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# Relation of ALS 1 and ALS3 genes and fluconazole resistance in *Candida albicans* isolated from vaginal candidacies

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ABSTRACT

*Candida albicans* is an opportunistic fungi that is able to thrive in many host niches, including the skin, mucosal, surfaces, the blood stream and internal organs. Agglutinin-like sequence (ALS) genes which could play a role in forming biofilms, adherence to host surfaces as a virulence factor and antifungal drug resistant. The purpose of the present study was to evaluate the presence ALS1 and ALS3 genes in clinical strains of *C.albicans* isolated from women with vaginal candidiasis symptom. Vaginal swabs were collected from patients suffering from vaginal candidiasis and admitted to health care centers (Tehran, Iran) from June 2011 until June 2012 and cultured on Sabaouraud Dextros Agar for 48 hours. A PCR-RFLP was used with MspI restriction enzyme for identification of *Candida albicans*. Susceptibility testing of 53 clinical isolates of *C.albicans* isolates was done against Fluconazole by using disk diffusion method. Total DNA was extracted from *C.albicans* isolates and PCR assay was used to evaluate the presence of ALS1, ALS3 and internal control (ACT1) genes. 53 clinical isolates out of 100 were identified as *C.albicans* by using PCR-RFLP. Three (5.7%) of them were susceptible and 50 (94.3%) were resistant to fluconazole. 44 patients (83%) with vaginal candidiasis were positive for ALS1 gene and 48 ones (90.5%) were positive for ALS3. According our finding, a significant correlation was seen between the presence of ALS1 and ALS3 genes and fluconazole resistance in *C.albicans* isolates.

## 1. Introduction

*Candida albicans* is a pleiomorphic pathogenic fungus that causes many infections in humans including mucosal and systemic, often serious infections in immunocompromised patients (Calderone, 2002). Vulvovaginal candidiasis is an important gynecological disorder in women worldwide and *Candida albicans* is the etiological agent in over 80% of the cases (Sobel et al., 1998). Many *Candida* infections involve colonization of the vaginal mucosal layer. About three-quarter of all women suffer from vaginitis at least once during their life time (Sobel, 2007). Among the different antifungal agents available, the class of azoles has been used extensively during the past 20 years. Due to the frequent use of this agent, treatment failures

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were observed to be associated with emergence of azole-resistant *C.albicans* strains (Rex et al., 1995). Because of increasing antifungal resistance and creating recurrent candidiasis, identification and detection the genes responsible for drug resistance is very significant in clinical specimens which isolated from the patients. There are many molecular mechanisms involved in the development of secondary resistance to fluconazole in *C.albicans* such as Over-expression/mutations of target enzyme, lanosterol14a-demethylase; overexpression of efflux pumps such as CDR and MDR genes (Prasad et al., 1995; White, 1997; Lopez-Ribot et al., 1998).

It has been established that agglutinin-like sequence (ALS) genes family contribute to generate fluconazole resistance. Adherence of microorganisms to host surface is a critical step in the devel-opment of biofilm and initiation of infection. Adhesion of *C.albicans* is required to some adhesion molecules, such as the ALS family, hyphal wall protein (HWP) and cell wall glycoproteins (Nobile and Mitchell, 2006). The ALS family of *C.albicans* includes eight genes that encode large cell surface glycoproteins. Although they share a similar three-domain structure, sequence differences between the ALS proteins can be wide, suggesting that the proteins may have different functions (Hoyer, 2001). It has been shown that ALS1 and ALS3 genes have adhesive function in *C.albicans* and associated with the pathogenesis of *C.albicans* (Fu et al., 2002; Zhao et al., 2004).

In this study, we performed PCR (polymerase chain reaction) for amplification of ALS-specific primers to focus on presence of ALS1 and ALS3 genes in *C.albicans* isolated from vaginal specimens and evaluation of fluconazole resistance in these isolates.

## 2. Materials and Methods

### *2.1.Sample collection and identification of C.albicans*

One hundred vaginal samples of the participants which admitted to Gynecology and obstetrics clinics (Tehran, Iran) during a one-year period from September 2011 until September 2012 collected by using a sterile swab. Prior to sample collection the informed consent was obtained from all patients.

Samples cultured on Sabaouraud Dextros agar containing Chloramphenicol (Merck, Germany) to prevent growth of bacteria and incubated for 48 hours at 35°C. Microscopic observation of *Candida* pseudohypha or filamentous blastoconidia in direct preparations was proved *Candida* infection. Then all of the isolates were cultured on CHROM agar *Candida* (Merck, Germany) medium in order to distinguish their color. This microscopic feature could be a probable reason for pathogenic form of *Candida* in vaginal tissue.

### *2.1.DNA extraction and PCR-RFLP for identification of C. albicans*

Genomic DNA from clinical isolates and standard species of *Candida* were extracted by glass bead and lysis solution (Yamada et al., 2002). Briefly 10 mm3 of a fresh colony was transferred to a 1.5 ml ependorf tube and then 300 µl of lysis buffer containing (100 mM Tris pH 8, 10 mM pH 8, 100 mM NaCl, 1% SDS, Triton 2% X-100), 300 µl of phenol: chloroform (1:1) and 200 µl of glass beads, with a diameter of 1 mm, were added and the tube was vigorously shaken for 60 minutes, the sample was centrifuged for 5 minutes at 5000 rpm. The supernatant was transferred to a clean tube and 400 µl of chloroform was added. After centrifuging, the aqueous phase was transferred to a clean tube and then 1 volume of cold isopropanol and 5 of 3M sodium acetate (pH: 5.2) were added and was kept at -20°C for 10 minutes. After that, the samples were washed by 70% ethanol. Then 30 µl distilled water was added and the sample was kept at -20°C.

PCR was performed to amplify ITS1-5.8SITS2 segment in ribosomal DNA. The sequences of ITS1 and ITS2 primers were 5'-TCC GTA GGT GAA CCT GCG G-3' and 5'-TCC TCC GCT TAT TGA TAT GC-3', respectively. For PCR reaction, 2.5 µl of 10x PCR buffer, 1.5mM MgCl2, 0.5 µl of 10 mM dNTPs, 0.4µM primers, 1.25 units of Taq polymerase (Cinnagene, Iran), 1 µl of template DNA and molecular grade dH2o up to 25 µl. Thirty amplification cycles were performed in the thermo cycler (Bio Rad) at 95°C for 5 minutes, an annealing step at 55°C for 30 second, and an extension step at 72°C for 1 minute, with a final extension at 72°C for 5 min following the last cycle.

PCR-RFLP technique using MspI restriction enzyme (Fermentas) for identification of *Candida albicans* was used as previously described method (Mirhendi et al., 2006). The digested fragments electrophoresed through 1.8% agarose gel and then visualized by ethidium bromide staining.

### *2.2. PCR with specific primers for ALS1 and ALS3 genes*

The confirmed *C.albicans* isolates were used in PCR analysis for the presence ALS1 and ALS3 genes. ACT1 was used as a control housekeeping gene (Table 1). PCR reactions were carried out in a reaction mixture with total volume of 25 µl containing 20ng of genomic DNA, 20 µl sterile water, 2.5 µl 10x Taq polymerase buffer, 0.3 µl dNTPs (10 mmol l-1), 1 U Taq DNA polymerase and 25 pmol l-1 from each primer. PCR reactions were denatured for 5 minute at 94°C and subjected to 40 cycles at 94°C (30 s), 58°C (30 s) and 72°C (30 s). A final 7 minute extension at 72°C is completed the reaction.

### *2.3. Antifungal susceptibility test*

A disk diffusion method for *Candida albicans* isolates against fluconazole 25µg (Mast, England) on Muller-Hinton agar (Merck, Germany) was carried out according to the NCCLS guidelines (Gain et al., 2004). A suspension of isolated colony was adjusted to $10^6$ CFU/ml, after preparation of this turbidity the samples was plated on Muller-Hinton agar using sterile swap then fluconazole disk was placed on a plate and investigated after 18-24 hours of incubation in 37°C. The standard *C.albicans* resistant (ATCC 76615) and susceptible (ATCC 10231) strains were used as controls. Inhibition zone of disk for all isolates measured.

## 3. Results

Of 100 isolates which collected from women with vaginal candidiasis, fifty three isolates were identified primary as *C.albicans* by producing green color on CHROM agar *candida* medium and confirmed by PCR-RFLP method. ITS1/ITS2 primer pairs generated PCR products of 535bp for *C.albican* isolates. MspI enzyme produced 2 fragments for each of clinical and standard isolates of *C.albicans* in PCR-RFLP assay and was verified by electrophoresis is an expected 297 and 389 bp (Figure 1).

Among total 53 clinical isolates of *C.albicans*, 50 isolates (92.4%) were resistant to fluconazole (inhibition zone <14mm) while only 3 isolates (7.5%) were sensitive (inhibition zone>19mm) in disk diffusion method (Figure 3). The predicted amplification product for ALS1 and ALS3 gene were seen as 318 and 184 bp on 1% agarose gels, respectively (Figure 2). PCR analysis detected the presence of ALS1 in 44 (83%) and ALS3 in 48 (90.5%) of *C.albicans* isolates. The results of PCR analysis are shown in (Table 2).

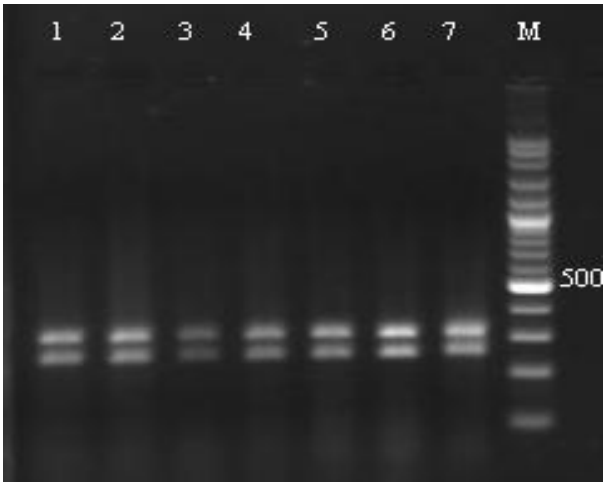


**Figure 1**. Lane 1-6:PCR-FRLP products of *C.albicans* clinical isolates with MspI (297 and 389 bp fragments); lane7:PCR-RFLP product of *C.albicans* standard strain ATCC10231.

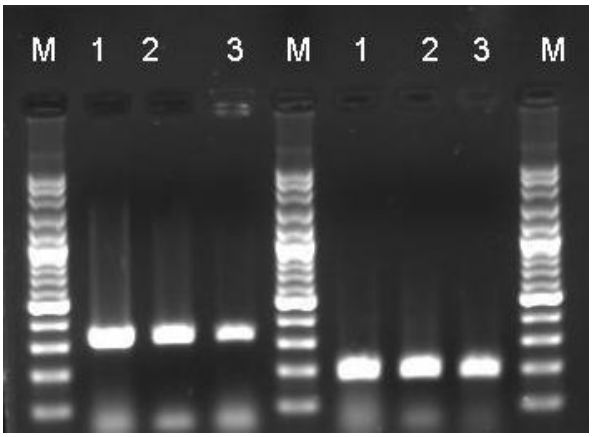


**Figure 2.** PCR product of amplified ALS1 and ALS3 genes.M: Molecular weight marker (100bp). Lane 1, 2 and 3(left): amplified ALS1 gene (318bp); lane1, 2 and 3 (right): amplified ALS1 gene (184bp).

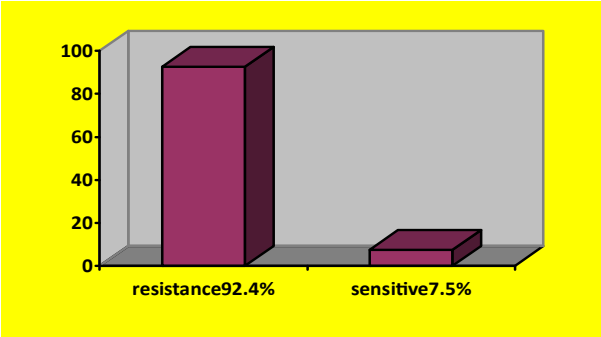


**Figure 3.** Percentage of sensitivity clinical isolates of *C.albicans* to fluconazole in disk diffusion method.

## 4. Discussion

In this study, the presence of ALS1 and ALS3 genes among the isolates were analyzed for the first time using specific primers in PCR methods and its relation with fluconazole resistance in these isolates. In disk diffusion method, 94.3% of *C.albicans* isolates were resistant to fluconazole which can be related to uncontrolled prescription of antifungal drugs without performing susceptibility testing of isolates. Our result indicated that there is a positive correlation between the presence of ALS1 and ALS3 genes in isolates of *C.albicans* and fluconazole resistance, because all of *C.albicans* isolates that were positive for amplification of ALS1 and ALS3 genes, were resistant to fluconazole.

43 (80.9%) of isolates that were positive for both ALS1 and ALS3 genes were also resistant to fluconazole. Moreover, 2(3.7%) of isolates had at least one ALS genes also were resistant (Table 2).

In our study, 3(5.5%) of isolates which were positive for at least one genes, were sensitive to fluconazole. It seems that other regulatory genes have contributed to the fluconazole resistance mechanisms. Also the role of Med31, Med20 and Srb9/Med13 transcriptional regulator genes in the expression of the relevant virulence genes including ALS gene family and biofilm formation should not be ignored (Uwamahoro et al., 2012)

It has been shown that *C.albicans* Tor1 gene plays an important role in regulation of expression of several virulence associated genes including ALS1 and ALS3 and other adhesions (Braun et al., 2001). According to our result, ALS gene family in *C.albicans* had a crucial role in fluconazole resistance since ALS genes involve in biofilm formation and adherence to host cells. It is suggested that the isolates of *candida* which have the ALS genes, is more virulent and their ability to initiate the infection is greater. However, more studies are required to understand the exact mechanisms involved (Chandra et al., 2001). In conclusion, although treatment failure is common in patients with vaginal candidiasis and antifungal resistance is a potential problem, the understanding of the correlation between resistance to antifungal treatment and the presence of special genes is useful. Other factors, such as the immune status of the patient and delay in diagnosis, may contribute to the poor response to treatment. Finally the it is suggested that more sensitive molecular methods are required to find out this correlation, due to a network regulatory genes that contribute to antifungal resistance in *C.albicans*.

**Table 1.** *ALS1*, *ALS3* and *ACT1* specific primers for PCR amplification

| Primer name | Sequence (5'→3') | PCR Product size (bP) | Accession number |
|---|---|---|---|
| ALS1-R | ACCAGAAGAAACAGCAGGTG | 318 | L25902 |
| ALS1-F | GACTAGTGAACCAACAAATACCAG | | |
| ALS3-F | CCAAGTGTTCCAACAACTGAA | 184 | AY223552 |
| ALS3-R | GAACCGGTTGTTGCTATGGT | | |
| ACT1-F | CCAGCTTTCTACGTTTCC | 200 | HM997110 |
| ACT1-R | CTGTAACCACGTTCAGAC | | |

**Table 2.** Frequency of presences of *ALS1* and *AlS3* genes of *C.albicans* isolates.

| Profile | Resistance to Fluconazole | Presences of ALS1/ALS3 | Percent (%) |
|---|---|---|---|
| 1 | Resistant | -/- | (5)9.4**%** |
| 2 | Resistant | +/+ | (39)73.5 |
| 3 | Resistant | +/+ | (1)1.8% |
| 4 | Resistant | +/+ | (3)5.6% |
| 5 | Resistant | -/+ | (2)3.7% |
| 6 | Sensitive | -/+ | (2)3.7**%** |
| 7 | Sensitive | +/+ | (1)1.8**%** |

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