Virulence Factors and Azole-Resistant Mechanism of *Candida Tropicalis* Isolated from Candidemia

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Abstract

**Background** Limited knowledge exists on the virulence factors of *Candida tropicalis* and the mechanisms of azole resistance that lead to an intensified pathogenicity and treatment failure. We aimed to evaluate the virulence factors and molecular mechanisms of azole resistance among *C. tropicalis* isolated from patients with candidemia.

**Materials and Methods** Several virulence factors, including extracellular enzymatic activities, cell surface hydrophobicity (CSH), and biofilm formation, were evaluated. Antifungal susceptibility pattern and expression level of *ERG11*, *UPC2*, *MDR1*, and *CDR1* genes of eight (4 fluconazole resistance and 4 fluconazole susceptible) clinical *C. tropicalis* isolates were assessed. The correlation between the virulence factors and antifungal susceptibility patterns was analyzed.

**Results** During a 4 year study, forty-five *C. tropicalis* isolates were recovered from candidemia patients. The isolates expressed different frequencies of virulence determinants as follows: coagulase 4 (8.9%), phospholipase 5 (11.1%), proteinase 31 (68.9%), esterase 43 (95.6%), hemolysin 44 (97.8%), biofilm formation 45 (100%) and CSH 45(100%). All the isolates were susceptible to amphotericin B and showed the highest resistance to voriconazole. There was a significant positive correlation between miconafungin minimum inhibitory concentrations (MICs) and hemolysin production ($r_s = 0.316$). However, we found a negative correlation between fluconazole MICs and esterase production ($r_s = -0.383$). We observed the high expression of *ERG11* and *UPC2* genes in fluconazole-resistant *C. tropicalis* isolates.

**Conclusion** *C. tropicalis* isolated from candidemia patients extensively displayed capacities for biofilm formation, hemolysis, esterase activity, and hydrophobicity. In addition, the overexpression of *ERG11* and *UPC2* genes was considered one of the possible mechanisms of azole resistance.
Keywords Candida tropicalis · Virulence factors · Biofilm · Candidemia · Azole resistance

Introduction

Nosocomial bloodstream infections (BSIs) caused by Candida species are the fourth and sixth causes of BSIs in the USA and Europe, respectively [1–4]. Candida albicans is the most common agent causing candidemia. However, the increasing mortality rate due to non-albicans Candida (NAC) species reveals their importance [5–7]. C. tropicalis is one of the most common agents causing candidemia in some developing countries [8, 9]. The significant link between primary colonization and disseminated invasive infections caused by Candida spp. can be associated with their virulence factors [10]. Many virulence factors have been represented for candidemia development, including extracellular secreted enzymes (phospholipase, hemolysin, coagulase, and proteinase) and biofilm formation [11, 12]. These factors can intensify candidemia by helping Candida spp. escape from the immune system to damage the host tissue [13]. Another reason for the therapeutic failure of candidemia is the widespread use of antifungals, leading to increasing resistance of clinical C. tropicalis isolates to some antifungals, especially azoles.[14, 15]. Among the predictors, determining the outcome of candidemia is the susceptibility of Candida spp. to antifungal agents [1]. Some factors that contribute to the azole resistance of Candida spp. include mutations and/or overexpression of the following genes: ERG11 (encodes cytochrome P450 lanosterol 14a-demethylase); UPC2 (encodes the transcription factor of ERG11 gene); CDR1 (encodes efflux protein of ATP binding-cassette (ABC) family); and MDR1 (encodes efflux protein of major facilitator superfamily (MFS) family) [16, 17]. Hence, the attention to virulence factors, patterns of antifungal susceptibility, and azole-resistant mechanism of C. tropicalis isolates can be used to establish positive management approaches to control candidemia based on the new therapy. This study aimed to evaluate the virulence factors (extracellular enzymatic activities, cell surface hydrophobicity, and biofilm formation), the pattern of antifungal susceptibility, and the expression of ERG11, UPC2, MDR1, and CDR1 genes in C. tropicalis isolated from candidemia patients in Tehran, Iran.

Materials and Methods

Identifying the Yeast Isolates

In this study C. tropicalis isolates were recovered from blood samples of candidemia patients admitted to two tertiary care training hospitals (Imam Khomeini Hospital Complex and Shariati Hospital) in Tehran, Iran. All the isolates were identified using morphological and molecular techniques [18, 19]. Briefly, after sub-culturing yeasts isolated from blood samples on CHROMagar Candida plates (Merck, Germany), we confirmed all the isolates using the multiplex PCR method. Informed consent was obtained from each patient, person earmarked to accompany the patient, or a close family member (often the spouse or parents). Isolates investigated in this study were the part of studies that had been approved by the local ethical committees of Tarbiat Modares University (Code: IR.MODARES.REC.1398.109) and Tehran University of Medical Sciences (Code: IR.TUMS.SPH.REC.1400.117).

Determining Hemolysin Production

Hemolytic activity of the C. tropicalis isolates was determined using a blood agar assay as described by Manns et al. [20]. Seven milliliters of fresh sheep blood were added to 100 ml SDA supplemented with glucose 3% (w/v). The final pH of the medium was adjusted to 5.6 ± 0.2. The prepared media plates were inoculated with 10 µl suspension (10^6 yeast cells/ml) of both the test and the control Candida isolates. The plates were then incubated at 37 °C in 5% CO2 for 48 h. Then, we examined the plates and determined the hemolytic index (Hz value) as the ratio of the diameter of the colony to that of the translucent zone of hemolysis (mm). The interpretation of the results was as follow: high activity ≥ 0.59; medium activity 0.6–0.79; low activity 0.8–0.99; no activity ≤ 1 [2, 21]. C. albicans (ATCC 14053) was used as the positive control, while C. parapsilosis (ATCC 22019) was used as the negative control. The assay was performed in duplicate on three separate occasions for each isolate.
Determining Proteinase Production

The proteinase activity of the isolates was evaluated, according to Staib et al. [22]. The C. tropicalis suspension was prepared from overnight cultures, of which 10 μl containing $1 \times 10^6$ Candida cells /ml was used to inoculate the bovine serum albumin (BSA) agar plate, composed of BSA solution 1%, dextrose 2%, KH$_2$PO$_4$ 0.1%, MgSO$_4$ 0.05%, and agar 2%. We incubated the plates at 37 °C for 72 h. Thereafter, we used 20% trichloroacetic acid to fix the plate for 15 min and then stained it with 1.25% amido black for 30 min. Finally, we used 15% acetic acid to decolorize the setup before determining the zone (Pz) around the colonies. The classification of proteinase activity was as represented for the hemolysin activity. C. albicans (ATCC 14053) was used as the positive control, while C. glabrata (ATCC 90030) was used as the negative control. The assay was performed in duplicate on three separate occasions for each isolate.

Determining Phospholipase Production

The phospholipase activity of the C. tropicalis isolates was evaluated according to the technique described previously [23]. Briefly, the egg yolk agar plate was prepared using 13.0 g of SDA, 11.7 g of NaCl, 0.11 g of CaCl$_2$, and 10% sterile egg yolk (all in 184 ml distilled water). All components were mixed and sterilized before adding the egg yolk. The egg yolk was centrifuged at 500 rpm for 10 min at room temperature, and 20 ml of the supernatant was added to the sterilized medium. Ten microliters of C. tropicalis suspension containing $10^6$ cells/ml were inoculated onto the egg yolk plates. After drying the plates at room temperature, we incubated the plates at 37 °C for 48 h. The classification of phospholipase activity was as represented for the hemolysin activity. C. albicans (ATCC 14053) was used as the positive control, while C. parapsilosis (ATCC 22019) was used as the negative control. The assay was performed in duplicate on three separate occasions for each isolate.

Determining Esterase Activity

Esterase activity of the isolates was evaluated using the Tween 80 opacity test, according to Slifkin et al. [24]. To 1000 ml of distilled water, 10 g peptone, 5 g NaCl, 0.1 g CaCl$_2$, and 15 g agar were dissolved; pH adjusted to 6.8 and then autoclaved. Then, 5 mL of sterile Tween 80 was added to the media after cooling (at 50 °C) and dispensed into 90 mm plates. Ten microliters of C. tropicalis suspension (1 $\times 10^6$ cells /ml) were spotted on each plate and incubated for two days at 37 °C. The classification of esterase activity was as represented for the hemolysin activity. C. albicans (ATCC 14053) was used as a positive control. The assay was performed in duplicate on three separate occasions for each isolate.

Cellular Surface Hydrophobicity (CSH)

Candida cells grown overnight at 37 °C, were harvested and washed twice with phosphate-buffered saline (PBS) pH = 7.4. Candida cell suspension displaying an optical density (OD) of 1.0 at 600 nm was prepared in PBS (A$_0$). We took 3 ml of this Candida suspension and overlaid it with 0.4 ml of the hydrophobic hydrocarbon, xylene (ADWIC, Qalyubiya, Egypt) and incubated in a water bath at 37 °C for 10 min, vortexed for 30 s, and then returned to the bath for 30 min. The lower aqueous phase was carefully transferred to a clean tube. Any traces were removed by vortexing for 30 s. The absorbance at 520 nm was then measured after fast vortexing. The CSH was expressed as the percentage decrease in the optical density of the test suspension compared with that of the control. The greater the differences in the absorbances, the more hydrophobic were the yeast cells. Each strain was tested in triplicate on three independent occasions. The CSH was interpreted as follows: 0–9.99% = low, 10%–19.99% = moderate, > 20% = high [21].

Biofilm Assay

Biofilm assay for C. tropicalis was formed on the bottom of 96-well microtiter plates, and each biofilm was adapted in triplicates as previously described [25]. Briefly, a full loop of freshly picked colonies from the SDA medium was inoculated into Sabouraud dextrose broth (SDB; Merck, Germany) medium and was incubated for 18–24 h at 37 °C. After washing 24-h culture with PBS, cell suspension containing $10^6$ cells /ml was prepared in RPMI 1640 (with L-glutamine and without bicarbonate) with morpholinepropanesulfonic acid (MOPS, Sigma-Aldrich). A 100 μl of this
suspension was inoculated in each well. After incubation at 37 °C for 48 h, wells were carefully washed three times with sterile PBS to remove planktonic cells. Then, the biofilm of each well was fixed with 200 μl of 99% (v/v) methanol for 15 min. After washing the plates with dH2O, 200 μl of 0.1% (v/v) crystal violet (CV) solution was added into each well for 20 min at room temperature. In the next step, the extra CV was aspirated, and microplates were washed twice with dH2O, then air-dried. Finally, to dissolve the CV bounded to cells, 200 μl of 33% (v/v) acetic acid was added into each well. Biofilm biomass was measured in the wavelength of 595 nm by the microplate reader (STAT FAX 2100), and the well with the Candida cell-free media was considered as a blank and negative control. The cutoff value was calculated according to the average ODs of negative controls in addition to three standard deviations of negative controls. Biofilm results were interpreted based on the following: categories containing ODc = No biofilm producer, ODc < OD ≤ 2 × ODc = Low biofilm producer, 2 × ODc < OD ≤ 4 × ODc = Moderate biofilm producer, 4 × ODc < OD = High biofilm producer. In this study, the cutoff value (ODc) was 0.243.

Determining Coagulase Activity

Coagulase activities were evaluated as described previously by Yijit et al. [26]. Briefly, C. tropicalis cells were inoculated into SDB and incubated aerobically at 37 °C for 18–24 h. A test tube containing 0.5 ml of EDTA rabbit plasma was inoculated with 100 μl of the overnight broth and incubated aerobically at 37 °C for different time points containing (2–4 – 6 – 24 h). The coagulase activity was interpreted as follows: positive (the presence of platelet clumping) and negative (the absence of platelet clumping). Staphylococcus aureus (ATCC 25923) was used as the positive control, and Staphylococcus epidermidis (ATCC 14990) was the negative control. The assay was performed in duplicate on three separate occasions for each isolate.

Antifungal Susceptibility Test

Antifungal susceptibility patterns of the C. tropicalis isolates testing were evaluated according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI) M27-A3/S4 [27, 28]. We tested the isolates against the following antifungal drugs: fluconazole (FLZ, Pfizer, Groton, CT, USA), itraconazole (ITR, Janssen Research Foundation, Beerse, Belgium), voriconazole (VOR, Pfizer, Central Research, Sandwich, United Kingdom), amphotericin B (AMB, Bristol-Myers-Squib, Woerden, Netherlands), caspofungin (CFG, Merck Sharp & Dohme, Haarlem), micafungin (MCF, Astellas Pharma, Ibaraki, Japan), and anidulafungin (AND, Pfizer, Central Research, Sandwich, United Kingdom). Briefly, 100 μl of Candida suspension (containing 0.5–2.5 × 10^3 cells/ml) was added to the first ten wells in a raw 96-wells round bottom microtitre plate, each containing 100 μl of drug diluted in RPMI 1640 (up to the lowest concentration). The eleventh well is positive control containing 100 μl of cell suspension and 100 μl of RPMI; the twelfth well is negative control containing only 200 μl RPMI. We incubated the plates at 37 °C for 24 to 48 h, and the results were read as follows: for azoles and echinocandins, the drug concentration at which 50% yeast cells growth was inhibited, and for AMB, at which 100% growth was inhibited were recorded as minimal inhibitory concentration (MIC). The C. krusei (ATCC 6258) and C. parapsilosis (ATCC 22,019) were used as quality control strains. Interpretation of results was based on the species-specific clinical breakpoints (CBPs) for FLZ, VOR, AND, MCF, and CFG or epidemiological cutoff values (ECVs) for AMB and ITR due to the lack of defined CBPs (Table 2) [28, 29].

RNA Extraction, cDNA Synthesis, and Real-Time PCR

The extraction of total RNA from eight C. tropicalis clinical isolates treated with FLZ based on the range of susceptibility to FLZ (4 susceptible and 4 resistant isolates) was performed by the RNAX plus kit (Sina clone, Iran). To gain the mass of C. tropicalis cells, isolates were cultured in 24-well plates, and the total RNA was extracted from cultures of logarithmic phase based on the manufacturer instructions. The determination of RNA concentrations was done by spectrophotometric measurements (Biochrom WPA Biowave II, UK). Extracted RNA was employed to synthesize first-strand complementary DNAs (cDNAs) by the cDNA synthesis kit ( Parsstous, Iran). The expression levels of ERG11, UPC2, MDRI, and
CDR1 genes were evaluated by real-time PCR. Real-time PCR was done in triplicates by Rotor-Gene 3000 real-time PCR system (Applied Biosystem, USA). Each reaction with a final volume of 20 contains 10 μl Syber Green master mix (Pars tous, Iran), 1 μl forward primer, 1 μl reverse primer, 2 μl cDNA, and 6 μl distilled water. The reactions were conducted in the following order: denaturation at 95 °C for 30 s, followed by 40 cycles consisting of 5 s at 95 °C and 21 s at 60 °C. The expression level of each gene was calculated by the -ΔΔCt method and ACT gene as internal control [16]. The primer design was performed according to the relevant gene sequences of \textit{C. tropicalis} (Table 1).

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 6.05 software. We used simple frequencies to describe the virulence factors and the antifungal susceptibility profile of the \textit{C. tropicalis} isolates. The correlation between the virulence factors and MIC values was determined using Spearman’s rank correlation. Continuous variables were compared using the student’s t-test. We considered \(P\) values ≤ 0.05 to be statistically significant in all tests.

Results

During the four-year period of this study, forty-five \textit{C. tropicalis} isolates were collected. The relationship between the virulence factors produced and different levels of their activities among 45 clinical isolates of \textit{C. tropicalis} is shown in Table 2. Four (8.9%) of the isolates were coagulase-positive, 5 (11.1%) were phospholipase-positive, and 31 (68.9%) were protease-positive, whereas all the evaluated isolates 45 (100%) showed biofilm formation and CSH, 44 (98%) produced hemolysin, and 43 (95.6%) produced esterase. Among hydrolytic enzymes, most 41 (91.2%) isolates indicated high hemolytic activity. However, 43 (95.6%) and 36 (80%) isolates were determined to have low or undetectable phospholipase and protease activities, respectively. Interestingly, although few isolates displayed high phospholipase activity, the majority 39 (86.7%) of isolates indicated high esterase activity. While more than 60% of isolates produced high biofilm and CSH, there was no significant correlation between CSH and biofilm mass \((P = 0.912, r_s = -0.017)\). Table 3 shows the antifungal susceptibility patterns of the 45 \textit{C. tropicalis} clinical isolates. The isolates showed the highest resistance (13.3%) and dose-dependent susceptibility (22.2%) to VOR, but all (100%) were sensitive to AMB. Four FLZ- resistant isolates of \textit{C. tropicalis} revealed overexpression of \textit{UPC2} and especially \textit{ERG11} gene compared with four FLZ-susceptible isolates of \textit{C. tropicalis} (mRNA fold change of \textit{ERG11} 3.155 versus 1.083, \(P < 0.05\); mRNA fold change of \textit{UPC2} 2.751 versus 1.096, \(P < 0.01\)). In contrast, on the expression levels of \textit{CDR1} and \textit{MDR1} genes, no significant difference was observed between 4 FLZ-resistant \textit{C. tropicalis} isolates and the 4 FLZ-susceptible \textit{C. tropicalis} isolates (Fig. 1). Correlations between antifungal susceptibility profiles and virulence factors of \textit{C. tropicalis} are shown in Table 4. Spearman’s correlation revealed a statistically significant positive correlation between MICs of MCF and hemolysin production \((P < 0.05, r_s = 0.316)\). On the

### Table 1 Primer sequences for genes expression

| Primer name | Primer sequences | Product length (bp) |
|-------------|------------------|-------------------|
| \textit{ERG11}-F | TCTGCTTCCACTTCTGCCTG | 117 |
| \textit{ERG11}-R | ATCGTTCAAGTACCACCCCT | 117 |
| \textit{CDR1}-F | CCTGAACTGATGCCACGGT | 154 |
| \textit{CDR1}-R | AAGTTTGCCCTGATGGTCGT | 154 |
| \textit{MDR1}-F | TGCCTGCTCTTTGACCCGTT | 160 |
| \textit{MDR1}-R | AGTTTAATGCTGACAGCAG | 160 |
| \textit{UPC2}-F | CAGTTGAACAAACAACAAACAA | 208 |
| \textit{UPC2}-R | TAAATCCCTAAACCTGAAAGA | 208 |
| \textit{ACT}-F | TGCTCCAGAAGAACCACAG | 187 |
| \textit{ACT}-R | ACCATCACCAGAATCCAGA | 187 |
other hand, it showed negative correlations between MICs of MCF and proteinase \((P < 0.05, r_s = -0.360)\), and phospholipase \((P < 0.05, r_s = -0.393)\) secretion. Similarly, a significant negative correlation was found between MICs of FLZ and esterase production \((P < 0.05, r_s = -0.383)\).

Discussion

During recent years, candidemia by NAC species has been considered an emerging problem [18]. In addition, the mortality rate of candidemia due to \(C.\) tropicalis is high compared to other NAC species [18, 30]. The colonization and tissue invasion by \(Candida\) spp. are associated with their ability to produce virulence factors, including extracellular secreted enzymes and biofilm formation [31]. Although the majority of virulence factor studies are about \(C.\) albicans, few studies evaluated virulence factors of NAC species. The ability of phospholipases to hydrolyze at least one ester linkage in glycerophospholipids of cell membrane leads to the invasion of host mucosal epithelia [32]. In our study, 11.1% of \(C.\) tropicalis isolates produced phospholipase. Similarly, other studies reported that phospholipase activity of \(C.\) tropicalis isolates recovered from blood samples was

**Table 2** Levels of activity of different virulence factors produced by 45 clinical \(C.\) tropicalis isolates recovered from candidemia patients

| Virulence factors \((n = 45)\) | Activity levels |
|-------------------------------|----------------|
|                              | High \(^a\) (%) | Medium \(^b\) (%) | Low \(^c\) (%) | No activity \(^d\) (%) |
|-------------------------------|----------------|----------------|--------------|----------------------|
| Phospholipase                 | 1 (2.2)        | 1 (2.2)        | 3 (6.7)      | 40 (88.9)           |
| Proteinase                    | 6 (13.3)       | 4 (8.9)        | 21 (46.7)    | 15 (33.3)           |
| Esterase                      | 39 (86.7)      | 3 (6.7)        | 1 (2.2)      | 2 (4.4)             |
| Hemolysin                     | 41 (91.2)      | 2 (4.4)        | 1 (2.2)      | 1 (2.2)             |
| Biofilm                       | 29 (64.5)      | 15 (33.3)      | 1 (2.2)      | -                   |
| CSH                           | 30 (66.7)      | 9 (20)         | 6 (13.3)     | -                   |

\(^a\)Pz value, ≤ 0.59; Biofilm, 0.972 < OD; % CSH, > 20%

\(^b\)Pz value, 0.6–0.79; Biofilm, 0.486 < OD ≤ 0.972; % CSH, 10%–19.99%

\(^c\)Pz value, 0.8–0.99; Biofilm, 0.243 < OD ≤ 0.486, % CSH, 0 and 9.99%

\(^d\)Pz value, 1; Biofilm, OD < 0.243

CSH, cell surface hydrophobicity; OD, Pz value, the colony diameter divided by diameter of the colony plus zone diameter

**Table 3** Antifungal susceptibility results of 45 clinical \(C.\) tropicalis isolates recovered from candidemia

| Antifungal agents | MIC (µg/ml) | Category (%) | CBPs (µg/ml) | ECV (µg/ml) |
|-------------------|-------------|--------------|--------------|-------------|
|                   | MIC\(_{50}\) | MIC\(_{90}\) | GM | S/WT | SDD | I | R | S | SDD | I | R |
| Fluconazole       | 0.75        | 6            | 1  | 75.7 | 15.5 | 8.8 | ≤ 2        | 4   | –   | ≥ 8 | –   |
| Voriconazole      | 0.125       | 1            | 0.1 | 64.5 | 22.2 | 13.3 | ≤ 0.12     | 0.25–0.5 | –   | ≥ 1 | –   |
| Itraconazole      | 0.5         | 2            | 0.5 | 93.4 | –    | 6.6 | –          | –   | –   | –   | 0.5 |
| Caspofungin       | 0.062       | 0.37         | 0.1 | 91.2 | 2.2  | 6.6 | ≤ 0.25     | –   | 0.5 | ≥ 1 | –   |
| Micafungin        | 0.062       | 0.25         | 0   | 95.6 | 2.2  | 2.2 | ≤ 0.25     | –   | 0.5 | ≥ 1 | –   |
| Anidulafungin     | 0.04        | 0.18         | 0   | 97.8 | 2.2  | –   | ≤ 0.25     | –   | 0.5 | ≥ 1 | –   |
| Amphotericin b    | 0.5         | 1            | 0.67 | 100  | –    | –   | –          | –   | –   | –   | 2   |

CBPs, clinical breakpoints; ECVs, epidemiologic cutoff values; GM, geometric mean; S, susceptible; SDD, susceptible dose-dependent; I, intermediate; R, resistant; WT, wild-type; NWT, non-wild-type; MIC, minimum inhibitory concentration
low or undetectable [2, 33–35]. We also reported that most phospholipase-undetectable \textit{C. tropicalis} isolates had the production capacity of esterase as carboxyl ester hydrolase. This is in agreement with Sriphannam et al., and Atalay et al.’s studies [33, 36]. Proteinases by degrading proteins associated with the host immune system such as albumin, mucin, antibodies, complement, and interleukins can protect \textit{Candida} spp. from host defense [13]. In this study, low and negative proteinase activities were observed in more than 70\% \textit{C. tropicalis} isolates. Similarly, in other previous studies, the majority of \textit{C. tropicalis} isolates recovered from blood exhibited low or undetectable proteinase activity [2, 6, 33, 34, 36].

The iron absorption by the activity of hemolysin is one of the critical factors for the survival and growth of pathogenic microorganisms in the host [37]. Our findings demonstrated that 44 (97.8\%) \textit{C. tropicalis} isolates produced hemolytic factor with strong activity in 41 (91.2\%) of isolates that corroborates Canel et al. study [2]. Besides, Franca et al. compared the hemolytic activity of 14 \textit{C. tropicalis} and 34 \textit{C. parapsilosis} isolates with reference to anatomic sites and found that \textit{C. tropicalis} isolates from blood had statistically higher hemolytic activity \((P < 0.05)\) than \textit{C. parapsilosis} [38]. Coagulase activity in \textit{Candida} spp. is a virulence factor that facilitates spread and its roles in pathogenicity need not be overemphasized. The low coagulase activity in this study coincided with Mushi1 MF et al. and Deorukhkar SC et al. that indicated coagulase activity of \textit{C. tropicalis} strains isolated from different specimens was low or undetectable [32, 34, 39]. In contrast, Rodrigues et al. reported that coagulase activity of \textit{C. tropicalis} isolates was high (83\%) [40]. The possible reasons for this contradiction can be attributed to the limited sample size, and/ or innate biological alterations in tested isolates. Biofilm formation is another virulence factor that helps fungal cells to escape the host immune system [41]. In the current study, all the evaluated isolates produced biofilm. Similarly, the studies of Sachin et al. and Sriphannam et al. revealed that the majority of \textit{C. tropicalis} blood isolates produce biofilm [32, 36]. Also, Negri et al. study indicated that the ability of \textit{C. tropicalis} clinical strains to form in vitro biofilm on silicon is extensive [42, 43]. CSH, a marker related to the composition of the cell wall, and

![Fig. 1](image.png)

**Fig. 1** Patterns of \textit{ERG11, CDR1, MDR1,} and \textit{UPC2} expression in groups of FLZ-susceptible and FLZ-resistant \textit{C. tropicalis} isolates. The quantification of each gene was done by -\Delta\Delta\textit{CT} and the control gene (\textit{ACT}). Error bars reveal the SDs. *, **, and *** correspond to \(p < 0.05, p < 0.01,\) and \(p < 0.001,\) respectively

| Antifungals       | Proteinase | Hemolysin | Esterase | CSH       | Phospholipase | Biofilm |
|-------------------|------------|-----------|----------|-----------|--------------|---------|
| Fluconazole       | – 0.055 (0.719) | – 0.112 (0.463) | – 0.383 (0.011) | 0.029 (0.848) | – 0.077 (0.617) | – 0.003 (0.982) |
| Itraconazole      | – 0.117 (0.444) | – 0.146 (0.337) | – 0.239 (0.122) | 0.103 (0.502) | – 0.147 (0.334) | – 0.093 (0.545) |
| Voriconazole      | – 0.081 (0.597) | – 0.255 (0.091) | – 0.018 (0.909) | 0.032 (0.834) | – 0.084 (0.584) | 0.073 (0.633) |
| Amphotericin b    | 0.128 (0.402) | 0.029 (0.852) | 0.135 (0.388) | 0.114 (0.457) | 0.170 (0.265) | 0.177 (0.245) |
| Caspofungin       | 0.059 (0.701) | 0.279 (0.064) | – 0.035 (0.823) | 0.106 (0.488) | – 0.067 (0.663) | 0.194 (0.201) |
| Micafungin        | – 0.360 (0.015) | **0.316 (0.035)** | – 0.135 (0.387) | 0.279 (0.063) | – **0.393 (0.008)** | 0.136 (0.374) |
| Anidulafungin     | 0.099 (0.518) | 0.029 (0.851) | – 0.055 (0.725) | 0.020 (0.894) | 0.156 (0.307) | 0.273 (0.069) |

Numbers in bold are statistically significant.
biofilm formation ability are usually evaluated together [7]. A previous study that evaluated the virulence profile of 184 Candida spp. showed C. tropicalis strains, among the different Candida spp., with the most biofilm biomass did not have any correlation with its high hydrophobicity [7]. This study agrees with our results in which high hydrophobicity (91%) of C. tropicalis isolates did not correlate with their biofilm mass. Among the attributes of Candida spp., hydrophobicity and biofilm formation protect the yeast cells from environmental stresses and human host defenses with a subsequent decrease in susceptibility to antifungal agents leading to treatment failures [44]. Moreover, susceptibility to antifungal agents, among other factors, determines the outcome of established candidemia. In the current study, we evaluated the susceptibility of the C. tropicalis clinical isolates against the three classes of antifungal agents. The highest resistance rate of our isolates associated with VOR (13.3%) is consistent with previous studies in India and Iran [18, 35]. Generally, previous studies reported that echinocandins have a reasonable impact on C. tropicalis isolates, while our isolates revealed low resistance to echinocandin [8, 45]. Consistent with our findings, the studies of Khan et al., Bessetti et al., and Deorukhkar et al. revealed that all C. tropicalis blood isolates were susceptible to amphotericin B [1, 34, 46]. Available data regarding the azole-resistant mechanism of C. tropicalis strains compared with other species of Candida are not enough. In our study, the expression of the ERG11 gene in FLZ-resistant C. tropicalis isolates was higher than FLZ-susceptible C. tropicalis isolates, as found in other studies conducted in France and China [16, 17, 47]. Furthermore, Choi et al. reported that high expression level of the ERG11 gene was observed in FLZ- non and -less susceptible C. tropicalis isolates compared with FLZ-susceptible [48]. Upregulation of ERG genes can contribute to enhanced expression of the UPC2 gene as a transcription factor [17]. In the present study, the UPC2 expression in FLZ-resistant C. tropicalis isolates was higher than FLZ-susceptible that supports Jiang et al. study [17]. Therefore, it can be proposed that ERG11 and UPC2 genes have an effective impact on the molecular mechanisms behind FLZ-resistant C. tropicalis isolates. In contrast, our study did not find any difference in the expression levels of CDR1 and MDRI genes between resistant and susceptible C. tropicalis isolates, an observation that corroborates Jiang et al. study [16]. However, Barchiesi et al. revealed that the resistance of C. tropicalis isolates was the connection to overexpression of CDR1 and MDRI genes [49]. One explanation for this controversy is that the mechanism of acquisition of resistance to FLZ in C. tropicalis in vitro can differ from our clinical isolates. Furthermore, collected isolates of C. tropicalis may be heterogeneous. This work has some limitations, such as our inability to investigate point mutations of involved genes in C. tropicalis isolates as an alternative mechanism of azole resistance and virulence factors based on in vivo and molecular methods.

In conclusion, our finding showed high activities of hemolysin, esterase, CSH, and biofilm formation contribute to the C. tropicalis pathogenesis and making therapeutic strategies harder in patients with candidemia. All the isolates were susceptible to AMB. We have also indicated the involvement of ERG11 and UPC2 genes in the mechanism of FLZ resistance in C. tropicalis isolates.

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Declarations

Conflict of interest The authors declare that they have no competing interests.

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