# Molecular Studies on Cytochrome P-450 Lanosterol 14 Alpha-Demethylase Gene of Fluconazole Resistant *Candida albicans* Clinical Isolates

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**Resistance to azole antifungals continues to be a significant problem in *Candida albicans*. One of the major mechanisms of azole resistance in *C. albicans* has been reported to be mutations in the *ERGII* gene encoding the azole target enzyme cytochrome P-450-dependent lanosterol 14 α-demethylase. This study was carried out to explore the presence of any point mutation in *ERGII* gene sequence of some *C.albicans* clinical isolates resistant to fluconazole. Three hundred C. *albicans* isolates, from various specimens were examined. These isolates were tested for antifungal susceptibility to fluconazole. The minimum inhibitory concentrations (MICs) of resistant *C. albicans* isolates were done by NCCLs macrobroth dilution method and E test. *C. albicans* ATCC 90028 was used as a control susceptible strain in all tests. The resistant *C. albicans* isolates were tested by PCR to amplify the entire *ERGII* gene. The isolates were further tested for point mutation concerning the *ERGII* gene by single-stranded conformation polymorphism (SSCP) analysis and DNA sequencing. Sixteen (5.4%) out of 300 of the isolates were resistant to fluconazole by disc diffusion and macrobroth dilution (MICs ranged from 64 → 128 μg/ml). E test showed 4 (25%) sensitive isolates (MIC 4-8 μg/ml) and 12 (75%) resistant (MIC 64 → 256 μg/ml). Point mutation (fragment variations) in *ERGII* gene was detected in 7(43.75%) isolates by SSCP technique. DNA sequencing of the PCR amplified *ERGII* fragments of four isolates that showed band mutation by SSCP revealed; 25 nucleotide changes, resulting in 4 amino acid substitutions in regions covered by fragments that did and did not show variation by SSCP. Accordingly, the sensitivity of the SSCP technique was (55.5%). Ten of these changes were also present in strain ATCC90028, other mutations exist in resistant isolates only and these mutations could play a role in drug resistance alone or in association with other mechanisms particularly when they resulted in an amino acid substitution. Although, PCR-SSCP analysis can give false positive results, since not all mutations detected can cause amino acid changes (silent mutations), our results concerning DNA sequencing were confirmative for strains showing positive PCR-SSCP results.**

## INTRODUCTION

The genus Candida is one of the most important genera of medically important yeasts. Although many Candida species can cause human disease,(1) *C. albicans* is clearly the major agent inciting human mycotic infections and is capable of causing diseases ranging from superficial disorders to invasive rapidly fatal infection in immunocompromised patients.(2) The introduction of orally active antifungal azole drugs since 1980, particularly fluconazole in 1990-1992, was a significant development allowing treatment of systemic fungal infection without the problem of nephrotoxicity associated with amphotericine B treatment.(3) The azoles prevent the synthesis of ergosterol, a major component of fungal plasma membrane, by inhibiting the cytochrome p-450 dependent enzyme lanosterol 14 α-demethylase (*Erg II p*) ,(4) the product of the *ERGII* gene (also called *CYP51* gene). Cytochrome p450 proteins are the products of a gene superfamily of heme binding monooxygenases found in so many diverse species from protists to mammals.(5) They are characterized spectrophotometrically by the presence of an absorbance peak at 450 nm when the reduced hemeprotein is gassed with carbon monoxide, hence the name p450 to designate a pigment absorbing light at 450 nm.(6)

Azole resistance appears to be emerging as the major problem in patients treated for yeast infections.(7) Many different types of mechanisms are known to contribute to a drug resistance phenotype in eukaryotic cells. The mechanisms could be; Alterations in drug import, alteration in intracellular drug processing (modification and degradation), alteration in the target enzyme (point mutation, overexpression of the Erg II gene), and alterations in other enzymes in the ergosterol biosynthetic pathway. Drug efflux from the cells is another component of resistance in *C.albicans*. Northern blot analysis have shown that genes from two families of efflux pumps

(the *MDR I* and *CDR* gene families) are overexpressed in azole-resistant isolates.[7,8,9]

Many techniques have been developed to analyze the presence of mutations in a DNA target. Screening for single base pair mutations of structural and regulatory genes by single-stranded conformation polymorphisms (SSCP) may be a useful tool in understanding mechanisms of antifungal drug resistance.[10,11] It is based on the fact that single-stranded DNA has a sequence specific secondary structure. Sequence differences as small as a single base change can affect this secondary structure and can be detected by electrophoresis in a non-denaturing polyacrylamide gel.[12] Of importance, combination of PCR that amplify the specific region of genes where most of drug resistant associated mutations are located and automated DNA sequencing have been applied to the genotypic detection of drug resistance. It is considered to be the gold standard method to which all the molecular techniques are compared.[13]

The purpose of this study was to explore the presence of any point mutation in *ERGII* gene sequence of *C. albicans* clinical isolates resistant to fluconazole, as a possible mechanism of resistance to this antifungal drug.

## MATERIALS & METHODS

Three hundred isolates of *Candida spp.* from different clinical specimens referred to the routine microbiology laboratory of Alexandria Main University Hospital were tested.

A control strain of *C. albicans* that is susceptible to fluconazole (ATCC 90028) was also included in this study.

I. **Identification of the isolates as Candida albicans:** Identification was carried out using the Germ tube test,[14] rice agar tween 80 test,[15] growth on CHROM agar *Candida*[16] and their assimilation pattern using the API 20 C kit.

II. **Antifungal susceptibility testing:**

(a) All *C. albicans* isolates were tested for antifungal susceptibility against fluconazole 25 µg using disc diffusion method.[17] *C. albicans* isolates, resistant to fluconazole were also tested for antifungal susceptibility against intraconazole 10 µg, ketoconazole 10 µg & amphotericin B 10 µg.

(b) Determination of minimum inhibitory concentrations(MICs) of Fluconazole: The MICs of resistant *C. albicans* isolates were performed by NCCLs macrobroth dilution method[18] and E test.[19] Candida albicans ATCC 90028 was used as a control susceptible strain in all tests.

- Macrodilution antifungal susceptibility test: Briefly, candida isolates at a final concentration of 0.5-2.5 X $10^3$ org./ml were incubated in air at 35°C for 48 hours with serial double fold dilutions of fluconazole from 0.125 to 128 µg/ml. The MIC endpoints where read visually following 24 and 48 hours of incubation and were defined as the lowest concentration that produced a prominent reduction in growth ( 50-80%) compared with that of the drug free growth control. Interpretations of MICs were assigned according to the NCCLs criteria. Trailing growth was defined as a susceptible MIC after 24 hour incubation and a resistant MIC after 48 hour incubation.[20]

- E-test: E-test strips was available commercially from AB Biodisk, Solna, Sweden. It is based on a combination of the concepts of both dilution and diffusion tests, the strip was placed on the surface of inoculated RPMI agar and then incubated for 24 hours. As the strip contains a predefined continous gradient of fluconazole from 256 to 0.016 µg/ml, it is possible to obtain a reproducible, quantitative MIC reading.[19,21]

**III- DNA extraction from resistant isolates:**

DNA extraction was carried out as reported earlier.[22] The azole resistant strains were grown in LB medium (Bacto-tryptone 10g, Bacto-yeast extract 5g, NaCl 10g, Distilled water 1 liter) overnight in shake culture, at 37°C. Cells were collected by centrifugation, then washed and resuspended in 500 ml SET buffer. β-mercaptoethanol (1µl) and zymolyase enzyme, 100 units (10 units/µl, Arthrobacter luteus, Sigma, USA) were added for spheroblast formation. The mixture was incubated for 60 minutes at 30°C. Spheroblasts were collected by centrifugation, then suspended in 1ml Tris-EDTA (TE) buffer and lysed with the addition of 0.1ml 10% sodium dodecyl sulphate (SDS) (sigma, USA), then incubated

at 56°C for 30 min. Equal volume of phenol: chloroform (1:1) was added to remove proteins. Equal volume of isopropanol (Placebo, France) was added to the DNA solution mixed well and stored at -20°C for 15-20 minutes to precipitate DNA. The DNA pellet was collected by centrifugation, resuspended in 50 µl sterile, (DNase-free water) and stored at -20°C until use.

**IV-Detection of *ERGII* gene by polymerase chain reaction (PCR):**

Amplification of the *ERGII* gene from the genome of resistant isolates of *C. albicans*, as well as the (ATCC 90028) strain was carried out by a PCR procedure using primers designed to amplify the full length (1400 bp) of the *ERGII* gene.[21,23] The forward primer (FWD1) sequence was:
5`-ACG CGT CGA CAA TAT GGC TAT TGT TGA AAC TGTC-3` and the reverse primer (REV5) sequence was:
5` GCG GAT CCT TAA AAC ATA CAA GTT TCT CTT TT-3`
The PCR reaction was performed in a 50 µl reaction mixture, which consisted of 25µl PCR master mix (Qiagen), 30 pmole of each primer (10 pmole/ml), 3µl of template DNA containing up to 100 ng of target DNA. Amplification was carried out using a thermal cycler (Progene, techne Ltd., Cambridge, UK). Thermocycling consisted of an initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 50°C for 1 minute and extension at 72°C for 1.5 minutes, followed by 1 cycle of another extension at 72°C for 5 minutes. PCR products were detected on 1% agarose gel electrophoresis run at 80 volts for 1 hour. The gel previously stained with ethidium bromide was visualized using UV transilluminator for bands of 1.4 kbp as determined by DNA base bair marker (Lambda DNA Hind III 250bp, Promega, USA) run at the same time.

**V-PCR-SSCP analysis: [23]**

Five PCR reactions were carried out for *ERGII* gene of each resistant isolate, using 5 pairs of primers, each pair of primers was designed to amplify approximately 300bp of the *ERGII* gene, according to the *C. albicans* genomic DNA sequence. Primers included in every reaction were as follows. (Figure 1)

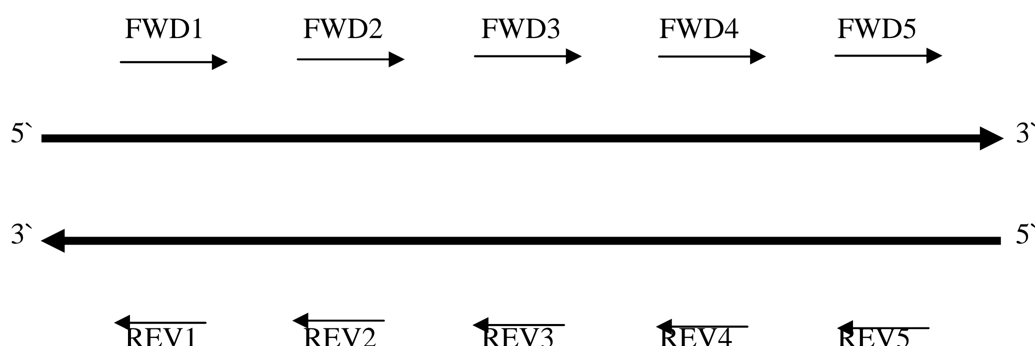


**Figure (1):** Schematic presentation of the primers used for PCR-SSCP

**Reaction 1: FWD1 sequence**
(5`-ACG CGT CGA CAA TAT GGC TAT TGT TGA AAC TGT C-3`) and REV1 sequence.
(5`-CCT TTT GGA CCT AAA TAA ACC GTC-3`).

**Reaction 2: FWD2 sequence**
(5`-CGG TTT ATT TAG GTC CAA AAG G-3`).

**Reaction 3: FWD3 sequence**
(5`-GCC AAT GTT ATG AAA ACT CAA CCA G-3`). and REV3 sequence
(5`-GAT CAG TCA TTT TCA CAC CA-3`)

**Reaction 4: FWD4 sequence**
(5`-TGG TGT GAA AAT GAC TGA TC 3`). and REV4 sequence
(5`-CTA GTA TGA GCA TAA CCT GGA GAA AC-3`)

**Reaction 5: FWD5 sequence**
(5`-CTC CAG GTT ATG CTC ATA CTA GTG-3`) and REV5 sequence.
(5`-GCG GAT CCT TAA AAC ATA CAA GTT TCT CTT TT-3`).

A 25µl PCR mixture was prepared as follows; 100 ng of genomic DNA (4µl), 30 pmole of each primer (10 pmole/µl), and12.5µl of PCR master mix (2x). Amplification was carried out using a thermal cycler. Thermocycling consisted of an initial denaturation at 95°C for 2.5 minutes, followed by 30 cycles of: denaturation at 94°C for 45 seconds, annealing at 50°C for 45 seconds, extension at 72°C for 30 seconds, followed by 1 cycle of extension at 72°C for 5 minutes. PCR products were analyzed through 6% polyacrylamide native gels (30% acrylamide stock solution, 5XTBE, 10% ammonium persulphate, sigma USA, and TEMED (N,N,N`,N` tetramythelene diamine). The gel assembly was immersed in 1% TBE buffer in a Biorad tank (Biorad Mini-Protean II$^{TM}$). Ten µl of the 300bp PCR products were mixed with 10µl sample buffer (95% formamide, 20 mM EDTA, pH 8.0, 0.05% xylene cyanol and 0.05% bromophenol blue). The mixtures were heated at 95°C for 5 minutes, quickly cooled on ice, then loaded onto the gel (20x20 cm) and electrophoresis was applied at room temperature using constant voltage of 250 volts for 4 hours. DNA bands were visualized through silver staining.

**VI-DNA sequence analysis**

The obtained PCR products of selected isolates were purified with a Wizard PCR Preps DNA purification system (Promega, USA)[24], separated by agarose gel electrophoresis and visualized on the UV transilluminator. The purified PCR products were sequenced by the dideoxynucleotide chain termination method according to Sanger *et al.*[25], using *ERGII* specific primers (FWD1 and REV5) and the previously described PCR cycling conditions. DNA sequence was performed using an ABI PRISM sequencer (model 310, version 3.4) and ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit with ampli-Taq DNA polymerase at both the University of Düsseldorff and the Biological Products and Vaccine Holding Company (VACSERA, Cairo, Egypt).

The obtained nucleotide sequences were then analyzed using nucleotide BLAST (Basic Local Alignment Search Tool) search engine database. The nucleotide sequences were translated as well to amino acid open reading frame sequences using the Clone Manager computer software program. Moreover, the nucleotide sequences and amino acid open reading frame sequences of the sequenced isolates were aligned with each other along with all published sequences from the database as well as with the sequence of the control susceptible strain (ATCC 90028), using Multalin search program, in order to detect any mutation in the sequenced isolates as described earlier.[26]

## RESULTS

Out of 300 isolates of *C. albicans* tested for susceptibility to fluconazole, 16 isolates (5.4%) were resistant. These isolates were isolated from patients with hematological malignancies and had a history of antifungal drug intake (fluconazole and amphotericin B). The susceptibility pattern of the 16 flucorazole resistant isolates to other antifungal drugs is shown in table (I); 75% were resistant to itraconazole, but, all the 16 isolates were sensitive to ketoconazole, and amphotericin B.

**Table (I):** Susceptibility pattern of the 16 *C.albicans* resistant isolates.

| Antifungal Drugs | Isolates | | | |
|---|---|---|---|---|
| | Resistant | | Sensitive | |
| | No. | % | No. | % |
| -Fluconazole | 16 | 100 | ND | 0 |
| -Ketoconazole | ND | 0 | 16 | 100 |
| -Itraconazole | 12 | 75 | 4 | 25 |
| -Amphotericin B | ND | ND | 16 | 100 |

*ND: not detected

The MICs of fluconazole of the 16 resistant isolates are shown in table (II). The break points have been defined as follows: susceptible, MIC 8 μg/ml, resistant, MIC ≥ 64 μg/ml, trailer; susceptible after 24h incubation (MIC ≤ 8μg/ml) and resistant after 48h incubation (MIC ≥ 64 μg/ml). It was found that 12 isolates showed resistance to fluconazole by macroboth dilution (MIC 64->128 μg/ml) and E test (MIC 64-> 256 μg/ml). The MIC of the other four *C.albicans* isolates showed discrepancies between their 24 and 48 hours values (24hrs, 0.5-μg/ml, 48hrs, 32-128 μg/ml) by macrobroth dilution method, while E test showed MIC values (4-8μg/ml) of these isolates.

**Table (II):** Comparison between MICs of resistant isolates by macrobroth dilution and E test.

| **Isolate No.** | **Macrobroth dilution MIC μg/ml** | | **E test (μg/ml)** | **Interpretation** |
|---|---|---|---|---|
| | **24h** | **48h** | | |
| 1 | 128 | 128 | >256 | R |
| 2 | 64 | 64 | 128 | R |
| 3 | 64 | >128 | >256 | R |
| 4 | 1 | 128 | 8 | Trailer |
| 5 | 1 | 64 | 4 | Trailer |
| 6 | 64 | 64 | 128 | R |
| 7 | 64 | 64 | 128 | R |
| 8 | 2 | 64 | 8 | Trailer |
| 9 | 128 | >128 | >256 | R |
| 10 | 128 | >128 | >256 | R |
| 11 | 64 | 64 | 128 | R |
| 12 | 64 | 64 | 128 | R |
| 13 | 64 | 64 | 64 | R |
| 14 | 0.5 | 64 | 8 | Trailer |
| 15 | 128 | 128 | >256 | R |
| 16 | 128 | 128 | >256 | R |

*R: resistant (MIC ≥ 64 μg/ml).
Trailer: susceptible after 24h incubation (MIC ≤ 8 μg/ml) and resistant after 48h incubation (MIC ≥ 64 μg/ml).

Fluconazole-resistant *C. albicans* isolates were then subjected to polymerase chain reaction (PCR) to amplify the *ERGII* gene. Bands of expected size (1400bp) were produced from all tested resistant isolates as well as the control susceptible strain (ATCC 90028) (figure II). The PCR products detected after amplification of 5 different fragments belonging to the *ERGII* gene are shown in figure III.

DNA fragment variations detected by PCR-SSCP among these isolates are summarized in table (III). These band variations were compared to the control susceptible strain (ATCC 90028) (Table III and figure IV). Differences in the PCR-SSCP analysis were observed in 7 isolates (43.75%): Two in isolates 1,11,15 and 16, three in isolates 9 and 16, four in isolates 3,9 and 10. No band variation was detected in 9 isolates (56.25%).

Correlation between the presence of DNA band variations and MIC results among the 16 resistant isolates is shown in table IV. PCR-SSCP detected fragment variations in 7 isolates (1,3,9,10,11,15, and 16) that were also resistant to fluconazole by both macrobroth dilution and E test, while no fragment variations were detected by PCR-SSCP in 9 isolates as follows: four isolates (4,5,8 and 14) were sensitive by E test, but resistant by macrobroth dilution method, while five isolates (2,6,7,12, and 13) were resistant by both E test and macrobroth dilution method.

Table (III): Fragment variations detected using PCR-SSCP* among *C.albicans* fluconazole resistant isolates

| Isolate No. | Fragment No. showing variation | Primers used | Nucleotide position (from-to)(bp) | Number | % |
|---|---|---|---|---|---|
| 1 | 2 | FWD2-REV2 | 278-280 | | |
| 3 | 4 | FWD4-REV4 | 863-1236 | | |
| 9 | 3 and 4 | FWD3-REV4 | 556-1236 | | |
| 10 | 4 | FWD4-REV4 | 863-1236 | 7 | 43.75 |
| 11 | 2 | FWD2-REV2 | 278-580 | | |
| 15 | 2 | FWD2-REV2 | 278-580 | | |
| 16 | 2 and 3 | FWD2-REV3 | 278-883 | | |
| 2 | No change | | | | |
| 4 | No change | | | | |
| 5 | No change | | | | |
| 6 | No change | | | | |
| 7 | No change | ------------- | -------------- | 9 | 56.25 |
| 8 | No change | | | | |
| 12 | No change | | | | |
| 13 | No change | | | | |
| 14 | No change | | | | |
| Total | | | | 16 | 100 |

Table (IV): Correlation between DNA fragment variation using PCR-SSCP technique and MICs by both macrobroth dilution and E test among *C. albicans* fluconazole resistant isolates.

| Isolate No. | Fragment No. showing variation | MIC (μg/ml) | | |
|---|---|---|---|---|
| | | Macrobroth dilution | | E test |
| | | 24hrs | 48hrs | |
| 1 | 2 | 128 | 128 | >256 |
| 3 | 4 | 64 | >128 | >256 |
| 9 | 3 and 4 | 128 | >128 | >256 |
| 10 | 4 | 128 | >128 | >256 |
| 11 | 2 | 64 | 64 | 128 |
| 15 | 2 | 128 | 128 | >256 |
| 16 | 2 and 3 | 128 | 128 | >256 |
| 2 | No variation | 64 | 64 | 128 |
| 4 | No variation | 1 | 128 | 8 |
| 5 | No variation | 1 | 64 | 4 |
| 6 | No variation | 64 | 64 | 128 |
| 7 | No variation | 64 | 64 | 128 |
| 8 | No variation | 2 | 64 | 8 |
| 12 | No variation | 64 | 64 | 128 |
| 13 | No variation | 64 | 64 | 64 |
| 14 | No variation | 0.5 | 64 | 8 |

Direct sequencing of the entire *ERG11* genes were determined from isolates 9, 10, 15 and 16 by using the PCR fragments. These nucleotides sequences were translated to amino acid open reading frame using a program described in materials and methods. Both nucleotide and amino acid sequence data were compared to a published *ERG11* gene sequence as well as the control susceptible strain (ATCC 90028) gene sequence. Twenty five nucleotide substitutions, resulting in 4 amino acid changes were identified. These are: T315C, T348A **(D116E)**, A357G, A383C**(K128T)**, C411T, A589G, A595T, T609G, T618G, G622A, G629T, A634T, C643A, C658T, C996T, A1020G, C1110T,

T1203C, T1205C(**V402A**),-1214T(**S405F**), T1296C, T1302C, A1440G, T1470G. Ten of these changes were also present in strain ATCC 90028, when compared to a published ERG11 gene sequence. These are: T315C, T348 **(D116E)**, A383C **(K128T)**, C658T, C996T, A1020G, C1110T, T1296C, A1440G, T1470G, (table V).

The correlation between SSCP technique and DNA sequence analysis revealed that, SSCP detected 20 out of 25 mutations derived from 6 PCR fragments. The sensitivity of the test was (55.5%) and the specificity was (44.5%), table (VI).

Table (V): Nucleotide and amino acid substitutions in *ERGII* genes derived from isolates 9,10,15 and 16 in comparison to the control sensitive strain (ATCC 90028).

| **Isolate No.** / **Base change** | **ATCC 90028** | **9** | **10** | **15** | **16** |
|---|---|---|---|---|---|
| T315C | + | - | + | - | - |
| T348A | - | - | + | + | + |
| **(D116E)** | | | | | |
| A357G | - | - | + | + | - |
| A383C | - | - | - | + | + |
| **(K128T)** | | - | + | + | + |
| C411T | - | | | | |
| C658T | + | + | + | + | + |
| A1020G | + | + | + | - | - |
| C1110T | + | + | + | - | - |
| A1440G | + | + | - | - | - |
| T1470C | + | + | - | - | - |
| C996T | + | + | + | - | - |
| T1296C | + | + | + | - | - |
| A583G | - | - | - | - | + |
| A589G | - | - | - | - | + |
| A595T | - | - | - | - | + |
| T609G | - | - | - | - | + |
| T618G | + | - | - | - | + |
| G622A | - | - | - | - | + |
| G629T | - | - | - | - | + |
| A634T | - | - | - | - | + |
| C643A | - | - | - | - | + |
| T1203C | - | - | + | - | - |
| T1205C | - | - | + | - | - |
| **(V402A)** | | | | | |
| X1214T | - | - | + | - | - |
| **(S405F)** | | | | | |
| T1302C | + | - | + | - | - |

C = Cytosine T= Thymine A= Adenine
G= Guanine *T315 C means: T has changed to C
D= Aspartic acid E= Glutamic acid K= Lysine
T= Threonine V= Valine A= Alanine
S= Serine F= Phenyl alanine
*Bold type indicates that the mutation at the corresponding site created an amino acid substitution.

Table (VI): Detection of mutation by SSCP technique compared to DNA sequence analysis among 20 PCR fragments of *C.albicans* isolates (9,10,15 and 16).

| **DNA Sequence** / **SSCP** | **Positive** | **Negative** |
|---|---|---|
| Positive | 6(55.5%) | 0(0.0%) |
| Negative | 5(45.5%) | 9(100.0%) |
| Total | 11 | 9 |

Sensitivity: 55.5%, Specificity: 100.0%, Positive predictive value: 100.0%, Negative predictive value: 64.3%, Accuracy: 75.0%

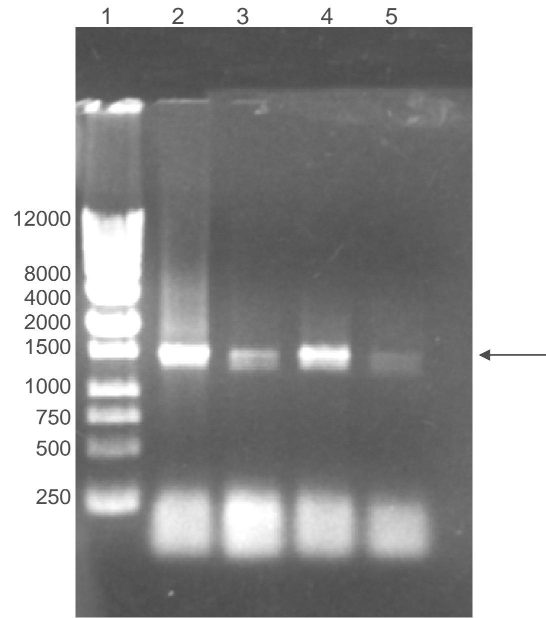


**Figure (II):** Agarose gel electrophoresis (1%) to analyze PCR products of *C.albicans ERG11* gene. Lanes 2, 3, 4 and 5 represent PCR products (~1.4 kbp) developed when using genomic DNA of *C.albicans* of four different isolates (9, 10, 15, and 16) respectively. Lane 1 represents a DNA base pair marker (Hind III 250 bp ladder). The arrow points at PCR products.

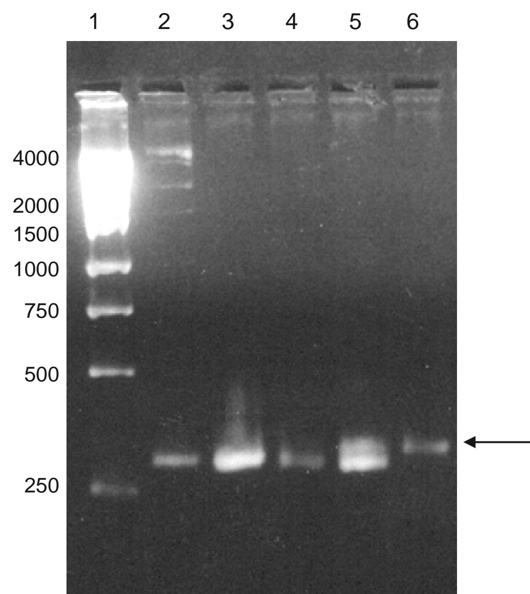


**Figure (III):** Agarose gel electrophoresis (3%) to analyze PCR products that were developed using the *ERG11* internal primers illustrated in figure I. Lanes 2-6 represent PCR products (~300 bp). Lane 1 represents a DNA base pair marker (Hind III 250 bp ladder). The arrow points at PCR products

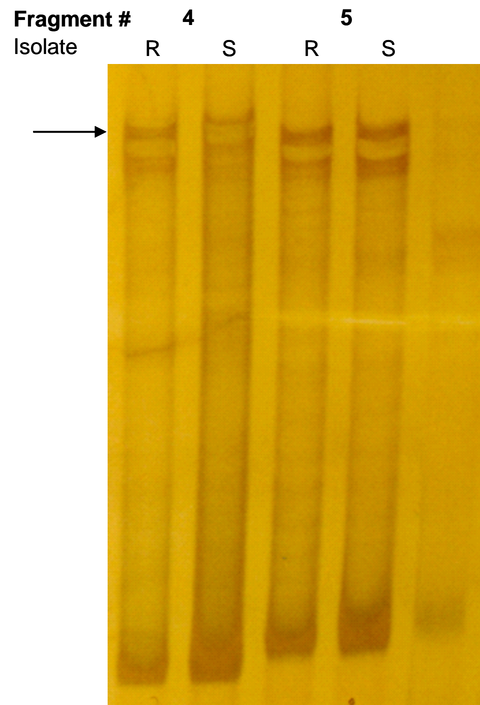


**Figure (IV):** SSCP analysis of DNA fragments 4 and 5 from the control susceptible strain (ATCC 90028) [S] and from isolate (3) (resistant [R]). The arrow points at fragment 4 that shows band variation. Upper bands represent the single stranded DNA, the lower bands represent the reannealed double stranded DNA.

## DISCUSSION

*C. albicans* is both a ubiquitous commensal and an important opportunistic human pathogen causing common ailments such as thrush and vaginitis, as well as chronic conditions in immunocompromised patients.[27] These infections are treated with antifungal agents, particularly the triazole derivative fluconazole. However, due to the repeated use of this agent, the number of cases of in vitro resistance correlating with clinical failure has risen significantly.[28,29,30] The multiplicity of mechanisms of resistance to azole antifungal agents represents a set of biological tools that enables yeast cells to develop resistance by using different combination of mechanisms.[31,32,33]

Our study revealed that fluconazole resistance was present in 16 (5.4%).When compared to a study conducted by law D *et al*, who estimated that >33% of patients with AIDs had an oral commensal strain of candida, that is azole resistant.[34] Also, the prevalence of azole resistance has been estimated to be 21 to 32% in symptomatic patients and up to 14% in asymptomatic patients in studies conducted by Michaelis S and Berkower C, Maenza JR *et al* and Revankar SG *et al*.[35,36,37] Even though C. albicans is still the most common pathogen among candida species, the spectrum of candida infections has been increased to include more of other species. The emergence of non- albicans candida species infection may be due to an increased population of immunosuppressed patients, an increased usage of antifungal agents and an improved diagnosis of candida species.[38] In a study conducted by Nguyen *et al*, an increased incidence of non-albicans species with increased azole MICs arose over 3.5 years study.[39]

Primary fluconazole resistance in cancer patients has been well documented. It was observed by Franz R *et al*. that, mutations leading to drug resistance are random and it is therefore not surprising that fluconazole resistant *C.albicans* strains have also been isolated from patients who were not treated with fluconazole.[40] The development of secondary resistance has been confirmed with reports of disseminated disease due to resistant *C. albicans* developing in patients with leukemia and bone marrow transplant.[37]

In our study, the 16 fluconazole resistant isolates were isolated from patients suffering from hematological malignancies. They received fluconazole and amphotericin B during their chemotherapy treatment. This was in agreement with three studies conducted by Marr KA *et al*,[41] Mori T *et al*,[42] and Notle FS *et al*,[43] who reported that 4 patients with hematological disorders were known to have developed fungemia with azole resistant C. albicans.

In the present study, the MICs of 12 resistant isolates by NCCLs macrobroth dilution method were matching with the MICs obtained by E test, while 4 isolates were resistant by NCCLs method (MIC at 48 hrs was 64-128 μg/ml) and sensitive by E test (MIC was 4-8 μg/ml), resulting in an agreement of 75% between both methods. In general, our data confirm the previous conclusions of Bolmstrom that there was a good correlation between MIC obtained by E test and broth dilution methods. The determination of the end points for the azoles is a significant factor in the validity of MIC results for these drugs. Occasionaly, NCCLs testing of yeasts with fluconazole produces trailing end points; partial growth inhibition, usually at 48 hours. This may be due to the fungistatic activity of the azoles, which allows several generations of growth before significant inhibition occurs.[44] This would explain, in the present study, the discrepancies between MICs at 24 and 48 hours observed in 4 isolates where 24 hrs MICs were (0.5-2μg/ml) while 48 hrs MIC, were (64-128 μg/ml). Therefore, the NCCLs method results obtained at 24 hrs would be more appropriate for these isolates,[44] which will be consistent with their E test MIC values (4-8 μg/ml).

The purpose of the present study was to explore the presence of any point mutation, as a possible mechanism of fluconazole resistance, in the 16 C. albicans fluconazole resistant isolates.

PCR-SSCP analysis and DNA sequencing were used to recognize DNA sequences that have been mutated in the series, in comparison to a control susceptible strain (ATCC 90028). Differences in the PCR-SSCP analysis between the resistant isolates and the control susceptible strain revealed fragment changes in 7(43.75%) isolates, while no changes could be detected in the remaining 9(56.25%) isolates. On the contrary, in the study conducted by Wenli W *et al*, who studied 32 fluconazole resistant *C. albicans* strains for resistance mechanisms, all the 32 tested strains showed positive results by PCR-SSCP.[45] It is likely that other factors contributing to azole resistance must be operating in these 9 isolates, such as overexpression of ERG11 gene, up-regulation of efflux pumps (CDR and MDR1) genes, especially that 12 isolates (1,2,3,6,7,9,10,11,12,13,15 and 16) showed cross-resistance to both fluconazole and itraconazole. This may suggest overexpression of CDR genes, as the efflux pumps encoded by these genes appear to confer resistance to multiple azoles. Among these 9 isolates, 4 (4,5,8 and 14) were susceptible to fluconazole by E test (MIC 4-8 μg/ml), and showed trailing end point by NCCLS method, which was consistent with the absence of any band variation by SSCP analysis. All these observations may indicate absence of resistance to fluconazole.

In the present study, ERG11 genes from both susceptible control strain (ATCC 90028) and fluconazole-resistant isolates (9,10,15 and 16) were sequenced. Comparison with the published sequences for ERG11 revealed that the isolates contained several silent nucleotide changes, as expected for different strains of Candida in regions covered by fragments that did and did not show variation by SSCP. Our results showed that SSCP detected 20 out of 25 mutations from 6 out of 11 PCR fragments, which consisted of several single nucleotide substitutions and one insertion. This was in agreement with a study conducted by Grazer Y *et al*, where most of the mutations (twelve single base mutations and insertions were detected from six out of eight PCR fragments) in 52 natural *C. albicans* isolates from Duke University medical centre, were detected by SSCP analysis.[46]

Globally, eleven amino acid substitutions were found to be associated with a resistant phenotype: D116E, G450E, G307S, Y132H, D446N, G464S, F126I, K143R, S405F, F449S and T229A.[31] In the present study, four amino acid changes were identified; D116E, K128T, V402A and S405F. However, D116E and K128T were present in both susceptible control strain and resistant isolates (10,15 and 16), thus, do not

account for resistance in this series. This is consistent with studies performed by Asai K *et al*,[47] Marr KA *et al*,[48] and Sanglard D *et al*.[49] The fact that many of the mutations described here were also found independently by others in the ERG11 genes from other isolates obtained in different geographic locations, illustrates that there may be preferential amino acid positions able to confer a phenotype of resistance to fluconazole and other azole derivatives. These mutations repeatedly identified by different groups may represent "hot spots" for the development of azole resistance.[31,49] Mutations described here affect ERG11 and produce what might be alterations in its affinity for azoles through two possibilities: (i) the amino acid affected by the mutation is in direct contact with some part of the azole molecule, thus the substitution results in a less efficient binding of the azole to the mutated protein. Or (ii) the amino acid substitution displaces the 3-dimensional arrangement of structures important for the optimal binding in affinity of the mutated protein for azoles, hereby,[48] azoles can not interact with the haem iron or this interaction is interfered.[44] A novel amino acid substitution (V402A), that has not yet been reported, was identified in isolate 10. This mutation could influence fluconazole resistance in this isolate, when compared to a previously unknown a.a. change (V404L) observed in TIMM3165 strain studied by Maebashi *et al*.[50] They suggested that V404L could influence fluconazole resistance in strain TIMM3165, and this is supported by the fact that the S405F substitution, which is positioned just after V404L, is close to the substrate and azole-binding pocket in Erg 11p. This prediction is consistent with Wenli W *et al*,[45] who proposed that, since the mutations found in their study were located near the target position of the azoles, one or more mutational alterations (alone or in combination) might lead to expression of an enzyme highly resistant to the inhibitory action of fluconazole which in turn is responsible for the fluconazole resistant trait in their strains.

Since the present study was limited to the study of point mutations in ERG11 gene implicated in the development of fluconazole resistance, and since it is unclear whether these substitutions alone can confer fluconazole resistance in the studied isolates, it should be noted that other mechanisms (changes in the ergosterol biosynthetic pathway, overexpression of multidrug efflux pump genes) and other yet uncharacterized resistance mechanisms, may be operational in these series of isolates, which may contribute to the overall decrease in susceptibility.[51] In conclusion, PCR-SSCP analysis can give false positive results, because not all mutations detected can cause amino acid changes (silent mutations). DNA sequencing is thus recommended for strains showing positive PCR-SSCP results. The SSCP technique may be a rapid and relatively inexpensive screening method to be used in conjunction with conventional drug susceptibility testing for larger collections of isolates when DNA sequencing instruments are not available.

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# دراسات جزيئية على جين سيتوكروم ب – ٤٥٠ لانوستيرول ١٤ ألفا ديميثايليز لعزلات فطر الكانديدا ألبيكانز المقاومة لعقار الفلوكونازول

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