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# Identification of Azole Resistance Markers in Clinical Isolates of *Candida tropicalis* Using cDNA-AFLP Method

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*Background:* Global reports have highlighted the increasing prevalence of *Candida tropicalis* infections as well as organism's drug resistance. This study aimed at identifying azole resistance markers in clinical isolates of *C. tropicalis*, which will be a great resource for developing new drugs. *Methods:* Two susceptible and resistant isolates of *C. tropicalis* were recovered from an epidemiological investigation of candidiasis in immunocompromised patients. *C. tropicalis* ATCC 750 was used as reference strain. Antifungal susceptibility to fluconazole and itraconazole was determined using Clinical and Laboratory Standards Institute (CLSI) method. Complementary DNA-amplified fragment length polymorphism (cDNA-AFLP) technology and real-time reverse-transcriptase (RT) PCR were used for identification of potential genes involved in azole resistance of *C. tropicalis* clinical isolates. *Results:* Five genes encoding the following enzymes were identified as superoxide dismutase (*SOD*) implicated in antioxidant defense, ornithine aminotransferase (*OAT*), acetyl ornithine aminotransferase (*ACOAT*), adenosylmethionine-8-amino-7-oxononanoate aminotransferase (*DAPA AT*), and 4-aminobutyrate aminotransferase (*ABAT*)—belonging to pyridoxal phosphate (PLP) dependent enzymes and acting in an important physiological role in many fungal-cell cycles. Real-time RT-PCR confirmed mRNA level of the aforementioned genes. *Conclusion:* Our findings showed that factors such as PLP-dependent enzymes and *SOD* might be implicated in drug resistance in *C. tropicalis* clinical isolate. Therefore, further studies are required to explore the accurate biological functions of the mentioned genes that would be helpful for effective drug development. J. Clin. Lab. Anal. 30:266–272,2016.

**Key words:** azole resistance; candidiasis; differential display; real-time RT-PCR

## INTRODUCTION

*Candida* species are the most common cause of opportunistic fungal infections (1). Infections due to opportunistic fungal pathogens have increased mainly over the past two decades, related to increasing patient populations such as persons with AIDS, individuals undergoing solid organ and bone marrow transplantation, and immunosuppressed and cancer patients (2, 3). *Candida albicans* is the most significant agent of candidiasis in immunocompromised patients (4, 5).



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*C. tropicalis* has long been proposed to be an agent of invasive fungal infections in patients with cancer, leukemia and in hematopoietic stem cell transplantation recipients (6, 7). It has been well determined that among non-*albicans Candida* species *C. tropicalis* is the third leading cause of *Candida* infections as well as the second in respiratory specimens (3, 4).

There are currently three main classes of antifungal agents for treatment of candidiasis: the polyenes, azoles, and echinocandins (8). Fluconazole is the most highly used azole drug (9). Drug resistance is an important issue for treating infections caused by *Candida* spp. (7). Clinical isolates of *C. tropicalis* resistant to azoles, particularly to fluconazole, are increasingly reported (3,5,10). According to the literature, fluconazole resistance rate is dramatically increasing among clinical isolates of *C. tropicalis*, a third leading etiologic agent in candidiasis (11, 12).

The cellular and molecular foundations of resistance are strongly dependent on the method of antifungal activity. Molecular resistance may contain detection of point mutation, gene conversions, and gene amplifications causing overexpression or mitotic recombination (10, 13). Although the molecular mechanisms of drug resistance in the *C. tropicalis* are not completely established, azole resistance mechanisms have been studied basically in *C. albicans* and *C. glabrata* (14, 15). Lanostrol 14-α demethylase is encoded by the *ERG11* gene. Involvement of overexpression and point mutation in *ERG11* have been reported in azole resistance (16,17). The efflux pumps such as ATP-blinding cassette (ABC) transporters and major facilitator super family (MFS) proteins including *Candida* drug resistance (*CDR*) 1 and multidrug resistance (*MDR*) 1 genes have been related to azole resistance in several *Candida* species, especially *C. albicans* strains (18).

Complementary DNA-amplified fragment length polymorphism (cDNA-AFLP) method is one of the appropriate transcriptomic methods for gene discovery that does not require prior sequence knowledge of the genes (19). This method is used for the identification of new genes in various organisms such as *Candida* yeast (19, 20). In this study, cDNA -AFLP method was utilized for identification of molecular resistance markers in *C. tropicalis* isolates from immunocompromised patients.

## MATERIALS AND METHODS

### Yeast Isolates

Two clinical isolates of *C. tropicalis* composed of isolates sensitive and resistant to fluconazole and itraconazole were used in this study. These isolates were previously recovered from oropharyngeal samples of immunocompromised patients and stored in culture collection of Department of Medical Mycology and Parasitology of Tehran University of Medical Sciences.

*C. tropicalis* ATCC 750 was used as a reference. All clinical isolates were identified by standard mycological and biochemical methods including colony color in CHROM agar *Candida* (CHROMagar, Paris, France) and API20C AUX system (Bio Merieux, Lyon, France).

### Culture Conditions and Drug Susceptibility Testing

Three strains of *C. tropicalis* including two clinical isolates and a reference strain were cultured on a yeast extract peptone dextrose (YEPD) agar plate containing 5 g/l yeast extract (Baltimore Biological Laboratory, Washington, USA), 10 g/l peptone (Merck, Frankfurt, Germany), 20 g/l dextrose (Merck, Frankfurt, Germany), and 20 g/l agar (Biolife, Milan, Italy), and then the plates were incubated for 72 h at 37°C. Later a single colony of each isolate and reference strain was transferred in YEPD broth and incubated for 24 h at 37°C.

The in vitro susceptibility of two clinical isolates to fluconazole and itraconazole was determined by broth microdilution method according to CLSI M27-A3 standard (21). *C. krusei* ATCC 6258 was used for quality control. After 48 h of incubation at 35°C, minimum inhibitory concentrations (MICs) were determined visually with the aid of mirror by comparison of growth in the wells containing the drug with the drug-free controls. The MICs of azoles were determined as lowest concentration supporting ≥50% growth inhibition compared to the growth in control wells. All tests were performed at least two times. The MICs of fluconazole and itraconazole for resistant isolate were ≥64 and ≥16 μg/ml, respectively.

### RNA Extraction and cDNA Synthesis

Total RNA was extracted from the log phase using the RNeasy protect mini kit (Qiagen, Hilden, Germany), following the mechanical infraction of the yeast cells by sonication with acid-washed glass beads (0.45–0.52 mm diameter). To avoid any genomic contamination, all RNA samples were treated with RNase-free DNase (Qiagen, Hilden, Germany) as explained by the manufacturer. The quantity and quality of RNA were determined using nanodrop (ND-1000, Thermo Fisher Scientific, Waltham, Massachusetts, USA) and the electrophoresis on 1.5% agarose gel.

The single-strand cDNA (sscDNA) for each of the clinical isolates and the reference strain was synthesized using 6 μg RNA, 20 pmol/μl random hexamer (Fermentas, Burlington, Canada), 20 pmol/μl oligo-dT (Fermentas, Burlington, Canada), and 10 mM of dNTP mix (Fermentas, Burlington, Canada) incubated at 65°C for 10 min and then cooled on ice followed by addition of 20 U Ribolock (Fermentas, Burlington, Canada), 7.5 μl of 5×

**TABLE 1. Adaptors Used in cDNA-AFLP Method**

| Adaptors | Sequence (5′–3′) |
|---|---|
| ADEcoR I | ACCGACGTCGACTATCCATGAAG |
| adEcoR I | AATTCTTCATGG |
| Pre amp | ACCGACGTCGACTATCCATGAAGAATTC |
| S1Ecor I | ACCGACGTCGACTATCCATGAAGAATTCC |
| S2Ecor I | ACCGACGTCGACTATCCATGAAGAATTCG |
| S3Ecor I | ACCGACGTCGACTATCCATGAAGAATTCA |
| S4Ecor I | ACCGACGTCGACTATCCATGAAGAATTCT |

reverse-transcriptase (RT) buffer (Fermentas, Burlington, Canada) and 200 U of Moloney Murine Leukemia virus (M -MuLV) RT enzyme (Fermentas, Burlington, Canada) and then incubated at 72°C for 10 min followed by incubation at 42°C for 60 min. The accuracy of cDNA was checked with actin (*ACT*) gene primers as housekeeping (Table 2). The PCR condition was initial denaturation step for 5 min at 94°C, 30 cycles of denaturation for 30 sec at 94°C, annealing for 30 sec at 62°C, an extension for 7 min at 72°C with a final extension of 72°C.

Double-strand cDNA (dscDNA) was synthesized using 40 U DNA *polymerase I* (Fermentas, Burlington, Canada) and 10 mM dNTP mix at 16°C for 3 h and was precipitated with ethanol. The quantity and quality of synthesized dscDNA were determined using nanodrop (ND-1000, Thermo Fisher Scientific, Waltham, Massachusetts, USA) and the electrophoresis on 2% agarose gel.

## cDNA-AFLP

cDNA-AFLP method was performed as explained previously with some modifications (20). Two micrograms purified dscDNA was digested with 5U EcoR1 restriction enzyme (Fermentas, Burlington, Canada) for 4 h at 37°C, and the enzyme was inactivated at 80°C for 20 min. The digested components were ligated to AFLP adaptors (Table 1), 8 μg ADEcoR1 and 4 μg adEcoR1 in a final volume of 60 μl reactions. Briefly, it was conducted 1 min at 50°C, decreasing to 10°C over 1 h (i.e., 1°C/1.5 min) and then 6 U T4 DNA Ligase (Takara, Tokyo, Japan) was added and incubated at 16°C for 16 h.

The preamplification was performed with preamp primers (Table 1) on ligated fragments according to the following PCR conditions: 5 min of denaturation at 94°C; 30 cycles of 94°C for 30 sec; 63°C, 30 sec; 72°C, 30 sec; and final extension at 72°C for 5 min. The sensitive amplification was performed using sensitive primers with the same preamplification PCR conditions. These primers included adaptor sequences in addition of one new nucleotide at the 3′ end (Table 1). The PCR products resulting from this step were separated by 8% nondenaturated polyacrylamide gel electrophoresis (PAGE) and stained with silver nitrate. Then, the gels were studied for the existence of differentially expressed transcription-derived fragments (TDFs).

## Isolation, Cloning, and Sequencing of TDFs

Differentiated TDFs were extracted from the PAGE and reamplified with appropriate sensitive primers. The PCR products were checked using electrophoresis on 1.5% agarose gel. Then, they were cloned using TOPO TA cloning kit (Invitrogen, Carlsbad, California, USA) and screened with universal primers, M13 forward (−20) and M13 reverse (Table 3), according to the following PCR condition: 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and 72°C for 7 min. The PCR products were checked by agarose (Merck, Frankfurt, Germany) gel electrophoresis. The recombinant plasmids containing unknown DNA were sequenced using M13 forward and reverse primers. Sequence results were analyzed in nonredundant nucleic and protein databases BLAST (www. ncbi. nlm. nih. gov/ BLAST/).

## Real-Time RT-PCR Analysis

Real-time RT-PCR was carried out to detect the mRNA expression level of TDF-derived genes between two resistant and sensitive *C. tropicalis* isolates by specific primers designed by primer3-Bio Tools-University of Massachusetts Medical (biotools. Umassmed. edu/ bioapps/ primer3-www. cgi, Table 2). The sscDNA for each of the clinical isolates and the reference strain was synthesized from 6 μg of total RNA using Quantitect Reverse Transcription kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. RT-PCR was performed in 25 μl reactions containing 2.5 μl cDNA target, 300 nM forward and reverse primers, and 1× Real-Time PCR Master Mix E3 containing EvaGreen, dUTP, and ROX (500 nM) (GeneON, Ludwigshafen, Germany). Tests were performed in triplicate by StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, California, USA).

The PCR condition was as follows: denaturation at 95°C for 60 sec and 40 cycles of denaturation at 95°C for 15 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec. The actin gene was used as a housekeeping gene and expression level of each gene was calculated by the $2^{-\Delta\Delta Ct}$ method as previously described (22). Serial dilutions of the sscDNA were used for calculation of the efficiencies of the primer sets on real-time PCR.

## Statistical Analysis

All tests were performed at least two times and the results are explained as the mean ± SDs. The value of differences was defined by Student's *t*-test and the acceptable level of significance was 95% ($P < 0.05$).

**TABLE 2. Primers Used in This Study**

| Gene name | Primer | Nucleotide sequence (5′–3′) | GenBank accession no. | Product size (bp) |
|---|---|---|---|---|
| *ACT* | ACT F | GAAGATCTTGTCTGAACGTGGTT | AJ389059.1 | 120 |
| | ACT R | GAGGTTTGCATTTCTTGTTCG | | |
| *SOD* | SOD F | AGGTGGTGGTCAACATCCAG | XM-002550104 | 131 |
| | SOD R | AAGCCCAACCAGAACCTTG | | |
| *OAT* | OAT F | GGTATTGTTGTTCCGCCAGA | XM-002545168.1 | 146 |
| | OAT R | TTCACCCCCTTTGAGTGTTC | | |
| *ACOAT* | ACOAT F | TCACCTTTAATCCCAGGAG | XM-002547867.1 | 128 |
| | ACOAT R | GGATTAACTCCACCTTCACC | | |
| *ABAT* | ABAT F | GTTTATCTGGTGCCGATGCT | XM-002547219.1 | 147 |
| | ABAT R | CCAAATCTGGAGAACCTGGA | | |
| *DAPA AT* | DAPAAT F | ACCGGTGTCTGGAAATCTCA | XM-002546330.1 | 129 |
| | DAPAAT R | TTCAATCACTCCCACAGCAC | | |

## RESULTS

### Antifungal susceptibility

Drug sensitivity of *C. tropicalis* isolates to fluconazole and itraconazole was determined CLSI guideline (document M27-A3). Results showed that MIC values of fluconazole were ≥64 and 1 μg/ml and for itraconazole were ≥16 and 0.25 μg/ml in resistant and sensitive isolates, respectively. The MIC values of fluconazole and itraconazole for resistant isolate were about 64 times higher than for the sensitive isolate.

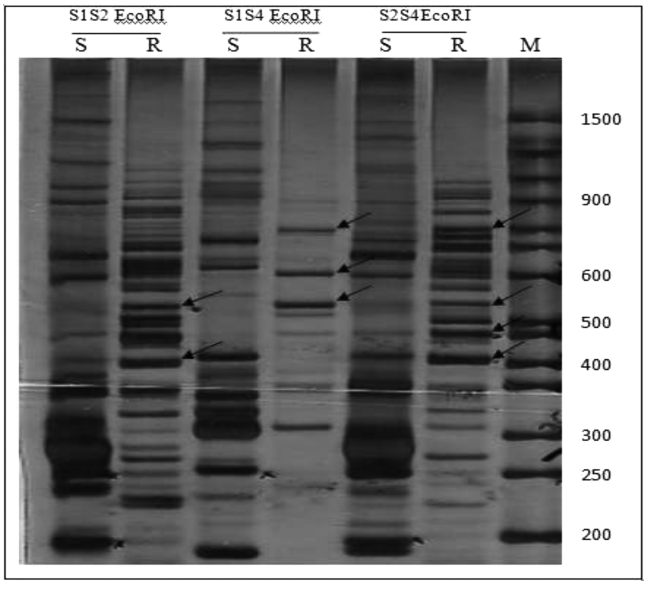


**Fig. 1.** An illustration representative of cDNA-AFLP on PAGE. Sensitive amplification of cDNA-AFLP on a PAGE from three different primer combinations: S1S2EcoRI, S1S4EcoRI, S2S4EcoRI. The arrows show differentially expressed TDFs in resistant and sensitive *C. tropicalis* isolates. M, (50 bp) molecular weight marker; S, sensitive; R, resistant.

### cDNA-AFLP Analysis of Clinical Isolates

To detect the mRNA expression level related to molecular resistance, a cDNA-AFLP technique was used on drug-resistant and drug-sensitive *C. tropicalis* isolates. To study the validity of cDNA, the actin gene was used as a housekeeping gene and RT-PCR using specific primers related to actin (120 bp) was conducted (data not shown). cDNA-AFLP results determined differential expression of TDFs on 8% nondenaturating PAGE using silver staining by 10 differential primer combinations (Fig. 1). Because of restriction enzyme digestion in this method, several transcripts may have the similar size. Accordingly, several bands may be one gene or one band may contain more than one gene. Fourteen TDFs were extracted from the cDNA-AFLP on PAGE and reamplified.

Finally, each favorable band was identified by cloning and DNA sequencing. The BLAST analysis indicated that six sequences had remarkable similarities with genome database ($E$-value $\leq 10^{-5}$). Five target genes containing superoxide dismutase (*SOD*), ornithine aminotransferase (*OAT*), acetyl ornithine aminotransferase (*ACOAT*), adenosylmethionine-8-amino-7-oxononanoate aminotransferase (*DAPA AT*), 4-aminobutyrate aminotransferase (*ABAT*) and one unknown gene coding hypothetical proteins were detected with different mRNA expression levels in resistant *C. tropicalis* isolate compared to sensitive isolate and sensitive reference strain. Some characteristics of these genes are presented in Table 3.

Real-time RT-PCR was used to investigate the different mRNA expression of target genes in the resistant and sensitive *C. tropicalis* clinical isolates (Fig. 2). Data show a very considerable upregulation of *SOD* (32.221-fold), *OAT* (88.208-fold), *ACOAT* (69.277-fold), *DAPA AT* (59.933-fold), and *ABAT* (59.933-fold) in resistant clinical isolate compared to sensitive clinical isolate ($P < 0.05$).

**TABLE 3. Sequences Identified by cDNA-AFLP**

| Code no. | Annotation | Length (bp) | Accession no. |
|---|---|---|---|
| 1 | SOD | 234 | XP 002550150.1 |
| 2 | OAT | 436 | XP 002545232.1 |
| 3 | ACOAT | 448 | XP 002547913.1 |
| 4 | DAPA AT | 433 | XP 002546376.1 |
| 5 | ABAT | 471 | XP 002547265.1 |
| 6 | Hypothetical proteins | 147 | XP 002547165.1 |

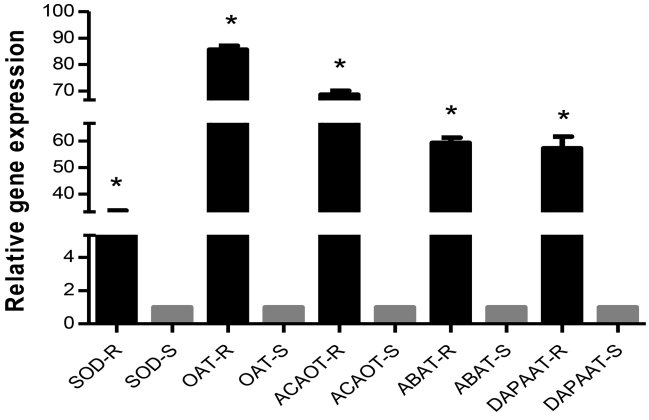


**Fig. 2.** Relative gene expression pattern of target genes in resistant and sensitive *Candida tropicalis* isolates by real-time RTPCR. The expression of actin gene was used to normalize the data. R, resistant; S, sensitive. Values are mean ± SD. $^{*}P < 0.05$.

## DISCCUSION

Among non-*albicans Candida* species, *C. tropicalis* represents the third most common species but the second in respiratory specimens (3, 4). Resistance to azoles, especially to fluconazole, in clinical isolates of *C. tropicalis* is increasingly reported (11, 12). Antifungal resistance of *Candida* species is a great problem in treating infections caused by these agents (7).

This study describes the role of five target genes including *SOD*, *OAT*, *ACOAT*, *DAPA AT*, and *ABAT* on the azole resistance of *C. tropicalis*, which were identified using cDNA-AFLP. Our results demonstrate upregulation of these five genes in resistant *C. tropicalis* clinical isolate for the first time. According to this study, these five genes seem to be potential new markers in molecular resistance that confirmed upregulation of expression level of these genes by real-time RT-PCR.

SOD catalyzes the conversion of superoxide radical to hydrogen peroxide and dioxygen (23). According to the metal cofactor, four classes of SOD exist: there are iron (Fe SOD), manganese (Mn SOD), copper and zinc (CuZn SOD), and nickel (Ni SOD) types of enzymes (23, 24). Most antifungal drugs were shown to induce cell death in yeast with apoptotic mechanism by the generation of high intracellular reactive oxygen species (ROS) levels (25, 26).

*ROS* is one of the major causes of damage to DNA, proteins and lipids, and mutations (27, 28). In this study we report the upregulation of *SOD* in resistant *C. tropicalis* clinical isolate. *SODs* play a significant role in protecting cells from the superoxide ion toxicity (23, 29). Several studies have described the effects of *Cu/Zn SOD* on the morphological phenotype, oxygen metabolism, and virulence of *C. albicans* (23, 24).

*SOD5* gene, which seems to be an important cofactor in the virulence, switches from yeast to hyphal forms and protects *C. albicans* cell against intracellularly generated superoxide radicals (24). *SOD5* expression levels were increased when cells grew in the presence of high salt concentrations. Martchenko et al. reported there is a correlation between generation and detoxification of superoxide in respiratory of *C. albicans* (24).

It has been suggested that different factors such as antioxidant defense systems of *C. albicans* play critical roles for resistance against host immune response (23). Hwang et al. showed that the *Cu/Zn SOD* deficient strain revealed an increased susceptibility to fungicidal damage by macrophages and attenuated virulence in a mouse model for systemic candidiasis. Our results are in accordance with the above-mentioned studies that indicated upregulation of *SOD* in resistant *C. tropicalis* clinical isolate.

*OAT*, *ACOAT*, *DAPA AT*, and *ABAT* belong to pyridoxal phosphate (PLP) dependent aspartate aminotransferase superfamily. Aspartate aminotransferase is a key enzyme in the nitrogen metabolism of all organisms (30, 31). Various studies determined that all proteins belonging to this family are involved in different biological processes including transamination (movement of amino groups), decarboxylation (removing COOH groups), racemization (redistribution of enantiomers), and various side-chain reactions depending on the enzyme involved (30–32).

These enzymes have been considered to play important physiological role of fungal-cell cycle (33, 34). *ABAT* metabolic machinery is interfaced with some of the major facets of fungal-cell cycle. These include nitrogen and energy metabolism, sporulation, differentiation, and development; and the most important facets include nitrogen and carbon source, energy metabolism, sporulation, differentiation, and development (33). It has been understood that some biomolecules including putrescine, spermidine, and spermine produced by the *OAT* and *ABAT* enzymes known as compounds implicated in developmental processes and stress tolerance (33, 35–37). Fungi have an unusually high requirement of polyamines as essential components for cell growth (33, 35).

It has been reported that overexpression and genetic alterations of various genes such as *ERG11* depend on azole

resistance (16, 17). The efflux pumps such as ABC transporters and MFS proteins including *CDR1* and *MDR1* genes have been related to azole resistance in several *Candida* species, especially in *C. albicans* strains (18).

The most ubiquitous mechanism resistant to drug toxicity is the elimination of drugs out of the cell using ABC membrane transporters. These molecular pumps actively translocate drugs through the cell membrane by using the energy obtained from ATP hydrolysis (38). The *OAT*, *ACOAT*, *DAPA AT,* and *ABAT* enzymes play a main role in the production of tricarboxylic acid cycle (TCA cycle) precursors. Therefore, overexpression of these genes leads to the production of high amounts of ATP and energy in fungal cells, so that a part of the ATPs may be used by multidrug efflux pumps for translocation of Azole drugs out of the yeast cells.

We used cDNA-AFLP method for the identification of target genes involved in azole resistance of *C. tropicalis* clinical isolate. We discovered five genes including *SOD*, *OAT*, *ACOAT*, *ABAT,* and *DAPA AT* as well as one hypothetical protein. This is the first report of indication of the above-mentioned genes in azole resistance of *C. tropicalis* clinical isolate. Our findings showed that factors such as PLP-dependent enzymes and *SOD* might be implicated in drug resistance in *C. tropicalis* clinical isolate. Therefore further studies are required to explore the accurate biological functions of the mentioned genes, which would be helpful for effective drug development.

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