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Original article

Evaluation of ERG-11 gene expression in azole resistant *Candida* isolates from various clinical specimens in ICUs

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ABSTRACT

Background: *Candida* infection is considered one of the most dangerous infections in intensive care unit patients (ICU). Resistance to azoles is a major challenging condition in treating candida infections. Mutations or overexpression of the ERG11 gene may contribute to azoles resistance. **Methods:** This prospective study evaluated 300 clinical samples including sputum, blood, urine, wound swabs, and pus obtained from different ICU patients. Different *candida* species were identified phenotypically by conventional methods and biochemical analysis by API 20 C kit (api® Candida) and confirmed by VITEK 2 compact system using identification yeast ID-YST cards. Antifungal susceptibility testing was performed using the VITEK 2 compact system followed by detection of ERG11-2 gene overexpression by RT-PCR in azoles resistant *candida* species. **Results:** A total of 165 candida species were detected from 300 clinical samples. *Candida albicans* (*C. albicans)* was identified in 59.4%. Most of *C. albicans* and *non albicans* were isolated from sputum samples. Only 17% of the overall isolated *Candida* species were resistant to fluconazole while 5% were resistant to voriconazole. Overexpression of the ERG11 gene was detected only in 10 isolates. **Conclusion:** Increased ERG11 gene expression may be one of the leading causes of azoles resistance among *candida* species in ICU patients.

Introduction

Candida species are the main leading cause of fungal infections that are usually associated with wide range of infections either mild mucocutaneous candidiasis or severe invasive candidiasis with high morbidity and mortality rate [1].

Multiple risk factors like prolonged use of broad-spectrum antibiotics, immunosuppression, and the use of invasive devices are associated with high incidence of *Candida* infections in intensive care unit (ICU) patients [2].

Most cases of candidiasis are caused by *Candida albicans* (*C. albicans)*. Nowadays, non-albicans Candida species (NAC) such as *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, and *C. krusei* have been reported by several studies as a major cause of candida infection [3,4].

Azole antifungals have been commonly used as first-line therapy for empiric treatment of candida infections [5]. Azoles inhibit lanosterol 14α-demethylase enzyme which is encoded by the ERG11 gene that is responsible for the synthesis of

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ergosterol essential for fungal cell membrane function [6].

The widespread and long-term use of azoles such as fluconazole has been associated with the emergence of azole resistant candida isolates [7]. Mutations or alterations in the expression of the ERG11 gene are involved as one of the molecular mechanisms of acquired azoles resistance [8]. Overexpression of ERG11 above normal results in an increased synthesis of the encoded enzyme, lanosterol 14α-demethylase, the primary target of azole drugs. As a result of the elevated enzyme levels, the intracellular concentration of azoles is insufficient to inhibit its function [9].

The antifungal susceptibility patterns of candida species are usually different; some NACs exhibit intrinsic resistance to certain antifungal agents. Therefore, accurate identification of isolates and antifungal sensitivity is essential for accurate diagnosis, proper selection of the appropriate treatment, reducing mortality, control outbreaks, and performing epidemiological investigations [10,11].

The epidemiological data regarding the distribution, the susceptibility pattern, and molecular mechanism of azole resistance in candida spp are scarce in Tanta university hospital. So this study was designed to identify the distribution of different candida species in ICUs, their antifungal susceptibility testing, and evaluation of ERG 11 gene expression in azoles resistant candida species obtained from different clinical samples.

Materials and Methods

This prospective study was conducted in the department of Medical Microbiology and Immunology, faculty of medicine, Tanta university from January 2020 to September 2020. All procedures were carried out under the tenets of the Helsinki Declaration. Written consent was obtained by all participants after discussing the procedure and possible benefits and risks. The study was approved by the Institutional Human ethical committee of Tanta University.

Sample collection

A total of 300 clinical samples including sputum (87), blood (30), urine (98) wound swabs (35), and pus (50) were collected from patients admitted to different ICUs (anesthesiology, surgery, and internal medicine) of Tanta University Hospitals, Egypt according to standard procedures related to each sample under complete aseptic technique [12]. Specimens were transferred immediately to the medical Microbiology and Immunology Department, faculty of medicine, Tanta University. Patients with increased risk of fungal infection such as prolonged use of antibiotics, immunosuppressed patients, or patients with intravascular devices were included while infections other than *candida* were not enrolled in the present study. Demographic data were collected together with a complete clinical examination and any risk factor was identified.

Isolation, identification and antifungal susceptibility testing of *candida* species

All clinical samples were inoculated in Sabouraud dextrose agar (SDA) (Oxoid, Basingstoke, UK) at 37ºC aerobically for 24 to 48 hours [13]. Blood samples were inoculated on blood culture bottles (Oxoid) and were subsequently subcultured on (SDA).

Different *Candida* species were identified phenotypically by conventional methods (colony morphology, Gram stain, sugar fermentation, and germ tube test) and biochemical analysis by API 20 C kit (api® *Candida*) (BioMerieux, France) which were performed according to manufacturers' instructions.

Identification of each isolate down to species level was confirmed by VITEK 2 compact system using identification yeast ID-YST cards. Antifungal susceptibility testing for amphotericin B, flucytosine, fluconazole, voriconazole, caspofungin, and micafungin were determined for all *candida* isolates by the automated VITEK 2 compact system (bioMérieux, France) using AST-YST cards cards following the manufacturer's instructions.

Briefly, about 2– 3 colonies of 24-hours *Candida* cultures were inoculated into 5-mL glass tubes containing 3 mL of 10% saline, adjusted to 2.0 McFarland standards (acceptable range of 1.8 to 2.2) using the DensiChek (Biomerieux, Marcy l'Etoile, France) instrument. The Vitek 2 ID, AST-YST cards cards were automatically filled with the prepared culture suspension, loaded cassettes were then placed into the Vitek 2 instrument. The cards were incubated at 35.5 °C for 18 h, and data were collected at 15-min intervals during the entire incubation period and final identification and breakpoint minimum inhibitory concentration (MIC) values used to categories *Candida* species, susceptible, intermediate, or resistant to each drug were investigated using version 7.0 software, an advanced expert system (AES) designed to evaluate the results produced by the VITEK

A standard strain of *C. albicans* (ATCC 10231) was used to evaluate the performance of the VITEK machine.

All azole resistant *Candida* isolates were transferred to Brain heart infusion broth (Oxoid, Basingstoke, UK) containing 10% glycerol and stored at - 80 °C until use.

Evaluation of ERG-11 gene overexpression by real time-PCR(RT-PCR)

All *candida* species with azoles resistance were subjected to detection ERG-11 gene overexpression by RT-PCR.

RNA extraction

All samples were extracted according to the manufacture procedures of the QIAzol Lysis reagent (Qiagen, Cat. No. 79306). Briefly,1.5 ml of bacterial suspension was centrifuged to pellet the cells then 1 ml QIAzol Lysis Reagent was added and the homogenate was incubated at room temperature (15–25°C) for 15 minutes. 200 ul of chloroform were added then mixed by vortexing and was incubated in ice for 15 minutes. Tubes were centrifuged for 10 minutes at 12,000 x g. The upper aqueous phase (containing RNA) was transferred to a new tube. An equal volume of absolute ethanol was added to the aqueous phase mixed gently by inverting the tube upside down. The tube was incubated at -20° C for 1 hour then centrifuged for 10 minutes at 12,000 xg. The supernatant was discarded, and the pellet was washed with 1 ml of 70 % ethanol. After decanting the ethanol, the pellet was air-dried. The pellet was dissolved in 25 ul nuclease-free water.

cDNA synthesis

All RNA samples were converted into cDNA using Revert Aid First Strand cDNA Synthesis, (Thermo Scientific, Cat. No.1622) as follow:

RT Reaction Mix was prepared as the following: RNA Template 3 ul, Nuclease free water 8 ul, Random Hexamer Primer 1 ul,5X Reaction Buffer 4 µl, RiboLock RNase Inhibitor (20 U/µL) 1 µl,10 mM dNTP Mix 2 µl, and RevertAid M-MuLV RT (200 U/µL) 1 µl

Reaction program: Reaction tubes were placed in a thermal cycler and programmed as follows: 25°C for 5 min. 42°C for 60 m 70°C for 5 min.

CYBR green assay

The real-time PCR was performed for ERG-11 and (ACTIN) ACT1 genes according to manufacture instructions of Maxima SYBR Green qPCR Master Mix, (Thermo Scientific, Cat. No. K0241).

PCR reaction set up

PCR reaction mix. was prepared separately for each assay (ERG-11 and ACT1) as follows: Maxima SYBR Green qPCR Master Mix. (2X) 10 ul. Forward Primer (10 pmol) 0.8 ul. Reverse Primer (10 pmol) 0.8 ul. cDNA sample (tenfold diluted sample) 8.0 ul. Nuclease–free water 0.4 ul.

- The primers used to span the entire ERG11 open reading frame:5'-GTT GAA ACT GTC ATT GAT GG (forward) 5'-TCAGAA CAC TGA ATC GAA AG (reverse) [11].
- The primers for the ACT: 5´-GAT TTT GTC TGA ACG TGG TAA CAG-3´ (forward) 5´-GGAGTT GAA AGT GGT TTG GTC AAT AC-3´(reverse) [14].

Reaction program

The reaction plate was placed in a thermal cycler and programmed as follows: Initial denaturation: 95°C for 15 min. Denaturation: 93°C for 30 sec. Annealing and extension: 60°C for 1 min. prepared reaction tubes were cycled on Applied Biosystems 7500 Fast Real-Time PCR system.

Statistical analysis

Statistical presentation and analysis of the study was done by the use of the mean, number, percentage, student t- test and the analysis of variance [ANOVA] tests by (Statistical Package for Social Science; SPSS, Chicago, IL, USA). Comparison between two groups in quantitative data was performed using the unpaired Student T-test. Student t test and Paired t test were applied to compare 2 continuous parametric variables respectively. p-value $\leq$ 0.05 is considered significant.

Results

A total of 165 *candida* species were isolated from 300 clinical samples. *Candida albicans* was identified in 98 samples (59.4%), however, *non-albicans* species were isolated in 67 samples (40.6%), most cases of *Candida* infection were detected in older age patients between 51 to 60 years old, more females were reported in cases with *Candida* infection representing 57% and 66% of *C. albicans* and *non-albicans* infection respectively. Risk factors for candida infection included prolonged stay in ICU for more than one week, systemic antibiotic therapy, diabetes mellitus, chronic diseases and different invasive devices as a urinary catheter or central line, this is shown in **table (1)**.

Table 2 illustrates the prevalence of *Candida* species in different clinical samples. Most of *C. albicans* were isolated from sputum followed by urine, pus, wound, and blood representing 41.83%, 35.71%, 10.2%, 7.14%, and 5.1% respectively. Furthermore, a higher prevalence of *non-albicans* species was detected in sputum (35.8%) followed by urine (25.34%), pus (19.4%), blood (10.44%), and wound (8.95%).

As regarding azoles susceptibility to isolated *Candida* species **(Table 3)**, 17% of the overall isolated *Candida* species were resistant to

fluconazole while 5% only were resistant to voriconazole. In *C. albicans* species, four cases were resistant to fluconazole while all cases were sensitive to voriconazole. Concerning *non-albicans* species, most cases of *C. tropicalis* and *glabrate* were sensitive to both fluconazole and voriconazole, in contrast, all cases of *C. Krusi* were resistant to fluconazole. All cases of *C. parapsilosis* were sensitive to fluconazole and voriconazole. The two cases of *C. famata* were not tested as its MIC values to all antifungal drugs were not detected by VITEK.

Among 36 resistant isolates, only 10 isolates showed overexpression of ERG-11 gene in comparison to sensitive isolates as illustrated in **tables (4,5)** and **figure 1 (A, B)**.

The study detected that all azole resistant candida spp. were associated with prolonged stay in ICUs (more than one week). Other coexistent comorbidities and risk factors like systemic antibiotic therapy, invasive devices, diabetes, previous exposure to antifungal agents and chronic diseases were present in variable degrees as shown in **table (6)**

Table 1. Sociodemographic data of different clinical specimens.

		Candida albicans (n=98)	**Non albicans (n=67)**	***p*- value**
Age	21 – 30	10 (10%)	10 (15%)	0.361
	31 – 40	25 (26%)	16 (24%)	0.812
	41 – 50	18 (18%)	10 (15%)	0.563
	51 – 60	35 (36%)	26 (39%)	0.686
	61 – 70	10 (10%)	5 (7%)	0.547
Gender	Male	42 (43%)	23 (34%)	0.271
	Female	56 (57%)	44 (66%)	
Risk factors	ICU stay (> 1 week)	74 (74%)	58 (80%)	0.081
	Diabetes	53 (54%)	28 (42%)	0.121
	Under antibiotic therapy	98 (100%)	67 (100%)	-
	Under antifungal therapy	16 (16%)	13 (19%)	0.610
	Underlying chronic diseases	85 (87%)	50 (75%)	0.048*
	Invasive devices	90 (92%)	63 (94%)	0.594

Table 2. Distribution of *Candida* species in different clinical specimens.

Clinical specimens / **Species**	**Blood (n=12)**	**Wound (n=16)**	**Pus (n=20)**	**Sputum (n=65)**	**Urine (n=52)**	**Total 165**
C. albicans	5 (5.1%)	10 (10.2%)	7 (7.14%)	41 (41.83%)	35 (35.71%)	98 (59.4%)
C. non albicans	7 (10.44%)	6 (8.95%)	13 (19.4%)	24 (35.8%)	17 (25.34%)	67(40.6%)
C. tropicalis	4	4	5	10	7	30(18.18%)
C. glabrata	1	0	4	5	5	15 (9.09%)
C. krusi	0	2	2	6	4	14 (8.48%)
C. parapsilosis	0	0	2	3	1	6 (3.6%)
C. famata	2	0	0	0	0	2 (1.2%)

Table 3. Distribution of *Candida* species according azole susceptibility pattern.

Drugs / Species		Fluconazole		Voriconazole	
		S	R	S	R
C. albicans (n=98)		94	4	98	0
C. tropicalis (n=30)		25	5	29	1
C. glabrata (n=15)		10	5	13	2
C. krusi (n=14)		0	14	9	5
C. parapsilosis (n=6)		6	0	6	0
Totally yeast tested	(n=163)	135	28	155	8
	%	83%	17%	95%	5%

S: sensitive R:resistant

Table 4. ERG-11 gene expression in sensitive and resistant isolates by RT-PCR.

	ERG11 gene expression in sensitive isolates	**ERG11 gene expression in resistant isolates**
Mean	3.56	7.81
t. test	3.182	
***p*- value**	0.013*	

*significant

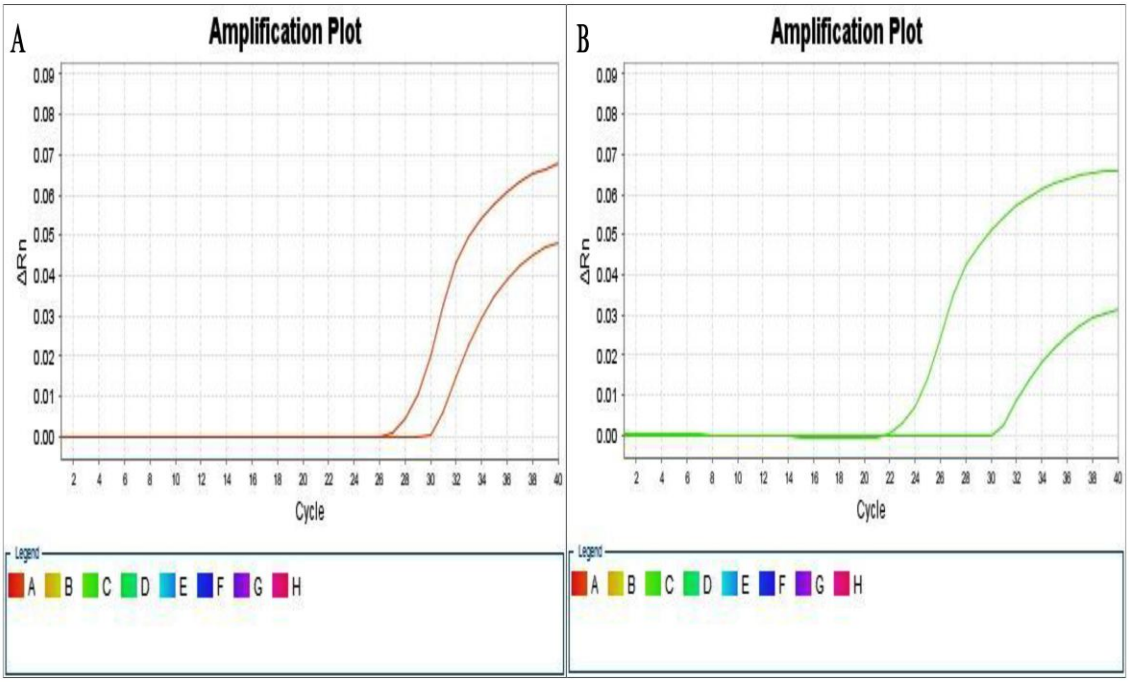
Table 5. Prevalence of overexpressed ERG-11gene in resistant isolates.

Over expression of ERG 11 gene in resistant isolates	**N**	**%**
Yes	10	27.8
No	26	72.2
Total	36	100

Table 6. Different risk factors and associated comorbidities in azole resistant candida species.

Comorbidities	**Fluconazole resistant candida species (n=28)**	**Voriconazole resistant candida species (n=8)**
ICU stay (> 1 week)+ antibiotic therapy (n=36)	28(100%)	8(100%)
ICU stay (> 1 week)+ Invasive devices (n=32)	26 (93%)	7 (87.5%)
ICU stay (> 1 week)+ Diabetes (n= 30)	23 (82%)	7 (87.5%)
ICU stay (> 1 week)+ antifungal therapy (n=18)	13 (46.5%)	5 (62.5%)
ICU stay (> 1 week)+ chronic diseases (n=10)	9 (32%)	1(12.5%)

Figure 1. (A) This figure shows ERG-11gene expression in correlation with ACT1 gene expression in sensitive isolate. (B) This figure shows ERG11gene expression in correlation with ACT1 gene expression in esistant isolate.



Discussion

As the spectrum of yeasts and their antifungal susceptibility profile are poorly identified, treatment of fungal infections has remained empirical and some fungal species exhibit intrinsic resistance to some antifungal agents, rapid and accurate identification of *candida* species is essential for proper selection of antifungal agents and subsequent management [15].

Candida tropicalis, *C. krusei C. glabrata*, and *C. parapsilosis* are the prominent causes of candidiasis following *C. albicans*. The distribution of these *non-albicans* species differs throughout the world mostly due to the medical state of patients, geographic distribution, age, and gender [16].

The present study reported there were no significant distribution differences between *C. albicans* and the *non-albicans* regarding age and gender (p>0.05). There was a higher predilection of *Candida* species in females which was in concordance with findings reported by **Vijay et al.** [17], but inconsistent with results reported by **Strollo et al.** and **Zeng et al.** who detected more frequent invasive *Candida* infections in males with advanced age [18,19]. In contrast to our findings **Marak and Dhanashree** found a nearly equal distribution of *Candida* species among males and females [20].

In our study, *Candida* species was more common in patients between 51 to 60 years old similar to a study conducted by **Marak and Dhanashree** [20]. In contrast, surveys conducted by **Emeribe et al.** found that the maximum *Candida* infection occurred within 21–40 years old [21].

Concerning the present study, underlying chronic diseases are the only associated risk factor for the higher prevalence of *C. albicans.*

In this study, a total of 165 *Candida* isolates were obtained from different clinical specimens over a 9 months duration. Most *C. albicans* were isolated from sputum followed by urine, pus, wound, and blood representing 41.83%, 35.71%, 10.2%, 7.14%, and 5.1% respectively. Furthermore, a higher prevalence of *non-albicans* species was detected in sputum (35.8%) followed by urine (25.34%), pus (19.4%), blood (10.44%), and wound (8.95%). Our study revealed that the majority of *Candida* species were isolated from sputum and urine which indicates a higher incidence of *candida* species induced urinary and respiratory tract infections. This is coincident with a study conducted by **Khadka et al.** [22].

This study detected that *C. albicans* was the most predominant species representing (59.4%), among the NAC species *C. tropicalis* (18.18%) was the most common isolates followed by *C. glabrata* (9.09%), *C. krusi* (8.48%), *C. parapsilosis* (3.6%) and *C. famata* (1.2%) respectively.

Similar to our study, **Khadka et al.** reported *C. albicans* as the major isolates. Among the NAC species, *C. tropicalis* was most prevalent

followed by *C. glabrata* and *C. krusei* respectively [22]. Furthermore, **Sasso et al.** [23] reported *C. albicans* as the most commonly isolated species.

However, *C. parapsilosis* was reported as the most commonly isolated yeast in another study conducted by **Sahal and Bilkay** [24]. Moreover, the predominance of NAC species was demonstrated by many previous studies [25].

In the present study, *C. tropicalis* was the second dominant species which was inconsistent with many other studies where *C. krusei* was the second predominant NAC species [26,27].

In our study, concerning azole susceptibility, among the *Candida* isolates tested, 83% were susceptible to fluconazole, 17% were resistant. and 95% were susceptible to Voriconazole and 5% were resistant. Our findings agree with **Ghaddar et al.** [28] who detected nearly similar results. Furthermore, **Zeng et al.** reported a total of 18.6% of the candida isolates were resistant to fluconazole. Voriconazole resistance was observed in 18.5% of all the *Candida* isolates [19].

Concerning azole resistance, our study showed that the resistance to fluconazole was higher than Voriconazole. This high level of fluconazole resistance is mostly due to the overuse of this antifungal agent and also its empirical therapy. Since fluconazole is most commonly used in the treatment of invasive yeast infections, the increase in resistance to fluconazole among candida species is alarming [29]. Hence, the necessity for specification and antifungal susceptibility before treatment with the antifungal drug is crucial.

In contrast, the azole resistance rates in our study were lower than those reported by other studies [20].

Azole resistance among *Candida* species may result from overexpression of the ERG11 gene independently or in combination with other resistant mutations. Overexpression of the ERG11 gene has been noted in *C. Albicans, C. parapsilosis,* and *C. tropicalis* [30].

Regarding our study, among the 36 azole-resistant isolates, only 10 isolates showed overexpression of the ERG11 gene in comparison to sensitive isolates, which agrees with **Rosana et al.** [31].

Furthermore, **Fathi et al.** found ERG11 gene expression in the azole-resistant isolates group was more than its expression in the azole-susceptible group [32]. On the contrary, **Zhang et al.** did not find any difference in the expression of ERG11 genes between the fluconazole-susceptible and resistant isolates [33].

The present study detected that all patients with azole resistance were admitted to ICU for more than 1 week and were under systemic antibiotic therapy, this is similar to other study in which prolonged ICU stay was the main risk factor for azole resistance [34]. In addition, our study detected that diabetes increased the risk of azole resistance which is quite similar to another study [35] that reported diabetes as an independent factor for azole resistant candida infection. Previous antifungal exposure is another risk factor detected in our study associated with azole resistance, this agrees with study performed by **Pulcrano et al.** [36] who detected antifungal agents as a stress factor for azole resistant candida infection.

One of the limitations of this study is the small number of cases and the lack of correlation between the empirical use of antifungal agents and ERG11 gene expression in candida species resistant to azoles, hence further studies on larger number of cases are needed to assess the effect of haphazard use of antifungal agents and expression of ERG11 gene.

Conclusions

The study reported that candida spp. are significant pathogens in ICU patients. *C. albicans*, *C. tropicalis*, and *C. glabrata* were the most predominant findings. The fluconazole resistance was higher than voriconazole. ERG11 gene expression was more predominant in azole resistant than azole-sensitive isolates which highlights its importance in developing resistance to azole in ICUs.

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Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms to publish their clinical data in this journal without showing their name or initials.

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Conflict of interest

The author have no conflicts of interest.

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