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GENE EXPRESSION STUDY OF ANTIDRUG RESISTANCE GENE (ERG11) IN *CANDIDA ALBICANS* ISOLATED FROM DIFFERENT CLINICAL INFECTION CASES BY USING REVERSE TRANSCRIPTION REAL-TIME PCR

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ABSTRACT : The main aim of our study was to investigate gene expression of theERG11 antidrug resistance gene in *Candida albicans* isolates taken from candida infection in the calf, immunocompromised patients and vaginal infection in women. The experiment consists of four groups,the first group (T1) included*Candida albicans*isolates from calf.The second group (T2) included *Candida albicans*isolates from immunocompromised patient.The third group (T3) included *Candida albicans* isolates from vagina. The fourth group (T4)(control group) included *Candida albicans* isolates from normal individual. All the isolates are submitted to the extraction of RNA, test gene expression then transferred to cDNA to reading the results that related with gene expression of ERG11 gene by comparison between the groups depending on reverse transcription Real-Time PCR technique by using specific primers designed for this purpose. The results revealed that (T2) had higher gene expression and had significant differenceas compared with other groups. However, T2 is (56.83±19.01), While (T1) and (T3) hadn't any significant differences with (T4), it was (9.28±3.16) and (6.81±2.20) respectively. In conclusion, The isolates were taken from the immunocompromised patient had higher gene expression while other Groups don't show any significant difference.

Key words : Antidrug resistance gene, *Candida albicans*, ERG11 gene, Real-Time PCR.

INTRODUCTION

Candidiasis is almost common fungal disease caused by *Candida albicans*, the candida infection found in immunocompromised patients, particularly in immunodeficiency virus (HIV)-infected patients (Pohan, 2006; De Repentigny *et al*, 2004 and Wingeter *et al*, 2007).

Candida albicans is an important opportunistic fungal pathogen of animals and humans and the major cause many of diseases such as oropharyngeal candidiasis (OPC) in the calf, children, women and AIDS patients (Odd, 1988; Johnson and Cobb, 2010).

Candida albicans is an opportunistic fungus, can cause a disease called candidiasis in animals and infected AIDS humanpatient (Lvyin *et al*, 2015; Guo *et al*, 2013; Kim and Sudbery, 2011).

Candida albicans become most common inhuman and animals, it can spread, develop and form advanced defences mechanism against all thing prevent its distribution, wherever it can resist primary and secondary immune-response such as resistance genes that enhance it to survivalsand increase thelevel of gene expression of some antifungal resistances gene (Franz *et al*, 1998).

ERG11 is antidrug most common gene distributed among *Candida albicans* isolates. This gene encodes protein (enzyme) called lanosterol 14a-demethylase (CYP51), this enzyme causes a structural change of the affinity of fluconazole, it makes *Candida albicans* isolates more resist to some antifungal drugs like fluconazole (White *et al*, 2002; Wang *et al*, 2009; Sanglard and Odds, 2002 and Silva *et al*, 2016).

The gene encodes a protein, when gene expression increases, it will provide encoding protein (enzyme) more amount (Vandeputte *et al*, 2007). Resistance is molecular mechanisms responsible for the development of resistance against azoledrugs in Candida isolates (Barker and Rogers, 2006; Berila *et al*, 2009 and Carvalho *et al*, 2006).

Therefore, the detection of ERG11 gene in clinical *C. albicans* isolates can provide us new information about the resistance to antifungal drugs. The genetic and

molecular reports are made asection of antifungal drugs is more developed. Therefore, study ERG11gene in *C. albicans* clinical isolates is very important.

MATERIALS AND METHODS

Bacterial isolates

Sixteen sample of *Candida albicans* were provided byAl-Diwaniya hospital,four isolates from Candidiasis immunocompromised patients, four isolated from Candidiasis of vaginal infection and four from oral Candidiasis infection of the calf.

Total RNA extraction

RNA extracted from *Candida albicans* isolatesby usekit called (Accuzol ® reagent kit. Bioneer. Made in Korea). It's prepared based on company directions as follows 200µl of 4 hours incubation *Candida albicans* isolates on LB broth was placed in a sterile 1.5ml Eppendorf tube and 1 ml of Accuzol reagent was added and mixed well by using micropestle then the tubes were shaken vigorously for 1minute. After that, chloroform (200 µl) added to all tube and shakes it for fifteen seconds. The final products incubated at (-4) for five min. then centrifuged at 12000 rpm at 4°C for 15 min. The super layer Trans to a new mini tube and isopropanol (500 µl) was mixed. The final mixture added and inverts the tube for 4-5 times and incubated at 4°C for 10 min. Then centrifuged use at 12000 rpm 4°C for 10 min. Remove upper layer and add Ethanol (1ml 80%) and use vortex for shaking. Then, submitted to the centrifuge at (12000) rpm 4°C for 5 min. Then remove the upper layer and the RNA pellet left to dry.

Last step, DEPC water (50 µl) for dissolving RNA pellet then RNA store at (-20). Extracted RNA measure by using Nanodrop (THERMO. Made in the USA).

DNase I Treatment

The final products of RNA treated with enzyme named DNase I enzyme to discard all remained DNA by use (DNase I enzyme kit) and prepared depending on directions of Promega company, as given in Table 1.

The mixture incubating at 37°C for 30 min. Then, stop solution (1 µl) was mixed and keep at 65°C for 10 min. For stopping the action of DNase enzyme.

cDNA synthesis

Using kit called AccuPower® RocktScript RT PreMix for transmission RNA to cDNA depend on instructions Bioneer company, made in Korea and prepared depending on company directions as given in Table 2.

This RT PreMix was placed in AccuPower Rocket Script RT PreMix tubes that containlyophilised Reverse transcription enzyme in the form. Then dissolved completely by vortex and briefly spinning down.

Converting process(RNA to cDNA) is done in a thermocycler apparatus as given in Table 3.

Quantitative Real-Time PCR (qPCR)

QPCRwas performed for detection and quantification of relative gene expression ofantidrug resistance gene (ERG11) in *Candida albicans* isolates from clinical infection cases in human whereas, was carried out by using ($2^{-\Delta\Delta CT}$ Livak method) (Livak and Schmittgen, 2001). The qPCR reaction was done on a Real-Time PCR system (BioRad. the USA) by using SYBER Green dye qPCR master mix that used in detection and amplification of target genes and GAPDH housekeeping gene for normalisation of gene expression. The Primers were designed using the primer3 plus (Primers sequences are listed in Table 4.

Table 1 : DNase I treatment master mix preparation.

Mix	Volume
Total RNA 1µg	10µl
DNase I enzyme	2 µl
10X buffer	4 µl
DEPC water	4 µl
Total	20 µl

Table 2 : RT master mix for cDNA synthesis.

RT master mix	Volume
Total RNA 100ng/ul	10 µl
Random Hexamer primer (10pmol)	1 µl
DEPC water	9 µl
Total	20 µl

Table 3 : Thermocycler conditions for cDNA synthesis.

The Step	The Temperature	The Time
cDNA synthesis	(50 °C)	one hour
inactivation by Heat	(95 °C)	five min.

Table 4 : RT-qPCR primers with their sequence.

Primer		Sequence	Amplicon size	GenBank
ERG11	F	GAGACGTGATGCTGCTCAAAAG	85bp	XM_711668.2
	R	TGGATCAATATCACCACGTTCTC		
GAPDH	F	TGCTAAAGCCGTTGGTAAGG	88bp	XM_714816.1
	R	AACGGAAACATCGGTGGTTG		

Table 5 : qPCR master mix preparation.

qPCR master mix	volume
cDNA template (10ng)	5µL
Forward primer (10pmol)	2 µL
Reverse primer(10pmol)	2 µL
2X green star master mix	25 µL
DEPC water	16 µL
Total	50 µL

QPCR master mix was prepared for HLA target gene and GAPDH housekeeping gene according to (AccuPower™ 2XGreen Star qPCR master mix kit. Bioneer. Korea) instructions as given in Table 5.

After that, qPCR master mix reaction component that mentioned above placed in qPCR white tube strips and mixed by Exispin vortex centrifuge for three minutes, then the strips placed in Miniopticon Real-Time PCR system BioRad. The USA as following thermocycler conditions (Table 6).

RESULTS

All the isolates tested and examined by using Reverse Transcription Real-Time PCR assay to detect the gene expression of Erg11 gene between the isolates, as it showed in Table 7 and Figs. 1-3 the second group (T2) are established as higher gene expression when compared with another group, it contain this gene by 56.83%, so this promised these isolates were resistant to antifungal drugs.

DISCUSSION

Many of the reports detected the gene expression of ERG11 gene in *Candida* spp isolates. Some the reports used Reverse Transcriptase Polymerase Chain Reaction techniqueto investigate gene expression ERG11gene in *Candida* spp isolates, which is agreement with our study (Tavakoli *et al*, 2010; AL-ameri *et al*, 2014 and Lee *et al*, 2004).

According to current results, immunocompromised patients (T2) have higher gene expression as compared with other groups (T1) and (T3). Where (T2) recorded (56.83±19.01), While (T1) and (T3)are (9.28±3.16), (6.81±2.20), respectively.

ERG11 is a gene responsible for drugs resistance against azole antibiotic group. Twenty-three isolates carry ERG11 genedetected (Xu *et al*, 2015).

The result agrees with Rosana *et al* (2015) in Indonesia, where the over use and misuse of the fluconazole could lead to the emergence of resistance. ERG11 gene encode aprotein, this protein has resistance chemical against fluconazole antifungal in *Candida albicans* that isolated from HIV patients. The highest gene over expression of ERG11 was found in resistant *C. albicans*.

Also agreement with results of He *et al* (2015), where the mRNA levels of ERG11 gene in Itraconazole-resistant isolates. It shows higher expression compared with other isolates. The highest over expression of ERG11 individually was found in isolates that were resistant to single fluconazole (Yasmon and Lestari, 2015; Chen *et al*, 2010).

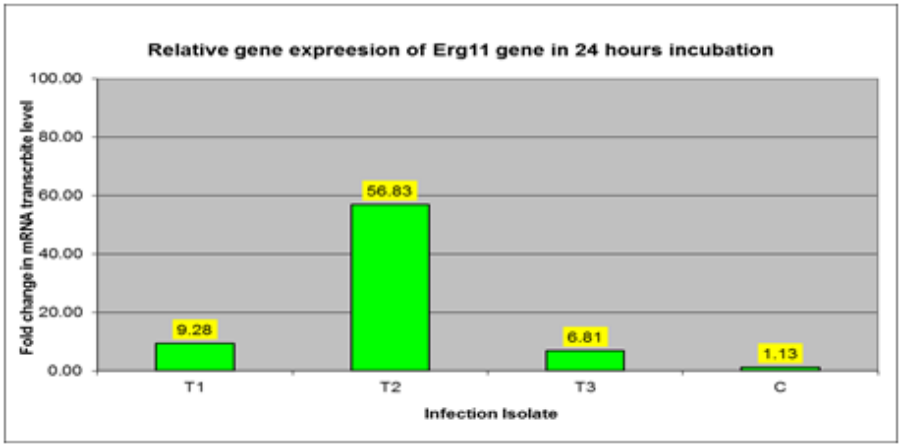

Fig. 1 : Chart show values of gene expression of Erg11 gene.

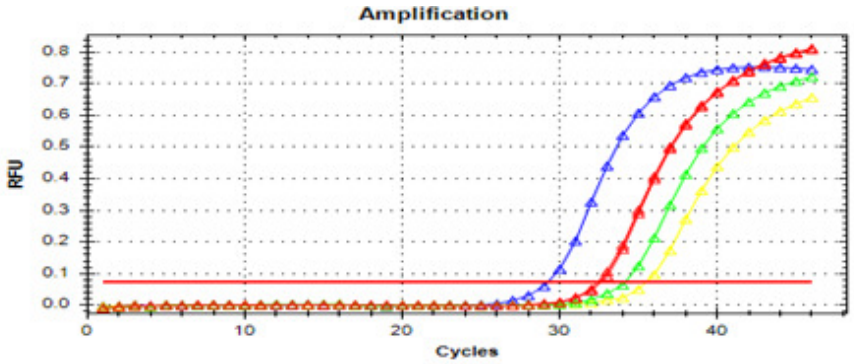

Fig. 2 : Real-Time PCR amplification plots based SYBER green for detection Erg11 producing *Candida albicansi* isolate. Where, the red plot (T1), blue plot (T2), green plot (T3) and yellow plot (C).

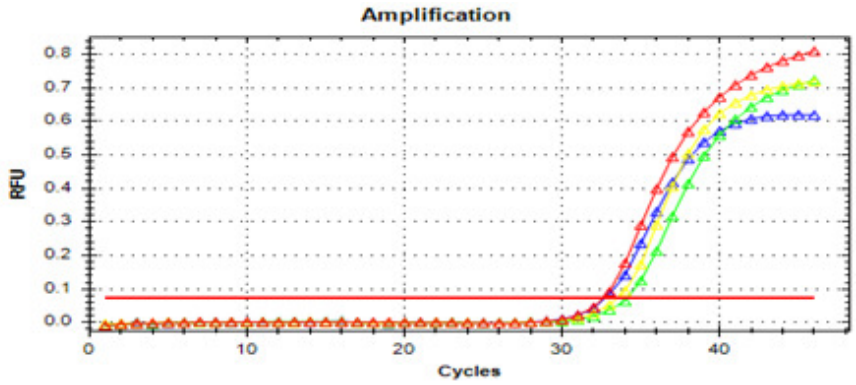

Fig. 3 : Real-Time PCR amplification plots based SYBER green for detection GAPDH producing *Candida albicansi* isolate. Where the red plot (T1), blue plot (T2), green plot (T3) and yellow plot (C).

Table 6 : QPCR thermocycler conditions.

qPCR step	The Temperature	The Time	Repeat cycle
The Initial Denaturation	50 °C	1 hour	1
The Denaturation	95 °C	20 sec	40
The Annealing	60 °C	30 sec	
The Melting	60-95°C	0.5 sec	1

A common use of fluconazolehas the potential to lead to the emergence of resistance in Candida isolates, which have been detected in many countries (Orru *et al*, 2008; Morio *et al*, 2010 and Perea *et al*, 2001). Furthermore, Perea *et al* (2001) found 35% of *Candida albicans* isolates have overexpressed in ERG11gene. However, Loeffler *et al* (2009) found the isolates expressed the ERG11 gene at high levels in highest MIC of fluconazole.

Ge *et al* (2010) reported ERG11gene in *C. albicans* isolated from (23) vaginal isolates, it revealed a high level of gene expression. ERG11 gene is not only responsible for all fluconazole resistance, where other genes contributed to fluconazole resistance, which explains some susceptibility isolates, but it hasn't ERG11 gene. Also, up- or down-regulation of ERG11 gene was not stable (White *et al*, 2002; Marr *et al*, 1998; Park and Perlin, 2005).

The ability of gene expression does not only vary among different species of *Candida* spp, but differs among the strains of same species isolated from different body sites and different patient (Deorukhkar and Saini, 2015 and Ramos *et al*, 2015). 50% of *Candida* isolates showed over expression in one or more of the genes (Sourour *et al*, 2017). The expression of drug resistance gene in *Candida* isolates causes reduced azole susceptibility; the expression depends on several factors such as overuse, misuse and genetic determinant all that will result in a variety of level of expression in different places and time (Li *et al*, 2012). Also, there are some genes that responsible efflux mechanism will be more expression to remove antifungal outside of the cell (Souza *et al*, 2015).

The difference in gene expression levels may be contributed to the contrast in immune status, where the immunocompromised patient is usually

Table 7 : The groups, and the mean of gene expression of Erg11.

Gene expression for (Erg11)							
Treatment Isolate	CT Erg11	CT GAPDH	ΔCT (Test)	ΔCT (contr)	ΔΔCT	Fold change (2^-ΔΔCT)	Mean
T1	31.62	33.79	-2.17	1.97	-4.140	17.63	9.28
T1	32.48	32.66	-.18	1.97	-2.150	4.44	
T1	31.34	32.80	-1.46	1.97	-3.430	10.78	
T1	32.67	32.80	-.13	1.97	-2.100	4.29	
T2	31.56	33.48	-1.92	1.97	-3.890	14.83	56.83
T2	30.34	33.48	-3.14	1.97	-5.110	34.54	
T2	29.23	33.77	-4.54	1.97	-6.510	91.14	
T2	29.30	33.77	-4.47	1.97	-6.440	86.82	
T3	33.62	33.90	-.28	1.97	-2.250	4.76	6.81
T3	33.04	33.90	-.86	1.97	-2.830	7.11	
T3	32.19	33.90	-1.71	1.97	-3.680	12.82	
T3	33.38	32.77	.61	1.97	-1.360	2.57	
C	35.50	33.67	1.83	1.97	-0.140	1.10	1.13
C	35.48	34.00	1.48	1.97	-0.490	1.40	
C	34.78	33.48	1.30	1.97	-0.670	1.59	
C	36.90	33.63	3.27	1.97	1.300	0.41	
Mean C	35.67	33.70	1.97				

T1: *Candida albicans* from Candidiasis infection of calf
T2*: Candida albicans* from Candidiasis immunocompromised patient
T3: *Candida albicans* from Candidiasis of vaginal infection
C: *Candida albicans* from normal individual.

going to clinic centres and hospital more routinely than other patients like women and owners of the calf. Therefore, they get antifungal drugs more than other patients which certainly will cause changes in gene expression.

No doubt that the environmental factors have direct affection on theexpression level of the genes. The immunological factors are one of them, the immunological titer may be playing a great role in an expression where high immune titer will reduce and limited overexpression, and therefore immune titer is high in women followed by children then immunocompromised patient, where candida spp isolates from immunocompromised patient show higher gene expression.

In the end, using antifungal drugs, the patient and immune status interfere with the level of gene expression.

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