | – | – | – | – | – |
|              | Luliconazole | 0.00097–0.125 | 0.0156 | 0.031 | 0.010 | – | – | – | – | – | – | – |
| Candida glabrata (3) | Amphotericin B | 0.5 | – | – | – | – | – | – | 100 | – | – | – |
|              | Nystatin | 8 | – | – | – | – | – | – | – | – | – | – |
|              | Caspofungin | 0.125 | – | – | – | – | – | – | 100 | – | – | – |
|              | Voriconazole | 0.25 | – | – | – | – | – | – | – | – | – | – |
|              | Fluconazole | 4 | – | – | – | – | – | – | 100 | – | – | – |
|              | Itraconazole | 8 | – | – | – | – | – | – | 100 | – | – | – |
|              | Econazole | 4 | – | – | – | – | – | – | – | – | – | – |
|              | Luliconazole | 0.00097 | – | – | – | – | – | – | – | – | – | – |
| Candida tropicalis (1) | Amphotericin B | 0.062 | – | – | – | – | – | – | 100 | – | – | – |
|              | Nystatin | 4 | – | – | – | – | – | – | – | – | – | – |
|              | Caspofungin | 0.5 | – | – | – | – | – | – | 100 | – | – | – |
|              | Voriconazole | 0.0156 | – | – | – | – | – | – | 100 | – | – | – |
|              | Fluconazole | 0.5 | – | – | – | – | – | – | 100 | – | – | – |
|              | Itraconazole | 0.25 | – | – | – | – | – | – | 100 | – | – | – |
|              | Econazole | 0.5 | – | – | – | – | – | – | – | – | – | – |
|              | Luliconazole | 0.031 | – | – | – | – | – | – | – | – | – | – |

*MIC<sub>50</sub> and MIC<sub>90</sub> values could not be calculated because of the small number of isolates (<10).

### TABLE 3  Enzymatic activity of isolated species

| Candida spp. | Enzymes | Enzymatic activity |
|--------------|---------|--------------------|
|              |         | Negative | Weak | Mild | Strong | Very strong | Minimum | Maximum | Mean |
| Candida albicans (19) | Hemolysin | 5.3% | 10.5% | 15.8% | – | – | 68.4% | 0.44 | 1 | 0.59 |
|              | Proteinase | 31.6% | – | 21.1% | 10.5% | – | 36.8% | 0.3 | 1 | 0.79 |
|              | Phospholipase | – | 5.3% | 21.1% | 26.3% | – | 47.4% | 0.2 | 0.92 | 0.66 |
|              | Esterase | – | – | – | 5.3% | – | 94.7% | 0.1 | 1 | 0.39 |
| Candida glabrata (3) | Hemolysin | – | – | 33.3% | – | – | 66.6% | 0.3 | 0.87 | 0.623 |
|              | Proteinase | 33.3% | – | 66.7% | – | – | 66.6% | 0.3 | 1 | 0.876 |
|              | Phospholipase | 33.3% | 33.3% | 33.3% | – | – | 0.88 | 1 | 0.939 |
|              | Esterase | 66.7% | – | – | – | 33.3% | 0.42 | 1 | 0.806 |
| Candida tropicalis (1) | Hemolysin | – | – | – | – | – | 100% | – | – | – |
|              | Proteinase | 100% | – | – | – | – | – | – | – | – |
|              | Phospholipase | – | – | – | – | – | 100% | – | – | – |
|              | Esterase | – | – | – | – | – | 100% | – | – | – |
were strong phospholipase producers. Very strong hemolysin and proteinase activity was seen in 13 (68.4%) and seven (36.8%) C. albicans strains, respectively. Eighteen (94.7%) C. albicans strains presented very strong esterase activity with a mean value of 0.39. There were no significant differences between genotypes in the frequency of hemolysin (p ≤ 0.8), proteinase (p ≤ 0.1), or phospholipase activity (p ≤ 0.4), whereas a significant difference between genotypes was found for esterase activity, with p ≤ 0.04 (Table 3).

4 | DISCUSSION

Esophageal candidiasis is an opportunistic infection frequently reported in immunocompromised patients, although a higher occurrence was also reported in the immunocompetent population with underlying conditions. Once host protection mechanisms are compromised, Candida can proliferate on the esophageal mucosa and form adherent white to yellowish plaques. In a case-control study of patients with esophageal candidiasis (>65 years old) a 6-month mortality rate of 47% compared to 5% in controls was reported. In some rare cases, esophageal candidiasis may result in hemorrhagic esophageal ulcers, invasive candidiasis, and esophageal squamous cell carcinoma.

In the present study, the prevalence of esophageal candidiasis was 2.4%. The occurrence rate of esophageal candidiasis ranged from 0.0007% to 5.6% in previous inquiries. The prevalence reported by Hani et al. was distinctly lower compared with other studies. The highest rate was reported by Mushi et al. The variation in prevalence rates in different studies is mostly related to differences in the study population and predisposing factors.

Esophageal candidiasis is diagnosed by biopsy or esophageal brushing through endoscopy examination. Nonetheless, the determination of etiologic agents and antifungal susceptibility tests should be taken into account because some Candida species such as C. glabrata and C. krusei are intrinsically resistant to azole agents. According to our findings, the most frequent isolate was C. albicans (82%) followed by C. glabrata (13%), which was in line with other studies.

We found no reports about the ABC genotyping of C. albicans isolates from esophageal specimens. Therefore, the closest reports were related to isolates from mucosal sites and the oral cavity. We found all three types (A, B, and C) of C. albicans in our study, and genotype A was the predominant one. We also found 2 isolates belonging to genotype C. In contrast, according to the study by Sardi et al., the majority of C. albicans isolates (51.6%) belonged to genotype B, and 48.4% were genotype A; these authors found no genotype C isolates. Odds et al. described genotype C as a rare genotype found in only 9% (173/1931) of C. albicans isolates. In a study with diverse clinical samples, Rosca et al. found no significant correlation between genotype (A at 52.92%, B at 17.85%, C at 29.20%) and the infection site. It thus appears that genotype A is the most frequent genotype in clinical specimens.

Treatment for esophageal candidiasis always includes systemic antifungal agents. The most commonly used medication to treat esophageal candidiasis is oral fluconazole, with itraconazole, voriconazole, and amphotericin B among other treatment options. Caspofungin may be preferred over amphotericin B because the former has fewer side effects. In the present study, isolated species exhibited no resistance to selected antifungal agents. Three isolates of C. albicans and one C. tropicalis isolate showed intermediate susceptibility to caspofungin. Shields et al. noted that the MIC for anidulafungin or micafungin might be a more reliable predictor of treatment failure in C. glabrata invasive candidiasis, but provided no data regarding C. albicans. Lakshmy et al. reported resistance rates of 6% to fluconazole and 4% to itraconazole in C. albicans isolates, and their non-C. albicans isolates were resistant to fluconazole. These authors did not report any resistance to amphotericin B. Salehi et al. did not distinguish between C. albicans and non-albicans isolates in their report and found MICs ranging from 0.125 to 8.0 μg/ml for fluconazole, 0.008 to 0.75 μg/ml for itraconazole, and 0.007 to 0.75 μg/ml for amphotericin B. Luliconazole, as a new member of the azole family, has broad antifungal activity. We found that MIC ranged from 0.0097 to 0.125 μg/ml for C. albicans isolates. Taghipour et al. reported that within the range of 0.016 to 2.0 μg/ml, all their C. albicans isolates were sensitive to luliconazole. In view of the urgent need for alternative antifungal agents due to ever-increasing reports of resistant isolates, systemic formulations and in vivo studies of luliconazole are needed.

Because fluconazole is still a first-line antifungal agent for esophageal candidiasis, we investigated C. albicans isolates that exhibited trailing effects to identify variations in antifungal susceptibility profile and phenotype. Trailing growth can reflect the degree of azole tolerance; however, no definite molecular explanation has been found for this phenomenon. In the present study, the susceptibility rate across three generations was similar for fluconazole and other drugs. In a similar study, Hauzer et al. found no increase in fluconazole susceptibility in microcolonies over three generations. Although in vitro studies support the notion that microcolonies can be disregarded, there is as yet not enough in vivo evidence for the harmlessness of this phenomenon.

The ability of C. albicans to produce extracellular enzymes may be linked to its virulence. Candida can produce different enzymes, and their amount and strength vary depending on the isolation site. In the present study, the most active enzyme was esterase, which showed very strong activity in 18 (94.7%) isolates of C. albicans. Furthermore, 13 (68.4%) isolates showed very strong hemolysin activity, whereas the least active enzyme was proteinase. Slikkin et al. reported that all C. albicans in their analysis demonstrated esterase activity, which was a useful way to distinguish Candida spp. Charaghani et al. reported higher hemolytic and proteinase activities in oral isolates. Dabiri et al. reported higher proteinase (50%) and phospholipase activity (100%) compared with the present study. Nouraei et al. reported significant differences in hemolysin, proteinase, and phospholipase activity between albicans and non-albicans isolates. An interesting comparison of heterozygous and homozygous strains of C. albicans demonstrated
that the latter had higher phospholipase and proteinase activity than heterozygous strains. Comparisons of ABC genotyping and enzyme production in the present study showed a significant difference between genotypes only for esterase production (p ≤ 0.04). Because of the low number of non-albicans isolates in our sample, we were unable to compare them with C. albicans isolates.

*Candida albicans* can switch between several morphological phenotypes in response to a variety of environmental signals—a trait which is essential for their life as a commensal or pathogenic organism. reported that white colonies demonstrated low proteinase activity, whereas gray colonies had higher proteinase activity. These authors also noted that white colonies were more virulent than gray and opaque colonies. In line with their study, most of the gray colonies in the present study had strong or very strong proteinase activity. In contrast, about half of our *C. albicans* isolates had no or only mild proteinase activity. Also, most of the *C. albicans* isolates in our study were white, that is, the phenotype that was most virulent in the ex vivo study by Tao et al. In this connection, we note that phenotype switching over generations in five of our isolates did not affect trailing.

In conclusion, there is limited information about the characteristics of etiologic agents of esophageal candidiasis or yeast factors operative in the pathogenesis of esophageal candidiasis, and in vivo experiments are scarce. The prevalence of esophageal candidiasis varies in different studies due to differences in study populations and predisposing factors. We found no resistance among our *C. albicans* isolates to selected antifungals, but because of the low number of isolates studied here, there is a need for larger studies. However, all three *C. glabrata* isolates remained non-wild type for voriconazole and itraconazole resistance. New antifungals such as luliconazole may be an effective alternative in cases of antifungal resistance, and further in vivo studies should be done. The predominance of genotype A, the white phenotype, and strong hemolysin activity may indicate a high prevalence of pathogenicity in these isolates.

The present study had some limitations. Because of the COVID-19 pandemic, sampling was limited and the number of isolates available for analysis was consequently low. We suggest a larger population for further research. Because most of the patients were not hospitalized, we could not investigate their predisposing factors. On the other hand, the present study is the first, to our knowledge, to determine the frequency of phenotype switching and ABC genotypes in *C. albicans* as the predominant clinical isolate.

**CONFLICT OF INTEREST**
The authors state that there is no competitive concern of any nature with this manuscript.

**AUTHOR CONTRIBUTIONS**
AZM involved in study design, supervision, data interpretation, and manuscript editing. HJ involved in experimentation, data collection, analysis, literature search, and manuscript writing. MG involved in experimentation, data collection, and predisposing factors. We found no resistance among our *C. albicans* isolates.

**DATA AVAILABILITY STATEMENT**
Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

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