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Detection of ERG11 gene in fluconazole resistant urinary *candida* isolates

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Abstract

Candida species resistant to fluconazole and voriconazole were screened for the presence of ERG11gene by polymerase chain reaction (PCR). Also, the association of this gene with the demonstration of *Candida* virulence factors; biofilm formation, phospholipase and proteinases activities were studied. A total of 61 *Candida* isolates were collected from urine specimens. *Candida* species were identified by API 20 C Aux test. Extracellular phospholipase, secretory aspartyl proteinase and biofilm formation were determined. ERG11 gene was detected by PCR. *C. albicans* was identified in 34.5%, *C. glabrata* in 29.5% and *C. tropicalis* and *C. krusei* in 18% each. *Candida* species was resistant to fluconazole and voriconazole in 55.7% and 27.9%, respectively. Seventeen (50%) of fluconazole resistant *Candida* isolates were sensitive to voriconazole. The most frequently *Candida* species revealed fluconazole resistance were *C. glabrata* (47.1%), *C. krusei* (29.4%), and C*. tropicalis* and *C. albicans* (11.8% each). Biofilm formation, phospholipase and proteinase activity were determined in 41.2%, 67.6% and 35.3% of fluconazole resistant *Candida* isolates, respectively. *Erg 11* gene was determined in 82.4% of fluconazole resistant *Candida* isolates and prominent in *C. glabrata* (93.75%), followed by *C. krusei* (90%), *C. tropicalis* (75%) and *C. albicans* (25%). *Erg 11* gene was detected in 64.7% (11/17) of fluconazole resistant-voriconazole sensitive *Candida* isolates. Regarding, correlation of *Erg11* gene positivity and virulence factors among fluconazole resistant *Candida* isolates, 34.5% exhibited biofilm formation and 62.1% and 31% showed phospholipase and proteinase activities, respectively. There were statistically significant difference concerning the association of proteinase activities and Erg 11 gene expression among fluconazole resistance *Candida* isolates (*P*=0.04). The study emphasizes the high prevalence of Erg11 gene among fluconazole resistant *Candida* species. There was association between the proteinase activity, fluconazole resistance and the presence of *Erg11* among *Candida* species. Voriconazole maintains better activity towards *Candida* species and represent an alternative therapy.

Keywords: ERG11 Gene, Fluconazole, Urinary, *Candida*, PCR

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Introduction

Candida species are significant opportunistic pathogens that have been associated with high morbidity and mortality especially in immunocompromised patients. Although *Candida albicans* (*C. albicans)* is the most pathogenic species often isolated from patients with invasive infections, non-*albicans Candida* (NAC) infections have been increased dramatically in recent years.[1] *Candida albicans, Candida glabrata, Candida tropicalis, Candida parapsilosis*, and *Candida krusei* are estimated to cause more than 90% of invasive fungal infections.[1,2]

Global awareness of fungal disease should be increased as the infection is still underestimated although *Candida* spp. are the fourth most common cause of bloodstream infections in hospitalized patients and the third common cause of central-line associated invasive infections among intensive care unit (ICU) patients.[1] In addition, NAC have the potential to cause outbreaks, recurrent infections and resistance to antifungal drugs as some species such as *C. glabrata* and *Candida krusei* have higher minimum inhibitory concentration (MIC) values towards antifungal azoles.[2,3]

Candida is a common fungus of the human microbiota.[4] *Candida* species has adapted variety of mechanisms to establish itself both as commensal and as pathogen in humans. This is attributed to a set of virulence factors, including the ability to escape host defense, phenotyping switching, adhesion, biofilm formation and secretion of coagulase and hydrolytic enzymes.[5,6,7,8] Development of new antifungal drugs and better therapeutic approaches to candidiasis can be achieved in the near future with continuing progress in the understanding of the mechanisms of *Candida* pathogenesis.[9]

Azole antifungals particularly fluconazole, is considered as the first line drug for *Candida* infections and are commonly prescribed for treatment and prevention due to fewer side effects and low cost.[10] Fluconazole acts primarily on ergosterol biosynthesis by targeting 14-α-lanosterol demethylase encoded by *ERG11* gene leading to inhibition of conversion of lanosterol to ergosterol. Unfortunately, the widespread continuous use of fluconazole enhanced the resistance to the drug. [11] Voriconazole is a second-generation broad-spectrum triazole antifungal agent with activity against a wide range of yeasts and filamentous fungi. It is used to treat severe invasive fungal infections.[12]

Different mechanisms of azole resistance exist, and more than one mechanism can be present in azole-resistant strains.[13] Understanding the mechanisms underlying fluconazole resistance is critical for managing our limited antifungal repertoire and keeps fluconazole a possible option to treat many *Candida* infections and rationale design of newer antifungals and target-based molecular approaches.[2] The major azole resistance mechanisms include (i) decreased permeability of cell membrane, which decreases the amount of drug entering the cell; (ii) the overexpression and/or (iii) mutation of the azole drug target Erg11p, (iv) the inactivation of *ERG6*, and (v) increased cellular export of azoles by up-regulated drug efflux transporters.[14,15,13,16] Point mutations in *ERG11* reducing the ability of azoles to interact with or bind at the enzyme's target site, thereby reducing the effectiveness of the drug.[13] Several mutations of the *ERG11* gene have been associated with fluconazole resistance in *C. albicans, C. parapsilosis, C. krusei* and *C.tropicalis.*[17] *ERG*11 upregulation also can occur via mechanisms that include gain of function mutations in transcription factors such as zinc-cluster transcription factor that targets genes, including those of the ergosterol biosynthesis pathway, thereby decreasing the ability of azoles to inhibit its action.[13] Overexpression of *ERG11* increases the Erg11p enzyme copy number and results in elevated ergosterol synthesis, which overwhelms the capacity of the antifungal drug.[18]

Therefore, the search for the *ERG11* gene in clinically relevant *Candida* species can provide a better understanding of the molecular mechanisms involved in resistance to antifungal agents and aid in epidemiological research. In addition, the genetic and molecular

characterization of resistant *Candida* species could help in the search for new bioactive molecules with antifungal activity. Thus, the aim of the study was to screen *Candida* species resistant to fluconazole and voriconazole for the presence of *ERG11* by polymerase chain reaction (PCR). Also, to correlate the presence of this gene with the demonstration of *Candida* virulence factors, biofilm formation, phospholipase and proteinases activities.

Materials and Methods

Candida Isolates Identification

The study included 61 *Candida* clinical strains, isolated from inpatient and outpatient clinics of Theodor Bilharz Research Institute (TBRI) in the period from March 2019 till December 2019. Urine samples were collected as midstream morning sample or from tip of catheter and processed for microbiological examination. *Candida* isolates were identified morphologically by Gram-stain, germ tube test, subculture on chromogenic medium (CHROM agar, France) and biochemically using API 20 C Aux test (BioMerieux, Durham, USA), according to the manufacturer's instructions. *Candida* isolates were stored in glycerol broth at -70^{o}C. A fresh culture was obtained by subculturing the isolates on Sabouraud Dextrose Agar (Oxoid, England) for 48 h at 37 °C before use. Standard strain *C. albicans ATCC10231* (Microbiologics, USA) was included as a control.

Antifungal Susceptibility testing

Disk diffusion test

-The antifungal susceptibility of isolates was determined by the disk diffusion method using Fluconazole (25 µg) (Bio-Rad, liofilchem, Italy) on Muller Hinton Agar (MHA) (Hi-Media, Mumbai, India) supplemented with 2% Glucose and Methylene Blue dye 0.5 µg/ml (GMB) as per the Clinical laboratory Institute guidelines (CLSI).[19]

-MICs determination of fluconazole and voriconazole

The minimal inhibitory concentration (MICs) of fluconazole and voriconazole were detected by using the E test (AB Biodisk, Solna, Sweden) and was performed according to the manufacturer's instructions. In brief, the inoculum concentration was adjusted to 0.5 McFarland standards for *Candida* species. Then, 0.5 mL of this suspension was inoculated onto plates containing RPMI 1640 agar (1.5%) with 2% glucose using a cotton swab. Within a period of 15 minutes, the E test strips were applied. The plates were incubated at 35 °C and read after 24 and 48 hours. Interpretation of break points for fluconazole and voriconazole were according to CLSI guidelines.[19]

Determination of virulence determinants among Candida spp. isolates

-Detection of Extracellular Phospholipase Activity

An aliquot (10µl) of the yeasts suspension was inoculated onto Sabouraud egg yolk agar (Oxoid, England) and incubated at 37°C for four days. Colony diameter and precipitation zone plus colony diameter were measured and interpreted for each isolate according to Mahmoudabadi et al., 2010,[20] The standard strain *C. albicans* (ATCC 10231) (Microbiologics, USA) was used as control.

-Detection of Aspartic Proteinase Activity

Candida isolates were suspended in saline to produce turbidity equivalent to a 0.5 McFarland standard. A 6 mm sterile filter paper discs were impregnated with 10 µl of the suspension and placed on the surface of bovine serum albumin agar plates. The plates were incubated at 30°C up to 7 days. Enzyme activities were scored according to the criteria by Patil et al., 2014,[21] Standard strain *C. albicans* (ATCC 10231) was used as control.

-Quantification of biofilm formation by crystal violet

Quantitative analysis of biofilm production was based on the method described previously.[22] Briefly, a 20 µl aliquot of *Candida* cell suspension containing 3x10^{7} CFU/ml was inoculated into wells of microtiter plate containing 180 µl Sabouraud glucose broth (Oxoid, England) and incubated at 35 °C for 24 hours without agitation. The biofilm coated wells were washed twice with 200 µl of sterile

distilled water and stained with 110 µl of 0.4% aqueous crystal violet solution for 45 minutes. Afterwards, the wells were washed 4 times with 350 µl of sterile distilled water and destained with 200 µl of 95% ethanol. After 45 minutes of destaining, 100 µl of destained solution was transferred to a new plate. The amount of the crystal violet stain in the destained solution was measured with a microtiter plate reader (automated Titertek Multiskan Plus lab system, Germany) at 595 nm. Interpretation of the results obtained was done according to Stepanovic *et al.,* 2000[23] classification. The standard strain *C. albicans* (ATCC 10231) was used as control.

Molecular Detection of Erg11 gene

DNA Extraction was preformed using a QIAamp DNA Mini purification kit (Qiagen, USA), according to the manufacturer's instructions. Detection of the ERG11 gene was based on using a conventional PCR assay system (Bio Rad T100 thermal cycler, USA). The PCR reaction mixture (25 µL total volume) contained 12.5 µL PCR master mix, 1 µL forward and reverse primers (10 µM each), 1 µL of DNA template (40 ng/µL), and 10.5 µL deionized water (Thermoscientific, USA). ERG11 primers sequences were as follow: 5'- GTT GAA ACT GTC ATT GAT GG (forward) and 5'-TCA GAA CAC TGA ATC GAA AG (reverse). An initial denaturation at 92 °C for 3 min followed by 30 cycles, each of denaturation at 92 °C for one minute, annealing at 43 °C for one minute and 1 min of extension at 72 °C. This was followed by a final extension at 72 °C for 10 min. For each PCR run, a negative control was also included containing the reaction buffer, dNTPs, Taq polymerase without the target DNA. DNA extract of the reference strain *C. albicans* ATCC10231 was included in each run as a positive control. PCR product was separated on 2% agarose gel with ethidium bromide and 100bp ladder used as DNA molecular weight standard.[24,25,26]

Ethical considerations

The study protocol was reviewed and approved by the Research Ethics Committee of the TBRI (No. PT 496, dated October 2019). All specimens included in the study were archived and codes were used instead of patient's names.

Statistical Methods

Data were statistically described in terms of frequencies (number of cases) and relative frequencies (percentages). A probability value (*P*-value) less than 0.05 was considered statistically significant. All statistical calculations were done using computer programs Microsoft Excel 2013 (Microsoft Corporation, NY., USA) and SPSS (Statistical Package for the Social Science; IBM SPSS statistic) version 20 for Microsoft Windows.

Results

The study included 61 *Candida* strains isolated from urine specimens. They were identified as *C. albicans* 34.5% (21/61), *C. glabrata* 29.5% (18/61), *C. tropicalis* and *C. krusei* 18% each (11/61). The susceptibility of 61 Candida species to fluconazole showed resistance in 55.7% (34/61), susceptible to dose dependent (S-DD) in 11.4% (7/61) and sensitive in 31.1% (19/61) (Table 1).

Table 1. *In vitro* Activities of Fluconazole against Isolates of *Candida* Species by E- test.

Candida Species	Sensitive			S-DD			Resistant		
	MICs (µg/ml)	No.	%	MICs (µg/ml)	No.	%	MICs (µg/ml)	No.	%
C. albicans (n=21)	2-8	14	66.7	16-32	3	14.3	>256	4	19
C. glabrata (n=18)	0	0	0	32	2	11.1	>256	16	88.9
C. tropicalis (n=11)	8-12	5	45.4	16-32	2	18.2	>256	4	36.4
C. krusei (n=11)	8	1	9.1	0	0	0	>256	10	90.9
Total (n=61)	2-12	20	32.8	16-32	7	11.5	>256	34	55.7

S-DD: Susceptible to dose dependent.

Of the fluconazole resistant *Candida* isolates 85.3% (29/34) had MICs >256µg/ml. As regard the susceptibility to voriconazole, 27.9% (17/61) of the isolates were resistant and 72.1% (44/61) were sensitive (Table 2). Of the fluconazole resistant *Candida* isolates 50% (17/34) were sensitive to voriconazole with MICs ranged from 0.5 to 1µg/ml. The most frequently *Candida* species revealed fluconazole resistance were *C. glabrata* 47.1% (16/34), followed by *C. krusei* 29.4% (10/34), and then *C. tropicalis* and *C. albicans* were equally detected 11.8% each (4/34).

Table 2. *In vitro* Activities of Voriconazole against Isolates of *Candida* Species by E test.

Candida Species	Sensitive			Resistant		
	MICs (µg/ml)	No.	%	MICs (µg/ml)	No.	%
C. albicans (n=21)	0.064-1	21	100	0	0	0
C. glabrata (n=18)	0.5-1	4	22.2	2->32	14	77.8
C. tropicalis (n=11)	0.25-1	9	81.8	2->32	2	18.2
C. krusei (n=11)	0.5-1	10	90.9	2	1	9.1
Total (n=61)	0.064-1	44	72.1	2->32	17	27.9

Regarding *Candida* virulence factors, biofilm formation was detected in 39.3% (24/61), phospholipase activity in 72.1% (44/61) and proteinase activity in 44.3% (27/61) of *Candida* species. Biofilm formation, phospholipase and proteinase activity were determined in 41.2% (14/34), 67.6% (23/34) and 35.3% (12/34), respectively of fluconazole resistant *Candida* isolates. There was no statistically significant difference regarding the association of the expression of virulence factors and fluconazole resistance among *Candida* isolates (P> 0.05).

Erg 11 gene was determined in 82.4 % (28/34) of fluconazole resistant *Candida* isolates. Erg 11 gene was prominent in *C. glabrata* 93.75% (15/16), followed by *C. krusei* 90% (9/10), then *C. tropicalis* 75% (3/4) and lastly *C. albicans* 25% (1/4). Erg 11 gene was detected in 64.7% (11/17) of fluconazole resistant-voriconazole sensitive *Candida* isolates (Table 3, Figure1). Regarding, correlation of Erg11 gene positivity and virulence factors among fluconazole resistant *Candida* isolates, 34.5% (10/29) exhibited biofilm formation and 62.1% (18/29) and 31% (9/29), respectively showed phospholipase and proteinase activities (Table 4). There were statistically significant differences concerning the association of proteinase activities and Erg 11 gene expression among fluconazole resistance *Candida* isolates (P=0.04). However, there was no statistically significant difference regarding the association of biofilm formation and phospholipase activity and Erg 11 gene expression in fluconazole resistance *Candida* isolates (P> 0.05).

Table 3. MICs of fluconazole and Voriconazole and Erg 11 Gene Detection among Fluconazole Resistant *Candida* Isolates.

Sample No.	Candida Species	E test				Erg11 Gene
		Fluconazole		Voriconazole		
2	*C. glabrata*	>256	R	>32	R	Positive
4	*C. glabrata*	>256	R	>32	R	Positive
6	*C. tropicalis*	64	R	1	S	Negative
7	*C. glabrata*	>256	R	>32	R	Positive
8	*C. glabrata*	>256	R	>32	R	Positive

Table 3. Continued.

Sample No.	*Candida* Species	E test				Erg11 Gene
		Fluconazole		Voriconazole		
9	*C. glabrata*	>256	R	>32	R	Positive
10	*C. krusei*	>256	R	0.75	S	Positive
14	*C. glabrata*	>256	R	>32	R	Positive
15	*C. tropicalis*	>256	R	>32	R	Positive
16	*C. glabrata*	>256	R	>32	R	Positive
17	*C. krusei*	>256	R	2	R	Positive
22	*C. glabrata*	>256	R	>32	R	Positive
27	*C. krusei*	>256	R	0.75	S	Positive
29	*C. albicans*	>256	R	0.19	S	Negative
32	*C. krusei*	>256	R	0.75	S	Positive
34	*C. krusei*	64	R	0.5	S	Positive
35	*C. albicans*	48	R	0.75	S	Positive
36	*C. tropicalis*	>256	R	0.19	S	Positive
37	*C. glabrata*	>256	R	0.5	S	Negative
38	*C. glabrata*	>256	R	1.5	R	Positive
39	*C. glabrata*	48	R	1	S	Positive
40	*C. glabrata*	>256	R	>32	R	Positive
41	*C. krusei*	>256	R	0.5	S	Positive
45	*C. glabrata*	>256	R	2	R	Positive
50	*C. tropicalis*	>256	R	4	R	Positive
51	*C. glabrata*	>256	R	>32	R	Positive
52	*C. albicans*	64	R	1	S	Negative
53	*C. krusei*	>256	R	0.5	S	Positive
54	*C. albicans*	>256	R	0.75	S	Negative
55	*C. glabrata*	>256	R	>32	R	Positive
56	*C. krusei*	>256	R	0.5	S	Positive
57	*C. krusei*	>256	R	0.5	S	Positive
58	*C. glabrata*	>256	R	>32	R	Positive
60	*C. krusei*	>256	R	0.75	S	Negative

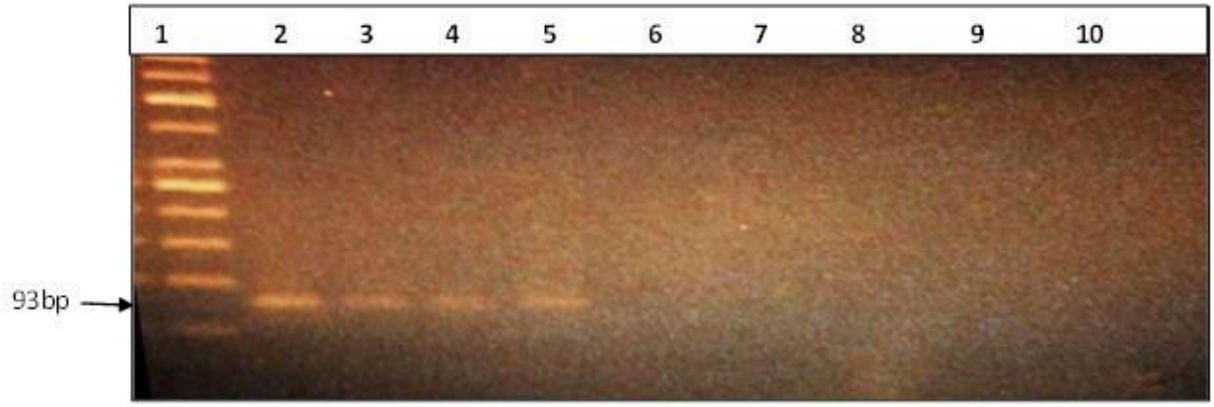

Figure 1. Agarose gel 2% for electrophoresis of PCR products of *Candida*. Lane 1: Molecular weight marker (50 bp). Lanes 3–5 are positive Erg11 gene. Lane 2 positive control. Lane 6 negative control. The arrow points to the expected amplicon band (93 bp).

Table 4. Erg 11 Gene Detection and Virulence Factors among 34 Fluconazole Resistant *Candida* Isolates.

Sample No.	*Candida* Species	Vor	Erg 11 gene	Virulence Factors		
				Biofilm	Phospholipase	Proteinase
2	*C. glabrata*	R	Positive	Negative	Positive	Negative
4	*C. glabrata*	R	Positive	Negative	Negative	Negative
6	*C. tropicalis*	S	Negative	Positive	Positive	Positive
7	*C. glabrata*	R	Positive	Negative	Positive	Positive
8	*C. glabrata*	R	Positive	Negative	Positive	Negative
9	*C. glabrata*	R	Positive	Negative	Positive	Negative
10	*C. krusei*	S	Positive	Negative	Negative	Positive
14	*C. glabrata*	R	Positive	Negative	Positive	Negative
15	*C. tropicalis*	R	Positive	Negative	Negative	Positive
16	*C. glabrata*	R	Positive	Positive	Positive	Positive
17	*C. krusei*	R	Positive	Positive	Positive	Negative
22	*C. glabrata*	R	Positive	Negative	Positive	Negative
27	*C. krusei*	S	Positive	Positive	Positive	Negative
29	*C. albicans*	S	Negative	Negative	Positive	Positive
32	*C. krusei*	S	Positive	Positive	Positive	Negative
34	*C. krusei*	S	Positive	Negative	Negative	Negative
35	*C. albicans*	S	Positive	Positive	Positive	Positive
36	*C. tropicalis*	S	Positive	Negative	Negative	Positive
37	*C. glabrata*	S	Negative	Negative	Negative	Negative
38	*C. glabrata*	R	Positive	Negative	Positive	Negative
39	*C. glabrata*	S	Positive	Negative	Negative	Negative
40	*C. glabrata*	R	Positive	Negative	Positive	Positive
41	*C. krusei*	S	Positive	Positive	Negative	Negative
45	*C. glabrata*	R	Positive	Positive	Positive	Positive
50	*C. tropicalis*	R	Positive	Negative	Positive	Negative
51	*C. glabrata*	R	Positive	Negative	Negative	Negative

Table 4. Continued.

Sample No.	*Candida* Species	Vor	Erg 11 gene	Virulence Factors		
				Biofilm	Phospholipase	Proteinase
52	*C. albicans*	S	Negative	Positive	Positive	Negative
53	*C. krusei*	S	Positive	Positive	Positive	Negative
54	*C. albicans*	S	Negative	Positive	Positive	Positive
55	*C. glabrata*	R	Positive	Negative	Negative	Negative
56	*C. krusei*	S	Positive	Positive	Positive	Negative
57	*C. krusei*	S	Positive	Positive	Positive	Negative
58	*C. glabrata*	R	Positive	Negative	Negative	Positive
60	*C. krusei*	S	Negative	Positive	Positive	Negative

Vor: voriconazole.

Discussion

The infection of *C. albicans* continues to be a major cause of high mortality among immunocompromised and hospitalized patients and the most common etiologic agent of fungal-related biofilm infection.[27] Although *C. albicans* remains the most common pathogenic fungi encountered, the frequency of isolation of non-*Candida* albicans *Candida* species, i.e., C. *tropicalis, C. krusei, C. glabrata* and *C. parapsilosis*, has increased.[15,16] The incidence of resistance to *Candida* treatment has surged during the recent decades. Emergence of fluconazole-resistant *Candida* species has been progressively reported in the last 30 years.[18]

The present study identified *C. albicans* to be the predominant causative agent of candiduria (34.5%) followed by the non-albicans strains; *C. glabrata* (29.5%) while *C. tropicalis* and *C. krusei* (18%) were equally detected. Our study results are in agreement with that of Badawi *et al*., 2004[28] and Yassin *et al.,* 2020[29] ascertained that *C. albicans* represented the predominant strain followed by *C. glabrata* and *C. tropicalis*. Hassaneen *et al*., 2014[30] reported close incidence rates of *C. glabrata* (21.4%) and *C. tropicalis* (7.1%) isolated from the urine of patients admitted with urinary tract infections to the Zagazig University Hospitals in Egypt. In Kuwait, *C. albicans* was the most prominent species recovered from urine cultures from candiduric patients in a tertiary care hospital.[31] Although NAC are emerging as potential pathogens responsible for candiduria,[32] *C. albicans* is still being reported as the dominant species infecting the urinary tract of not only Egyptian patients but also patients in several Arab countries.[33, 34, 35, 36, 37, 38]

E-test on Sabouraud dextrose agar was reported as a simple method for MICs determination and could detect S-DD strains in case of azoles.[28] In the present study fluconazole susceptibly was screened by disk diffusion technique whereas fluconazole and voriconazole E-test was used for determination of MICs. Totally, 55.7 % of isolates were resistant to fluconazole. Higher resistance rate was observed for NAC species than for *C. albicans* where 66.7% were susceptible to fluconazole similar to those reported by Sandhu *et al*., 2017[39] where the sensitivity rate was 73%. Antifungal resistance among *C. albicans* is uncommon in accordance with other researchers who reported sensitivity of *Candida* species to fluconazole ranging from 63.3% to 95%, respectively.[40,41,42,43] However, in a recent study, 93% of *C. albicans* isolates were resistant to fluconazole.[44] With increased use of fluconazole, several studies were concerned with emergence of fluconazole resistant NAC especially *C. tropicalis* which possessed moderate level of fluconazole resistance.[45,46] In our study, out of 11 *C. tropicalis* strains 45.5% were sensitive and two were S-DD. A study conducted by Edward *et al*., 2020[47] found that 100% of *C. tropicalis* fluconazole were resistant. Furthermore, out of 18 *C. glabrata*, 16 were

resistant to fluconazole and only 2 strains were S-DD. These are in accordance with another study by Lima *et al*., 2017[48] who mentioned that all *C. glabrata* isolates were either resistance (5.6%) or dose-dependent susceptibility (94.4%) to fluconazole. Globally, *C. glabrata* showed higher resistance rates (7.7%-11.9%) than other *Candida* species.[49] Only one strain of *C. krusei* was susceptible to fluconazole with 90.9% resistant to fluconazole. Various national and international studies have reported total fluconazole resistant *C. krusei* isolates. In fact, *C. krusei* has an innate resistance to fluconazole, as reported by several studies, demonstrated the innate resistance of *C. krusei* to this antifungal drug.[50,51] Fluconazole is the most commonly prescribed azole agent for candiduria, which may explain the increased resistance rates and susceptible dose dependence observed for this antifungal agent in our study which may suggest the future emergence of these species with resistant isolates.

In the present study, the overall sensitivity to voriconazole of both *C. albicans* and NAC species was 72.1%. This is in accordance with other reports, where it was in the range of 76.6% to 100%.[41, 42,43] Similar results for voriconazole sensitivity were also obtained by Padawer *et al*., 2015 [52] from Israel, Alkilani *et al*., 2016[38] from Egypt and Khadke *et al*., 2017[53] from Nepal. Many authors have evaluated voriconazole activity to be better than that of fluconazole and have considered voriconazole as a better alternative than fluconazole for the primary therapy of candiduria.[54]

The observed fluconazole resistance in *Candida* spp. has drawn attention in recent years due to resultant serious infection and failure of treatment.[55] This may be attributable to various virulence traits; adhesion to surfaces and secretion of extracellular hydrolases, proteinases and phospholipases.[56] The most important hydrolytic enzymes produced by *Candida* species include phospholipases and proteinases. In the current study, production of hydrolytic enzymes was evaluated as a major part of *Candida* species pathogenicity determinants. Proteinase and phospholipase were detected in 44.3% and 72.1%, respectively of *Candida* isolates. A study conducted on 200 Egyptian candiduric patients admitted to the ICU of our Institute's Hospital, phospholipase production was in line with our results (68.6%) while protinease production was higher than in our study (91.4%).[57] Another study stated that phospholipase activity was seen in 58.1% of isolates while proteinase production was seen in 37.8% of *Candida* isolates.[58] Conversely, Jose *et al*., 2015[59] showed highest percentage of phospholipase (50%) and proteinase (75%) enzyme production among *Candida* isolates. Hydrolytic enzyme production was correlated to fluconazole resistance by comparative analysis of the sensitive and resistant strains. Of the total 34 resistant strains of *Candida*, 67.7% and 35.5% were phospholipase and proteinase producers, respectively.[59] In agreement with our study, Edward *et al*., 2020 [47] noted that roughly half of these enzyme- producing isolates (40-46%) were resistant to fluconazole. The previous reports demonstrated that fluconazole resistance was associated with the acquisition of superior virulence traits by *Candida* species, phospholipase and proteinases secretion being among these traits.[60]

A major virulence factor of *C. albicans* is its ability to form biofilms, a closely packed community of cells that can grow on both abiotic and biotic substrates, including implanted medical devices and mucosal surfaces. Biofilms are extremely hard to eradicate and resistant to conventional antifungal treatment.[4] In the present study, of the total, 34 resistant strains of *Candida* 41.2% were found to be biofilm producers. In correlation with our study the fluconazole resistance was detected in 47.4% of the biofilm producers.[7] There was no correlation between the biofilm formation and fluconazole susceptibility ($P > 0.05$), which was in agreement with other studies.[61,47]

In all fungal species, *Erg11* (gene encoding Erg11-p or lanosterol 14αdemethylase) is an essential gene for ergosterol synthesis. *Erg11* gene over expression results in conformational changes that reduces the effective binding between azoles and their target and is commonly associated with drug resistance in clinical isolates.[62] In our study *Erg11* was

observed in 82.4% of fluconazole resistant isolates where 93.75% of *C. glabrata* harbored the gene and only amplified in 25% of *C. albicans*. It was reported by Teymuri *et al*., 2015[63] that all fluconazole-resistant *C. albicans* isolates showed up regulation of *Erg11*. On the other hand, Abdelhamid *et al*., 2018[64] detected *Erg 11* by real time PCR in 11.1% (2/18) among *Candida* isolates resistant and susceptible-dose dependent to fluconazole (One susceptible dose dependent *Candida albicans* isolate and one resistant *Candida glabrata* isolate). In another study, *Erg11* overexpression was detected in only 37% (five out of 14) of the isolates.[65]

The significance of increased *Erg11* expression in fluconazole-resistant NAC is variable. Likewise, several studies have identified higher expression of *Erg11* in fluconazole-resistant clinical isolates of *C. tropicalis* as compared to fluconazole-susceptible isolates.[66] Moreover, *Erg11* expression was even higher among a subset of fluconazole-resistant isolates that were also resistant to itraconazole and voriconazole. These results were recently echoed by a similar study characterizing 35 ℃. *tropicalis* isolates from Korean university hospitals, nine of which were fluconazole-non-susceptible.[67] Unlike in other species of *Candida*, *Erg11* does not appear to play a major role in fluconazole resistance and there are only two clinical isolates reported to display overexpression of *Erg11*.[68] The contribution of this overexpression to fluconazole resistance in these isolates is unclear. The same is true for *C. krusei*; a single report of increased *Erg11* expression observed in four clinical isolates.[69] Unfortunately, due to non-availability of sequencing machine in our environment, it was not possible to determine the level of expression of the *Erg11* genes.

The changes in expression of *Erg 11* were shown to be involved in drug resistance in biofilms.[70] In our study, 34.5 % exhibited biofilm formation with *Erg11* positive. Shi *et al*., 2019[70] stated that there was no difference in expression between biofilm-associated cells and planktonic cells. However, upon the addition of fluconazole, both groups showed increased *Erg11* expression.[72] There were statistically significant difference concerning the association of proteinase activities and Erg 11 gene expression among fluconazole resistance *Candida* isolates. This was in accordance with Feng *et al.,* 2021[72] who reported that the expression of secreted aspartyl proteinase 2 (SAP2) and *ERG11* was significantly higher in the resistant strains compared with the sensitive strains, and there was a positive liner correlation between SAP2 and *ERG11* messenger RNA expression.

In conclusion, the present study highlighted the high prevalence of resistance to antifungal drugs among *Candida* species. *C. glabrata* showed maximum resistance among all the *Candida* species so *in vitro* susceptibility testing to antifungal agents and species identification is crucial for better selection of antifungal agents in treatment of fungal infections. Moreover, there was association between the virulence factors, antifungal resistance, and the presence of *Erg11* among different *Candida* species. Our study confirmed that voriconazole maintains better activity towards *Candida* species and represent a better alternative therapy in cases of candiduria.

Author Contributions

ME was responsible for the main concept of the research and was a contributor in writing the manuscript and revised it. HB analyzed, interpreted the data, and revised the manuscript. DG performed the microbiological identification and interpreted the results and was a contributor in writing the manuscript. DS analyzed, interpreted the data and was a major contributor in writing and revising the manuscript. HD performed the molecular detection and revised the manuscript. AE performed the molecular extraction and was responsible for follow up of data and revised the manuscript. All authors read and approved the final manuscript.

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Ethical approval

The study protocol was reviewed and approved by the Research Ethics Committee of the TBRI (No. PT 496, dated October 2019).

Informed consent

A signed consent form was obtained from each study participant.

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References

1. Pappas, P.G., Kauffman, C.A., Andes D.R. (2016). Clinical practice guideline for the management of candidiasis. *Clinical Infectious Diseases*, 62 (4): e1-e50.
2. Berkow, E.L., Lockhart, S.R. (2017). Fluconazole resistance in *Candida* species: a current perspective. *Infect Drug Resist*, 31(10):237-245.
3. Healey, K.R., Perlin, D.S. (2018). Fungal resistance to echinocandins and the MDR phenomenon in *Candida* glabrata. *J Fungi,* 4:105.
4. Ponde, N.O., Lortal, L., Ramage, G. et al (2021). *Candida albicans* biofilms and polymicrobial interactions. *Crit Rev Microbiol*, 47(1):91-111.
5. Subramanya, S.H., Baral, B.P., Sharan, N.K. et al. (2017). Antifungal susceptibility and phenotypic virulence markers of *Candida* species isolated from Nepal. *BMC Res Notes*; 10 (1):543.
6. Chen, E., Benso, B., Seleem, D. et al. (2018). Fungal-Host Interaction: Curcumin Modulates Proteolytic Enzyme Activity of *Candida albicans* and Inflammatory Host Response *in Vitro. Int J Dent*, 2393146.
7. Kadry, A.A., El-Ganiny, A.M., El-Baz, A.M. (2018). Relationship between Sap prevalence and biofilm formation among resistant clinical isolates of *Candida albicans. Afr Health Sci,* 18(4): 1166-1174.
8. Vieira de Melo, A.P., Zuza-Alves, D.L., da Silva-Rocha, W.P. et al. (2019). Virulence factors of *Candida* spp. obtained from blood cultures of patients with candidemia attended at tertiary hospitals in Northeast Brazil. *J Mycol Med*, S1156-5233(18)30304-4.
9. Mba, I.E., Nweze, E.I. (2020). Mechanism of *Candida* pathogenesis: revisiting the vital drivers. *Eur J Clin Microbiol Infect Dis*, 39 (10):1797-1819.
10. Wiederhold, N.P. (2017). Antifungal resistance: current trends and future strategies to combat. *Infection and drug resistance,* 10: 249.
11. Benedetti, V.P., Savi, D.C., Aluizio, R. et al. (2019). ERG11 gene polymorphisms and susceptibility to fluconazole in *Candida* isolates from diabetic and kidney transplant patients. *Revista da Sociedade Brasileira de Medicina Tropical,* 52.
12. Li, T.-y., Liu, W., Chen, K. et al. (2017). The influence of combination use of CYP450 inducers on the pharmacokinetics of voriconazole: a systematic review. *The Journal of Clinical Pharmacy and Therapeutics*, 42(2):135-146.
13. Sanglard, D., Coste, A.T. (2016). Activity of isavuconazole and other azoles against *Candida* clinical isolates and yeast model systems with known azole resistance mechanisms. *Antimicrob Agents Chemother*, 60:229 –238.
14. Jensen, R.H., Astvad, K.M., Silva, L.V. et al. (2015). Stepwise emergence of azole, echinocandin and amphotericin B multidrug resistance in vivo in *Candida albicans* orchestrated by multiple genetic alterations. *J Antimicrob Chemother*, 70(S9):2551-2555.
15. Shao, J., Shi, G., Wang, T. et al. (2016). Antiproliferation of Berberine in Combination with Fluconazole from the Perspectives of Reactive Oxygen Species, Ergosterol and Drug Efflux in a Fluconazole-Resistant *Candida tropicalis* Isolate. *Front Microbiol*, 23; 7: 1516.
16. Reis de Sá, L.F., Toledo, F.T., Gonçalves, A.C. et al., (2017). Synthetic Organotellurium Compounds Sensitize Drug-Resistant *Candida albicans* Clinical Isolates to Fluconazole. *Antimicrob Agents Chemother*, 61(1): e01231-16.
17. Cheng, J.-W., Liao, K., Kudinha, T. et al. (2017). Molecular epidemiology and azole resistance mechanism study of *Candida* guilliermondii from a Chinese surveillance system. *Sci Rep*, 7: 907.
18. Alizadeh, F., Khodavandi, A., Zalakian, S. (2017). Quantitation of ergosterol content and gene expression profile of ERG11 gene in fluconazole-resistant *Candida albicans. Curr Med Mycol*, 3(1): 13-19.
19. Clinical and Laboratory Standards Institute (2017). M27: Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard-4th ed.; *CLSI: Wayne*, PA, USA.
20. Mahmoudabadi A.Z., Zarrin, M., Miry, S. (2010). Phospholipase activity of *Candida albicans* isolated from vagina and urine samples. *Jundishapur J Microbiol*, 3(4): 169-173.

21. Patil, S., Ugargol, A.R., Srikanth, N.S. (2014). Comparison of two methods or detection of secreted aspartyl proteinase in urinary isolates of *Candida* species. *National Journal of medical research,* 4: 119-121.
22. Jin, Y., Yip, H.K., Samaranayake, Y.H., et al. (2003). Biofilm-forming ability of *Candida albicans* is unlikely to contribute to high levels of oral yeast carriage in cases of human immunodeficiency virus infection. *J Clin Microbiol*; 41:2961-2967.
23. Stepanovic, S., Vukovic, D., Dakic, I., et al. (2000). A modified microtiter-plate test for quantification of staphylococcal biofilm formation. *J Microbiol Methods*, 40(2): 175-179.
24. Perea, S., López-Ribot, J.L., Kirkpatrick, W.R. (2001). Prevalence of molecular mechanisms of resistance to azole antifungal agents in *Candida albicans* strains displaying high-level fluconazole resistance isolated from human immunodeficiency virus-infected patients. *Antimicrob Agents Chemother*, 45(10):2676-84.
25. Chau, A.S., Mendrick C.A., Sabatelli, F.J. et al. (2004). Application of real-time quantitative PCR to molecular analysis of *Candida albicans* strains exhibiting reduced susceptibility to azoles. *Antimicrob Agents Chemother*, 48(6):2124-2131.
26. Manastır, L., Ergon, M.C., Yücesoy, M. (2011). Investigation of mutations in Erg11 gene of fluconazole resistant *Candida albicans* isolates from Turkish hospitals. *Mycoses*, 54(2):99-104.
27. Jia, W., Zhang, H., Li, C. et al. (2016). The calcineruin inhibitor cyclosporine a synergistically enhances the susceptibility of *Candida albicans* biofilms to fluconazole by multiple mechanisms. *BMC Microbiology*; 16:113
28. Badawi, H., Kamel, A. I., Fam, N. et al., (2004). *Candida* Urinary Infections: Emerging Species, Antifungal Susceptibility Trends and Antibody Response. Egypt. *J Med. Microbiol*, 13(1): 1-14.
29. Yassin, M.T., Mostafa, A.A., Al-Askar, A.A., et al., (2020). In vitro antifungal resistance profile of *Candida* strains isolated from Saudi women suffering from vulvovaginitis. *Eur J Med Res*, 25(1):1.
30. Hassaneen, A.M., Ghonaim, R.A., Hassanin HM. et al. (2014). Different aspects of candiduria as an important nosocomial infection. Med J Cairo Univ, 82 (1):199-204.
31. Alfouzan, W., Dhar, R., Ashkanani, H., et al., (2015). Species spectrum and antifungal susceptibility profile of vaginal isolates of *Candida* in Kuwait. J Mycol Med, 25(1):23-28.
32. Kauffman CA (2005). Candiduria. *Clin Infect Dis*, 41(6):S371- S376.
33. Sallam, A., Lynn, W., McCluskey, P. et al. (2006). Endogenous *Candida* endophthalmitis. Expert *Review of Anti-infective Therapy*; 4(4): 675-685.
34. Bukhary ZA (2008). Candiduria: a review of clinical significance and management. *Saudi J Kidney Dis Transpl*, 19(3):350-60.
35. Omar, M., Fam, N., El Leithy, T. et al. (2008). Virulence factors and susceptibility patterns of *Candida* species isolated from patients with obstructive uropathy and bladder cancer. Egypt. *J. Med. Microbiol*, 17(2):317-328.
36. Alhussaini, M.S., El-Tahtawi, N.F., Moharram, A.M. (2013). Phenotypic and Molecular Characterization of *Candida* Species in Urine Samples from Renal Failure Patients. Science *Journal of Clinical Medicine*, 2 (1): 14-25.
37. Awad, E.T., Mohamad, E.A. (2014). The use of Chromagar for detection of *Candida albicans* and non albicans Nosocomial infections of immunocompromised patients in Shebin El-Kom teaching hospital: risk factors and an analysis of microbiological data. Egypt *J Med Microbiol,* 23(3), 37-45.
38. Alkilani, A.A., El Shalakany, A.H., El-Masry, E.A. et al (2016). Nosocomial Candiduria in Critically Ill Patients Admitted to Intensive Care Units in Menoufia University Hospitals, Egypt. *Journal of Advances in Medicine and Medical Research*, 15(9):1-15.
39. Sandhu, R., Dahiya, S., Sayal, P. et al. (2017). Increased role of non-albicans *Candida*, potential risk factors, and attributable mortality in hospitalized patients. *J Health Res Rev*, 4:78-83.
40. Mohammed, O., Haman, N., Ahmed, M.E. (2018). Diversity and prevalence of different *Candida* species among Egyptian cancer patients. *Advanced Research Journal of Microbiology*, 5 (3):196-202.
41. Shaik, N., Penmetcha, U., Myneni, R.B. et al. (2016). A study of identification and antifungal susceptibility pattern of *Candida* species isolated from various clinical specimens in a tertiary care teaching hospital, Chinakakani, Guntur, Andhra Pradesh, South India. *Int J Curr Microbiol App Sci*, 5(7):71-91.
42. Gupta, S., Goyal, R.K. (2016). Species distribution and antifungal drug susceptibility of *Candida* in clinical isolates from a tertiary care centre at Bareilly. *IOSR*, 16(1):57-61.
43. Malhotra, S. (2017). Occurrence of candiduria in paediatric patients and its antifungal susceptibility in a tertiary care centre. *J Infect Dis Med,* 2:108.

44. Zare-Khafri, M., Alizadeh, F., Nouripour-Sisakht, S. et al. (2020). Inhibitory effect of magnetic iron-oxide nanoparticles on the pattern of expression of lanosterol 14 α-demethylase (ERG11) in fluconazole-resistant colonizing isolate of *Candida albicans*. *IET Nanobiotechnol*, 14(5):375-381.
45. Kashid, R.A., Belawadi, S., Devi, G. et al (2012). Incidence of non-*Candida Albicans* in patients with urinary tract infection with special reference to speciation and antifungal susceptibility. *J Evol Med Dent Sci*, 1(4): 572-577.
46. Toner, L., Papa, N., Aliyu, S.H. et al. (2016). *Candida* growth in urine cultures: a contemporary analysis of species and antifungal susceptibility profiles. *QJM*, 109(5):325-9.
47. Edward, E.A., Mohamed, N.M., Zakaria, A.S. (2020). Resensitization of Fluconazole-Resistant Urinary *Candida* spp. Isolates by Amikacin through Downregulation of Efflux Pump Genes. *Pol J Microbiol*, 69(1):73-84.
48. Lima, G.M.E., Nunes, M.O., Chang, M.R., et al. (2017). Identification and antifungal susceptibility of *Candida* species isolated from the urine of patients in a university hospital in Brazil. Rev. *Inst. Med. trop. S. Paulo*, 59:1-8.
49. Canela, H.M.S., Cardoso, B., Vitali, L.H. et al (2018). Prevalence, virulence factors and antifungal susceptibility of *Candida* spp. isolated from bloodstream infections in a tertiary care hospital in Brazil. *Mycoses*, 61(1):11-21.
50. Junqueira, J.C., Jorge, A.O., Barbosa, J.O. et al. (2012). Photodynamic inactivation of biofilms formed by *Candida* spp., Trichosporon mucoides, and Kodamaea ohmeri by cationic nanoemulsion of zinc 2,9,16,23-tetrakis(phenylthio)-29H, 31H-phthalocyanine (ZnPc). *Lasers Med Sci*, 27(6):1205-1212.
51. Patil, S., Rao, R.S., Majumdar, B. et al. (2015). Clinical Appearance of Oral *Candida* Infection and Therapeutic Strategies. *Front Microbiol*; 6:1391.
52. Padawer, D., Pastukh, N., Nitzan, O. et al. (2015). Catheter-associated candiduria: Risk factors, medical interventions, and antifungal susceptibility. *Am J Infect Control*, 43(7):e19-22.
53. Khadka, S., Sherchand, J.B., Pokhrel, B.M. et al. (2017). Isolation, speciation and antifungal susceptibility testing of *Candida* isolates from various clinical specimens at a tertiary care hospital, Nepal. *BMC Res Notes*, 10(1):218.
54. Pfaller, M.A., Masser, S.A., Boyken, L. et al. (2004). *In vitro* activities of Voriconazole, Posaconazloe, fluconazole against 4169 clinical isolates of *Candida* species and Cryptococcus neoformans, collected during 2001 and 2002 in the ARTEMIS global antifungal surveillance program. *Diagn Microbiol Infect Dis*, 48:201-5.
55. Mayer, F.L., Wilson, D., Hube, B. (2013). *Candida albicans* pathogenicity mechanisms. *Virulence*, 4(2):119-128.
56. Höfs, S., Mogavero, S., Hube, B. (2016). Interaction of *Candida albicans* with host cells: virulence factors, host defense, escape strategies, and the microbiota. *J Microbiol*, 54 (3):149-69.
57. Ashour, S.M., Kheiralla, Z.M., Maklad, S.S. et al. (2015). Relationship between virulence factors of *Candida* species with candiduria and myeloperoxidase concentrations. *Int J Curr Microbiol Appl Sci*, 4(1):108–123.
58. Aher, C.S. (2014). Species distribution, virulence factors and antifungal susceptibility profile of *Candida* isolated from oropharyngeal lesions of HIV infected patients. *Int J Curr Microbiol App Sci,* 3(1): 453-460.
59. Jose, N.V., Mudhigeti, N., Asir, J. et al. (2015). Detection of virulence factors and phenotypic characterization of *Candida* isolates from clinical specimens*. J Curr Res Sci Med*, 1:27-31.
60. Ying, S., Chunyang, L. (2012). Correlation between phospholipase of *Candida albican*s and resistance to fluconazole. *Mycoses*; 55(1):50-55.
61. Marak, M.B., Dhanashree, B. (2018). Antifungal susceptibility and biofilm production of *Candida* spp. isolated from clinical samples*. International journal of microbiology*, 2018:1-5.
62. Wang, Y., Ren, Y.C., Zhang, Z.T. et al. (2015). *Candida funiuensi* sp. nov., a cellobiose-fermenting yeast species isolated from rotten wood. *Int J Syst Evol Microbiol*, 65(6):1755-1758.
63. Teymuri, M., Mamishi, S., Pourakbari, B. et al. (2015). Investigation of ERG11 gene expression among fluconazole-resistant *Candida albicans:* first report from an Iranian referral pediatric hospital. *British journal of biomedical science*; 72(1):28-31.
64. Abdelhamid, D.H., Eltohamy, O.A., Saad, R.S. et al. (2018). Antifungal susceptibility profile and molecular detection of ERG11 resistant gene in *Candida* species associated with vulvovaginal candidiasis. QJM: *An International Journal of Medicine*, 111(1): 200-032.
65. Chen, L., Xu, Y., Zhou, C. et al. (2010). Overexpression of CDR1 and CDR2 Genes Plays an Important Role in Fluconazole Resistance in *Candida* albicans with G487T and T916C Mutations. *Journal of International Medical Research*, 536-545

66. Jiang C, Dong D, Yu B, Cai G, Wang X, Ji Y, Peng Y(2013). Mechanisms of azole resistance in 52 clinical isolates of Candida tropicalis in China. *J Antimicrob Chemother*, 68(4):778-785.
67. Choi, M.J., Won, E.J., Shin, J.H. et al. (2016). Resistance Mechanisms and Clinical Features of Fluconazole-Nonsusceptible *Candida tropicalis* Isolates Compared with Fluconazole-Less-Susceptible Isolates. *Antimicrob Agents Chemother*; 60(6):3653-3661.
68. Tavakoli, M., Zaini, F., Kordbacheh, M. et al. (2010). Upregulation of the ERG11 gene in *Candida* krusei by azoles. *Daru*, 18(4):276-280.
69. Borecká-Melkusová, S., Moran, G.P., Sullivan, D.J. et al. (2009). The expression of genes involved in the ergosterol biosynthesis pathway in *Candida albicans* and *Candida dubliniensis* biofilms exposed to fluconazole. *Mycoses*, 52(2):118-128.
70. Shi, C., Liu, J., Li, W. et al. (2019). Expression of fluconazole resistance-associated genes in biofilm from 23 clinical isolates of *Candida albicans. Brazilian Journal of Microbiology*, 50(1): 157-163.
71. Nailis, H., Kucharíková, S., Řičicová, M. et al. (2010). Real-time PCR expression profiling of genes encoding potential virulence factors in *Candida albicans* biofilms: identification of model-dependent and-independent gene expression. *BMC microbiology*, 10:1-11.
72. Feng, W., Yang, J., Ma, Y. et al. (2021). The effects of secreted aspartyl proteinase inhibitor ritonavir on azoles-resistant strains of *Candida albicans* as well as regulatory role of SAP2 and ERG11. *Immun Inflamm Dis*, 9(3):667-680.