Original Article

Differential Expression Levels of Agglutinin-Like Sequence, Lipase, and Secreted Aspartyl Protease Genes in *Candida tropicalis* Treated with Fluconazole Alone and in Combination with Clotrimazole

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Abstract

Background: The frequency of opportunistic fungal infections in immunocompromised patients, especially by *Candida* species, has sharply increased in the last few decades. As the number of antifungal drugs available for the treatment of candidiasis is limited, combination therapy has been employed as one of the most commonly used techniques to alleviate this problem. **Aims:** The main aim of this study was to explore the antifungal activity of fluconazole in combination with clotrimazole on expression levels of virulence genes, agglutinin-like sequences (*ALS1* and *ALS2*), lipases (*LIP1* and *LIP4*) and secreted aspartyl proteases (*SAP2* and *SAP4*) in *Candida tropicalis*. **Methods:** Ten infected clinical isolates obtained from recurrent vulvovaginal candidiasis patients were used in this study. The broth microdilution assay was utilized to investigate antifungal susceptibilities to fluconazole alone and in combination with clotrimazole and the synergistic effects were interpreted with reference to the fractional inhibitory concentration (FIC) index model. The expression levels of *ALS1, ALS2, LIP1, LIP4, SAP2* and *SAP4* genes were quantified by real-time RT–PCR. **Results:** Antifungal susceptibility results showed that isolates were resistant to at least one type of azole antifungals. The combination of fluconazole with clotrimazole revealed synergistic effects against *C. tropicalis* isolates with FIC_{90} index ranging from 0.011 to 0.43. The results indicated that combination of fluconazole with clotrimazole could cause a down-regulation of gene expression of *ALS1, SAP2, LIP4, SAP4, LIP1* and *ALS2* genes, respectively. **Conclusions:** Fluconazole in combination with clotrimazole may diminish the virulence properties of *C. tropicalis*.

Keywords: *Antifungals, Candida tropicalis, clotrimazole, fluconazole*

Introduction

Candida tropicalis has been identified as one of the most virulent species of the *Candida*-non-*albicans* group. The distribution and frequency of *Candida*-non-*albicans* species vary geographically. *C. tropicalis* has been widely considered the second to fourth among the most commonly isolated species.[1-5]

Various factors have been reported to contribute to the virulence of *C. tropicalis* including adhesion to medical devices and host surfaces, biofilm formation, yeast-to-hyphae transition (morphogenesis), phenotypic switching, thigmotropism and the secretion of hydrolytic enzymes, including secreted aspartyl proteases (SAPs), esterases, lipases, phospholipases, and hemolysins.[5-8]

As an important step in pathogenesis, adhesion leads to tissue damage and invasive infections. The agglutinin-like sequence (ALS) protein family form important adhesion molecules. The composition of the *ALS* family of *C. tropicalis* includes 16 genes which encode cell-surface glycoproteins contribute to adhesion.[5,8,9] In addition, the activity of hydrolytic enzymes has been associated with infections thought to facilitate the disruption of host tissues, degrade defense proteins, and enhance the efficiency of acquisition nutrients. Moreover, the family of Saps comprising one subfamily of four genes (SAPT1-4) is recognized as an important virulence factor to the pathogenesis of *C. tropicalis*. In *C. tropicalis*, the family of lipases consists of 10 members (LIP1-10), which are involved in the triacylglycerols hydrolysis and synthesis.[5,10-12]

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Various imidazole antifungal drugs such as clotrimazole have been claimed to inhibit *Candida* growth. Moreover, triazoles such as fluconazole represent the wide spectra of antifungal activity against fungi. Although azole antifungals are members belonging to the same class of antifungal drugs, they represent largely different chemical properties which impact the pharmacokinetics and spectrum of activities. All azole antifungals inhibit the function of the cytochrome P450 system to some degree of specificity.[13,14] The increasing rate of azole drug-resistance of *C. tropicalis* has been documented. The number of antifungal agents is limited and their mostly fungistatic activity facilitates selection of antibiotic-resistant strains.[1,3,15] Thus, advances in the development of candidiasis therapies have focused on the use of antifungal agents in combination.[16] In this study, we first investigated the antifungal susceptibilities to fluconazole alone and in combination with clotrimazole using the broth microdilution method interpreting the synergistic effects by the fractional inhibitory concentration (FIC) index model. Finally, the antifungal effect of fluconazole in combination with clotrimazole on expression levels of *ALS1*, *ALS2, LIP1*, *LIP4*, *SAP2*, and *SAP4* genes in *C. tropicalis* were examined.

Materials and Methods

Candida tropicalis and growth conditions

Candida tropicalis ATCC 750 and ten infected clinical isolates obtained from recurrent (defined as 3 or more per year) vulvovaginal candidiasis patients who had previously used clotrimazole in the past 5 years were used in this study. All isolates were maintained on Sabouraud Dextrose Broth (Merck Research Laboratories, Darmstadt, Germany). Before experiments, the isolates were grown overnight at 35°C on Sabouraud Dextrose Agar (Merck Research Laboratories) plates.[17]

Antifungal agents

The antifungal agents used in the present study, i.e., fluconazole and clotrimazole, were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The antifungal agents were then dissolved and the stock solutions were diluted based on CLSI M27-A3 guidelines.[18]

Preparation of the *Candida tropicalis* cell inocula

The *C. tropicalis* cell density of five colonies more than 1 mm in diameter of propagated cells, suspended in 5 ml sterile phosphate-buffered saline was estimated through measurement of the optical density at 530 nm. The cell suspension measured at OD_{530} was made in Roswell Park Memorial Institute-1640 medium (Sigma-Aldrich) and prepared in accordance with CLSI M27-A3 guidelines. Briefly, the cell suspension (a concentration of 1–5 × 10^6 colony-forming units (CFU)/ml) was diluted to 5 × 10^2–2.5 × 10^3 CFU/ml and the viability of the yeast cells was measured through the viable pour plate counting method.[18]

Broth microdilution assay

The broth microdilution assay of fluconazole alone and in combination with clotrimazole was performed in accordance with the CLSI (M27-A3, M27-S4) guidelines with a few adaptations.[18,19] Briefly, 5 × 10^2–2.5 × 10^3 CFU/ml inoculum was added to the wells of a U-bottom polystyrene 96-well microdilution plates. One hundred µl of the two-fold dilution of each antifungal agent (alone or in combination) dilution was added to the respective wells with the cell suspension. Plates were incubated for 24 h at 35°C. The minimal inhibitory concentrations (MICs) were determined using a Stat Fax 303 Reader (Awareness Technology, Inc., USA) as the lowest concentration of antifungal agents required for 50% and 90% growth inhibition compared to the controlled growth. Clearly, the antifungal agent interactions were interpreted by the FIC index model.[20,21]

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

The qRT-PCR analysis was based on the procedure previously described.[22] The inoculum of 1-5 × 10^6 CFU/ml *C. tropicalis* ATCC 750 strain was treated with fluconazole alone and in combination with clotrimazole at concentrations of 2 × MIC and 1 × MIC. Next, the mixture under study was pelleted at 3000 rpm for 10 min. For each treatment, the total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's recommended protocol. The RNA quality was observed using the formaldehyde-denaturing agarose gel electrophoresis. The $OD_{260/280}$ and $OD_{260/230}$ were measured with a NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies Inc., Wilmington, DE). RNAse-free DNase I (Fermentas, USA) was used for the removal of genomic DNA contamination. Single-stranded cDNA was synthesized using M-MuLV reverse transcriptase and random hexamer oligonucleotides (Fermentas, USA) in accordance with the manufacturer's instructions. The synthesized cDNA formed in each treatment was amplified with primers [Table 1] by PCR using ™SYBR Green qPCR Master Mix (Fermentas, EU) in a Bio-Rad MiniOpticon™ system (USA). The cycling conditions included an initial step at 50°C for 2 min; holding at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 s and subsequently annealing at 55°C for 1 min. Finally, the melting reaction occurred at 72°C–99°C. The results from amplifications were quantified by the Pfaffl method.[22]

Ethical issues

The Research Ethics Committees of our institute, Islamic Azad University of Yasooj, Iran (Ethical code 1213342) approved the study. The study protocol conformed to the ethical guidelines of the 2008 Declaration of Helsinki. Informed consent was obtained from patients.

Statistical analysis

Results represent the mean of the three independent experiments ± standard deviations. Data were subjected to analysis of variance. The comparison of two means was calculated using the Tukey's *post hoc* test. Value of $P \leq 0.05$ was considered statistically significant. Statistical analyses were performed using the software SPSS 21.0 for Windows (SPSS Inc. Chicago, IL, USA).

Results

The age range of studied patients was 23–48 years old. The overall isolation rate of *Candida* species from vulvovaginal samples was 93.50%. The incidence rate of *C. tropicalis* was found in 10 of 187 (5.35%) of *Candida* species.

The antifungal effect assay setup was based on CLSI guidelines for the broth microdilution assay and the MICs of fluconazole alone and in combination with clotrimazole was determined against *C. tropicalis* [Table 2]. From among the 10-infected clinical isolates of *C. tropicalis*, 6 (60%) were resistant to fluconazole (MIC ≥8 μg/ml) and 10 (100%) were resistant to clotrimazole (MIC ≥1 μg/ml). A comparison between these values for fluconazole alone and in combination with clotrimazole against *C. tropicalis* showed that the combination of fluconazole with clotrimazole can be decreased from its MIC value. It is also noteworthy to mention that the FIC index values of all *C. tropicalis* tested (100%) were synergistic effects (FIC ≤ 0.5) in the presence of fluconazole in combination with clotrimazole. Fluconazole exhibits synergy with clotrimazole with a FIC_{90} index ranged from 0.011 to 0.43 in *C. tropicalis* isolates.

Table 1: Oligonucleotide primers used for quantitative real-time polymerase chain reaction

Primer	Sequence (5'-3')	Reference
ALS1	Forward GGGCTCTGGTCGTGATGT	[8]
	Reverse GTGAGGGAATGAGTCTTG	
ALS2	Forward ACTCGTGCCTATACCTAC	[8]
	Reverse TTGTTGCCGTAATGGTGG	
LIP1	Forward TGGGCAGCACCAATCAAAT	[8]
	Reverse GGGTAGACAATCGGGACA	
LIP4	Forward TTGACTGTGCTCCTTCCT	[8]
	Reverse GCTTTGGACCTTCGTAAT	
SAP2	Forward GCTGGTTTCTGTGCTTTG	[8]
	Reverse CCACGTAGGCATGTCTTA	
SAP4	Forward CTTCACCTCCTGGTTTCATTTC	[8]
	Reverse TCAACTACCCATAAATCAGAGG	
ACT1	Forward GACCGAAGCTCCAATGAATC	[23]
	Reverse AATTGGGACAACGTGGGTAA	

The analysis of the effect of fluconazole alone and in combination with clotrimazole against *C. tropicalis* ATCC 750 on their expression levels of *ALS1*, *ALS2, LIP1*, *LIP4*, *SAP2,* and *SAP4* genes revealed that the overall genes expression levels of the treatments differed significantly ($P < 0.05$). The results of the qRT-PCR analysis showed that fluconazole alone caused down-regulation in the expression levels of *SAP2*, *SAP4* and *LIP4* ($P < 0.05$) and the expression levels of *ALS1*, *ALS2,* and *LIP1* represented no significant changes ($P = 0.20$). The expression of *LIP1*, *LIP4*, *SAP2,* and *SAP4* genes was decreased on clotrimazole challenge. The combination of fluconazole with clotrimazole showed down-regulation of genes compared to the untreated control ($P < 0.05$; Figure 1).

The results showed that fluconazole in combination with clotrimazole at concentrations of 2 × MIC and 1 × MIC down-regulated the expression levels of *ALS1* by 3.75- and 3.13-fold and *ALS2* by 2.00- and 1.89-fold, respectively ($P < 0.05$). The expression levels of *LIP1*and *LIP4* genes were down-regulated by 2.18- and 1.95-fold and 2.90- and 1.55-fold in *C. tropicalis* treated with 2 × MIC and 1 × MIC of fluconazole in combination with clotrimazole, respectively ($P < 0.05$). The expression

Table 2: Minimal inhibitory concentration (μg/ml) and fractional inhibitory concentration values of fluconazole alone and in combination with clotrimazole against *Candida tropicalis*

Isolates/antifungal agents	Fluconazole		Clotrimazole		Fluconazole/clotrimazole				Outcome
	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	FIC_{90}	FIC_{50}	
Candida tropicalis ATCC 750	3.50±0.10	0.50±0.08	0.60±0.09	0.35±0.05	0.20±0.09	0.065±0.09	0.39	0.32	Synergy
CI-1	12.50±0.20	1.80±0.09	1.50±0.01	0.20±0.02	0.30±0.05	0.09±0.002	0.22	0.50	Synergy
CI-2	12.30±0.10	1.60±0.02	1.40±0.30	0.39±0.00	0.20±0.03	0.065±0.003	0.16	0.21	Synergy
CI-3	3.30±0.20	0.60±0.01	2.10±0.01	0.50±0.09	0.40±0.02	0.09±0.005	0.31	0.33	Synergy
CI-4	12.60±0.30	1.50±0.00	2.00±0.02	0.32±0.01	0.20±0.04	0.062±0.001	0.12	0.23	Synergy
CI-5	3.50±0.20	0.70±0.08	2.25±0.09	0.38±0.08	0.50±0.05	0.07±0.009	0.36	0.28	Synergy
CI-6	12.50±0.30	1.50±0.02	1.50±0.02	0.47±0.02	0.25±0.01	0.09±0.001	0.19	0.25	Synergy
CI-7	3.60±0.10	0.60±0.01	2.20±0.20	0.50±0.01	0.40±0.02	0.086±0.001	0.28	0.31	Synergy
CI-8	3.50±0.40	0.50±0.02	1.70±0.30	0.40±0.01	0.50±0.02	0.091±0.009	0.43	0.41	Synergy
CI-9	12.50±0.20	1.70±0.01	1.90±0.20	0.35±0.09	0.018±0.005	0.096±0.002	0.011	0.33	Synergy
CI-10	12.90±0.30	1.80±0.07	1.80±0.10	0.43±0.01	0.30±0.01	0.08±0.001	0.19	0.23	Synergy

Data are means±SD of three-independent experiments. SD: Standard deviation, MIC: Minimal inhibitory concentration, FIC: Fractional inhibitory concentration; CI: Clinical isolates of *Candida tropicalis*

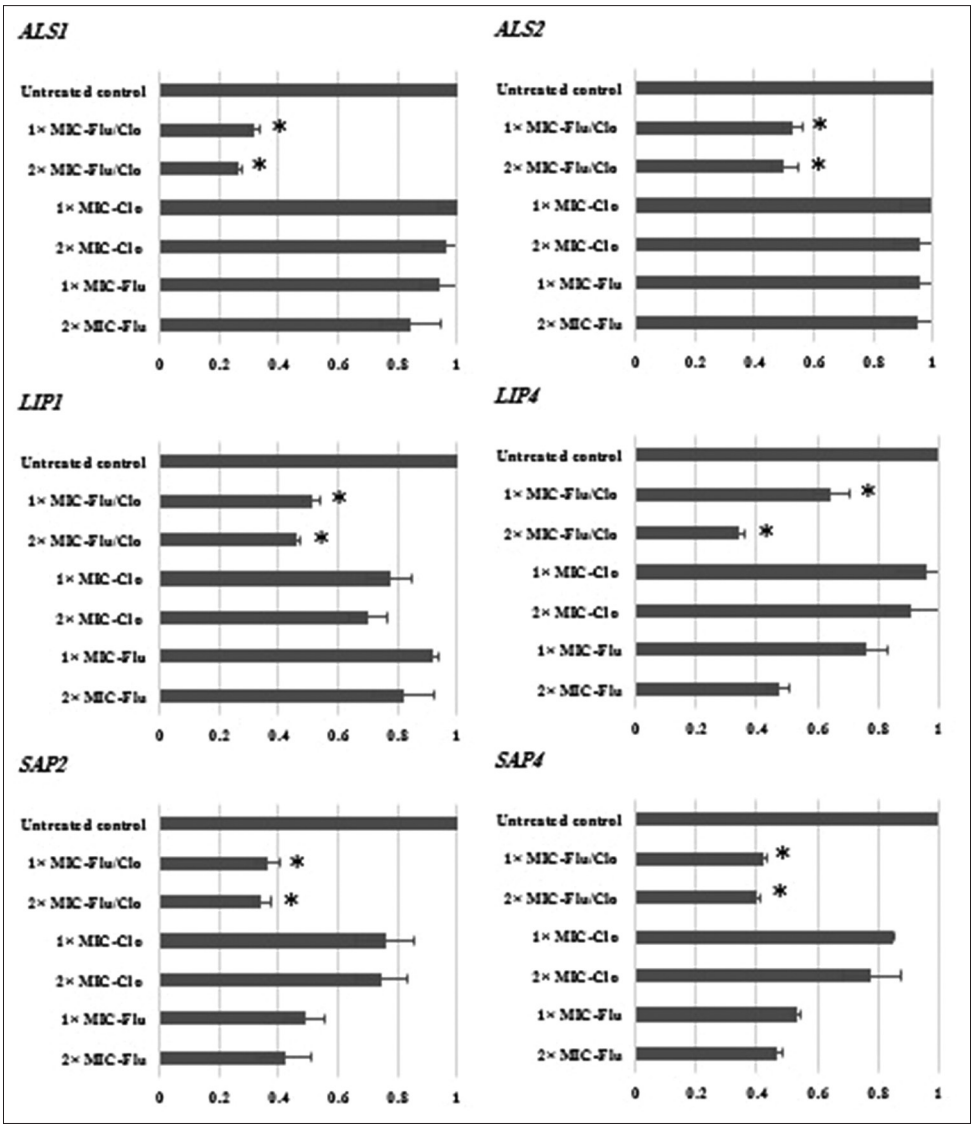

Figure 1: Expression analysis of agglutinin-like sequence 1, agglutinin-like sequence 2, *lipase 1, lipase 4,* secreted aspartyl protease 2 and secreted aspartyl protease 4 genes in *Candida tropicalis* ATCC 750 treated with 2 × minimal inhibitory concentration and 1 × minimal inhibitory concentration of fluconazole alone and in combination with clotrimazole by quantitative reverse transcription polymerase chain reaction. (*) means significant reduction of gene expression to untreated control at level <0.05

level of *SAP2* was down-regulated by 2.91- and 2.74-fold and *SAP4* down-regulated by 2.51- and 2.038-fold with 2 × MIC and 1 × MIC of fluconazole in combination with clotrimazole treatment, respectively ($P < 0.05$). However, the combination of fluconazole with clotrimazole could cause a down-regulation of gene expression of *ALS1, SAP2, LIP4, SAP4, LIP1,* and *ALS2* genes, respectively ($P < 0.05$).

Discussion

In the past decades, there has been renewed interest in investigating the combination therapy effect of antifungal agents for the treatment of candidal infections that are difficult to treat. Combination therapy is the potential strategy used to reduce resistance development, minimize their side effects/toxicities, increase the rate or extent of killing by synergy and may broaden the spectrum of activity.[24-26]

Azoles are the initial treatment of choice for antifungal treatment of candidiasis. Nevertheless, the development of antifungal resistance, drug-drug interactions, and severe side effects/toxicities has limited their effective therapeutic for fungal diseases. Therefore, the development of the effectiveness and acceptability therapeutic strategies for fungal diseases is warranted to overcome antifungal agent resistance and its side effect/toxicity.[24,27,28] In the present study, we observed significant results of resistance to clotrimazole in infected clinical isolates of *C. tropicalis* obtained from recurrent vulvovaginal candidiasis patients with previous exposure to clotrimazole. Similar results were obtained by Pelletier *et al.*[29] for the HIV-infected pediatric population receiving this azole. Diaz *et al*.[30]

showed resistance to clotrimazole of vaginal *C. tropicalis* isolates.

The results from the present research show that fluconazole in combination with clotrimazole exhibit potent antifungal synergy against all clinical isolates of *C. tropicalis*. Combination therapy of fluconazole with clotrimazole was as effective as single dose fluconazole in vulvovaginal mycoses.[31] Gharibi *et al*.[32] evaluated the effectiveness of fluconazole and clotrimazole combination in the treatment of recurrent vaginal candidiasis caused by *C. albicans*. They observed that for the treatment of patients with recurrent vulvovaginal candidiasis, fluconazole in combination with clotrimazole can be more effective than other treatments. Our results show that *in vitro* combination of fluconazole with clotrimazole could be effective against *C. tropicalis*.

The gene expression analysis of *C. tropicalis* treated with fluconazole alone and in combination with clotrimazole revealed that fluconazole in combination with clotrimazole could down-regulate the expression levels of genes. The results from the present research also revealed that the expression levels of *ALS1*, *ALS2, LIP1*, *LIP4*, *SAP2,* and *SAP4* were affected by fluconazole in combination with clotrimazole in *C. tropicalis* which is in agreement with the recent experimental results of Khodavandi *et al*.[33] The findings revealed that fluconazole in combination with amphotericin B significantly down-regulated the expression of *PLB* and *SAP2* genes in *C. tropicalis.* Moreover, the expression levels of *ALS1* and *ALS2* differed significantly in *C. tropicalis* treated with the combination of the two azoles. This may occur due to distinct adherence and biological functions of *ALS* family genes. Research suggests that the *ALS* family form important adhesion molecules for C. *tropicalis* pathogenicity.[5,8,9] Roudbarmohammadi *et al*.[34] investigated the expression of *ALS1* and *ALS3* genes in *C. albicans* isolated from vulvovaginal candidiasis. The results indicated that the expression of *ALS1* and *ALS3* genes was greater than that of the control group.

Our results showed that fluconazole alone and in combination with clotrimazole can induce changes in the expression levels of hydrolytic enzymes (*LIP1*, *LIP4*, *SAP2,* and *SAP4*), which are recognized as important virulence factors for C. *tropicalis* pathogenicity.[5,10-12] Our results consistent with those obtained by Gu *et al*.,[35] who showed that the combinations of fluconazole with fluoxetine cause a down-regulation of gene expression of SAP1-4 *C. albicans* strains. The expression of virulence genes (ALST1-3, *LIP1*, *LIP4*, and SAPT1-4) was investigated in *C. tropicalis* strains with diverse virulence. RT-PCR analysis showed that the expression of virulence genes was significantly different in the corresponding genes for most *C. tropicalis*.[8] Stehr *et al*.[36] investigated the expression pattern of *LIP1–LIP10* genes in *C. albicans* during experimental infections and in samples of patients with oral candidiasis. The findings of Stehr *et al*.[36] showed that individual lipase genes were differentially expressed in a mouse model of systemic candidiasis and in human specimens. Khodavandi *et al*.[37] showed that the expression of *SAP4* gene was down-regulated in *C. albicans* treated with fluconazole.

Conclusions

This study illustrates the potent synergist activity of fluconazole in combination with clotrimazole against *C. tropicalis* demonstrating the down-regulation of *ALS1*, *ALS2, LIP1*, *LIP4*, *SAP2,* and *SAP4* genes in C. *tropicalis* treated with fluconazole in combination with clotrimazole, as assessed in the qRT-PCR assay. Further research needs to be conducted to ascertain whether these events reflect the potential of fluconazole in combination with clotrimazole for the inhibition of virulence factors of *C. tropicalis* which differentially expresses specific gene. However, the present research demonstrated that the antifungal effect of fluconazole in combination with clotrimazole could be of great significance for the development of therapeutic strategies against resistant *C. tropicalis.*

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Conflicts of interest

There are no conflicts of interest.

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