



Evaluation natural cloning of azole- resistant genes CDR1,CDR2, MDR and ERG11 between clinical and soil isolates of *Candida albicans* based on gene expression

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Abstract : Candidiasis treatment failures in patients receiving prolonged azoles therapy, and these treatment failures have been demonstrated to be due to the emergence of azole-resistant *C. albicans* strains. Spread azole-resistant among *C. albicans* strains required to pursue the transition of azole-resistant genes between clinical isolates and soil of *C. albicans* from different sources. 88 clinical isolates of *C. albicans* were collected from patients hospitalized in Margan hospital, and 60 isolates were collected from garden soils of hospital. The aim of this study detected of azole-resistant genes via reverse transcription mRNA of 20 isolates of *C. albicans*, cDNA was amplified to determine the expression of CDR1, CDR2, MDR1, ERG11 and normalized with housekeeping gene ACT1 expression, and performed antifungal sensitivity test for Fluconazole, Miconazole, Caspofungin and evaluated the MIC via E-test of Fluconazole and Caspofungin. The result showed that most isolates of *C. albicans* from both sources are susceptible for Fluconazole, Miconazole, Caspofungin and the MIC of Fluconazole and Caspofungin was <0.02, 2 respectively. The results of this study emphasize of present four azole-resistant genes CDR1, CDR2, MDR1, ERG11 and ACT1 in most clinical and soil isolates showed PCR products: 286, 364, 201, 204 and 209bp respectively, most isolates susceptible to Caspofungin, Miconazole and fluconazole respectively. Our conclusion indicated of natural cloning possible the resistance genes among *C. albicans* population.

Key words: Natural cloning, azole-resistant, *Candida albicans*, CDR1, CDR2, MDR, ERG11, ACT1, cDNA, gene expression.

Introduction

Candida albicans are important opportunistic yeasts¹. Extensive researches were focused on antifungal resistance by clinical isolates of *C. albicans*, however, there have been numerous reports of treatment failures in patients receiving prolonged azole therapy, and these treatment failures have been demonstrated to be due to the emergence of azole-resistant *C. albicans*². Recently many studies referred to an increasing failure in *Candida* infections treatment because of the emergence of resistance to antifungal drugs were demonstrated the genes resistance via gene expression³.

The drug characteristic and altered sensitivity of the fungus to the drugs are among the causes why antifungal therapy does not work properly. Long-term or prophylactic treatment has given rise to *C. albicans* resistant isolates^{4,5}.

Several molecular mechanisms by which *C. albicans* develops resistance to antifungal drugs have been elucidated⁶. Azoles are widely used in treatment of candidiasis due to their bioavailability and safety. They

target the biosynthesis of ergosterol, the major sterol of the fungal membranes, ergosterol is necessary to provide suitable integrity and functionality of the fungal membrane⁵.

The azole antifungals target the products of ERG11, CDR1, CDR2, and MDR1. ERG11 gene produce lanosterol 14 α -demethylase is one of the enzymes in the bio synthesis of ergosterol, the major sterol of fungal membranes⁷. In the last decade, the widespread use of azole drugs has led to the rapid development of azole drug resistance in patients with recurring oral candidiasis. The development of resistance depends on a number of factors such as drug-drug interaction, dosages and scheduling, host factors, and factors intrinsic to *Candida*⁴. Many investigators have reported the increased overexpression of CDR gene⁷ and MDR1 gene³ in resistant clinical isolates of *C. albicans*. However, quite no research articles refer to evaluate or determined the transition azole resistance genes in between *C. albicans* isolates. The aim of this study antifungals sensitivity test for Fluconazole, Miconazole, Caspofungin and evaluated the MIC via E-test of Fluconazole and Caspofungin and detection of natural cloning of genes expression between clinical and soil sources via reverse transcription mRNA of 20 isolates of *C. albicans* collected from clinical and soils, cDNA was amplified to determine the expression of CDR1, CDR2, MDR1, ERG11 and normalized with housekeeping gene ACT1 expression.

Materials and methods

A total of 148 samples were collected :88 clinical sample(61 buckle swabs,27 vaginal swabs) and60 soil sample from hospital gardens soils). 150-200 g each for samples were collected from 5-15cm depth, in polyethylene bags⁸. The period of study from November 2014-January 2015.

Buckle and vaginal swab samples were streaked on the surface of Sabouraud dextrose agar (SDA) plate while soil sample cultured by dilution plate methods on and incubated for 48h at 37°C . based on single colony isolation method ,the isolates were initially identified as *C. albicans* based on CHROMagar⁹, and determined the confused between *C. albicans* and it *Ssp. dubliniensis*¹⁰ and molecular tools .The stoke cultures of isolates were maintained in slant tubes and subcultured monthly on SDA and incubate under 4°C.

Antifungal Susceptibility Tests

The MICs of Fluconazole ,Miconazole and Caspofungin were determined by the described by CLSI guidelines NCCLS (11), via Disk diffusion (DD) assay and were confirmed with E-test strips (AB Biodisk North America Inc.,) following the manufacturer's instructions. Agar plates of SDA were inoculated with *C. albicans* cells suspension(1.5×10^6) previously suspended in a saline solution (0.85%). The plates were leaved half an hour for liquid absorption^{12, 13}, and the incubated at 30°C for 24 h. After 24 h cultivation, the inhibitory diameter zone (dz) was measured based on Barry et al.¹⁴. E-test gradient strips of Fluconazole and Caspofungin with the concentration range from 0.02 to 3.2 lg/ml for Caspofungin and 0.016–256 lg/ml for fluconazole . The strips were stored frozen on -20°C until they were used in this study .We used the SDA to perform the test. The interpretative MIC breakpoints were followed^{15, 16}. The plates were incubated at 35°C, and the MICs were determined following incubation after 24 h and results were confirmed by second reading after 48 h.

Two sets of *C. albicans* (20 isolates for each) were grew in presence and absent of Fluconazole: 10ml of stock solutions of fluconazole (3.33 mg/ml in sterile distilled water)was prepared¹⁷. First set treated with work solution : composed of 0.5 ml of stock solution of fluconazole were inoculated with 0.5ml yeast cells suspension(1.5×10^6) previously suspended in a saline solution (0.85%) from a single colony in sterile tube, 0.5ml of work solution for each isolates were poured and striking by swab on SDA plate for each of 20 *C. albicans* isolates (15 clinical isolates and 5 isolates from soil source), second set growth in absent of fluconazole ,both plate sets were incubated at 30°C for 24h. and the extracted total RNA from isolates growth in presence of fluconazole and from isolates growth in second set as alternative to these not growth in first set¹⁸.

Extraction of DNA & RNA

Total nucleic acids from twenty isolates of *C. albicans* (15 clinical and 5 soil isolates) were manually extracted synchronous from isolates in the mid-logarithmic phase of growth based on Fredricksetal.¹⁸ and Imran and Al Asadi¹⁹. For cDNA assay, 10 μ L of total nucleic acid transferred to a new RNase-free micro centrifuge tube ,added 6 μ L of 1U/ μ L DNase1, 8 μ L of mM nucleas free MgCl₂ and 40 μ L nuclease-free water

.incubate the tubes at 37°C for 30 min. the tubes were heated at 75 °C for 10 min and then cooled on ice immediately, added 2 µL of transcriptase to each tube and incubate at 42 °C for one hour. Heated the tubes at 70 °C for 15 min for transcriptase denaturant and cooled the tubes on ice, 2 µL of RNase was added and incubate tubes at 37 °C for 10 min to remove residual of RNAs. And preserved the cDNA at -20 °C until use. Oligonucleotides were prepared to be complementary to the mRNAs for ACT1,ERG11,MDR, CDR1, and CDR2.All reagents were purchased from Promega Scientific Co. Sigma Chemical Co. , unless otherwise specified.

Confirmation of isolate species.

Most of the clinical isolates grew as green colonies on CHROMagar plates, suggesting that they are *C. albicans* were confirmed by simple PCR by two specific primer pair for *C.albicans* primer pairs INTI and CDBF28-CADBR125²⁰, and the PCR products were electrophoreses through an agarose gel. The electrophoresis pattern distinguishes one group of *C. albicans*. PCR reaction was performed based on INTI-F and INTI-R primer pair to amplified the target gene 310bp (table 1) .the PCR products were run through 1% agrose gel to differentiated the isolates of *C.albicans* and its *Ssp.dublinsiensis*¹⁰.

Table 1: list of oligonucleotieds of primers pairs included specific primers for *C.albicans* identification and antifungals resistance genes.

Primer	Sequence	Amplicons size(bp)
<i>INTI-F</i>	5'-A AGTATTTGGGAGAAGGGAAAGG G -3'	310
<i>INTI-R</i>	5'-AAAATGGGCATTAAGGAAAAGAGC -3'	
<i>CDBF28 -F</i>	5'-AAATGGGTTTGGTGCCAAATTA-3'	966
<i>CDBF125-R</i>	5'-AGCTAAATTCATAGCAGAAAGC-3'	
<i>ERG11 - F</i>	5TGG AGA CGT GAT GCT G -3'	204
<i>ERG11 - R</i>	5'-AGT ATG TTG ACC ACC CAT AA -3'	
<i>CDR1 - F</i>	5'- AAG AGA ACC ATT ACC AGG -3'	286
<i>CDR1 - R</i>	5'- AGG AAT CGA CGG ATC AC -3'	
<i>CDR2 - F</i>	5'- ATG CTG ATG CCC TAG T -3	364
<i>CDR2 - R</i>	5'-GCT TCC TTA GGA CAT GG -3'	
<i>MDR1 - F</i>	5'-GGA GTT TAG GTG CTG T -3'	201
<i>MDR1 - R</i>	5'-CGG TGA TGG CTC TCA A -3'	
<i>ACT1- F</i>	5'-CCA GCT TTC TAC GTT TCC -3'	209
<i>ACT1- R</i>	5'-CTG TAA CCA CGT TCA GAC -3'	

One µL of DNA(20µg/ml)from each of 20 *Candida* isolates were mixed with PCR mixture consisted of 12 µL of 2x Master Mix (Promega),2 µL of primers (10 pmole) and adjusted with molecular-grade water to final reaction volume 25 µL. The PCR conditions for primer pairs : INTI-F /INT2-R and CDBF28/CADBR125 primers were 95 °C for 3 min followed by 30 cycles 94 °C for 0.30 min ,annealing temperature 55 °C for 0.30 min. Extensions temperature 72 °C for 1 min. followed by final extension temperature 72 °C for 7 min. The PCR mixture was amplified by thermal cycler PCR System (Labnet, USA com.).

The PCR products for each target region were run on 1.2% agarose gel (Bio Basic Canada Inc.) Electrophoresis electrophoreses performed at 100 V. in TBE buffer. The gel was pre-stained with 0.05% ethidium bromide. The DNA and RNA bands were detected by Desktop Gel imager scope 21 ultraviolet transilluminator (Korea com.).

Results and discussion

A total of 100 *Candida* spp were isolated from clinical and soil samples, of which 60 were classified as *C.albicans* showed green color on CHROM agar medium. This medium which was demonstrated to be the presumptive test but less accurate evidence¹⁰, only *C.albicans* subjected for resistance genes detection others species were neglected .

Total DNA and RNA was successfully extracted by manual method. Three bands representing the 18S, 28S rRNA and 5, 5.8S was observed. Slightly smearing was observed in the RNA band patterns (Figure 1).

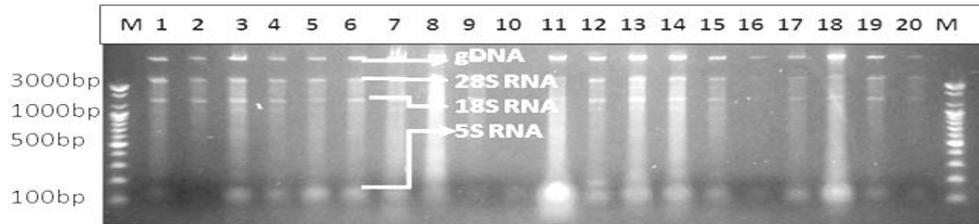


Figure 1: Profile gel electrophoresis of Nucleic acid (gDNA and RNA: 28S,18S and 5S RNA for 20 isolates of *Candida albicans*. M=molecular marker 100bp for each step.

Confirmed the identification of *C.albicans* by Molecular assay:

Molecular diagnosis for 20 isolates of *C.albicans* was performed by used specific primer pair INTI-F /INT2-R, all the isolate showed 310bp (Figure2), there are no PCR products (966bp) was showed when uses the specific primer pair CDBF28/CADBR125 as conformation test for presence of target region of *C.albicans* Ssp: *dublinensis* (data not showed).

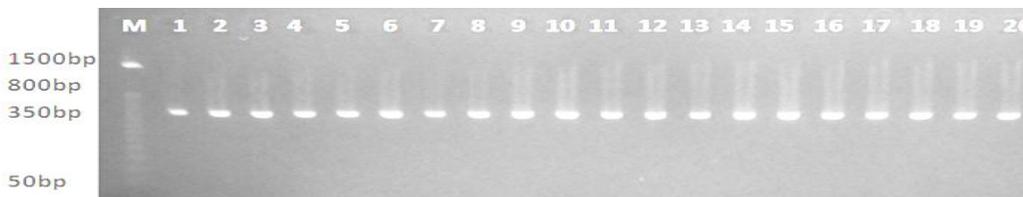
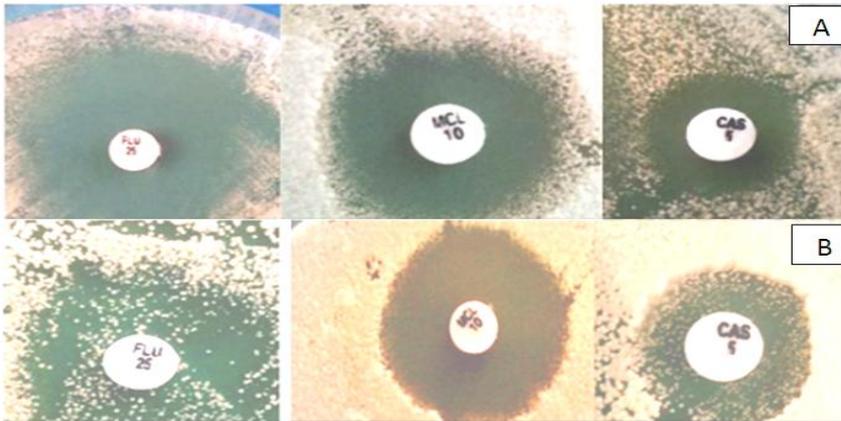


Figure 2: Profile gel electrophoresis of PCR products(310bp) amplified target region by specific primer pair INTI-F /INT2-R. lanes: 1-20 isolates of *C.albicans*, M=molecular marker 50bp for each step.

Susceptibility testing :

The results of the test highly percentage of both clinical and soils *C.albicans* isolates showed sensitivity in 24h of incubation periods but later (after 48-72h) many of them showed resistance (Figure3:A,B), these interpretative as evolve resistance due to selective pressure or to gene expression development .our results contracted with White et al.,⁷ and Lyons and White²¹.



Figure(3):Antifungals sensitivity of Caspofungin 5mg, Miconazole 10mg and Fluconazole 25mg based on diffusion methods for *C.albicans* ;A and B After 24h and 48h incubation period respectively.

The results of E-test showed the MIC was 2 and <0.02 for Fluconazole (Flu) and Caspofungin (CAS) respectively (Figure 4) .



Figure (4).E-test gradient strips of Fluconazole (Flu) and Caspofungin(CAS)agents showing susceptibility of *C.albicans* on SDA medium at 37°C and 48h.

The results of quantification of CDR1,CDR2,ERG11,MDR1 and ACT1 expression of *C.albicans* in the presence of fluconazol showed gene expression based PCR products of 20 isolates of *C.albicans* from clinical and soils sources, All 20 *C.albicans* as clinical and soil sources of CDR1 (show PCR product 286bp),while one clinical isolate show sensitivity to azole not have expressive gene of CDR2 others showed PCR product 364bp (Figure5:A&B) this resultse coincidence with Albertson et al.,²²,while 8clinical and 3 soil *C.albicans* isolates showed resistance gene of MDR other sensitive to azole .7 clinical isolate and 2 soil isolates source showed resistance to azole produced 204 bp of ERG11 gene expression while others not expression (Figure 5:C &D).while all isolates from both sources had housekeeping gene ACT1 produced PCR products 209bp,our results coincidence with previous studies (Lupetti et al.²³ ; Morschha et al.⁵and Frade et al.²⁴ .

The variation in gene expression between resistance gene may correlated with point mutations occur in in gene operator as mentioned by Jia et al.²⁵ .

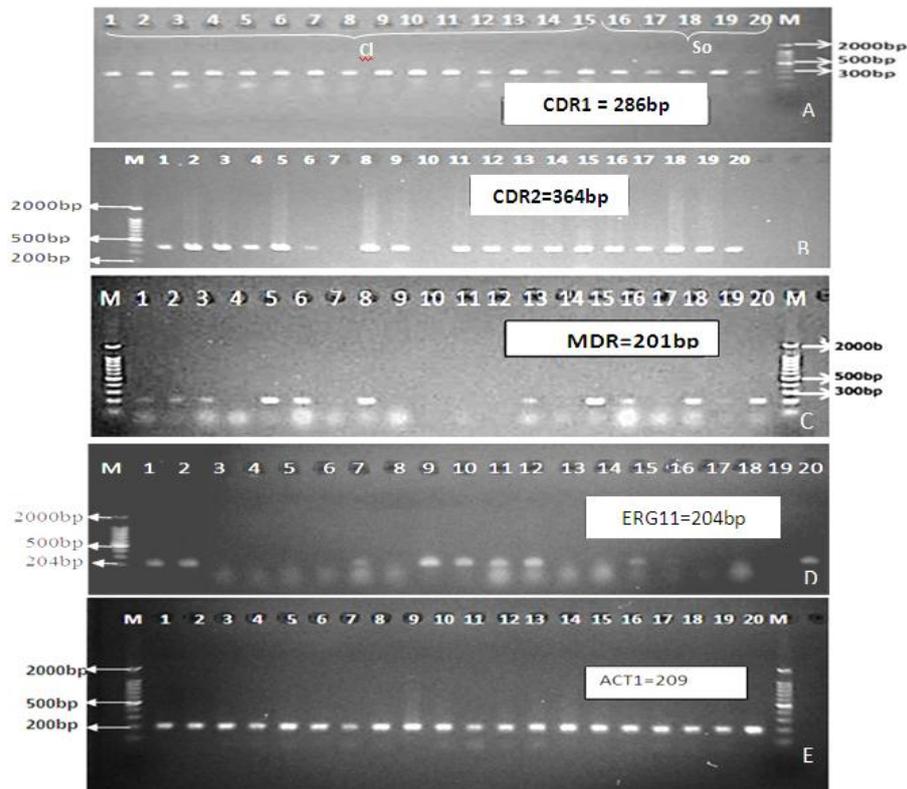


Figure 3: Profil gel electrophoresis of PCR products for resistance genes: CDR1(286bp), CDR2(364bp), MDR(201bp), ERG11(204bp),and normalized with housekeeping gene ACT1 (209bp) amplified CDR 1 resistance gene.Lanes:1-20 *C.albicans* isolates(1-15 clinical source of isolates,16-20 soil source of isolates). M=molecular marker 100bp for each step. Cl=clinical source of *C.albicans* isolates ,So= soil source of *C.albicans* isolates.

The results of this study attentiuted the frits one considered to compare and evaluted the gene expression between *C.albicans* from clinicl and soil sources while previous studies were performed the resistance mechanisms based on used matched sets of susceptible and resistant clinical isolates of the same strain ,This is necessary, as *C. albicans* is mostly clonal in nature²⁶ and isolates might differ considerably in their levels of expression of different genes. The purpose of the experiments described below is to use standard molecular techniques to screen for known resistance mechanisms . The CRD1 and CRD2 showed resistance for most of isolates under interast in this results may intrperated based on these gene are commonly overexpressed in resistance and suscepible isolates this results coincidence withLyons and White²¹.While the level of gene expression of both genes MDR and ERG11showed low expression in both sensetive and resistance isolates (Figure 1:C,D)also these phenomena reported by Lyons and White²¹.

One way to discover antifungal drug resistance mechanisms is to compare resistant clinical isolates with their susceptible parents^{7,27}. These studies have shown distinct patterns in the type and combinations of mechanisms that evolve in *C. albicans* populations to confer azole resistance. Often the patterns of mechanisms that evolve mimic those seen in clinical isolates, described above, validating this approach²⁸. Interestingly, the results pointed to probability of transience of resistance gene between clinical isolates and isolates colonized soil habitats due to matting or via plasmid infections carried gene resistance, many studies referred to cloning resistance genes ,the results of this study give attention to natural cloning for gene resistance between resistance and susceptible *C. albicans* isolates in nature due to highly uptake of antifungals by patients and dropping the west waters from hospitals and houses randomly in fields and on the side of roads in all cities without pretreatment in west treatment units may led to natural cloning between sensitive and resistance isolates and caused the increase of resistance *C.albicans* ,and led to failed of candidiasis treatment with antifungals our interpretation coincidence with Berrouane et al.²⁹, he was reported that some document resistance to azole antifungal agents in *C. glabrata* and *C. krusei* after prolonged exposure to these antifungals. Also this study indicated that multigene of antifungals resistance were transition or natural cloning in nature and led to increase

azole-resistance gene among environmental isolates of *Candida albicans* and this considered as a risk sources of spread azole drug resistance .

Ethical Approval

Both authors hereby declare that all actions have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

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