Expression pattern of Drug-Resistance Genes ERG11 and TAC1 in Candida albicans Clinical Isolates

Majid Zare-Bidaki
Birjand University of Medical Sciences
Anis Maleki
Birjand University of Medical Sciences
Nahid Ghanbarzadeh
Birjand University of Medical Sciences
Fatemeh Nikoomanesh (g.nikoomanesh@yahoo.com)
Birjand University of Medical Sciences https://orcid.org/0000-0002-2097-7973

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Abstract

Background:

Candida albicans (*C. albicans)* is an opportunistic fungus and the most common cause of vulvovaginal candidiasis (VVC). In recent years, the use of antifungal drugs has led to the incidence of drug resistance. The purpose of this study is two fold: to determine the relationship between risk factors and antifungal resistance among *C. albicans* isolates from women with VVC and to investigate the expression pattern of drug-resistance genes *ERG11* and *TAC1* in *C. albicans* isolates using real-time PCR.

Methods and Results:

This descriptive cross-sectional study was conducted on 50 *C. albicans* isolates from women with VVC. Antifungal susceptibility was tested for 4 azoles (fluconazole, itraconazole, voriconazole, clotrimazole) and amphotericin B via the microdilution methods per the testing standard M27-A3/S4 of the Clinical and Laboratory Standards Institute (CLSI). High susceptibility rates were recorded for itraconazole and voriconazole (68%), followed by ketoconazole (46%). Fluconazole had the smallest impact on *C. albicans* with 36% sensitivity The change in *ERG11* and *TAC1* genes expression was determined by qPCR, and the results revealed that *ERG11* and *TAC1* genes expression had overexpression in some fluconazole-sensitive isolates. The mRNA levels of *ERG11* and *TAC1* genes in fluconazole-resistant isolates compared to susceptible isolates increased by 1.1 and 3.1 times, respectively.

Conclusion:

The results revealed that drug resistance in *C. albicans* is not simply controlled by several genes but is a multi-factorial phenomenon, where several factors and mechanisms are involved in the process of drug resistance.

Introduction

Vulvovaginal candidiasis (VVC), is the most common mucosal infection caused by *Candida albicans* (*C. albicans)*, affects women of reproducing age (17-55years) [1]. Nearly 75 percent of women experience VVC at least once in their lifetime. Approximately, 40 to 50 percent of women have a history of VVC reinfection, and 5 percent of women develop recurrent vulvovaginal candidiasis (RVVC). The latter describes a situation where at least four discrete episodes occur in one year [2–4]. Although the disease is rarely life-threatening, it can lead to high-cost treatment, and in cases, infertility[5]. Even though the presence of itching and burning symptoms is regarded as a strong suspicion for the presence of VVC infection, these symptoms cannot differentiate between recurrent and acute types of the disease; therefore, it may be difficult to choose an appropriate treatment protocol [6].

Similar to other superficial fungal infections, VVC is typically treated with azole antifungal agents, which may include imidazoles (miconazole, clotrimazole, and ketoconazole) and/or triazoles (fluconazole and

itraconazole) [7]. While fluconazole is a first-line azole antifungal drug against VVC, susceptibility to this azole is decreased by various mechanisms of resistance [2, 8]. Indeed, the widespread use of azoles is assumed to be a factor that promotes drug resistance. Many factors are relevant to emerging azole resistance in *C. albicans*, including the expression of drug targets [9]. For instance, upregulation in lanosterol 14-α-demethylase encoded by the *ERG11*gene is essential for the functioning of a microorganism cell [10, 11]. Therefore, overexpression of several genes is associated with fluconazole resistance in *Candida* species. One of the mechanisms of resistance to azole drugs may result from the up-regulation of drug efflux pumps, which are encoded by the ATP-binding cassette transporter genes (*CDR1*, *CDR2*, and *MDR1*) [12, 13]. *TAC1* gene is a classical zinc cluster transcription factor. This gene regulates the expression of efflux pump genes directly [6].

According to the literature, there is a keen interest in research on molecular mechanisms of antifungal resistance [14, 15]. A greater understanding of mechanism-specific resistance and the biological factors relevant to resistance emergence is critical for developing better therapeutics and improving diagnostics and interventions that may overcome and prevent resistance [16].

This study attempted to test azole susceptibility among *C. albicans* isolates and to determine the relationship between risk factors and antifungal resistance of *C. albicans* clinical isolates. Moreover, in this study evaluated the relationship between *ERG11* and *TAC1* genes expressions and resistance to azoles to determine the pattern of molecular mechanism in *C. albicans* isolated from Iranian women with VVC.

Materials And Methods

Study design

This research was conducted on 50 *C. albicans* isolates obtained from women suspected of VVC, who were referred to the obstetrics and gynecological specialists in Birjand city from December 2018 to March 2019. In this regard, 250 patients suspected of VVC were identified and vaginal specimens were collected and were transferred to the laboratory for mycology diagnosis. The patient demographic characteristics including age, history of infection, symptoms, diabetes, method of contraception, and drug consumption were collected. Patients' identifying information was kept confidential. The study protocol was approved by the ethics committee of Birjand University of Medical Sciences, Iran (Ir.bums.REC.1398.350). All participants signed a written consent form. Besides, exclusion criteria comprised pregnant women, menstruating women, and women with a history of hysterectomy.

C. albicans **strains**

The study examined standard fungi strains, including *C. albicans* (ATCC10231) and 50 clinical isolates of *C. albicans* from women who suffered from VVC. The *C. albicans* isolates were confirmed using phenotyping method and confirmed by PCR.

Identification of Candida albicans **isolates**

The isolates for fungal culture was inoculated into Sabouraud Dextrose Agar (SDA Merck, Germany) medium and incubated at 30 °C. The identification of *C. albicans* was done by culture on CHROM agar (CAC, Becton Dickinson, Heidelberg, Germany) is ready to use (32^0C for 24-48h) with which a presumptive identification of *Candida* species can be made on the basis of the morphology and colors of the colonies (*C. albicans* strain produced light green colonies and smooth colony)[17].

Pcr Assay

To confirm the detection of *Candida albicans* species using PCR technique which as carried out as repoted previously [18]. In brief, PCR assay was optimized in a final volume of 25µl consists target primers used to identify were as follows: CALBF: $^{5'}$CCATGTCGAACGTAGCGTATGC$^{3'}$, CALBR-$^{5'}$AGATTATTGCCATGCCCTGAG$^{3'}$.

of *C. albicans* isolates, DNA template and master mix (2X Blue Load master Mix, DNAbiotech, Iran). Genomic DNA from clinical isolates and standard species of *candida* were extracted by glassbead and lysis solution according to the method described previously by Nikoomanesh et al [19]. The sequences of primer was BLAST in NCBI and approved. The primer was synthesized and shipped by Sinacolon Company (Tehran, Iran). Final volume of 25µl using Diethyl pyrocarbonate (DEPC) water. PCR amplification process was carried out with applied PeqSTAR 2X thermal cycler (PEQLAB, Germany) under the following condition: initial denaturation at 95^{o}C for 5min, followed by 35 cycle incubation at 95^{o}C for 45sec, 58^{o}C for 60sec and 72^{o}C for 60sec. and finally 72^{o}C for 5min as final extension. *C. albicans* (ATCC10231) was used for positive control. PCR products were analyzed by electrophoresis through a 2% agarose gel. (Gel Doc XR+, BioRad, USA). Species identification was achieved by discrimination of amplicon sizes (606bp).

Antifungal Susceptibility Testing (Afst)

Assays for susceptibility of *C. albicans* isolates were performed using broth microdilution as per the guidelines of the Clinical and Laboratory Standards Institute (CLSI). Briefly, all isolates were tested against antifungal drugs, including fluconazole, itraconazole, voriconazole, clotrimazole (Sigma-Aldrich, Canada), and amphotericin B (Sigma-Aldrich, Canada). The drugs were obtained from pure powder and prepared at concentrations specified in the M27-A3/S4 standard. The tests were performed in 96-well polystyrene microtitre plates.Antifungal drugs were diluted inspecific dilutions. Afterward, 100µL of RPMI1640 medium with different concentrations of antifungals was added to a series of wells. In the end, 0.5×10^3 to 2.5×10^3 CFU/ml were distributed in each well. The plates were closed and incubated at 35°C for 24 h. The final concentration was in the range 64-0.125 µg/ml for fluconazole and 16-0.03µg/ml for amphotericin B, itraconazole, voriconazole, and clotrimazole. Each *C. albicans* isolate was studied

twice for each antifungal drug. The susceptibility tests were interpreted according to the CLSI M-27 (A3/S4) standard. Lastly,*C. albicans* (ATCC10231) was used for quality control purposes [20].

Quantitative Real-time Pcr (Qrt-pcr)

In total, 50 isolates of *C. albicans* were evaluated for the expression of *ERG11*and *TAC1* genes using q RT-PCR. The primers used to amplify and identify expression of *ERG11* [14], *TAC1* [21], and housekeeping gene *Act1*[20] were as follows: *ERG11*-F: $^{5'}$ACCCTGAAGATTTTGATCCAACTAGATG-$^{3'}$,

ERG11-R: $^{5'}$- CCCAAACCCATAATCAACTTCATCAGA-$^{3'}$,

TAC1-F: $^{5'}$-TGGCAATGTATTTAGCAGATGAGG-$^{3'}$,

TAC1-R: $^{5'}$-TGCTTGAACTGAGGTGAATTTTG-$^{3'}$,

Act1-F: $^{5'}$-GACAATTTCTCTTTCAGCACTAGTAGTG-$^{3'}$,

Act1-R: $^{5'}$-GCTGGTAGAGAGACTTGACCAACC-$^{3'}$

The specific primers for *ERG11* and *TAC1*were designed by using Allele ID primer design software (version 7.5) The control strain ATCC 10231 was used for relative expression comparison.

Total RNA was extracted from cultured colonies of *C. albicans* isolates with glass beads and lysis buffer as described earlier [22]. The cDNA template was obtained from the total RNA usinga cDNA synthesis kit (Parstoos, Mashhad, Iran) as per the manufacturer's protocol. Quantitative real-time PCR was accomplished using AMPLIQON (Real Q plus 2 × master mixes Green High Rox, Sinnaclon, Tehran, Iran). To analyze PCR performance, the mixture containing 12.5 µl of master mix (Green High Rox), 0.25 µl of each specific primer pmol), and 4 µl of cDNA template (10 ng) were adjusted to a final volume of 25 µl using DEPC water. The PCR condition was started at 95°C for 5 min, followed by 40 cycles of 95°C for 15 s, 58°C for 30 s, and 72°C for 30 s. All steps were performed according to the ABI StepOne (Applied Biosystems, Rotkreus, Switzerland). All the experiments were carried out in triplicates. The expression of genes in RT-PCR was analyzed using REST 2009 software (Ver. 2.0.13) ($P < 0.001$).

Statistical Analysis

The collected data were statistically analyzed by SPSS (V. 22) software. Descriptive statistical tests, chi-square test, and t-test were used to analyze the data. The normality of numeric variables was checked by the Kolmogorov-Smirnov test. The association between antifungal resistance with potential risk factors and clinical symptoms was identified by the Chi-square test in univariate analysis. The significance level was set at $P < 0.05$.

Results

In this study,fifty *C. albicans* isolates were confirm by molecular identification *C. albicans*-specific primers which led to PCR product of 606bp for *Candida albicans*, and chromogenic medium were performed. According to the results of culturing on CHROM agar, 50 isolates which produced light green colonies.

Antifungal Susceptibility

The sensitivity of 50 *C. albicans* isolates to antifungals was tested for 1 polyene (amphotericin B) and 4 azoles (fluconazole, voriconazole, itraconazole, and ketoconazole) using the broth microdilution method. According to the Clinical and Laboratory Standards Institute (CLSI M27 (A3/S4) method, the *C. albicans* isolates were categorised as susceptible and resistance to fluconazole; MIC ≥ 8 µg/mL was considered susceptible, MIC ≤ 2 µg/mL was considered resistant, and MIC = 4 µg/mL was dose-dependent susceptibility. Similarly, the isolates were categorized for other azoles; MIC ≤ 0.12 µg/mL was considered susceptible (),MIC ≥ 1 µg/mL was considered resistant. About of amphotericin B ; MIC ≤ 2 µg/mL was considered susceptible, and MIC ≥ 2 µg/mL was considered resistant.

Amphotericin B exhibited an excellent efficacy against all isolates (100% sensitivity). Susceptibility to azoles varied among *C. albicans* isolates. The highest susceptibility was found for itraconazole and voriconazole (68%), followed by ketoconazole (46%) and fluconazole (36%). It was observed that fluconazole had the smallest impact on the *C. albicans* with 36% sensitivity. A detailed analysis of cross-resistance among the four tested azoles revealed that seven *C. albicans* isolates were resistant to all azoles. Antifungal susceptibility is displayed in Table 1.

Table 1
antifungal susceptibility patern of *C. albicans* isolates

Antifungals	**susceptible**		**Susceptible dose dependent**		**resistance**	
	N	%	N	%	N	%
Fluconazole	18	36.0	1	2.0	31	62.0
Itraconzole	34	68.0	0	0	16	32.0
Voriconazole	34	68.0	0	0	16	32.0
Clotrimazole	26	52.0	0	0	24	48.0
Amphotricine B	50	100	0	0	0	0

Risk Factors Associated Withthe Incidence Of Drug Resistance

The mean age (± SD) of study subjects was 31.22 ± 8.45 years. The clinical characteristics of study subjects are summarized in Table 2. Statistical analyses showed a significant difference between the

history of infection and the method of contraception with incidence of fluconazole resistance ($P < 0.05$). Individuals with a history of infection had a two-fold higher risk of developing fluconazole resistance than those who did not have such a history (OR: 2.22, $P = 0.006$). Besides, the patients who used a method of contraception were at a higher risk for incidence of fluconazole resistance (OR: 3.14, $P = 0.002$). However, no significant difference was seen among other characteristics and the incidence of resistance ($P > 0.05$).

Table 2
Comparison of the relationship between patients' risk factors and fluconazole resistance.

parameter	Total of patience N = 50		Susceptible Patience N = 18		Resistance Patience N = 32		Relative risk	OR	p-value
	N	(%)	N	(%)	N	(%)			
History of infection	19	38.0	9	50.0	22	68.8	1.25–3.96	2.22	**0.006**
Yes	31	62.0	9	50.0	10	31.2			
No									
Sign	19	26.0	12	66.7	25	78.1	0.107–0.568	0.24	**0.001**
Yes	37	74.0	6	33.3	7	21.9			
No									
Burning	12	24.0	4	22.2	8	25.0	0.614–2.275	1.18	0.61
Yes	38	76.0	14	77.8	24	75.0			
No									
Itching	12	24.0	4	22.2	8	25.0	0.614–2.275	1.18	0.61
Yes	38	76.0	14	77.8	24	75.0			
No									
Discharge	20	40.0	6	33.3	13	40.0	0.793–2.512	1.41	0.24
Yes	30	60.0	12	66.7	19	59.4			
No									
Lower abdominal pain	18	36.0	5	27.8	13	40.0	0.989–3.227	1.78	0.53
	32	64.0	13	72.2	19	59.4			
Yes									
No									
Intercross pain	12	24.0	4	22.2	9	28.1	0.724–2.626	1.37	0.32
Yes	38	76.0	14	77.8	23	71.9			
No									
Hemorrhage atypical	4	8.0	2	11.1	2	6.2	0.183–1.455	0.51	0.20
Yes	46	92.0	16	88.9	30	93.8			
No									

parameter	Total of patience N = 50		Susceptible Patience N = 18		Resistance Patience N = 32		Relative risk	OR	p-value
Method of contraception	39	78.0	16	89.0	23	71.9	1.466–6.752	3.14	**0.002**
Yes	11	22.0	2	11.1	9	28.1			
No									
Antibiotic used	4	8.0	2	11.1	2	6.2	0.687–5.457	1.93	0.20
Yes	46	92.0	16	88.9	30	93.8			
No									
Diabetes	2	4.0	-	-	2	6.2	-	-	-
Yes	48	96.0	-	-	30	93.8			
No									

Expression Of Antifungal Resistance Genes By Quantitative Real-time Pcr

The expression levels of antifungal resistance genes (*ERG11* and *TAC1*) in 50 *C. albicans* isolates susceptible and resistant to fluconazole were quantified and normalized relative to the housekeeping gene B-actin (*Act1*).

C. albicans isolates showed the highest resistance to fluconazole in this study; hence, they were selected for the evaluation of molecular mechanisms of resistance to fluconazole.

The rates of overexpression and lowexpression of *ERG11* gene among susceptible and resistant *C. albicans* isolates are shown in Fig. 1. The results show that overexpression of the *ERG11* gene was observed in all isolates of the resistant group (100%). Overexpression of the *TAC1* gene in the resistant group had the highest rate (96.8%) compared to the sensitive group (44.4%).The results of the t-test analysis showed a significant difference between the rate of overexpression and lowexpression of *ERG11* and *TAC1* genes ($P < 0.001$).

The mean relative gene expression levels of *ERG11* in susceptible and resistant isolates were 135.75 ± 150.4 and 170 ± 152.64, respectively. The mean relative gene expression levels of *TAC1* in susceptible and resistant isolates were 82 ± 128.21 and 173.85 ± 175.63, respectively.

Quantitative RT-PCR experiments revealed that the mean fold change in the expression levels of the *ERG11*gene was 1.1. The mean fold change in the expression levels of the *TAC1* gene was a 3.1 fold

change. There was a statistically significant difference in the levels of *ERG11* and *TAC1* expression in resistant isolates compared to susceptible isolates ($P < 0.001$) (Fig. 2).

Discussion

VVC is a widespread fungal infection that is induced primarily by *C. albicans* and affects reproductive-age women [2]. Although VVC is primarily a challenging therapy worldwide, some socioeconomic factors could affect its incidence [4]. Besides, service planning managers should utilize updated epidemiological data from across the world.

In the recent decade, the amount of fungal resistance to antifungal drugs has increased and has led to therapeutic problems [22]. In the present study, the 50 *C. albicans* isolates that were tested for susceptibility to antifungals belonged to the polyenes group (amphotericin B) and azoles group (fluconazole, voriconazole, itraconazole, and clotrimazole) via the microdilution method. The results obtained from amphotericin B exhibited an excellent susceptibility to all *C. albicans* isolates. This finding agrees with the results reported in studies by Eski et al. and Mohammadi et al. concerning an absence of amphotericin B-resistant isolates [23, 24]. Besides, susceptibility to different azoles drugs varied among *C. albicans* isolates, with lower susceptibility of *C. albicans* isolates to fluconazole (36%) followed by clotrimazole(52%). Meanwhile, itraconazole and voriconazole (68%) exerted similar effects.

Many studies have highlighted the resistance of *C. albicans* to azole antifungal drugs, especially fluconazole [2, 20–24]. A previous study conducted by Mohammadi et al. found the highest resistance to fluconazole (76%) compared to other antifungals (itraconazole 62%, ketoconazole 72%, clotrimazole 55%, voriconazole 6%, posuconazole7%, nystatin1%, and amphotericin B 0%) [24].

Lower susceptibility rates of *C. albicans* to fluconazole have been reported from Brazil (68%; 2011), India (48%; 2013), and Egypt (89%; 2016), respectively [26, 29, 30].

The widespread use of fluconazole against prophylaxis and *Candida* infections may be one of several factors contributing to antifungal resistance. *C. albicans* has developed multiple resistance mechanisms against azole antifungal drugs, leaving the way open to only a few antifungal agents available [31]. Therefore, it is of particular importance to investigate the mechanisms and patterns of antifungal resistance. In the current study, the analyses of the association between risk factors and incidence of fluconazole resistance showed higher rates of anti fungal resistance in women with a history of vaginitis. Moreover, the patients who had used a contraception method were at a greater risk to develop drug resistance (Table 2). As a result, the history of infection indicates that a failure in the treatment of primary infection leads to the emergence of resistant species. Also, any factor that predisposes to infection can lead to the emergence of resistant species.

Findings that arise from such research can contribute to designing and developing alternative therapeutic strategies. Additionally, understanding the molecular mechanism of antifungal resistance and the role of

genes relevant to drug resistance can be used to diagnose resistant isolates via molecular diagnostic tools.

Previous research has reported overexpression of ATP-binding cassette transporters, which are encoded by different genes, primarily *CDR1* and *CDR2*, in *C. albicans*. *TAC1* gene is the first transcription factor that regulates efflux pump family genes [32, 33]. However, it is well documented that the most significant factor involved in *C. albicans* resistance to azole antifungal agents is the overexpression of the *ERG11* gene [34, 35].

According to our results, *TAC1* gene expression was not merely increased in fluconazole-resistant isolates; it was also observed in sensitive isolates. The *TAC1*gene was found to have overexpression rates in resistant and sensitive isolates, respectively, with statistically significant differences between the two groups ($P < 0.001$). Moreover, fluconazole-sensitive isolates had *ERG11* gene overexpression. In fact, all resistant isolates had *ERG11* gene overexpression at significantly higher levels than the ATCC 10231 strain. Based on the results, we witnessed an overexpression of *ERG11* and *TAC1* in susceptible isolates. Despite the increase in gene expression in susceptible isolates, these isolates are sensitive to fluconazole.

In a previews study by Riberio et al. after fluconazole treatment, they observed *ERG11*gene overexpression in fluconazole-sensitive *C. albicans* isolates. They concluded that the overexpression of the *ERG11* gene is accompanied by the overexpression of other efflux pump genes and that the *ERG11* expression is not the only cause of increased resistance to fluconazole [36]. Teimouri et al. detected *ERG11* gene overexpression in eight resistant isolates of *C. albicans* from infants [37]. Another study conducted by Sasse et al. evaluated the expression of *TAC1* and *MRR1* transcription factors [38]. They investigated mutations in the gene sequence after they observed an increase in the expression of *TAC1* and *MRR1* genes. The results of this study showed that the increase in *TAC1* gene expression is sometimes up to 500-fold in the development of fluconazole resistance, arguably due to mutations in this gene. Shi et al. investigated the expression rates of efflux genes (*CDR1*, *CDR2*, *MDR1*, *Erg11*, and *TAC1*) in the biofilm formation process on fluconazole-resistant isolates of *C. albicans* [39]. The results showed that the increase in *TAC1* expression occurred in the early stages (first 8 hours) and that the expression increase was nearly two-fold. In a study by Chen 2010 and Xu in 2008, over 143 mutations,contributing to increased *ERG11* gene expression, were reported in fluconazole-resistant *C. albicans* isolates [40, 41].

The results obtained from this study, indicate that the occurrence of drug resistance can be directly related to increased expression of *TAC1* which as transcription gene. In addition to increasing the expression of drug resistance genes, the incidence of drug resistance can be directly related to patient risk factors such as the use of broad-spectrum antibiotics, immune system function, and underlying diseases such as diabetes [10]. As observed, there is a significant relationship between the resistance to azole resistance and history of infection or the use of contraceptive methods.

This study has some limitations. In this study, the mutations of the studied genes were not investigated due to limited facilities. Therefore, future research may aim to detect the frequency of definite mutations in the *C. albicans ERG11* and *TAC1* genes and its targets in resistant isolates.

Conclusion

The results showed that *C. albicans* isolates were more resistant to fluconazole than other antifungal agents. Also, the incidence of drug resistance is associated with some patient risk factors such as history of infection and the method of contraception. Molecular studies on *ERG11* and *TAC1* genes expression revealed that these genes had overexpression in some fluconazole-sensitive isolates. The results also revealed that drug resistance in *C. albicans* is not simply controlled by several genes but is a multi-factorial phenomenon, where several factors and mechanisms are involved in the process of drug resistance. It can be concluded that drug resistance is not merely controlled by genes but affectted mutations on genes expression.

Declarations

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Code availability: Not applicable

Author contributions statement

Carried out the statistical analysis and edited the final version of the manuscript by FN and AM. Preparation the paper, helped to carry out the study by MZB. Designed and supervised the study by NGH and FM.

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Figures

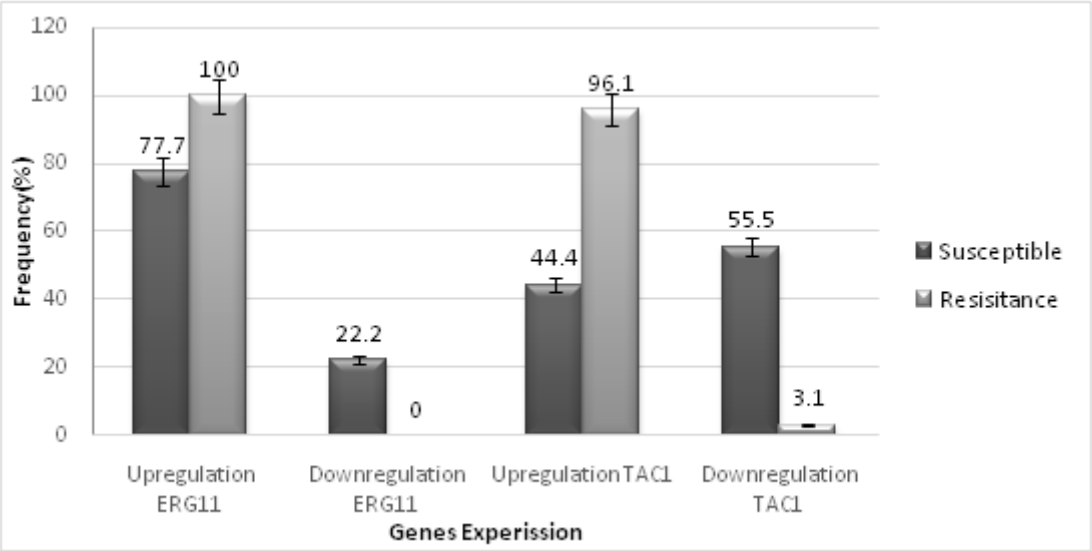

Figure 1

Comparison of Expression Decrease, Increased Expression in Expression of *ERG11* and *TAC1*Genes in Clinical isolates of *C. albicans*.

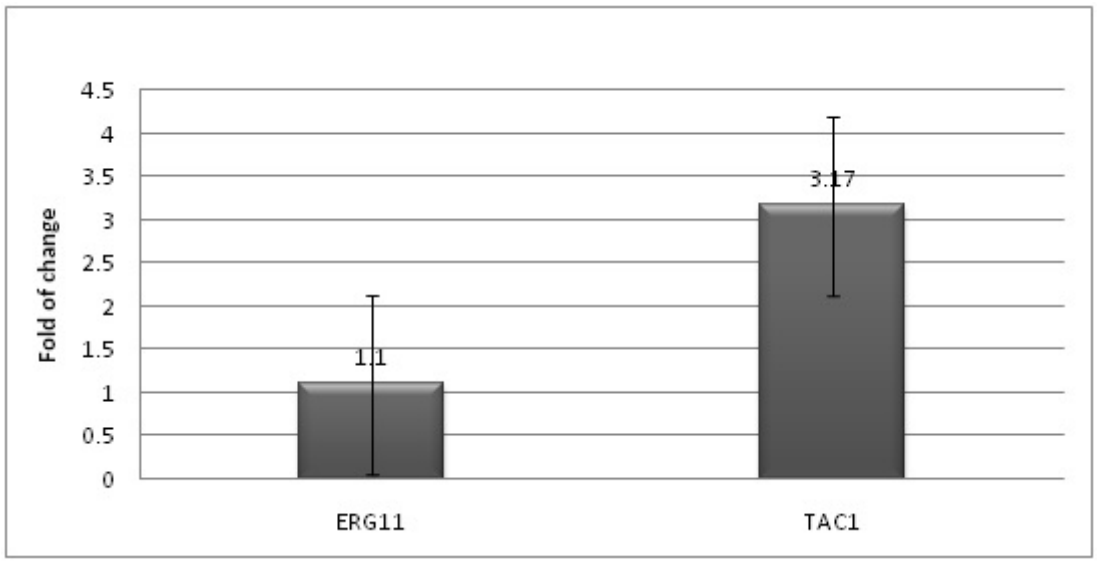

Figure 2

The fold change expression of *ERG11* and *TAC1* genes in resistant isolates compared to fluconazole sensitive isolates.