Microbes and Infectious Diseases 2023; 4(3): 994-1007

Microbes and Infectious Diseases

Journal homepage: https://mid.journals.ekb.eg/

Original article

Assessment of the accuracy of identification of clinical *Candida* species with reduced fluconazole susceptibility by rapid methods and their relative expression of major efflux pump genes

Aliaa Aboulela [1*], Samah Idris [1], Dalia Ragab [1], Rasha Emad [2], Gamal El-Sawaf [1]

1- Microbiology Department, Medical Research Institute, Alexandria University , Alexandria, Egypt.
2- Alexandria Main University Hospital, Alexandria, Egypt.

ARTICLE INFO

Article history:
Received 25 June 2023
Received in revised form 12 July 2023
Accepted 13 July 2023

Keywords:
C. albicans
C. tropicalis
Efflux pump expression
CDR1
CDR2 and *MDR1*
Fluconazole resistance

ABSTRACT

Background: *Candida* infections are commonly treated with fluconazole. Efflux pumps are one of the mechanisms for fluconazole resistance. This study assessed the accuracy of identification of *Candida* species with reduced fluconazole susceptibility and their relative expression of major efflux pump genes. **Methods:** *Candida* species (n=111) were collected from clinical samples, identified by Brilliance Candida Agar (BCA) and germ tube test, then tested for fluconazole susceptibility by disk diffusion. Confirmation of identification and susceptibility to fluconazole was done by Phoenix BD and broth microdilution methods, and the accuracy of preliminary methods was calculated. qPCR was performed to evaluate the gene expression of common efflux pump genes. **Results:** The most predominant were *C. albicans* (32%) and *C. tropicalis* (29%). Accuracy of BCA identification was 84% for both species. Reduced fluconazole susceptibility was detected in 8.3% of *C. albicans* and 40.6% of *C. tropicalis*. Accuracy of disk diffusion was 100% for *C. albicans* and 62% for *C. tropicalis*. qPCR showed upregulation of CDR2 efflux pump among non-susceptible *C. albicans* (1.99 ± 1.04), though not statistically significant. *CDR1* expression was at a basal level for *C. albicans* (0.9 ± 0.25) and *C. tropicalis* (0.69 ± 0.76). *MDR-1* was significantly downregulated in non-susceptible *C. tropicalis* (0.30 ± 0.63, p= 0.025). **Conclusion:** Brilliance Candida Agar identification requires verification. The disk diffusion method is of higher accuracy with *C. albicans* than with *C. tropicalis*. Overexpression of *CDR1* and *CDR2* in *C. albicans*, and *CDR1* and *MDR1* in *C. tropicalis* were not the main reasons behind reduced susceptibility to fluconazole.

Introduction

Candida species (spp.) are the fourth in frequency as causative agents of healthcare-associated infections. The clinical manifestations of *Candida* spp. infection can range from superficial to deep-seated ones, depending on the immune status of the host [1, 2].

Candida species can be broadly classified into: *C. albicans* and *Candida* non-albicans [3]. *C. tropicalis* is regarded as the second most potent

DOI: 10.21608/MID.2023.220044.1553
* *Corresponding author:* Aliaa Aboulela
E-mail address: *aliaagamaleldin@alexu.edu.eg*

species in terms of virulence, second only to *C. albicans* [4].

Various types of antifungal medications are utilized for the treatment of *Candida* infections. These include azoles, polyenes, echinocandins, and nucleoside analogs. The effectiveness of these drugs varies depending on several factors such as the site of infection and the susceptibility of the involved *Candida* species to the drug used [5].

For years, azoles have been the most widely utilized class of antifungal drugs for treating *Candida* infections. [6]. Fluconazole is the most used azole, for its high efficacy and relatively low cost. Azoles inhibit cytochrome P450 enzyme in the ergosterol biosynthesis pathway thus interrupting cell growth [7].

Antifungal resistance occurs when fungi develop the ability to grow in the presence of antifungal drugs that would normally eradicate them or impede their growth [8]. Depending on the species, azole resistance in *Candida* might be an intrinsic property as in *C. krusei* or acquired after prolonged exposure to the drug [9]. Fluconazole resistance may develop in *C. albicans* among patients with recurrent candidiasis due to compromised immune status [6].

Azole resistance is driven by several molecular mechanisms including *ERG11* gene mutations or upregulation, and increased expression of efflux pumps [10]. The two families of efflux proteins: ATP-binding cassette transporters (ABC-T) and the major facilitator superfamily transporters (MFS) have been linked with resistance to fluconazole [11]. Two proteins from the former family were extensively described to be associated with fluconazole resistance in *C. albicans* and *C. tropicalis*: the highly homologous *Candida* drug resistance 1 protein (*CDR1*) and the *Candida* drug resistance 2 protein (*C*DR2) [7, 12, 13]. Regarding the MFS transporters, only the Multidrug Resistance Regulator (*Mdr1*) has been related to fluconazole resistance [7, 14]

This study aimed to assess the accuracy of identification of clinical *Candida* species with reduced fluconazole susceptibility by the rapid methods (chromogenic agar and disk diffusion method) and to determine the relative expression of major efflux pump genes among these isolates.

Materials and methods

A total of 111 isolates of *Candida* species were collected from different clinical specimens, obtained from different clinical laboratories in Alexandria over the period from September 2019 to June 2021. The isolates were collected from 44 urine samples, 30 vaginal swabs, 26 sputum samples, 6 blood samples, 2 samples of bronchoalveolar lavage, 1 pus sample, 1 sample of nail scrapings, and 1 wound aspirate. All samples were streaked on Sabouraud dextrose agar (SDA, Oxoid) supplemented with chloramphenicol (0.1mg/ ml) and were incubated at 37 °C for 24-48 hours.

Identification of *Candida* species and fluconazole susceptibility testing

After microscopic examination, the colonies from SDA were then subcultured on chromogenic agar (Brilliant Candida Agar (BCA), Oxoid) for presumptive species identification. The germ-tube production test was then performed to confirm species identification for the most predominant species [15].

The two most predominant species (*C. albicans* and *C. tropicalis*) were tested for susceptibility to fluconazole using fluconazole 25 µg disks (Himedia, India) according to the CLSI guidelines. Susceptible dose-dependent (SDD) and resistant isolates were collectively considered to be of reduced susceptibility to fluconazole by the disk diffusion method [16].

The identity of all isolates with reduced susceptibility to fluconazole was verified by automated testing using the Phoenix BD system. The susceptibility of these isolates to fluconazole was further confirmed by the broth microdilution method [17]. The two isolates *C. albicans* ATCC 90028 and *C. albicans* ATCC 10231 were used for quality control.

RNA extraction and reverse transcription (RT-PCR)

Total RNA was extracted from *C. albicans* and *C. tropicalis* isolates that showed reduced susceptibility to fluconazole, and from susceptible isolates of the same species which represented the control (calibrator) group. Briefly, cells were harvested from a mid-logarithmic phase culture in Yeast Extract Peptone- Dextrose (YPD) broth. After initial fungal cell disruption by bead beating using 500-570 µm glass beads (ThermoScientific, Germany), RNA was extracted by RNAzol® RT (Molecular Research Center, Inc., USA) following the manufacturer's instructions. The extracted RNA was quantified by NanoDrop™ (ThermoScientific). cDNA was prepared from a total of 1 µg of extracted

RNA using Thermo scientific RevertAid First strand cDNA Synthesis Kit (ThermoScientific™, Lithuania), with Random Hexamer primers, according to the manufacturer's instructions.

Quantitative real-time PCR (qPCR)

Gene-specific primers **(Table S1)** were used to amplify the target efflux pumps genes in addition to the housekeeping gene ACT-1 for normalization. MAXIMA™ SYBER ® Green qPCR Master Mix (Thermo Scientific™, Lithuania) was used for qPCR, followed by melting curve analysis. The obtained CT values were used for the calculation of relative gene expression using the Delta-Delta CT method. The average CT values for the fluconazole-susceptible isolates were used as the calibrator.

Statistical analysis

The normality of data was tested visually by boxplots and statistically by the Shapiro-Wilk test. Accordingly, the descriptive statistics were presented in mean (SD) and median (IQR). In the case of *C. albicans*, data were parametric, hence, student t-test (FLU-S and FLU-R) and paired t-test (*CDR1* and *CDR2*) were used for group comparisons. As for *C. tropicalis*, data were non-parametric, hence Wilcoxon rank sum test/Mann-Whitney (FLU-S and FLU-R) and Wilcoxon signed rank test (*CDR1* and *MDR1*) were used for group comparisons. Data visualization was done in the form of boxplots.

For testing the agreement of results between different methods, interpretations for the kappa statistic were as follows: < 0.2 slight agreement, 0.2 - 0.4 fair agreement, 0.4 - 0.6 moderate agreement, 0.6 - 0.8 substantial agreement, > 0.8 almost perfect agreement (Sim & Wright, 2005).

Table S1. The Primer sequences used for Syber Green Real-Time PCR.

Gene	Primer	Sequence	Reference
C. tropicalis ACT1	Forward	5′-CGTCGGTAGACCAAGACACC-3′	This study
	Reverse	5′-CCCAGTTGGAGACAATACCGT-3′	
C. tropicalis CDR1	Forward	5′-TCGCCGTTTGCTGAAGAAGA-3′	This study
	Reverse	5′-GCAATCCCCAATTTCGATGGT-3′	
C. tropicalis MDR1	Forward	5′-GCAGTTACCTCATCTGGAGCA-3′	This study
	Reverse	5′-GCACCAAACAATGGGAACACA-3′	
C. albicans ACT1	Forward	5′-GCTTTTGGTGTTTGACGAGTTTCT-3′	(Ranji et al.,2020)
	Reverse	5′-GTGAGCCGGGAAATCTGTATAGTC -3′	
C. albicans CDR1	Forward	5′- GATTCTCAAACTGCCTGGTC -3′	(Zhang et al.,2019)
	Reverse	5′-CCAAAATAAGCCGTTCTTCCAC-3′	
C. albicans CDR2	Forward	5′-TCCGAGGTGGAGCACTTTC-3′	This study
	Reverse	5′-TGGACAACTGTGCTTCCAGG-3′	

Results

Presumptive species identification by chromogenic agar and germ tube test

The 111 isolates included 36 colonies identified as *C. albicans*, 32 colonies identified as *C. tropicalis*, 30 colonies identified as (*C. parapsilosis*/ *C. glabrata*, C. *kefyr*/ *C. lusitaniae*) and 2 colonies identified as *C. krusei*. Eleven isolates gave a mixture of colors indicating mixed species.

Germ tube production test was employed for confirmation of species identification of the 2 most predominant species; *C. albicans* and *C. tropicalis*. Results showed that the germ tube test and Brilliance Candida agar (BCA) were concordant in only 30 (83%) of the isolates identified as *C. albicans* by BCA and 29 (91%) of the isolates identified as *C. tropicalis* by BCA.

Screening of the isolates for reduced susceptibility to fluconazole by disk diffusion method

Reduced susceptibility to Fluconazole by disk diffusion method was detected in 3/36 isolates of *C. albicans* (8.3%), including 2 cases (5.6%) that were resistant and one case (2.8%) that was SDD. As for *C. tropicalis*, 13/32 isolates (40.6%) showed reduced susceptibility to Fluconazole, among which 6 cases (18.8%) were resistant, and 7 cases (21.8%) were SDD.

Confirmation of species identification by Phoenix BD automated system

Phoenix BD automated system was employed to verify the identity of all isolates of *C. albicans* (n=3) and *C. tropicalis* (n=13) showing reduced susceptibility to fluconazole by disk diffusion method, in addition to some randomly selected fluconazole susceptible isolates, for use as the control group in the subsequent gene expression analysis **(Table 1)**. The randomly selected group included 7 isolates identified as *C. albicans* by Chromogenic agar (BCA), 8 isolates identified as *C. tropicalis* by BCA, in addition to 1 isolate identified as either *C. parapsilosis, C. glabrata, C. kefyr, or C. lusitaniae* by BCA which was included to double check on species identification among this group by comparison to Phoenix BD results.

Among the 10 isolates that were identified as *C. albicans* by BCA, only 7 (70%) were verified to be *C. albicans* by the Phoenix BD system. The 3 isolates with reduced susceptibility to fluconazole belonged to this group. Two of the remaining 3 misidentified isolates by BCA were identified as *C. glabrata* and 1 was identified as *C. rugosa*, and this agreed with the negative result obtained from germ tube production testing.

As for the 21 isolates that were identified as *C. tropicalis* by BCA, only 16 (76%) were verified to be *C. tropicalis* by the Phoenix BD system. The 13 isolates with reduced susceptibility to fluconazole belonged to this group. The 5 misidentified isolates by BCA as *C. tropicalis* included 2 isolates that were identified as *C. albicans* by Phoenix BD, 2 identified as *C. glabrata*, and 1 as *C. krusei*.

Results of species identification by Phoenix BD were used as a reference for the calculation of the sensitivity and specificity of identification by the latter two presumptive methods. Overall, the results of Phoenix BD identification agreed with those of BCA and germ-tube test in 24/32 isolates (75%). Statistical analysis revealed that the identification of *C. albicans* by germ tube test was of 100% sensitivity, specificity, and accuracy **(Table S2).** The accuracy of BCA identification of *C. albicans* and *C. tropicalis* was similar (84%), with higher specificity (87%) for the former and higher sensitivity (100%) for the latter **(Tables S3 , S4).**

Confirmation of the susceptibility testing results of the isolates to fluconazole by broth microdilution method

The 3 isolates of *C. albicans* that showed reduced susceptibility to fluconazole by the disk diffusion method were also found to be of reduced susceptibility to fluconazole by the broth microdilution method. As for *C. tropicalis*, only 9 isolates were resistant to fluconazole by broth microdilution method, including one isolate that previously tested susceptible to the drug by the disk diffusion method **(Table S5).**

A statistically significant very good agreement (p= 0.0027) was observed between the disk diffusion method and broth microdilution method for the fluconazole susceptibility testing results in *C. albicans*. As for *C. tropicalis*, a slight statistical agreement was detected between the results of susceptibility testing by the 2 methods (p =0.375). The accuracy for fluconazole susceptibility testing by disk diffusion method was 100% with *C. albicans*, however, it was only 62% with *C. tropicalis* **(Tables S6 , S7).**

Molecular Analysis

The primary target of the current study was to detect the fold difference in gene expression of the efflux pumps *CDR1* and *CDR2* in fluconazole non-susceptible (FLU-R) isolates of *C. albicans* (n= 3) versus a matched control group of fluconazole susceptible (FLU-S) isolates (n= 3). Also, to detect fold difference in gene expression of the efflux pumps *CDR1* and *MDR1* in fluconazole non-susceptible *C. tropicalis* (n= 9) versus a fluconazole susceptible control group (n= 4).

Relative gene expression of *CDR1* and *CDR2* efflux pumps in *C. albicans*

In *C. albicans*, the mean fold difference in the expression of the *CDR1* efflux pump was at a basal level in both Fluconazole non-susceptible (0.73 ± 0.24) and susceptible (0.9 ± 0.25) isolates. Meanwhile, *CDR2* was upregulated (1.99 ± 1.04) in Fluconazole non-susceptible isolates, with a basal level of gene expression in susceptible isolates (1.22 ± 0.99) **(Table 2, Figure 1).**

Relative gene expression of *CDR1* and *MDR1* efflux pumps in *C. tropicalis*

In *C. tropicalis*, the mean fold difference in the expression of the *CDR1* efflux pump was at a basal level in both fluconazole non-susceptible (0.69 ± 0.76) and fluconazole-susceptible (1.08 ± 0.5) isolates. Meanwhile, *MDR1* was downregulated (0.30 ± 0.63) in fluconazole non-susceptible isolates, with a basal level of gene expression in susceptible isolates (1.16 ± 0.77), and this difference was statistically significant (p= 0.025) **(Table 3, Figure 2).**

Table 1. Results of identification of 32 isolates by BD Phoenix™ system.

Serial Number	Isolate ID	Chrome agar (BCA) identification	Germ-tube identification	BD Phoenix identification (n: 32)
1.	8	*C. tropicalis*	Negative	*C. tropicalis*
2.	13	*C. tropicalis*	Negative	*C. tropicalis*
3.	15	*C. tropicalis*	Negative	*C. tropicalis*
4.	26	*C. tropicalis*	Negative	*C. tropicalis*
5.	36	*C. tropicalis*	Negative	*C. tropicalis*
6.	41	*C. tropicalis*	Negative	*C. tropicalis*
7.	52	*C. tropicalis*	Negative	*C. tropicalis*
8.	56	*C. tropicalis*	Negative	*C. tropicalis*
9.	59	*C. tropicalis*	Negative	*C. tropicalis*
10.	94	*C. tropicalis*	Negative	*C. tropicalis*
11.	3M	*C. tropicalis*	Negative	*C. tropicalis*
12.	5M	*C. tropicalis*	Negative	*C. tropicalis*
13.	6M	*C. tropicalis*	Negative	*C. tropicalis*
14.	7M	*C. tropicalis*	Negative	*C. tropicalis*
15.	8M	*C. tropicalis*	Negative	*C. tropicalis*
16.	9M	*C. tropicalis*	Negative	*C. tropicalis*
17.	46	*C. tropicalis*	Positive	*C. albicans*
18.	65	*C. tropicalis*	Positive	*C. albicans*
19.	51	*C. tropicalis*	Negative	*C. glabrata*
20.	7	*C. tropicalis*	Negative	*C. glabrata*
21.	4M	*C. tropicalis*	Negative	*C. krusei*
22.	19	*C. albicans*	Positive	*C. albicans*
23.	25	*C. albicans*	Positive	*C. albicans*
24.	30	*C. albicans*	Positive	*C. albicans*
25.	71	*C. albicans*	Positive	*C. albicans*
26.	111	*C. albicans*	Positive	*C. albicans*
27.	113	*C. albicans*	Positive	*C. albicans*
28.	125	*C. albicans*	Positive	*C. albicans*
29.	83	*C. albicans*	Negative	*C. glabrata*
30.	118	*C. albicans*	Negative	*C. glabrata*
31.	60	*C. albicans*	Negative	*C. rugosa*
32.	84	*C. parapsilosis, C. glabrata, C. kefyr, or C. lusitaniae*	Negative	*C. glabrata*

Table 2. Comparison between fluconazole susceptible (FLU-S) and non-susceptible (FLU-R) isolates according to the fold change in gene expression of CDR1 and CDR2 efflux pump genes in *C. albicans.*

Efflux pump genes in *C. albicans*	FLU-S (n = 3)	FLU-R (n = 3)	T	*P*-value
CDR1				
Mean ± SD.	0.9 ± 0.25	0.73 ± 0.24	-0.859	0.439
Median (IQR.)	0.8 (0.8 to 1.0)	0.6 (0.6 to 0.8)		
CDR2				
Mean ± SD.	1.22 ± 0.99	1.99 ± 1.04	0.922	0.409
Median (IQR.)	0.9 (0.7 to 1.6)	2.4 (1.6 to 2.6)		
***P°*-value**	0.651	0.145		

SD: standard deviation. IQR: interquartile range, FLU-S: fluconazole sensitive, FLU-R: fluconazole resistant. t: Student t-test, *p*: p value for comparing between FLU-S and FLU-R. p_0: p value for Paired t-test for comparing between CDR1 and CDR2 in each group. *P*-values ≥ 0.05 is not statistically significant.

Table 3. Comparison between fluconazole susceptible (FLU-S) and non-susceptible (FLU-R) isolates according to the fold change in gene expression of CDR1 and MDR1 efflux pump genes in C. tropicalis.

Efflux pump genes in *C. tropicalis*	**FLU-S (n = 4)**	**FLU-R (n = 9)**	**U**	***P*-value**
CDR1				
Mean ± SD.	1.08 ± 0.5	0.69 ± 0.76	9	0.199
Median (IQR.)	1.0 (0.7 to 1.3)	0.4 (0.2 to 1.0)		
MDR1				
Mean ± SD.	1.16 ± 0.77	0.30 ± 0.63	3	**0.025***
Median (IQR.)	0.9 (0.8 to 1.3)	0.0 (0.0 to 0.2)		
***P*°-value**	0.875	0.0753		

SD: standard deviation. IQR: interquartile range, FLU-S: fluconazole sensitive, FLU-R: fluconazole-resistant, u: **Mann Whitney test/Wilcoxon rank sum test,** *p*: p value for comparing between **FLU-S** and **FLU-R,** p_0: p value for **Wilcoxon signed ranks test** for comparing between **CDR1** and **MDR1** in each group**,** *: Statistically significant at $p \leq 0.05$

Table S2. Sensitivity, specificity, and accuracy of germ tube production test in identification of *C. albicans* with reference to Phoenix BD identification.

C. albicans	**Phoenix BD**			**Sensitivity (95% CI#)**	**Specificity (95% CI)**	**PPV (95% CI)**	**NPV (95% CI)**	**Accuracy (95% CI)**
Germ tube test	Positive	Negative	**Total**					
Positive	9	0	9	1.00 (0.66, 1.00)	1.00 (0.85, 1.00)	1.00 (0.66, 1.00)	1.00 (0.85, 1.00)	1.00 (0.89, 1.00)
Negative	0	23	23					
Total	**9**	**23**	**32**					
□□(p)	5.656854 (**0.000000015***)							
Kappa	1							
Degree of agreement	Almost perfect							

Z (p): Z test statistic with its p-value, 95% CI: Intervals with 95% confidence Common interpretations for the kappa statistic are as follows: < 0.2 slight agreement, 0.2 - 0.4 fair agreement, 0.4 - 0.6 moderate agreement, 0.6 - 0.8 substantial agreement, > 0.8 almost perfect agreement (Sim & Wright, 2005)

Table S3. Sensitivity, specificity, and accuracy of Chromogenic agar (BCA) in identification of *C. albicans* with reference to BD Phoenix identification

C. albicans	**Phoenix BD**			**Sensitivity (95% CI#)**	**Specificity (95% CI)**	**PPV (95% CI)**	**NPV (95% CI)**	**Accuracy (95% CI)**
BCA	Positive	Negative	**Total**					
Positive	7	3	10	0.78 (0.40, 0.97)	0.87 (0.66, 0.97)	0.70 (0.35, 0.93)	0.91 (0.71, 0.99)	0.84 (0.67, 0.95)
Negative	2	20	22					
Total	**9**	**23**	**32**					
Z (p)	3.552084 (**0.00038***)							
Kappa	0.626							
Degree of agreement	Substantial agreement							

BCA: brilliance chromogenic agar, Z (p): Z test statistic with its p-value, 95% CI: Intervals with 95% confidence Common interpretations for the kappa statistic are as follows: < 0.2 slight agreement, 0.2 - 0.4 fair agreement, 0.4 - 0.6 moderate agreement, 0.6 - 0.8 substantial agreement, > 0.8 almost perfect agreement (Sim & Wright, 2005).

Table S4. Sensitivity, specificity, and accuracy of Chromogenic agar (BCA) in identification of *C. tropicalis* with reference to BD Phoenix identification.

C. tropicalis	**BD Phoenix**			**Sensitivity (95% CI#)**	**Specificity (95% CI)**	**PPV (95% CI)**	**NPV (95% CI)**	**Accuracy (95% CI)**
BCA	**Positive**	**Negative**	**Total**					
Positive	16	5	21	1.00 (0.79, 1.00)	0.69 (0.41, 0.89)	0.76 (0.53, 0.92)	1.00 (0.72, 1.00)	0.84 (0.67, 0.95)
Negative	0	11	11					
Total	**16**	**16**	**32**					
Z (p)	4.094131 (**0.00004***)							
Kappa	0.6875							
Degree of agreement	Substantial agreement							

BCA: brilliance chromogenic agar, Z (p): Z test statistic with its p-value, 95% CI: Intervals with 95% confidence Common interpretations for the kappa statistic are as follows: < 0.2 slight agreement, 0.2 - 0.4 fair agreement, 0.4 - 0.6 moderate agreement, 0.6 - 0.8 substantial agreement, > 0.8 almost perfect agreement (Sim & Wright, 2005).

Table S5. Results of fluconazole minimum inhibitory concentration (MIC) by broth microdilution method.

Species	**MIC (µg/ ml)**			
	Sensitive ≤ 2 µg/ ml		**Reduced susceptibility** > 2 µg/ ml	
C. albicans **(n=9)**	**No.**	**Percentage**	**No.**	**Percentage**
	6	67%	3	33%
Samples id	19, 30, 46, 65, 71, 113		25, 111, 125[a]	
C. tropicalis **(n=16)**	**No.**	**Percentage**	**No.**	**Percentage**
	7	44%	9	56%
Samples id	36, 41, 52, 56, 59, 94, 8M[a]		8, 13, 15, 26, 3M, 5M, 6M, 7M, 9M[a]	
Total	13		12	

[a:] The isolates in red and blue were resistant, and susceptible dose dependent to fluconazole by disk diffusion method, respectively.

Table S6. Agreement between the results of disk diffusion and broth microdilution results for susceptibility testing of fluconazole in *C. albicans.*

C. albicans (n= 9)	**Broth microdilution**			**Sensitivity (95% CI#)**	**Specificity (95% CI)**	**PPV (95% CI)**	**NPV (95% CI)**	**Accuracy (95% CI)**
Disk diffusion	**Non-susceptible (n = 3)**	**susceptible (n = 6)**	**Total**					
Fluconazole Non-susceptible	3	0	3	1.00 (0.29, 1.00)	1.00 (0.54, 1.00)	1.00 (0.29, 1.00)	1.00 (0.54, 1.00)	1.00 (0.66, 1.00)
Fluconazole Susceptible	0	6	6					
Total	**3**	**6**	**9**					
□□(p)	3 (**0.0027***)							
Kappa	1							
Degree of agreement	Almost perfect							

MIC: minimum inhibitory concentration, Z (p): Z test statistic with its p-value, 95% CI: Intervals with 95% confidence Common interpretations for the kappa statistic are as follows: < 0.2 slight agreement, 0.2 - 0.4 fair agreement, 0.4 - 0.6 moderate agreement, 0.6 - 0.8 substantial agreement, > 0.8 almost perfect agreement (Sim & Wright, 2005).

Table S7. Agreement between the results of disk diffusion and broth microdilution results for susceptibility testing of fluconazole in *C. tropicalis.*

C. tropicalis (n= 16)	**Broth microdilution**			**Sensitivity (95% CI#)**	**Specificity (95% CI)**	**PPV (95% CI)**	**NPV (95% CI)**	**Accuracy (95% CI)**
Disk diffusion	**Non-susceptible (n = 9)**	**Susceptible (n = 7)**	**Total**					
Fluconazole Non-susceptible	8	5	13	0.89 (0.52, 1.00)	0.29 (0.04, 0.71)	0.62 (0.32, 0.86)	0.67 (0.09, 0.99)	0.62 (0.35,0 .85)
Fluconazole Susceptible	1	2	3					
Total	**9**	**7**	**16**					
□□(p)	0.887667 (0.37472)							
Kappa	0.1864407							
Degree of agreement	Slight agreement							

MIC: minimum inhibitory concentration, Z: Z test statistic, 95% CI: Intervals with 95% confidence Common interpretations for the kappa statistic are as follows: < 0.2 slight agreement, 0.2 - 0.4 fair agreement, 0.4 - 0.6 moderate agreement, 0.6 - 0.8 substantial agreement, > 0.8 almost perfect agreement (Sim & Wright, 2005).

Figure 1. Boxplots showing differences in gene expressions of the efflux pump genes (CDR1 and CDR2) between fluconazole susceptible (FLU-S) and fluconazole non-susceptible (FLU-R) groups of C. albicans.

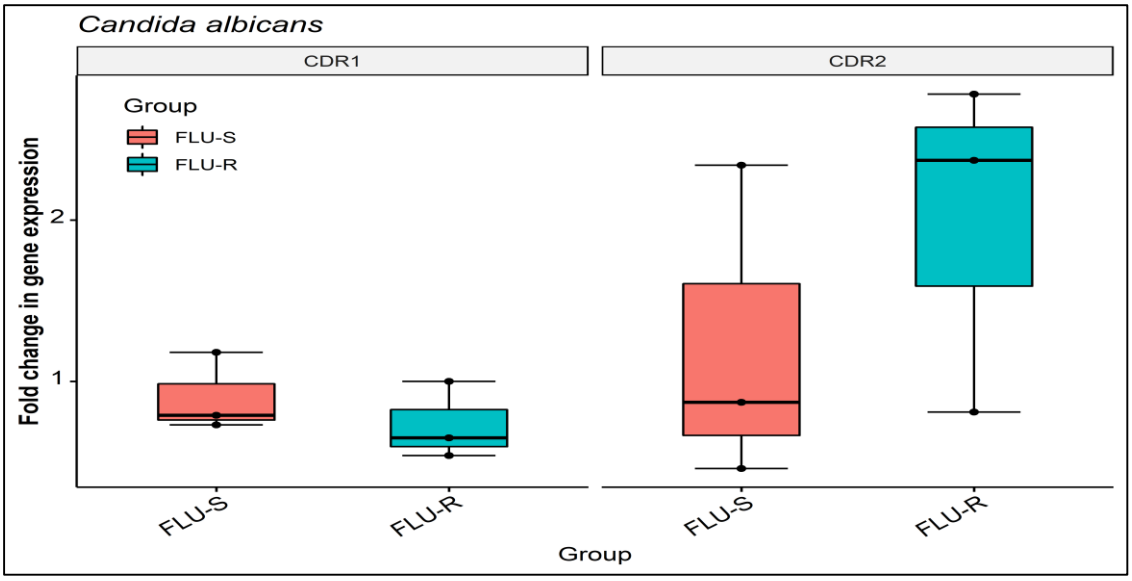
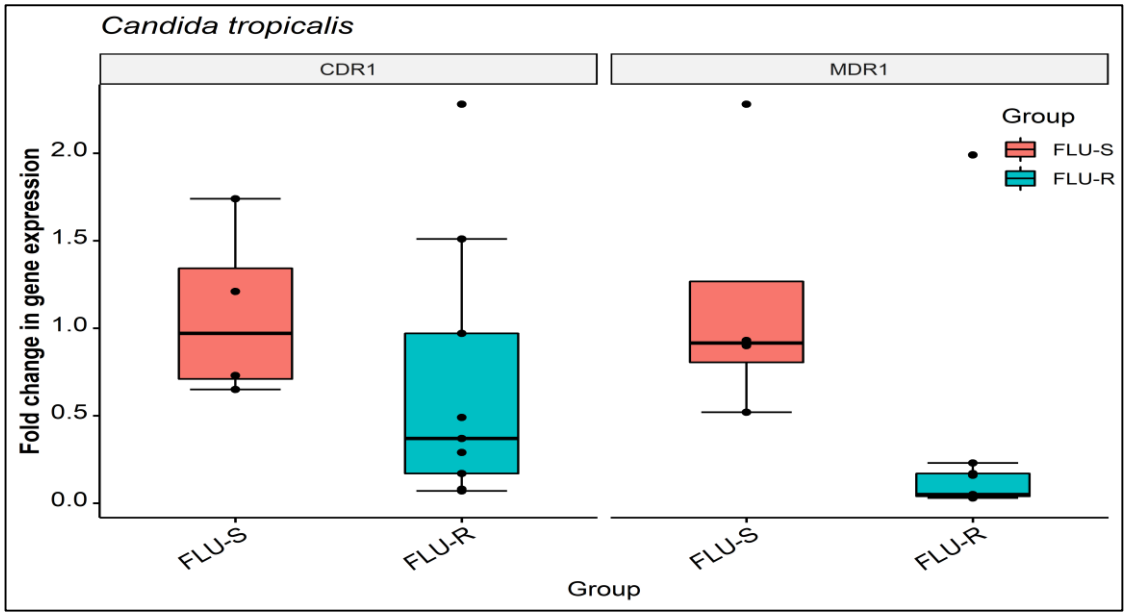


Figure 2. Boxplots showing differences in gene expressions of efflux pump genes (*CDR1* and *MDR1*) between fluconazole susceptible (FLU-S) and fluconazole non-susceptible (FLU-R) groups of *C. tropicalis.*



Discussion

Azoles are the most widely used class of antifungals. Fluconazole is usually the first choice for the treatment of *Candida* infections. Various molecular mechanisms underly fluconazole resistance, which might be intrinsic or acquired depending on the species of *Candida* [7, 11,18].

The purpose of the present study was to quantify the relative gene expression of drug efflux pumps among common clinical *Candida* species with non-susceptibility to fluconazole. Preliminary identification revealed that the most predominant species was *C. albicans* (n=36, 32%) while *C. tropicalis* ranked second and accounted for 29% (n=32). Verification of *C. albicans* and *C. tropicalis* species identification by germ tube production test revealed disagreement in 6/36 and 3/32 isolates of the two species, respectively. This implies that species identification should not rely solely on chromogenic media.

The predominance of *C. albicans* followed by *C. tropicalis* as the most common species causing clinical infection has been reported previously by several studies [19-21]. The proper identification of *Candida* species is crucial for therapeutic decision-making to discriminate between intrinsic and acquired resistance [7, 22].

Several studies relied on the disk diffusion method per se, or in combination with another method for MIC determination. For instance, **Jabeen et al.** [23] implemented this method for validation of the use of antifungal disks for direct testing of the susceptibility of *Candida* spp. in positive blood culture bottles. Moreover, **Yassin et al.** [24] and **Zarrinfar et al.** [25] implemented this method for the identification of resistant *Candida* strains among women suffering from vulvovaginitis.

In the current study, reduced susceptibility to fluconazole by disk diffusion method was much more observed with *C. tropicalis* at 40.6%, compared with 8.3% in *C. albicans*. The higher level of resistance to fluconazole in *C. tropicalis* compared with *C. albicans* was also reported in other regions [20, 26].

To further confirm the identity of the isolates under study, we considered Phoenix BD as the reference method. The strength of agreement between the identification of *C. albicans* by Phoenix BD system and germ-tube test was very good (p <0.001), with a lower value observed with BCA identification.

This high specificity (100%) of germ-tube identification of *C. albicans* observed in this study agrees with that reported by **Sheppard et al.** [27] who even suggested the use of this test for direct rapid identification of *C. albicans* from blood culture bottles.

This study showed that BCA resulted in the misidentification of *Candida* species as *C. albicans*

in 9.3% of the isolates and as *C. tropicalis* in 15.6%. This agreed with the findings of **Vera et al.** [28] who reported that chromogenic media results should not be relied upon as the sole method for species identification, and they should be accompanied by additional tests.

The misidentification of *C. albicans* isolates by BCA was previously reported by **Sariguzel et al.** at 5.26% [29] however, no isolates of *C. tropicalis* were misidentified by this chromogenic media in their study. Contrary to the findings of the current study, **Scharmann et al.** [30] reported that BCA resulted in the correct identification of all tested isolates of *C. albicans* and *C. tropicalis* after 48 hours incubation, however, they reported that at 24 hours incubation, only 36% of *C. albicans* and 67% of *C. tropicalis* could be identified.

The BCA had a higher sensitivity for the identification of *C. tropicalis* (100%) compared with *C. albicans* (78%). However, the specificity of BCA for the identification of *C. tropicalis* (69%) was less than its specificity for *C. albicans* (87%).

The selection of a reference method for the identification of *Candida* species to the species level has been a subject of research for many studies. In the current study, we employed BD Phoenix as our reference method due to the unavailability of MALDI-TOF in Alexandria, Egypt, and the high expenses of molecular identification by sequencing of the ITS1-5.8S-ITS2 region.

The study of **Marucco et al.** [31] evaluated the agreement between BD Phoenix and MALDI-TOF identification of *Candida* species. They reported that the two methods had a 100% and 97% concordance index for the identification of *C. albicans.* and *C. tropicalis*, respectively.

Candida species identification by BD Phoenix was also evaluated by **Posteraro et al.** [32] who relied on molecular identification as their gold standard reference method. They found that the results of BD Phoenix were 100% and 98.5% concordant with the results of molecular identification of *C. tropicalis*, and *C. albicans*, respectively.

The broth microdilution method is considered one of the reference methods for testing the susceptibility of yeasts to antifungals [7]. We implemented the broth microdilution method for confirmation of the fluconazole non-susceptibility results of the included *C. albicans* (n=9) and *C. tropicalis* (n= 16) clinical isolates which were of verified identity by Phoenix BD. Based on the obtained MIC values, this study found that the accuracy of the disk diffusion method for identifying fluconazole non-susceptibility was low in *C. tropicalis* (62%) compared with an accuracy of 100% for *C. albicans.*

To determine the contribution of efflux pumps to fluconazole non-susceptibility this study investigated the relative gene expression of 2 efflux pumps in *C. albicans*; CDR1 and CDR2, and 2 in *C. tropicalis*; *CDR1* and *MDR1*. These pumps were selected for being of well-documented association with azole antifungal resistance [22, 33].

This study revealed that the *CDR2* efflux pump was upregulated in the fluconazole non-susceptible isolates of *C. albicans* while *CDR1* expression was at a basal level and did not significantly differ from the fluconazole-susceptible isolates. These findings agreed with those reported by **Ariana et al.** [34] who concluded that the increased expression of *CDR1*, and *CDR2* did not play a significant role in the development of resistance to fluconazole.

On the contrary, a study in China revealed that relative gene expression of *CDR1* was significantly different between the fluconazole-resistant and susceptible isolates of *C. albicans* (p = 0.0193), while *CDR2* gene expression did not significantly differ among the 2 groups (p = 0.55) [35]. Likewise, an Iranian study also reported that *CDR1* gene expression was statistically significantly upregulated in the fluconazole resistant isolates of *C. albicans* compared to the Fluconazole susceptible group (p < 0.03). Meanwhile, *CDR2* relative gene expression was similar among the fluconazole-resistant and sensitive groups [36].

Regarding *C. tropicalis*, *CDR1* expression was also found to be at a basal level among fluconazole-susceptible and non-susceptible groups. Meanwhile, the *MDR-1* efflux pump was found to be downregulated among the isolates with reduced susceptibility to fluconazole with a basal level of expression among the fluconazole-susceptible isolates, and this was statistically significant (p= 0.025). This could potentially exclude the role of *MDR-1* as an active player causing reduced susceptibility to fluconazole among our isolates of *C. tropicalis.*

The findings of this work agreed in part with the results of **Fan et al.** [37] and **Jiang et al.**

[38] who also investigated the gene expression of *CDR1* and *MDR1* efflux pumps in azole-resistant clinical isolates of *C. tropicalis* and reported no statistically significant difference between the resistant and the susceptible groups. Both studies reported that the upregulation of multidrug resistance efflux pumps plays a minor role in azole resistance in *C. tropicalis*.

The results of **Jin et al.** [39] also partially agreed with the findings of the current study with regard to the gene expression level of *CDR1* in fluconazole-resistant isolates of *C. tropicalis*, as there was no statistically significant difference observed when compared with the fluconazole-susceptible group (p=0.262). However, they reported that the *MDR1* efflux pump was statistically significantly upregulated among their resistant isolates (p <0.05).

In contrast to the findings of this study, **Pandey et al.** [40] reported that the 2 efflux pumps *CDR1* and *MDR1* were statistically significantly more upregulated in the fluconazole-resistant isolates of *C. tropicalis* when compared with the fluconazole susceptible group (p <0.05).

Conclusion

We concluded that clinical *Candida* species identification by Brilliance Candida Agar (BCA) needs to be verified by a more reliable method due to the presence of some inaccuracy. BCA is of higher specificity in the identification of *C. albicans* and higher sensitivity for detecting *C. tropicalis.*

Fluconazole susceptibility testing by the disk diffusion method is of high accuracy with *C. albicans* and low accuracy for *C. tropicalis*.

The overexpression of the efflux pumps *CDR1* and *CDR2* in *C. albicans*, and *CDR1* and *MDR1* in *C. tropicalis* might not be the main players behind reduced susceptibility to fluconazole.

Ethical considerations

This study was approved by the Ethics Committee of the Medical Research Institute, Alexandria University (IORG#: IORG0008812).

Authors' contributions

The conception and design of the study: Aliaa Aboulela. The acquisition of data: Samah Idris. The analysis and interpretation of data: Rasha Emad, Aliaa Aboulela. The drafting of the article: Aliaa Aboulela. The critical revision for important intellectual content: Gamal Elsawaf, Dalia Ragab, Aliaa Aboulela. The final approval of the version to be submitted: All authors.

Conflicts of interest

There are no conflicts of interest to disclose.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

References

1- **Du H, Bing J, Hu T, Ennis CL, Nobile CJ, Huang G.** *Candida auris*: Epidemiology, biology, antifungal resistance, and virulence. PLoS pathogens 2020;16(10):e1008921. https://doi.org/10.1371/journal.ppat.1008921

2- Sarma S, Upadhyay S. Current perspective on emergence, diagnosis and drug resistance in *Candida auris*. Infection and drug resistance 2017:155-65.

3- **Ciurea CN, Kosovski I-B, Mare AD, Toma F, Pintea-Simon IA, Man A.** *Candida* and candidiasis—opportunism versus pathogenicity: a review of the virulence traits. Microorganisms 2020;8(6):857.

4- **Zuza-Alves DL, Silva-Rocha WP, Chaves GM.** An update on *Candida tropicalis* based on basic and clinical approaches. Frontiers in microbiology 2017; 8:1927.

5- **Pappas PG, Kauffman CA, Andes DR, Clancy CJ, Marr KA, Ostrosky-Zeichner L, et al.** Clinical practice guideline for the management of candidiasis: 2016 update by the Infectious Diseases Society of America. Clinical Infectious Diseases 2016;62(4):e1-e50.

6- **Arendrup MC, Patterson TF.** Multidrug-resistant *Candida*: epidemiology, molecular mechanisms, and treatment. The Journal of infectious diseases 2017;216(suppl_3):S445-S51.

7- **Berkow EL, Lockhart SR.** Fluconazole resistance in *Candida* species: a current

perspective. Infection and drug resistance 2017:237-45.

8- **Pristov K, Ghannoum M.** Resistance of *Candida* to azoles and echinocandins worldwide. Clinical Microbiology and Infection 2019;25(7):792-8.

9- **Tortorano AM, Prigitano A, Morroni G, Brescini L, Barchiesi F.** Candidemia: evolution of drug resistance and novel therapeutic approaches. Infection and Drug Resistance 2021:5543-53.

10- **Scorzoni L, de Paula e Silva AC, Marcos CM, Assato PA, de Melo WC, de Oliveira HC, et al.** Antifungal therapy: new advances in the understanding and treatment of mycosis. Frontiers in microbiology 2017; 8:36.

11- **Bhattacharya S, Sae-Tia S, Fries BC.** Candidiasis and mechanisms of antifungal resistance. Antibiotics 2020;9(6):312.

12- **Prasad R, Banerjee A, Khandelwal NK, Dhamgaye S.** The ABCs of *Candida albicans* multidrug transporter Cdr1. Eukaryotic cell 2015;14(12):1154-64.

13- **Chen PY, Chuang YC, Wu UI, Sun HY, Wang JT, Sheng WH, et al.** Mechanisms of azole resistance and trailing in *Candida tropicalis* bloodstream isolates. Journal of Fungi 2021;7(08):612.

14- **Logan A, Wolfe A, Williamson JC.** Antifungal resistance and the role of new therapeutic agents. Current Infectious Disease Reports 2022;24(9):105-16.

15- **Moya-Salazar J, Rojas R.** Comparative study for identification of *Candida albicans* with germ tube test in human serum and plasma. Clinical Microbiology and Infectious Diseases 2018;3(3):1-4.

16- **The Clinical & Laboratory Standards Institute (CLSI).** Method for Antifungal Disk Diffusion Susceptibility Testing of Yeasts. 3rd ed. CLSI guideline M44. Wayne, Pennsylvania, USA: CLSI; 2018.

17- **European Committee on Antimicrobial Susceptibility Testing (EUCAST).** Definitive document E. Def 7.3. 2. Method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for yeasts. Copenhagen, Denmark.: EUCAST; 2020.

18- **Pappas PG, Lionakis MS, Arendrup MC, Ostrosky-Zeichner L, Kullberg BJ.** Invasive Candidiasis. Nature Reviews Disease Primers 2018;4(1):1-20.

19- **Charles MP, Kali A, Joseph NM**. Performance of chromogenic media for *Candida* in rapid presumptive identification of *Candida* species from clinical materials. Pharmacognosy Research 2015;7(5s).

20- **Khadka S, Sherchand JB, Pokhrel BM, Parajuli K, Mishra SK, Sharma S, et al.** Isolation, speciation and antifungal susceptibility testing of *Candida* isolates from various clinical specimens at a tertiary care hospital, Nepal. BMC research notes 2017;10(1):1-5.

21- **Salem SAE-A.** Identification and in Vitro Susceptibility Pattern of Fungal Pathogens in Immunocompromised Patients with invasive Fungal Infections. Egyptian Journal of Medical Microbiology 2021;30(3):127-34.

22- **Whaley SG, Berkow EL, Rybak JM, Nishimoto AT, Barker KS, Rogers PD.** Azole antifungal resistance in *Candida albicans* and emerging non-albicans Candida species. Frontiers in microbiology 2017; 7:2173.

23- **Jabeen K, Kumar H, Farooqi J, Mehboob R, Brandt ME, Zafar A.** Agreement of direct antifungal susceptibility testing from positive blood culture bottles with the conventional

method for *Candida* species. Journal of Clinical Microbiology 2016;54(2):343-8.

24-Yassin MT, Mostafa AA, Al-Askar AA, Bdeer R. In vitro antifungal resistance profile of *Candida* strains isolated from Saudi women suffering from vulvovaginitis. European journal of medical research 2020;25(1):1-9.

25-Zarrinfar H, Kord Z, Fata A. High incidence of azole resistance among *Candida albicans* and C. glabrata isolates in Northeastern Iran. Current Medical Mycology 2021;7(3):18.

26-Jeon S, Shin JH, Lim HJ, Choi MJ, Byun SA, Lee D, et al. Disk diffusion susceptibility testing for the rapid detection of fluconazole resistance in *Candida* Isolates. Annals of laboratory medicine 2021;41(6):559-67.

27-Sheppard DC, Locas M-C, Restieri C, Laverdiere M. Utility of the germ tube test for direct identification of *Candida albicans* from positive blood culture bottles. Journal of Clinical Microbiology 2008;46(10):3508-9.

28-Vera L, Boyen F, De Visscher A, Vandenbroucke V, Vanantwerpen G, Govaere J. Limitations of a chromogenic agar plate for the identifying bacteria isolated from equine endometritis samples. Equine veterinary journal 2019;51(2):266-9.

29-Sariguzel FM, Berk E, Koc AN, Sav H, Demir G. Investigation of the relationship between virulence factors and genotype of *Candida* spp. isolated from blood cultures. The Journal of Infection in Developing Countries 2015;9(08):857-64.

30-Scharmann U, Kirchhoff L, Chapot VlS, Dziobaka J, Verhasselt HL, Stauf R, et al. Comparison of four commercially available chromogenic media to identify Candida albicans and other medically relevant *Candida* species. Mycoses 2020;63(8):823-31.

31-Marucco AP, Minervini P, Snitman GV, Sorge A, Guelfand LI, Moral LL, et al. Comparison of the identification results of *Candida* species obtained by BD Phoenix™ and Maldi-TOF (Bruker Microflex LT Biotyper 3.1). Revista argentina de microbiología 2018;50(4):337-40.

32-Posteraro B, Ruggeri A, De Carolis E, Torelli R, Vella A, De Maio F, et al. Comparative evaluation of BD Phoenix and Vitek 2 systems for species identification of common and uncommon pathogenic yeasts. Journal of clinical microbiology 2013;51(11):3841-5.

33-Brilhante RS, Paiva MA, Sampaio C, Castelo-Branco DS, Teixeira CE, Alencar LPd, et al. Azole resistance in *Candida* spp. isolated from Catú Lake, Ceará, Brazil: an efflux-pump-mediated mechanism. brazilian journal of microbiology 2016; 47:33-8.

34-Ariana N, Nazemi A, Nasrollahi Omran A. Using PCR to Compare the Expression of CDR1, CDR2, and MDR1 in *Candida albicans* Isolates Resistant and Susceptible to Fluconazole. Medical Laboratory Journal 2015;9(4):33-7.

35-Zhang H, Xu Q, Li S, Ying Y, Zhang Z, Zeng L, et al. Gene Expression Analysis of Key Players Associated with Fluconazole Resistance in *Candida albicans*. Jundishapur Journal of Microbiology 2019;12(7).

36-Maheronnaghsh M, Teimoori A, Dehghan P, Fatahinia M. The evaluation of the overexpression of the ERG-11, MDR-1, CDR-1, and CDR-2 genes in fluconazole-resistant *Candida albicans* isolated from Ahvazian cancer patients with oral candidiasis. Journal of Clinical Laboratory Analysis 2022;36(2):e24208.

37-Fan X, Xiao M, Zhang D, Huang J-J, Wang H, Hou X, et al. Molecular mechanisms of

azole resistance in *Candida tropicalis* isolates causing invasive candidiasis in China. Clinical Microbiology and Infection 2019;25(7):885-91.

38-**Jiang C, Dong D, Yu B, Cai G, Wang X, Ji Y, et al.** Mechanisms of azole resistance in 52 clinical isolates of *Candida tropicalis* in China. Journal of Antimicrobial Chemotherapy 2013;68(4):778-85.

39-**Jin L, Cao Z, Wang Q, Wang Y, Wang X, Chen H, et al.** MDR1 overexpression combined with ERG11 mutations induce high-level fluconazole resistance in *Candida tropicalis* clinical isolates. BMC Infectious Diseases 2018; 18:1-6.

40-**Pandey N, Tripathi M, Gupta MK, Tilak R.** Overexpression of efflux pump transporter genes and mutations in ERG11 pave the way to fluconazole resistance in *Candida tropicalis*: A study from a North India region. Journal of Global Antimicrobial Resistance 2020; 22:374-8.

Aboulela A, Idris S, Ragab D, Emad R, El-Sawaf G. Assessment of the accuracy of identification of clinical Candida species with reduced fluconazole susceptibility by rapid methods and their relative expression of major efflux pump genes. Microbes Infect Dis 2023; 4(3): 994-1007.