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OVEREXPRESSION OF EFFLUX PUMPS GENES IN RESISTANT *CANDIDA ALBICANS* CLINICAL ISOLATED FROM ORAL COLONIZATION IN IRANIAN HIV-POSITIVE PATIENTS

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ABSTRACT

Oral candidiasis is mainly treated with the use of Fluconazole. Treatment of oral candidiasis may be problematic due to either inherent resistance of *candida* species or acquired drug-resistance. Antifungal drugs including Azole could cause drug-resistance in *C. albicans* in two main mechanisms, these mechanisms include over expression of multi-drug resistance transport proteins such as MDR1 (a major facilitator) or CDR1 which is an ABC transporter. Fluconazole MIC in 66 clinical isolates of *C. albicans* were calculated by *broth microdilution* method and interpreted following *CLSI-M27-A3* guidelines. Then, 15 clinically resistant strains of *C. albicans* were used for total RNA extraction using hot phenol method. cDNA was created using the *MULV Reverse Transcriptase* and random hexamer primers stock. Expression levels of *MDR1* and *CDR1* genes in 15 resistance clinical isolates of *C. albicans* were measured by semi quantitative RT-PCR (qRT-PCR). Actin gene *ACT1*was used as control. We observed increased *mRNA* levels of *CDR1*, *MDR1* in 2 and 8 fluconazole-resistant isolates. The results showed that using q-RT-PCR, to determine the expression of resistance genes *MDR1* and *CDR1*, is appropriate.

INTRODUCTION

Candida albicans, as opportunistic fungi, can cause oral *candidiasis* at AIDS patients (Farah *et al*., 2010). Fluconazole as triazole drug has been used in patients with *Oral candidiasis* (Corrêa and Salgado, 2011). Exclusion of azole from drugs regiment of patients suffering from HIV, leads to infection with intrinsically-resistant *C. albicans* strains and non *C. albicans* species such as *C. glabrata*, *C. krusei* (Kontoyiannis and Lewis, 2002). Increment in the number of stains obtaining acquired resistance to a certain drug could be a result of three mechanism; selection, mutation and acquisition of characterization by genetic transmission (Hof, 2008). *C. albicans* can build up resistance to azole antifungal agent by various mechanisms. One of these mechanisms is a point Mutation in the Lanosterol demethylase (*ERG11*) gene which its product is targeted by azole. The conformational changes induced by the point mutation, unable proper binding of anti-fungal agent (Calderone *et al*., 2012). Another mechanism is the overexpression of molecules targeted by azole such as *ERG11*. Two types of transport pumps are involved with azole resistance; the ATP binding cassette (ABS) class of proteins and the proton motive force dependent major facilitators. ABC transporters belong to the gene family of efflux pumps associated with the movement of small molecules across the plasma membrane. Eight genes of ABC transporters have so far been determined in *C. albicans*. These genes include five *CDR* genes (*CDR1* to *CDR5*). Second classes of efflux pumps include major facilitators such as *MDR1*. These classes of transport proteins use the energy generated from the proton motive force in the plasma membrane and export drugs and other molecules from the cell (Barker *et al*., 2004; Cannon *et*

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al., 2009; Morschhäuser, 2010). Up regulation of these pumps has been correlated with resistance to azole drugs such as fluconazole Comprehensive analysis of C. albicans isolates from HIV positive/AIDS patients suffering from OPC and other aggressive diseases provided substantial proof that these mechanisms may work separately, consecutively and in harmony (Kanafani and Perfect, 2008). By the prevalence of *Candida* infections followed by their treatment by antifungal agents especially the azole compounds and resistance to such compounds, the necessity of using the methods of determining drug resistance have further been revealed (Sanguinetti *et al*., 2005). Susceptibilities of *candidiasis* strains to Triazole could be established by obtaining minimal inhibitory concentration (MICs) using broth microdilution (BMD), E-test strips or zone diameters using triazole discs (Pfaller *et al*., 2002), but the semi-quantitative RT-PCR analysis indicated the expression of resistance gene was measurable, Sq-RT-PCR method is suitable for determining gene expression.

MATERIALS AND METHODS

Patient's data

C. albicans strains were isolated from 66 clinical samples acquired from 8 different hospitals in Tehran city (Iran). All samples were obtained from patients suffering from AIDS which were hospitalized due to oral *candidiasis*. All patients have received high dose of fluconazole (10 mg/day). The clinical samples were collected from oral *candidiasis* 283 patients during two years, May 2009 to May 2011. This study included 66 positive *C. albicans* patients in wet mount slide and culture. From total of 66 patients, 41 were male and 25 were female. Informed consent was obtained from all patients. Oral candidiasis was confirmed by clinical evaluation, additionally, type and location of lesions were documented. All patients involved in this study showed reduced CD_4 cells at the time of examination, which were below 200 cells / mm^3.

Organisms and growth conditions

Clinical samples were taken from oral cavity of patients using sterile swap. Sabouraud Dextrose Agar / Chloramphenicol (Sc) was used for the selective isolation of *Candida* species and cultured media were incubated at 37°C for 5 days. In each sample, yeasts with different morphological characteristics were isolated and stored at -20°C for further identification. *C. albicans* isolated from samples were verified by Phenotypic (Corn Meal Agar, Chlamydospore and Germ tube) and Genotyping (PCR-RFLP) tests.

Antifungal Susceptibility Patterns

The Susceptibility testing of the antifungal fluconazole drug was conducted according to the manufacturer's instructions, which comply with the *Clinical and Laboratory Standards Institute CLSI-M27-A3* document (Cuenca-Estrella *et al*., 2010). The standard powder of fluconazole (Sigma Alderich, Company Germany) was prepared in 1ml Dimethyl Sulfoxide sterile (DMSO). The stock solution for fluconazole was prepared at the rate of *512 µg/mL.* For the susceptibility test, RPMI 1640 (with glutamine, bicarbonate-free, and containing phenol red as the pH indicator) (Sigma) was used as a medium. The final concentrations were in the range *0.125–256 µg/mL* for fluconazole. Each *C. albicans* was studied twice for fluconazole. Prior to testing, the isolates of *C. albicans* were grown on Sabouraud agar plates for 24 h at 37°C. The yeast suspensions were prepared in 0.85% Normal saline after 24h of incubation, obtaining an initial concentration of 1 to 5×10^6 cell/ml (adjusted spectrophotometrically (Eppendorf) at 625nm to match the turbidity of a 0.5 Mc Farland standard). These inoculums were diluted in RPMI 1640 medium, containing L-glutamine, and no sodium bicarbonate (Sigma Alderich, USA) morpholine-propanesulfonic acid was used as buffering agent (*Sigma-Aldrich*, Germany). The final cell density was 0.5×10^3 to 2.5×10^3 cell/ml. The cultured plates were incubated at 35°C for 48h. Visible fungal growth can be inhibited 50% by the MIC50 of fluconazole. MIC results were measured after 48 h of incubation. Two fungal strains were included in each assay for quality control. *C. albicans* ATCC10231 served as susceptible strain and *C. albicans* ATCC76615 was used as resistant strain to fluconazole.

Total RNA Extraction

The resistant isolates and quality control strains (*C. albicans*) were used for total RNA extraction. Total RNA was isolated using the Hot-Acidic-Phenol method. In the first Procedure, the yeast cell suspension is mixed with small (200 m diameter) glass beads and vigorously vortexed, followed by RNA extraction using Phenol: Chloroform: Isoamyl Alcohol (25:24:1) at room temperature (Schmitt *et al*., 1990). Organic solvents were used to eliminate protein contaminates and total RNA was recovered after Ethanol precipitation. The RNA concentration was determined by reading the absorbance at 260 nm by Spectrophotometry.

Synthesis of cDNA and Sq RT-PCR Amplification

Before synthesis of cDNA, the RNA of each resistant yeast strain was electrophoresed. Then the visualized total RNA was contaminated by genomic DNA. To eliminate DNA, samples were treated with DNaseI (Fermentas) (1U per 10 µl) and incubated at 37°C for 1 h. Random hexamers were used to generate cDNA. Total of 2 µg RNA was used as template for each reaction. Sterile DEPC-treated DDW was used to bring the volume up to 12 µl. The mixture was heated to 65°C for 5min and chilled on ice for 2 min. Then, 4 µl of reaction buffer, 2 µl of 10Mm dNTP mix, 1 µl RNase inhibitor (40 U/µL) and 1 MULV Reverse were added to each sample. The thermocycling conditions were as follows; at first, the mixture was incubated at 25°C for 5 min. Then it was incubate at 42°C for 60 min and at 70°C for 10 min to terminate the reaction. The cDNA was stored at 20°C freezer for further usage. Then, the template of cDNA was amplificated with *MDR1*, *CDR1* and *ACT1* primers. The primers were synthesized by Cinnagen Company (Iran). Thermal Cycler (Eppendorf) was used for SqRT-PCR. Master Mix (Fermentas) { Taq DNA polymerase (0.05 units/µl), $mgcl_2$ (4 mM), dNTPs (0.4mM)} was used. The initial denaturation was for 5 min at 94^oC. Total of 35 cycles was carried out as follows; denaturation for 45sec at 94°C, annealing for 50 sec at 58°C, and elongation for 55 sec at 72°C. The final elongation was for 10 min at 72°C. The list of oligonucleotide primers used in this study are available in Table 1 (Park and Perlin, 2005).

Table 1. Primers used for semi quantitative RT-PCR

Gene	Accession no Gene bank	Primer Sequence(5 to 3)	Amplicn size of PCR	Reference
CDR1	**X77589**	Forward primer5'-CTTAGTCAAACCACTGGATCG-3 Reverse primer 5'-CCAAAAGTGATGAAAAGGC-3'	85bp	
MDR1	**X53823**	Forward primer5'-TTCTTGGGTGGATTCTTTGC-3' Reverse primer 5'-GCACCTAAACTCCAAGCGGC-3'	113bp	15
ACT1	**X16377**	Forward primer5'- CCAGCTTTCTACGTTTCC-3' Reverse primer5'- CTGTAACCACGTTCAGAC-3'	209bp	

Table 2. The percent frequencies of susceptibility values against fluconazole in clinical isolates of *C. albicans*

Criteria	*Intermediate SDD(16-32 µg /ml)*	*Resistance (R>64 µg/mL)*	*Sensitive* (*S <16 µg/mL*)
Total isolates of C. albicans (66)	8	15	43
Percentage of frequency	12.12 %	22.73 %	65.15 %

Table 3. The expression of *MDR1* and *ACT1* genes in clinical resistant of *C. albicans*, susceptible control strain of *C. albicans ATCC10231* and resistant control strain of *C. albicans ATCC76615*

Lane (strain, gene)	MIC (µg/ml)	Concentration of RT-PCR product (ng/µl)	Ratio of MDR1 to ACT1 mRNA level	Fold of *MDR1* gene expression relative to susceptible control strain	Over expression MDR1
C. albicans ATCC10231, actin	8µg /ml	34.5	1.2	-	-
C. albicans ATCC10231, MDR1		40.1			
C. albicans ATCC76615,actin	*64µg /ml*	35.2	2.2	1.8	+
C. albicans ATCC76615,MDR1		75.8			
C. albicans R1, actin	64µg /ml	33.5	1.2	1	-
C. albicans R1, MDR1		41.1			
C. albicans R2, actin	128µg /ml	38.8	1.1	0.9	-
C. albicans R2, MDR1		43.1			
C. albicans R3, actin	64µg /ml	39.5	1.1	0.9	-
C. albicans R3, MDR1		43.8			
C. albicans R4, actin	64µg /ml	40.7	1	0.8	-
C. albicans R4, MDR1		42.1			
C. albicans R5, actin	64µg /ml	39.3	1	0.8	-
C. albicans R5, MDR1		40.1			
C. albicans R6, actin	128µg /ml	41.7	0.9	0.7	-
C. albicans R6, MDR1		40.1			
C. albicans R7 , actin	256µg /ml	33.9	2.1	1.9	+
C. albicans R7, MDR1		77.3			
C. albicans R8, actin	64µg /ml	42.9	0.9	0.7	-
C. albicans R8, MDR1		40.8			
C. albicans R9, actin	64µg /ml	43.5	1	0.8	-
C. albicans R9, MDR1		44.1			
C. albicans R10, actin	64µg /ml	32.7	0.9	0.7	-
C. albicans R10, MDR1		29.3			
C. albicans R11, actin	128µg /ml	39.5	1.1	0.9	-
C. albicans R11, MDR1		43.9			
C. albicans R12, actin	128µg /ml	34.7	2.3	1.7	+
C. albicans R12, MDR1		73.5			
C. albicans R13, actin	64µg /ml	34.5	1.2	1	-
C. albicans R13, MDR1		40.1			
C. albicans R14, actin	64µg /ml	43.5	1	0.8	-
C. albicans R14, MDR1		44.1			
C. albicans R15, actin	128µg /ml	40.9	0.07	0.05	-
C. albicans R15, MDR1		37.1			

⋆(R: Resistant, +: Overexpression showed, - : Not Overexpression showed)

Table 4. The expression of *CDR1* and *ACT1* genes in clinical resistant of *C. albicans*, susceptible control strain of *C. albicans ATCC10231 and* resistant control strain of *C. albicans ATCC76615*

Lane (strain, gene)	MIC (µg/ml)	Concentration of RT-PCR product (ng/µl)	Ratio of *CDR1* to *ACT1* mRNA level	Fold of *CDR1* gene expression relative to susceptible control strain	Over expression *CDR1*
C. albicans ATCC10231, actin	8µg /ml	40.8	1.2	-	-
C. albicans ATCC10231, CDR1		49.7			
C. albicans ATCC76615,actin	*64µg /ml*	*39.3*	*1.8*	*1.5*	+
C. albicans ATCC76615,CDR1		*69.7*			
C. albicans R1, actin	64µg /ml	37.8	1.2	1	-
C. albicans R1, CDR1		47.8			
C. albicans R2, actin	128µg /ml	38.4	1.7	1.4	+
C. albicans R2, CDR1		65.9			
C. albicans R3, actin	64µg /ml	38.8	1.1	0.9	-
C. albicans R3, CDR1		41.8			
C. albicans R4, actin	64µg /ml	38.5	1.7	1.4	+
C. albicans R4, CDR1		65.9			
C. albicans R5, actin	64µg /ml	37.8	1.2	1	-
C. albicans R5, CDR1		47.8			
C. albicans R6, actin	128µg /ml	38.4	1.8	1.5	+
C. albicans R6, CDR1		69.4			
C. albicans R7 , actin	256µg /ml	39.8	1.6	1.8	+
C. albicans R7, CDR1		69.4			
C. albicans R8, actin	64µg /ml	40.7	1.2	1	-
C. albicans R8, CDR1		48.9			
C. albicans R9, actin	64µg /ml	37.8	1.2	1	-
C. albicans R9, CDR1		47.8			
C. albicans R10, actin	64µg /ml	38.8	1.1	0.9	-
C. albicans R10, CDR1		45.8			
C. albicans R11, actin	128µg /ml	39.8	1.5	1.2	+
C. albicans R11, CDR1		63.4			
C. albicans R12, actin	128µg /ml	40.7	1.6	1.3	+
C. albicans R12, CDR1		66.9			
C. albicans R13, actin	64µg /ml	36.8	1.1	0.9	-
C. albicans R13, CDR1		43.8			
C. albicans R14, actin	64µg /ml	38.5	1.7	1.4	+
C. albicans R14, CDR1		65.9			
C. albicans R15, actin	128µg /ml	39.6	1.8	1.5	+
C. albicans R15, CDR1		70.9			

⋆(R: Resistant , + : Overexpression showed, - : Not Overexpression showed)

Gel electrophoresis of SqRT-PCR product was carried out using 1.8% Agarose-TBE buffer (Boric acid 27.5 gr, Tris base 54gr, EDTA 20ml (PH=8)). After electrophoresis, for determination of molecular weight and quantitative data of each band, UVIband software was used for analysis of image gel.

RESULTS

Patients' Data: Of the 283 patient, 66 (22.32%) were identified as *C. albicans* isolates. Male/female ratio was 41/25. Patients aged between 25 to 35 years, were more prevalent (23, 34.84 %), followed by ages 36 to 45 (14, 21.21 %). The rate of Oral *Candidiasis* in male patients was higher, compared with that of females.

Antifungal Susceptibility Patterns

A total of 66 *C. albicans* clinical isolates were collected. As shown in Table 2, MICs values for fluconazole were compared to the CLSI interpretative guideline CLSI M27-A3, On broth microdilution antifungal susceptibility testing. Any fungal isolate (with MIC value ≤ 8 µg/ml obtained in drug concentration) was considered as susceptible (S). Fungal growth at MIC ≥ 64 µg/ml was considered as resistant (R), and when MIC was concentration between 16 and 32 µg/ml, the isolate was considered as susceptible dose dependent (SDD). The standard isolates of *C. albicans* (ATCC 76615, as resistant strain, and ATCC 10231, as susceptible strain) were also used for quality control of each test. The microdilution broth was done in triplicates. Our results showed, with respect to fluconazole, 12.12 % of *C. albicans* were SDD and 22.73 % were resistant. In this study MIC50, total 66 clinical isolates of *C. albican*s was determined 0.25 µg/mL. MIC each of clinical resistant isolates of C. *albicans* was shown in Table 2. All of fluconazole-resistant *C. albicans* isolated from HIV patient who receiving 10 mg/day of fluconazole.

Synthesis of cDNA and Sq RT-PCR Amplification

In addition to assessing the sensitivity, RNA extraction of the 15 resistant clinical isolates was done. After electrophoresis, the bands were observed. Then after removal of genomic DNA from the total RNA and synthesis of cDNA, the PCR reaction was performed with treated and non treated total RNAs. The PCR results were good and acceptable. After cDNA synthesis, the PCR steps were done using primers specific to the *MDR1* and *CDR1* Genes encoding drug efflux pumps and housekeeping gene *ACT1*, as an internal control.

CDR1 and MDR1 expression in C. albicans isolates

After electrophoresis resolution, the quantitative analysis band density of the *MDR1* and *CDR1* gene's related to *ACT1* was done by using UVIband analysis software Figure 1. The

resistance profile of fifteen azole-resistant isolates, are listed in Table 3. Semi-quantitative reverse transcription-PCR was used to compare the gene expression profile of the fifteen isolates of *C. albicans* which showed resistance to azole with the standard azole-susceptible strain *C. albicans ATCC10231*. As presented in Figure 2, *MDR1* was expressed at a low level in the *C. albicans ATCC10231* strain, but a remarkable increase in expression was observed in two of the resistant isolates such as resistance *C.albicans ATCC76615*. Quantification of the mRNA level indirectly by measuring the intensity of the RT-PCR product revealed that the amounts of *MDR1* mRNA relative to *ACT1* (encoding the constitutively expressed housekeeping gene, actin) mRNA in drug resistant strains *C. albicans R7* and *C. albicans R12* were significantly higher at 1.7- and 1.9 fold compared to that of the *C. albicans ATCC10231* isolate Table 3.

The relative amounts of the *MDR1* mRNA in other drug-resistant strains were marginally equal or lower than that of the drug-susceptible *C. albicans ATCC10231* strain. *CDR1* was expressed at low level in *C. albicans ATCC 10231* strain. All eight resistant strains (*C. albicans R2, C. albicans R4, C. albicans R6, C. albicans R8, C. albicans R11, C. albicans R12, C. albicans R14, C. albicans R15*) overexpressed *CDR1* gene compared to the azole-susceptible *C. albicans ATCC10231* strain as shown in Figure 3 and 4. Quantification with UVIband analysis software revealed that the relative amounts of *CDR1* mRNA in drug-resistant strains were 1.4–1.8 folds higher than that of the drug-susceptible strain Table 4. The results indicated that some of the drug fluconazole-resistant C. *albicans* isolates had no over expression genes *MDR1* and *CDR1*, but only 2 isolates of resistant *C. albicans* (*C. albicans R7, C. albicans R12*) showed overexpression in both of *MDR1* and *CDR1* genes.

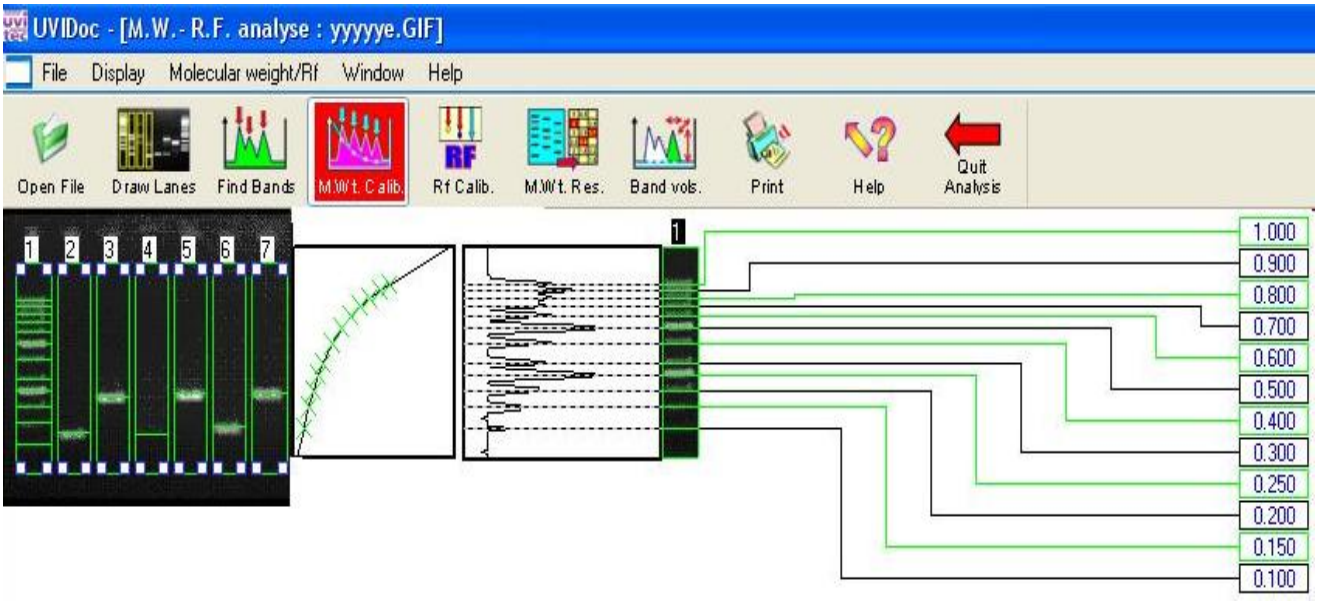

Figure 1. Calibration Molecular Weight of Marker (50bp Fermentas ≠SM0373) on gel electrophoresis image was down. Then Band density was measured by UVItec Analyze software

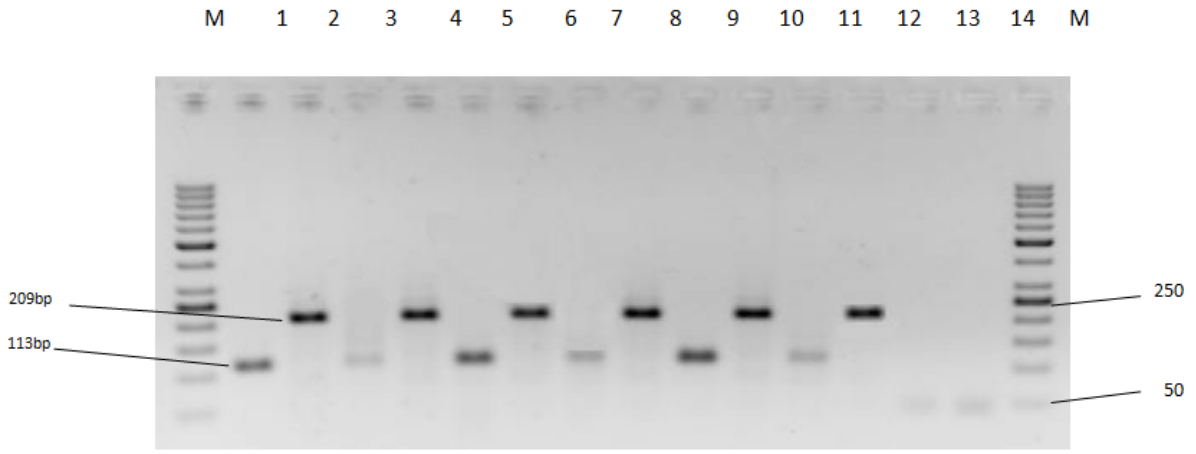

Figure 2. Gel electrophoresis of Sq-RT-PCR product. Lane M, Molecular Weight of marker 50 bp (50bp Fermentas ≠ SM0373). Lanes 2, 4, 6, 8, 10 and 12 : ACT1 amplicon. Lane 1, MDR1 amplicon (Resistant C. albicans ATCC76615) . Lane 3, MDR1 amplicon (Sensitive C. albicans ATCC10231). Lanes 5,7, 9 and 11 : MDR1 amplicon (Resistant clinical isolates of C. albicans). Lanes 13 and 14: Negative control amplicon

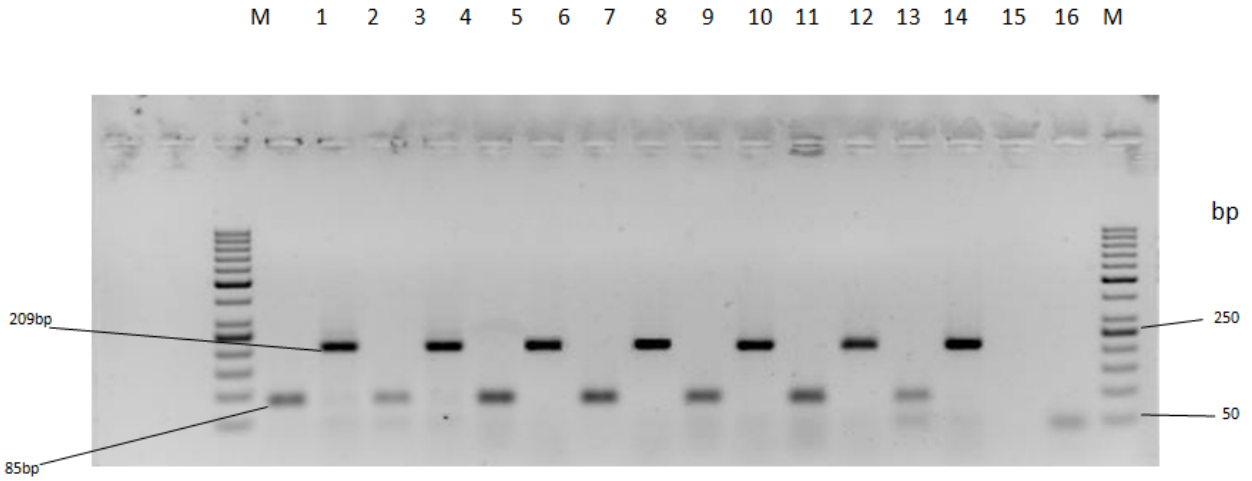

Figure 3. Gel electrophoresis of Sq-RT-PCR product. Lane M, Molecular Weight of Marker 50bp (50bp Fermentas ≠SM0373). Lanes 2, 4, 6, 8, 10, 12 and 14 : ACT1 amplicon. Lane 1, CDR1 amplicon (Resistant C. albicans ATCC76615). Lane 3, CDR1 amplicon (Sensitive C. albicans ATCC10231), Lanes 5, 7, 9,11 and 13 : CDR1 amplicon (Resistant clinical isolates of C. albicans). Lanes 15 and 16 : Negative control amplicon

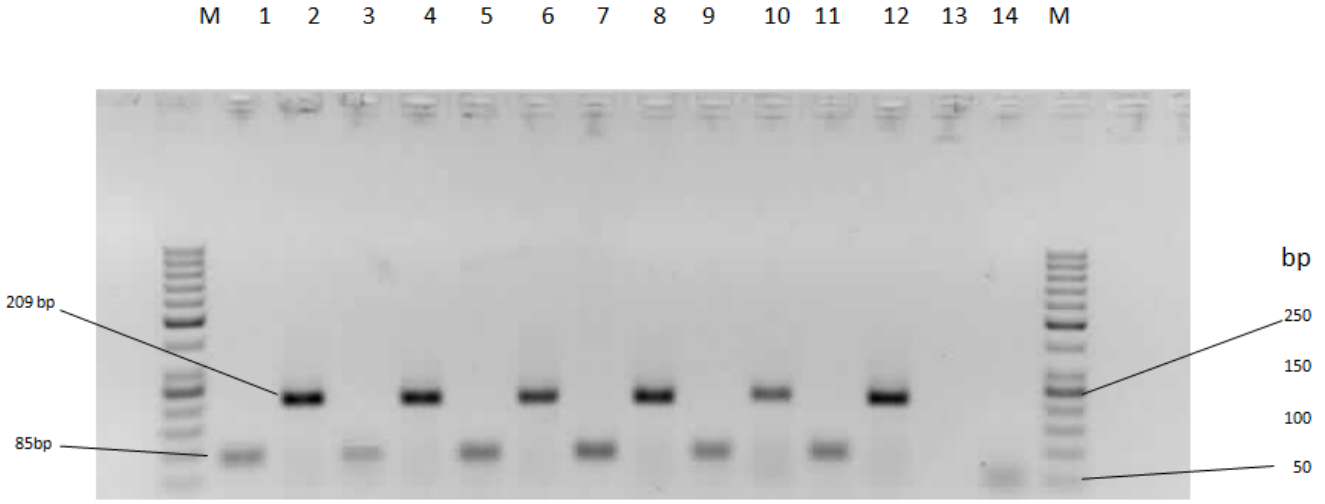

Figure 4. Gel electrophoresis of Sq-RT-PCR product. Lane M, Molecular Weight of Marker 50 bp (50bp Fermentas ≠SM0373). Lanes 2, 4, 6, 8, 10 and 12 : ACT1 amplicon. Lane 1, CDR1 amplicon (Resistant C. albicans ATCC76615). Lane 3, CDR1 amplicon (Sensitive C. albicans ATCC10231). Lanes 5, 7, 9 and 11 : CDR1 amplicon (Resistance clinical isolates of C. albicans). Lanes 13 and 14 : Negative control amplicon

DISCUSSION

Opportunistic pathogenic fungi such as fluconazole - resistant *C. albicans* are considered as one of the main cause's life-treating infections in immunodeficiency patients. *Oral candidiasis* is among the common infections in AIDS patients. The proposed treatment is by azoles, anti-fungal agents, especially by fluconazole as an efficient treatment method for infections caused by *C. albicans* yeast (Fichtenbaum *et al*., 2000). But the emergence of drug-resistances has challenged this method of treatment with severe problems. Therefore, it seems necessary to identify the species resistant to the antifungal agents (Kanafani and Perfect, 2008). In this research we aimed to characterize the C. albicans species resistant to fluconazole isolated form oral lesions of AIDS patients using SqRT-PCR emphasizing on expression profiles of MDR1 and CDR1 genes.

There are two major classes of efflux pumps, the ATP-binding cassette (ABS) transport proteins and the Major facilitator superfamily (MFS). Azole resistance in commonly accompanied by overexpression of efflux proteins. The resistance due to excessive drug release from the inside to the outside of the Cytoplasmic membrane (White *et al*., 1998). The importance of this study is that drug resistance is increasing. Therefore, identification of genes causing resistance to treatment is a better decision. Furthermore, the identification of genes likely to develop resistance to other azole is prevented. Only few researches have linked the overexpression of these efflux transporters to resistance mechanism of *C. albicans* in clinical strains. Magaldi *et al*. Evaluated the drug susceptibility of 108 *C. albicans* isolated from oral lesion of HIV patients by using disk diffusion method and reported % 10 fluconazole resistance among the isolates (Magaldi *et al*., 2001). Kabli *et al*. have also evaluated fluconazole susceptibility including 107 *C. albicans* isolated

from variation samples by using disk diffusion method and reported 26 % fluconazole resistance (Kabli, 2008). Maria *et al*. Evaluated the drug drug susceptibility of 52 *C. albicans* isolated from oral lesion of HIV patients by using broth microdilution method and reported 17.28 % fluconazole resistance among the isolates (Silva *et al*., 2002). The results obtained from the current study of the prevalence of fluconazole resistant *C. albicans* among HIV positive patients are also consistent with the findings of the above mentioned researchers. Prevalence of fluconazole-resistant *Candida* isolates in this study, among patients with AIDS by using broth dilution method is 22.73 %. Currently, only reference laboratories use sophisticated and expensive methods to unravel the molecular mechanism of drug resistance (Woodford and Sundsfjord, 2005). White et al. using northern blotting analysis demonstrated, in one isolate out of 17 strains of *C. albicans* isolated from patients suffering from AIDS, the co-overexpression of *MDR1* and *CDR1* genes that are responsible for drug resistance (White *et al*., 1998). Maebashi et al., using SDS-PAGE and immune-blotting, analyzed four fluconazole resistant *C. albicans* isolates and found that all of the isolates have had co-over expression of two drug resistant genes, (*CDR1* and *CDR2*), while none of them had over expression of the *MDR1* gene (Maebashi *et al*., 2001). Perea *et al*. using northern blotting and PCR assays experienced successful detection of the *MDR1* and *CDR1* drug resistant genes among 6 and 8 fluconazole resistant *C. albicans* isolates, respectively (Perea *et al*., 2001).

Chau et al. used RT-PCR method to evaluate the expression levels of the drug resistant genes (*MDR1* and *CDR1*) in 38 *C. albicans* strains isolated from oral lesion of AIDS patients. They found three isolates with over expression of the *MDR1* gene and fourteen isolates with over expression of the *CDR1* gene (Chau *et al*., 2004). Park *et al*. analysed the drug resistance of 59 *C. albicans* isolates and found over expression of both *MDR1* and *CDR1* genes among 15 and 13 isolates, respectively (Park and Perlin, 2005). In the current study, the percent of over expression of the genes responsible for fluconazole resistance of *CDR1* and *MDR1* genes was 50 % and 12.5 %, respectively. Many known or unknown factors may affect the specific drug resistant isolates of *C. albicans* in the broth microdilution methods but they may have normal or lower expression levels of the two drug resistant genes of *MDR1* and *CDR1*. The factors other than *MDR1* or *CDR1* genes that may lead to drug resistance include the expression of other resistance genes such as*ERG11, RTA2, FLU, PDR, CDR2* and the influence of other drug resistance mechanisms including cell variation, replacement of fluconazole resistant strains over susceptible ones, and many other unknown mechanisms (Jia *et al*., 2008; Mukherjee *et al*., 2003).

The results in our study mention that certain but not all clinically drug-resistant *C. albicans* strains may operate two or more mechanisms synergistically for conferring drug resistance to fluconazole drugs. An interesting phenomenon observed in this study was that overexpression of *CDR1*, *MDR1* mRNA correlates with the elevation of MICs of fluconazole Table 3 and 4. For instance, *C. albicans R7* with the highest MIC (256 µg /ml) to fluconazole among the 15 isolates had the greatest expression level of the *CDR1* and *MDR1* genes. The Sq-RT-PCR is the method of choice to evaluating the over expression of fluconazole resistant genes in the current study. This method, compared to others, has the advantages of time-saving and low cost. Identification rapid and precise of resistant isolates of *C. albicans* by using molecular assays provides a proper opportunity to physicians to overrule any improper prescriptions.

Conclusion

In this research we have showed that expression analysis can be an efficient and reliable method for classification of oral candidiasis showing resistance to fluconazole therapy. The obtained data indicate that there are high chances that HIV positive patients carry resistance strains of the yeast and alternative treatment strategies such as higher doses of fluconazole should be considered. A further study on this population is ongoing to determine the progression rate of drug-resistance oral-candidiasis.

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