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ORIGINAL ARTICLE

# Evaluation of virulence factors and azole resistance mechanisms of *Candida tropicalis* isolates from head and neck cancer patients with OPC

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## ABSTRACT

**Background and Objectives:** *Candida tropicalis* is one of the major non-*albicans* species causing nosocomial infection. There is limited data about mechanisms of azole-resistance and virulence factors of *Candida tropicalis*. This study was designed to investigate molecular mechanism of azole -resistance and major virulence factors of *C. tropicalis* isolated from oropharyngeal candidiasis in head and neck cancer patients.
**Materials and Methods:** After collecting 38 *C. tropicalis* clinical isolates, antifungal susceptibility pattern and the expression levels of *ERG11, CDR1, CDR2* and *MDR1* were evaluated. Moreover, proteinase and phospholipase activity and biofilm formation of the isolates were investigated as virulence factors.
**Results:** We detected fluconazole resistance in 7 *C. tropicalis* isolates. The expression levels of *CDR1, ERG11* and *MDR1* were increased respectively. Protease activity and biofilm formation were seen in all isolates. Five isolates did not exhibit phospholipase activity.
**Conclusion:** Taken together, the overexpressions of *ERG11, CDR1* and *MDR1* genes were found in fluconazole resistant *C. tropicalis*, isolated from oropharyngeal candidiasis patients. Also, voriconazole was an effective antifungal against *C. tropicalis* isolates. The observed high protease enzyme activity and biofilm formation suggested strong pathogenicity of these isolates.

**Keywords:** *Candida tropicalis*; Azole-resistance; Proteinase; Phospholipase; Biofilm

## INTRODUCTION

Oropharyngeal candidiasis (OPC) is the most common opportunistic infection that appears in immunodeficiency and other susceptible patients (1, 2). In head and neck malignancies patients, radiotherapy (RT) is one of the major treatment methods. RT in such cases increases oral fungal colonization, which may lead to significantly increased rates of oral fungal infections. Any variation in physiological and pathological conditions, particularly after RT in patients with malignancies, may cause the yeast to

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switch to its pathogen type (3).

Increased rates in prevalence of non-*albicans* species have concerned the researchers and they try to achieve effective approaches for management and controlling of this infection (4). *C. tropicalis* is rated between the second and third among the non-*albicans* species and it is considered to be the most frequent isolate from *Candida* infection patients (5). *C. tropicalis* includes more than 80% of the isolates collected from the clinical infections. However, contrary to *C. albicans,* the detection of *C. tropicalis* is often relevant to the development of profound fungal diseases (6). It has been demonstrated that *ERG11*, the encoding gene for lanosterol 14-α-demethylase, is the key azole-resistant determinant in *C. tropicalis.* Moreover, in addition to the mutations existing in *ERG11* gene, *UPC2*, and the *ERG11* expression regulator, *CDR1* and *MDR1* as efflux pumps are also of significant importance in azole-resistant species (7, 8).

Studies have shown that fluconazole susceptible dose-dependent *C. tropicalis* isolates could be replaced by isolates resistant to all azole drugs (9), indicating *C. tropicalis* as a harmful azole resistant species which needs to be targeted by surveillance (7). Several virulence factors are suggested to cause *C. tropicalis* infections, including secreted aspartyl proteinase (Sap), phospholipase (PL) activity and biofilm formation (10). It has been demonstrated that Sap from *C. tropicalis* acts as a virulence factor (11-14) which is related to the degradation of both mucosal and immune components. A correlation exists between high PL production and increased adherence capacity, increased mortality rate in animal models and damage of cell membrane in the host (2). Various virulence factors including Sap and PL determine the transformation of the yeast from commensal to pathogen, and PL function is to disrupt the membrane of epithelial cells and to allow the entrance of the hyphal tip to the cytoplasm (15). The first step of biofilm formation is considered to be the microbial adhesion that leads to initiation of *Candida* infection (10). This structure protects the microorganism from the environmental stresses and the host defense and decreases its susceptibility to the antimicrobial agents (4).

Very little is presently known about the azole resistance mechanisms and virulence factors such as proteinase production and biofilm formation of *C. tropicalis* isolates. Thus, the aim of this study was to investigate the molecular mechanism of azole resistance and virulence factors of *C. tropicalis* isolated from OPC in head and neck cancer patients.

## MATERIALS AND METHODS

**Patients and clinical isolates.** A total of 38 *C. tropicalis* isolates were collected from 116 head and neck cancer patients with OPC during the period of two years (July 2018 to October 2020) in Cancer Institute of Imam Khomeini Hospital in Tehran with the approval of the Ethics Committee of Pasteur Institute of Iran (IR.PII.REC.1398.052). The patients of all ages and sexes, who had received radiotherapy for two weeks were selected. The daily radiation dose was between 1.8 and 2 Gray in six fractions per week. The patients who received no antifungal and azole-prophylaxis before and during the radiotherapy were admitted in the study. The patients who used chemotherapy, corticosteroids, or recent use of antibiotics as well as patients who received antifungal therapy were excluded from the study. OPC was verified by finding white plaques on intraoral mucous layer, pain while eating and swallowing and confirmed by existence of yeasts and hyphae on KOH 10% preparation and positive culture of oral swabs (16). Sampling was carried out using sterile swabs.

After culturing on Sabouraud Dextrose Agar (SDA, E. Merck, Germany) medium for 48 h at 28°C, initial differentiation was done by phenotypic methods using culturing on CHROMagar *Candida* (CHROMagar Company, Paris, France) medium, germ tube and chlamydoconidia production and carbohydrate assimilation experiment using API 20C AUX (bioMérieux, France) kit; then confirmed by multiplex PCR technique (7). All *Candida* isolates were stored in 15% glycerol at -80°C until use.

**Identification by multiplex PCR.** After initial identification, the isolates were cultured on SDA medium for 48 h at 28°C. Total genomic DNA was extracted using phenol-chloroform-isoamyl alcohol method (17). Amplifications were performed by Taq PCR Master Mix, Ampliqon (Ampliqon, Denmark) in reaction volumes of 25 µl. The sequences of the specific primers are shown in Table 1. The PCR amplification conditions were 95°C for 5 min, 35 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s and a final extension at 72°C for 8 min. after electrophoresis, species identification was done based on PCR

**Table 1.** The primers used to multiplex-PCR experiments

| Species | Sequence (5'-3') | Size (bp) |
|---|---|---|
| *C. albicans* | 5'-AGATTATTGCCATGCCCTGAG-3'<br>5'-CCATGTCGAACGTAGCGTAT-3' | 606 |
| *C. glabrata* | 5'-ACCGTGCTTGCCTCTACA-3'<br>5'-GACATCTGAGCCTCGTCTGA-3' | 212 |
| *C. dubliniensis* | 5'-GTCGGACATATACCTCCAACTC-3'<br>5'-CCATGTCGAACGTAGCGTAT-3' | 718 |
| *C. parapsilosis* | 5'-TACACCAAGCGACTCAGC-3'<br>5'-ACCAGCTGCTTTGACTTG-3' | 490 |
| *C. tropicalis* | 5'-AGAACAAGAAAACAGTGAAG-CAA-3'<br>5'-CCATGTCGAACGTAGCGTAT-3' | 126 |

products sizes. All 126-bp bands were considered as *Candida* tropicalis (18).

**Antifungal susceptibility testing.** Specific antifungal drugs including fluconazole (FLC), voriconazole (VRC), itraconazole (ITC), amphotericin B (AMB) and caspofungin (CAS) were used for antifungal susceptibility testing according to the CLSI guidelines-document M27-A3 (19) of yeast. Various concentrations of antifungal drug in RPMI 1640 (Sigma Chemical Co., USA) were prepared and added to the 96-well microtiter plates. After being cultured for 48 h, *Candida* colonies were picked and fungal suspensions were prepared in inoculums sizes of 0.5-2.5 $\times 10^3$ cell/mL in RPMI 1640. Aliquots of 100 µL of fungal suspensions were added in wells of a microtiter plate containing 100 µL of various concentrations of the antifungals. The plate was incubated at 37°C and MICs endpoints were determined visually after 24 h. *C. albicans* ATCC10231 was used as a quality control. All the experiments performed in triplicate sets each.

**Biofilm formation.** The ability of biofilm formation was tested on each clinical isolates. Organisms were cultured on sabouraud dextrose agar plates for 24 h at 37°C. Saline washed cell suspension was prepared from each isolate and adjusted to a concentration of $10^6$ CFU/Ml (20). The fungal suspension at $10^6$ cells/ml concentrations in RPMI-1640 was added into flat-bottom 96-well microtiter plates to a final volume of 200 µl per each well; then incubated at 37°C for 90 min. After incubation time, planktonic cells were removed by discarding of upper medium and each well was washed with sterile phosphate saline buffer (PBS), followed by adding fresh medium. The biofilm formation was determined after 24 h at 37°C using 2, 3-bis (2-methoxy-4-nitro- 5-sulphophenyl)-2H-tetrazolium-5-carboxanilide. The metabolic activity was measured spectrophotometrically at 490 nm using a microplate reader. Each isolate was done in triplicate. To calculate the percent transmission (% ), the $\%_T$ value of each test sample was subtracted from the $\%_T$ of the reagent blank to obtain $\%_T$ bloc. The ability of biofilm formation of each isolate was scored as negative ($\%_T$ bloc < 10) or graded as 1+ ($\%_T$ bloc 10-20), 2+ ($\%_T$ bloc 20-35), 3+ ($\%_T$ bloc 35-50), and 4+ ($\%_T$ bloc ≥50). Based on this method of scoring, the isolates were divided into two categories including low biofilm producers (1+) and high biofilm producers (2+, 3+ or 4+) (21, 22).

**Phospholipase activity.** The phospholipase production of *Candida* isolates was evaluated by using the egg yolk agar plate method (23, 24). The test medium consisted of SDA, 57.3 g NaCl, 0.55 g $CaCl_2$ and 8% sterile egg yolk emulsion were added in. Each isolate was spot inoculated in triplicate. The plates were incubated at 37°C and the diameter of the colonies and the colonies plus precipitation zone (Pz) were evaluated after 3-8 days. The PL activity was calculated (23). The average of Pz was measured for each isolates. All the *Candida* isolates were grouped according to the Pz value as following: Pz = 1, no PL activity; 0.64 > Pz < 1, moderate PL activity (+); Pz < 0.64 high PL activity (++). By this classification, a high Pz value indicates low enzymatic activity.

**Proteinase activity.** Proteinase activity was evaluated using bovine serum albumin (BSA) agar containing yeast carbon base (1.17%), yeast extract (0.01%) and BSA (0.2%) (25). The pH of the medium was adjusted to 5.0, filtrated and added to autoclaved 2% agar. All *Candida* strains were spot inoculated in triplicate. The plates were incubated at 37°C for up to 5 days. The clearance zone around the colony was measured after incubation and the proteinase activity was evaluated by Pz values as described above.

**Gene expression by real-time PCR: RNA extraction.** The *ERG11, CDR1, CDR2, MDR1* gene expressions were evaluated in FLC resistant *C. tropicalis* isolates from head and neck cancer patients with OPC. Three FLC susceptible isolates were used as a control. Total RNA was extracted from the homoge-

nized fungal cells using glass beads and Guanidinium IsoThioCyanate (GITC) reagent, followed by treatment with RNase-free DNase (Thermo Fisher Scientific, USA) (26).

**cDNA synthesis.** Single stranded cDNA was prepared from a total of 1000 ng RNA, using Revert Aid M-MuLV reverse transcriptase with random hexamer primers, according to the manufacturer's protocol (RevertAid First Strand cDNA Synthesis Kit, Thermo Fisher scientific, USA).

**Real-time PCR.** Real-Time quantitative RT-PCR was carried out (27) using SYBR green master mix (Applied Biosystems), in a final volume of 25 µl for each reaction by quantitative RT-PCR of target cDNA, performed by a Rotor gene 6000 (Corbett), sequence detection system. The specific primer sets for Real-Time PCR were as the Table 1 (28). Real-Time PCR was followed with an initial incubation at 95°C for 60 s, 40 cycles of 95°C for 15 s and 55°C for 15s and 72°C for 45s (28). The reaction set also included negative and RT controls (water and RNA instead of the samples, respectively). All samples were carried out in triplicate. The results were analyzed by relative quantification, using *ACT1* expression as the reference gene. Gene expression level was calculated by $2^{-\Delta CT}$ method and compared with expression levels of FLC susceptible isolates. Fold increases (FI) were determined using the relative threshold method ($2^{-\Delta\Delta CT}$) (27).

**Statistical analysis.** All statistical analyses were carried out by One way ANOVA and Spearman analysis using GraphPad Prism 6 (GraphPad Prism Software Inc, USA). The differences with $P < 0.05$ were considered significant.

## RESULTS

**Clinical data.** The details concerning 38 *C. tropicalis* isolates and demographics data of the patients are described as follow: 27 (71.052%) isolates obtained from male and 11 (28.947%) isolates obtained from female patients. In total, 22 (57.89%) patients were in stage 2 and others were in stages 1 and 3. Most patients had hard plate SCC and Tongue SCC malignancy. The patient's information is shown in Table 3.

**Antifungal susceptibility profiles.** Antifungal susceptibility test was carried out according to the Clinical and Laboratory Standards Institute description. Interpretation of the results was based on the species-specific clinical breakpoints (CBPs) and epidemiological cutoff values (ECVs) for FLC, VRC, ITC, AMB and CAS (29) in this study. The antifungal susceptibility pattern of all *C. tropicalis* isolates were represented in Table 2. The results showed that 7 isolates (18.42%) from 38 *C. tropicalis* isolates represented resistance, 4 isolates (10.52%) represented susceptible dose dependent (SDD) and 27 isolates (71.05%) were susceptible to fluconazole (Table 2). Moreover, our results showed that the highest and the lowest resistance was seen in CAS and VRC isolates, respectively.

**Biofilm formation and phospholipase and proteinase activity.** As shown in Table 3, all of 38 clinical *C. tropicalis* isolates exhibited biofilm formation ability; so that 73.6% of them produced biofilm at the high level. Biofilm formation in all strains was more than the standard strain (i.e., *C. albicans* ATCC10231). All 38 *C. tropicalis* isolates indicated proteinase activity (31 isolates in high level and 7 isolates in low level). PL activity was seen in 25 isolates in high levels and in 8 isolates in low levels. No PL activity was detected in five *C. tropicalis* isolates. Out of 38 *C. tropicalis* isolates, 17 isolates represented biofilm formation, PL and proteinase activity in high levels (Table 3).

**Gene expression levels of efflux transporters.** The expression levels of *CDR1, CDR2, ERG11* and *MDR1* for 7 FLC-resistant, 4 SDD and 3 FLC-susceptible clinical *C. tropicalis* isolates were measured. The expression of the genes in resistant and SDD *C. tropicalis* isolates was compared with the mean of expression level of 3 FLC-susceptible isolates by quantitative

**Table 2.** The primers used to Real time-PCR experiments

| Gene | Primer | Sequence (5'-3') |
|---|---|---|
| ERG11 | F | 5'- CTACTCCCAAAAAAAACCATA -3' |
| | R | 5'- TAAACCTAATCCCAAGACATC -3' |
| CDR1 | F | 5'- TGGAAAGAGTTGGAGGGTATGTTA -3' |
| | R | 5'- TCCCAAGGTTTCGCCATC -3' |
| CDR2 | F | 5'-GCTTAGATGCCGCCACTG -3' |
| | R | 5'- AGCCCATTCTGATGAAATACTC -3' |
| MDR1 | F | 5'- TTGGCGTTAGAGGATTTACTTTGG -3' |
| | R | 5'- GAATGAAAACTTCTGGGAAAACTGG -3' |
| ACT1 | F | 5'-GACCGAAGCTCCAATGAATC -3' |
| | R | 5'-AATTGGGACAACGTGGGTAA -3' |

**Table 3.** Patient's information, *in vitro* antifungal susceptibility and protease and biofilm production for the clinical isolates of *C. tropicalis.*

| Isolate | Type of Cancer | Sex & Age | Stage | Antifungal Susceptibility (μg/ml) | | | | | Virulence factors | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | FLC | VRC | ITC | AMB | CAS | Phospholipase | Proteinase | Biofilm |
| 1 | Hard plate SCC | 54 M | 2 | >64 | 1 | 1 | 0.5 | 4 | ++ | ++ | ++ |
| 2 | Lip SCC | 32 M | 2 | 32 | 0.0125 | 0.0125 | 4 | 0.25 | ++ | ++ | ++ |
| 3 | Maxillary SCC | 63 M | 2 | 16 | 0.0125 | 0.5 | 0.0125 | 2 | ++ | ++ | ++ |
| 4 | Hard plate SCC | 27 F | 1 | 16 | 2 | 0.5 | 0.0313 | 0.25 | + | ++ | ++ |
| 5 | Nasopharyngeal cancer | 49 M | 3 | 8 | 0.125 | 0.0125 | 1 | 0.0625 | + | ++ | ++ |
| 6 | Lip SCC | 53 M | 2 | 8 | 0.5 | 0.5 | 0.5 | 0.0125 | ++ | ++ | ++ |
| 7 | Lip SCC | 66 M | 2 | 8 | 0.5 | 2 | 0.0125 | 2 | ++ | ++ | ++ |
| 8 | Maxillary SCC | 33 F | 3 | 4 | 0.5 | 0.0125 | 0.0313 | 0.0125 | ++ | ++ | ++ |
| 9 | Maxillary SCC | 79 M | 3 | 4 | 0.0313 | 0.0625 | 0.5 | 0.5 | + | ++ | ++ |
| 10 | Hard plate SCC | 46 M | 2 | 4 | 0.0125 | 0.0125 | 8 | 0.0625 | ++ | ++ | ++ |
| 11 | Hard plate SCC | 65M | 2 | 4 | 0.0313 | 0.25 | 0.5 | 1 | ++ | ++ | ++ |
| 12 | Tongue SCC | 26 M | 1 | 2 | 0.125 | 0.5 | 2 | 0.0125 | + | ++ | ++ |
| 13 | Face BCC | 58 M | 2 | 2 | 0.125 | 0.5 | 0.0125 | 0.25 | - | ++ | ++ |
| 14 | Tongue SCC | 49 M | 1 | 1 | 0.0625 | 0.0313 | 0.0625 | 0.0625 | + | ++ | + |
| 15 | Eye melanoma | 38 M | 2 | 0.5 | 0.0125 | 0.0625 | 1 | 0.25 | - | ++ | + |
| 16 | Tongue SCC | 81 F | 3 | 1 | 0.0125 | 0.5 | 1 | 0.0625 | ++ | + | ++ |
| 17 | Neck lymphoma | 28 M | 2 | 0.0125 | 0.25 | 0.25 | 1 | 8 | ++ | ++ | ++ |
| 18 | Eye melanoma | 62 M | 2 | 2 | 0.5 | 0.125 | 0.25 | 0.0313 | ++ | ++ | + |
| 19 | Neck lymphoma | 46 F | 1 | 0.5 | 0.5 | 1 | 0.0625 | 4 | ++ | ++ | ++ |
| 20 | Tongue SCC | 77 M | 2 | 0.0313 | 0.125 | 0.25 | 1 | 0.5 | ++ | ++ | + |
| 21 | Hard plate SCC | 56 M | 2 | 0.25 | 0.125 | 0.0125 | 0.0125 | 0.0125 | ++ | ++ | ++ |
| 22 | Lip SCC | 61 F | 1 | 1 | 0.0625 | 0.5 | 0.0125 | 0.5 | ++ | + | ++ |
| 23 | Face SCC | 55 F | 3 | 1 | 0.5 | 0.0125 | 0.0625 | 0.0625 | ++ | + | ++ |
| 24 | Face SCC | 34 F | 2 | 2 | 0.0625 | 0.0625 | 0.25 | 0.0125 | + | + | ++ |
| 25 | Tongue SCC | 78 M | 1 | 0.5 | 0.125 | 0.0313 | 0.250 | 0.5 | ++ | ++ | ++ |
| 26 | Tongue SCC | 44 F | 2 | 2 | 0.5 | 0.0125 | 4 | 8 | - | ++ | + |
| 27 | Neck lymphoma | 69 M | 2 | 1 | 0.0125 | 0.25 | 0.25 | 0.0313 | ++ | + | + |
| 28 | Oropharyngeal SCC | 76 M | 3 | 0.0313 | 0.25 | 0.0125 | 0.0125 | 0.0625 | - | ++ | + |
| 29 | Maxillary SCC | 54 M | 2 | 0.0125 | 0.0125 | 1 | 0.0125 | 0.0125 | ++ | ++ | + |
| 30 | Maxillary SCC | 41 M | 2 | 0.5 | 0.5 | 0.25 | 0.0625 | 0.25 | + | ++ | ++ |
| 31 | Hard plate SCC | 83 F | 2 | 2 | 0.0625 | 0.0625 | 0.0125 | 0.0313 | ++ | ++ | ++ |
| 32 | Thyroid cancer | 68 M | 1 | 0.5 | 0.0125 | 0.5 | 4 | 0.0125 | + | + | + |
| 33 | Hard plate SCC | 55 M | 2 | 1 | 0.5 | 0.5 | 0.0125 | 0.5 | ++ | ++ | ++ |
| 34 | Laryngeal cancer | 38 F | 3 | 0.0125 | 0.5 | 0.5 | 8 | 2 | ++ | ++ | + |
| 35 | Face BCC | 56 M | 2 | 0.0313 | 0.0125 | 0.5 | 0.25 | 0.0625 | ++ | + | ++ |
| 36 | Hard plate SCC | 71 F | 2 | 2 | 0.0625 | 0.5 | 0.0125 | 0.0625 | - | ++ | ++ |
| 37 | Neck lymphoma | 43 M | 3 | 0.5 | 0.0125 | 0.0313 | 0.0625 | 0.0125 | ++ | ++ | ++ |
| 38 | Parotid cancer | 72 M | 1 | 0.5 | 0.0125 | 0.0125 | 0.0625 | 4 | ++ | ++ | ++ |

The neutrophil counts have no change in these patients. BCC, face basal cell carcinoma; SCC, maxillary squamous cell carcinoma; FLC, fluconazole; ITC, itraconazole; VRC, voriconazole; AMB, amphotericin B; CAS, caspofungin.

PCR (30). All 7 FLC-resistant isolates of *C. tropicalis* represented high levels of *ERG11* and *CDR1* genes (mRNA fold change 3.966 versus 1.073 and 5.3 versus 1.047, respectively). The expression levels of *CDR2* represented no significant difference between FLC-resistant and FLC-susceptible isolates. *MDR1* showed in-

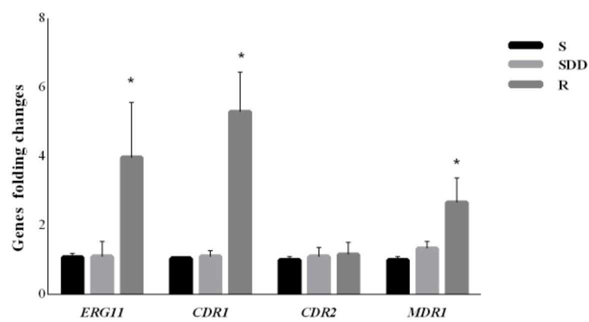


**Fig. 1.** Fold expression levels of *ERG11, CDR1, CDR2* and *MDR1* in FLC-resistant, SDD and susceptible *C. tropicalis* isolates. Expression levels of each target gene were quantified using the $2^{-\Delta\Delta Ct}$ method using the housekeeping gene ACT1 as a control. Each sample was processed in triplicate. Error bars show the standard deviations; $P < 0.0001$.

creased expression (mRNA fold change 2.66 versus 1) in FLC-resistant isolates. Also, in the expression of all tested genes, no significant difference in SDD *C. tropicalis* isolates was observed (Fig. 1); CT values of gene expression by Real-Time PCR are shown in Table 6.

## DISCUSSION

The increasing rate of *C. tropicalis* clinical isolation among the cancer patients has caused concerns for the public health authorities. *C. tropicalis* is considered as the most prevalent yeast in Asia (31-33). Although azole resistance and pathogenic factors have been widely investigated in several *Candida* spp., relevant studies are still insufficient regarding *C. tropicalis* in OPC in head and neck cancer patients (34). The ability to produce virulence factors including biofilm formation and extracellular secreted proteinase is associated with colonization and pathogenicity (35). In the present study, we investigated 38 clinical isolates of *C. tropicalis*, collected from head and neck cancer patients with OPC, with respect to proteinase and PL production, biofilm formation and FLC- resistance mechanism considering their efflux pumps. Our data showed that, out of 38 isolates of *C. tropicalis*, most were resistant to CAS (n=9); however, no isolate was resistant to all of the tested antifungals. Jiang et al. (36) have reported that VRC has a greater potency against *C. tropicalis*, compared to other antifungal drugs. In their study, no isolate was resistant to VRC; however, we found here that the lowest resistance was observed with respect to VRC, as two of the isolates were resistant to this agent.

The azole antifungals target lanosterol demethylase Erg11p, is a key enzyme in ergosterol biosynthesis pathway (37). It has been reported that the upregulation of *ERG11* has also been observed in several azole-resistant isolates. Moreover, azole resistance conferred by *ERG11* upregulation has been clearly demonstrated by the increasing *ERG11* copy number using a replicating vector (38). Also, Cdr1p/Cdr2p and Mdr1p are the ABC transporters and major facilitator superfamily members reported in *Candida* species which have been involved in azole resistance by upregulation of the corresponding genes (38). The overexpression of these efflux pumps is supposed to prevent the accumulation of effective concentrations of the azoles within the fungal cells (39).

There is little information about the molecular mechanisms of azole resistance in *C. tropicalis.* Jin et al. (40) have reported that *MDR1* overexpression combined with *ERG11* mutations cause high level fluconazole resistance in *C. tropicalis* clinical isolates. Similarly, Barchiesi et al. have demonstrated that *CDR1* and *MDR1* were over expressed in a FLC- resistant *C. tropicalis* isolate (41). However, other studies have reported no differences in *CDR1* and *MDR1* expression levels in azole-resistant *C. tropicalis.* For instance, Vandeputte et al. and Sasani et al. (42, 43) have found no overexpression in two transporter-related genes, namely *CDR1* and *MDR1* in clinical azole-resistant *C. tropicalis* isolates. In our study, the real-time RT–PCR quantification identified overexpressions of *CDR1*, while *MDR1* gene was overexpressed in fuconazole resistant *C. tropicalis* isolates.

Previous studies have shown the overexpression of *ERG11* gene associated with FLC- resistance in *C. tropicalis* isolates (34, 36). The overexpression of *ERG11* gene has also been reported in less and non-susceptible *C. tropicalis* isolates (44). It has been demonstrated that overexpression of *UPC2* gene as a transcription factor, can lead to upregulation of *ERG* genes, which cause resistance to FLC (36). These studies have proposed that *ERG11* and *UPC2* genes have an effective impact on the molecular mechanisms of FLC-resistant *C. tropicalis* isolates. Here, we found that the mRNA levels of *ERG11* gene in fluconazole resistant *C. tropicalis* isolates were higher than SDD and the susceptible isolates, in agreement to the previous studies (36, 43). Due to some limitations in conducting this study, the determination of the sequence and investigating the point mutations

of *ERG11* gene (as an alternative mechanism of azole resistance), were not carried out.

Many virulence factors such as secreted aspartyl proteinase, PL and biofilm formation are associated with the pathogenicity of *Candida* species (45). Various studies have been done on exoenzymes with focus on PLs and Saps (46) in *C. albicans*, unlike *C. tropicalis* which is the least studied. Additionally, some studies have reported high PL activity in *C. tropicalis* isolates while others have stated the absence of this activity (47, 48). These inconsistencies in the results may be due to the biological differences among the isolates tested (46). For instance, Ramos et al. have demonstrated that 3 *C. tropicalis* isolates obtained from patients presenting cutaneous candidiasis, had no PL activity, with proteinase activity in 2 isolates (15). Also, Costa et al. (2) have reported that proteinase activity could be observed in both *C. albicans* and *C. tropicalis*; however, PL activity was noted only in *C. albicans* isolates. They attributed the lack of PL activity in *C. tropicalis* to an inappropriate testing method. Deorokhkar et al. (46) have reported that almost 50% of the *C. tropicalis* isolates obtained from oropharyngeal swabs had PL activity. They demonstrated that PL was the major virulent factor expressed by *C. tropicalis* isolates. Moreover, they showed that almost all of these isolates represented proteinase activity (46). In the current study, all *C. tropicalis* isolates showed proteinase activity while 81.57% of them represented high proteinase activity and almost 85% of the isolates showed PL activity.

Biofilm formation is another key virulence factor of *Candida* species which plays an important role in escaping from the host immune system (49). Our results showed that all the evaluated isolates were capable of biofilm formation while 73.68% of them were high biofilm producers. Negri et al. (5) have demonstrated that all the tested *C. tropicalis* isolates were able to form biofilms in artificial urine; although, differences between the strains existed. Silva-Diaz et al. (4) have shown that from 184 *Candida* clinical isolates which they had obtained from different human reservoirs, *C. tropicalis* was one of the species with higher biomass production which was even more than *C. albicans*. Negri et al. (10) in 2010 reported that all *C. tropicalis* strains under their study were able to form biofilm that was consistence with our present results. However, no correlation between phospholipase, proteinase and biofilm formation with an antifungal susceptibility pattern was observed in our study (Tables 4 and 5).

Gene expression was investigated in 14 isolates (Table 6 and Fig.1), proteinase activity was almost equal and the statistical analysis showed no correlation between the proteinase activity and the genes expression. We calculated a correlation between the phospholipase activity and the genes expressions by spearman coefficient correlation (50) where the highest value of correlation coefficient between the phospholipase activity and *MDR1* was seen in the resistant (R) group, but due to the $P$ value (=0.076) being higher than $P< 0.05$, it was not statistically significant (Fig. 2).

**Table 5.** Virulence factors activity of 38 clinical *C. tropicalis* isolates

| Virulence factors | Activity n (%) | | |
|---|---|---|---|
| | High | Low | No activity |
| Biofilm | 28 (73.68) | 10 (26.31) | - |
| Phospholipase | 25 (65.78) | 8 (21.05) | 5 (13.15) |
| Proteinase | 31 (81.57) | 7 (18.42) | - |

**Table 4.** Antifungal susceptibility results of 38 clinical *C. tropicalis* isolates recovered from oropharyngeal candidiasis in head and neck cancer patients.

| Antifungal agents | Category (n%) | | | CBPs (µg/ml) | | | | ECV (µg/ml) |
|---|---|---|---|---|---|---|---|---|
| | S/WT | SDD/I | R/NWT | S | SDD | I | R | |
| Fluconazole | 27 (71.05) | 4 (10.52) | 7 (18.42) | 2 ≥ | 4 | - | 8 ≥ | - |
| Voriconazole | 24 (63.15) | 12 (31.57) | 2 (5.26) | 0.12 ≥ | 0.25–0.5 | - | 1 ≥ | - |
| Itraconazole | 34 (89.47) | - | 4 (10.52) | - | - | - | - | 0.5 |
| Caspofungin | 24 (63.15) | 5 (13.15) | 9 (23.68) | 0.25 ≥ | - | 0.5 | 1 ≥ | - |
| Amphotericin B | 33 (86.84) | - | 5 (13.15) | - | - | - | - | 2 |

WT: Wild Type; NWT: Non-Wild Type; CBPs: Clinical Breakpoints. S, susceptible; R, resistant; SDD, susceptible-dose-dependent; I, intermediate.

**Table 6.** CT values of gene expression by Real-Time PCR

| No. | FLC susceptibility | Mean CT | | | | |
|---|---|---|---|---|---|---|
| | | *ERG11* | *CDR1* | *CDR2* | *MDR1* | *ACT1* |
| 1 | R | 22.09 | 22.23 | 23.27 | 23.22 | 25.45 |
| 2 | R | 24.07 | 23.52 | 25.04 | 24.32 | 27.05 |
| 3 | R | 23.43 | 22.55 | 24.58 | 23.87 | 26.72 |
| 4 | R | 23.58 | 22.74 | 24.96 | 23.65 | 26.43 |
| 5 | R | 23.48 | 23.02 | 24.94 | 24.22 | 26.57 |
| 6 | R | 24.55 | 24.05 | 25.50 | 25.30 | 27.72 |
| 7 | R | 24.61 | 24.36 | 26.57 | 25.25 | 27.72 |
| 8 | SDD | 25.28 | 25.30 | 24.91 | 24.54 | 26.43 |
| 9 | SDD | 24.65 | 24.65 | 25.05 | 24.80 | 26.17 |
| 10 | SDD | 24.77 | 24.75 | 24.76 | 24.38 | 25.87 |
| 11 | SDD | 27.12 | 27.06 | 27.05 | 26.36 | 27.81 |
| 12 | S | 26.64 | 26.35 | 26.56 | 26.90 | 27.76 |
| 13 | S | 24.33 | 24.56 | 24.53 | 24.32 | 25.65 |
| 14 | S | 24.36 | 24.54 | 24.46 | 24.50 | 25.65 |

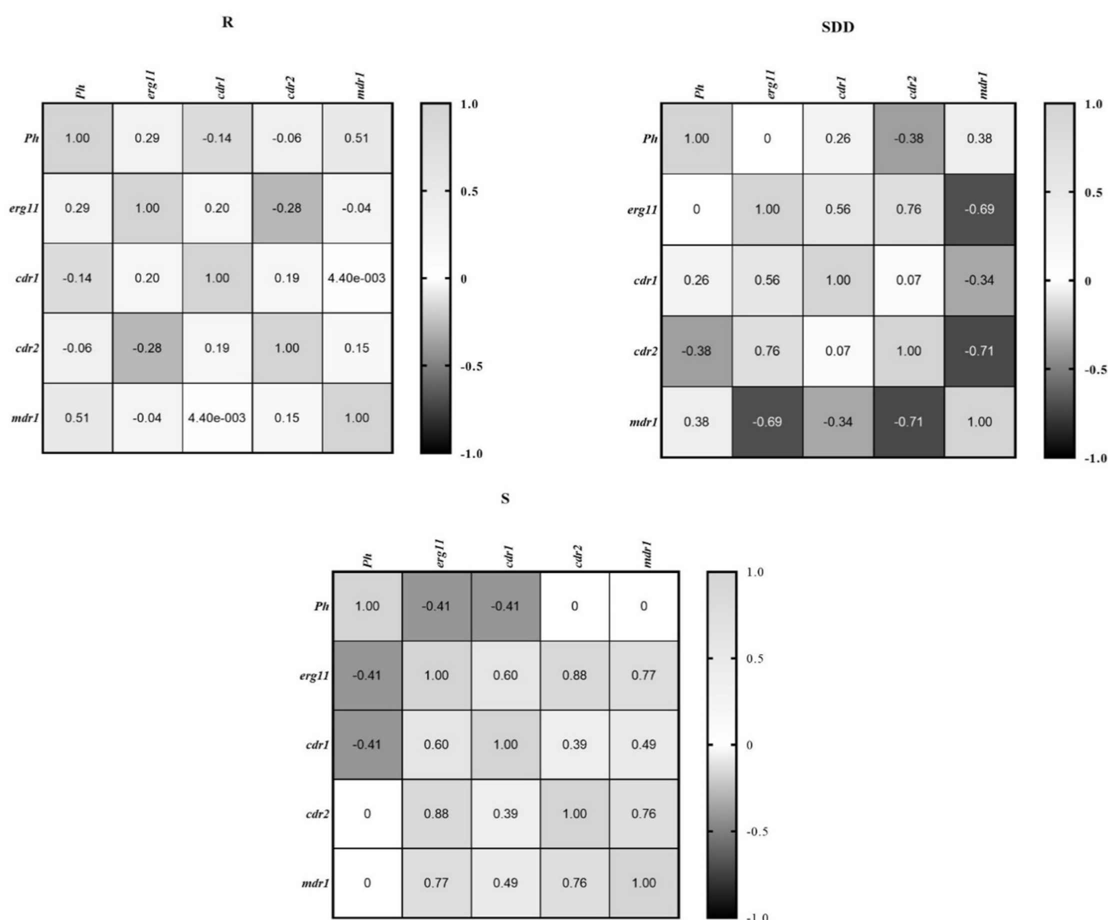


**Fig. 2.** Correlation coefficient between phospholipase activity and gene expression was measured by Spearman analysis by GraphPad Prism, the coefficient values was represented in each cells ($P$ <0.05). Ph, phospholipase activity; R, Resistant; SDD, Susceptible dose dependent; S, Susceptible.

## CONCLUSION

Taken together, voriconazole exhibited a good potency against *C. tropicalis* isolates. High activities of phospholipase, proteinase and biofilm formation indicated high pathogenicity potential of these isolates. We also determined that the overexpression of *ERG11, CDR1* and *MDR1* genes plays a greater role in fluconazole resistance than the mutation in *ERG11* gene.

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