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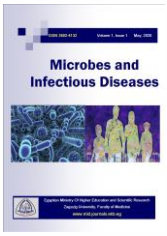

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Original article

ABC genotyping, virulence factors production and antifungal resistant pattern of *Candida albicans* isolated from immunocompromised patients with candidemia

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ABSTRACT

Background: *Candida albicans* is still the most common cause of candidemia in the majority of healthcare-acquired bloodstream fungal infections globally with a high morbidity, mortality and significant financial burden. Yeast genotyping is crucial for designing effective infection control strategies. In the current study, we aimed to detect the genotyping, some virulence factors and antifungal resistant pattern of c. albicans isolated from immunocompromised candidemic patients admitted in critical care units of Zagazig university hospitals (ZUHs), Egypt. **Methods**: *C. albicans* isolated from clinical samples were identified and characterized using the conventional mycological methods and Vitek 2 system is used for confirming the identification and for antifungal susceptibility test (AFST). ABC genotyping was used to characterize subgroups of c. albicans and virulence activities (phospholipase (Pls), protease (Prt), esterase (Est), hemolysin (Hs), coagulase (Cgl) and biofilm formation). **Results**: From a total of 55 isolates of *C. albicans*, genotype A was the predominant (50.9%). Phospholipase and proteinase activities were detected in 96.4% and 85.4%. Esterase, hemolysin and coagulase activities were detected in 83.6%, 74.5% and 60.0% respectively. 83.6% of isolates were biofilm-forming. Isolates of c. albicans were 100% sensitive to caspofungin, micafungin and miconazole, while 1.8% are resistant for each of amphotericin B and voriconazole, while flucytosine and fluconazole showed a higher resistant (3.6%) for each. **Conclusions**: ABC genotyping can classify *C. albicans* species. The association of genotype A and resistance to flucytosine and fluconazole need further assessment. Routine AFST to guide empiric to targeted therapy should be encouraged.

Introduction

Candidemia is the most prevalent invasive healthcare-acquired fatal fungal infections worldwide [1]. Several species of the genus candida can cause a broad range of systemic, mucocutaneous, and cutaneous infections [2]. Over 20 species have been found to be opportunistic human pathogens [3]. Among all infections in intensive care units worldwide, candida albicans is considered to be the third most common isolated microorganism [4], and represents a primary cause of all healthcare-acquired bloodstream infections with a crude mortality rate of over 40% [5]. *C. albicans* is an

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opportunistic pathogen, several microbial and host factors are contributed to its pathogenicity. Extracellular enzymes such as (proteinase, phospholipase, esterase, hemolysin and coagulase) and biofilm formation are among the microbial virulence factors that facilitate its tissue destruction, penetration, invasion, adherence, colonization, evasion of host immune response, and resistance to antifungals [6]. These virulence and pathogenic factors are released only in seriously immunocompromised and critically ill patients [5]. Various molecular typing techniques have been employed to genotype yeasts in epidemiological investigations for establishment of an effective infection control measures [7]. *C. albicans* are classified based on existence or absence of an intron in the 25 s rDNA, using the ABC genotyping PCR method into four genotypes: A (450 bp), B (840 bp), C (450 and 840 bp), E (1000 bp), and D (1080 bp), which is *C. dubliniensis* [8]. Primary or secondary antifungal drug resistance is of a major global concern, particularly in immunocompromised patients [9, 10]. There are currently only four classes of antifungal drugs; polyenes, triazoles, echinocandins, and pyrimidine analogues available to treat these potentially fatal fungal infections, thus antifungal susceptibility tests (AFST) are essential for directing empirical treatment toward targeted therapy [11]. The current study was carried out to detect genotyping of *C. albicans* causing candidemia in immunocompromised patients admitted in different ICUs of Zagazig University hospitals (ZUHs). Also, to investigate the distribution of some virulence determinants and antifungal resistant pattern among *c. albicans* isolates.

Subjects and Methods

This cross sectional study included a total of 55 candida albicans isolates collected from November 2023 to January 2024 from positive blood cultures of hospitalized patients underlying predisposing factors in various critical care units including (5) pediatric and (26) adult oncology, (9) surgery, (4) dialysis and (11) intensive care units of ZUHs, Egypt. Their age ranged from 17- 75 yrs. and 67% were males. The approvals were obtained from the participant and the study protocol was approved by Zagazig medical research ethical committee (IRB: 11242-20-11-2023).

Growth detection and identification of *C. albicans*:

The BACT/Alert system (BioMérieux, France), was used to recover candida strains from 55 positive blood cultures after subculturing on sabouraud dextrose agar medium (SDA) for fungal isolation and gram staining.

C. albicans was phenotypically identified by positive germ tube test and a growth on hypertonic sabouraud broth. The VITEK 2 compact system was used according to the manufacturer's instructions for further identification and MIC determination of each strain [12]. Also additional tests to investigate biofilm formation, virulence factors, and ABC genotyping were done.

Germ tube test:

This is a quick test used to distinguish species, a colony was added to 1 milliliter of human serum using a sterilized loop, then incubated aerobically for 3 hours at 37 ºC. Subsequently, the germ tube cells were observed using a microscope, exhibiting a breadth or length around 3 to 4 times the actual cell size. [13].

Hypertonic sabouraud broth

This test examine the ability of *C. albicans* to grow in an excess of NaCl, 1ml of sterile sabouraud broth supplemented with 6.5% NaCl was inoculated with 20 µL of 0.5 McFarland suspensions of each isolate then incubated at 28˚C for 96h and assessed visually for fungal growth every 24 hours. c. albicans [14].

Determination of c. albicans minimum inhibitory concentration (MIC)

The fully automated VITEK 2 compact system (AST-YS07 card; bioMérieux, France) was used according to the manufacturer's instructions to estimate MICs for all *C. albicans* isolates based on spectrophotometric determination. A card that contains two fold serial dilutions of antifungal agents (amphotericin B, fluconazole, flucytosine, caspofungin, micafungin, voriconazole, and miconazole) was inoculated with a fungal suspension, then was introduced to the system. A quality control of reference strains; *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 were used. The AFST results of the *C. albicans* are interpreted according to the CLSI species-specific clinical

breakpoints (SS-CBPs) according to CLSI M60 document for voriconazole, fluconazole, caspofungin, and micafungin [15], and on the epidemiological cut-off values (ECVs) as determined by the CLSI M59 document for amphotericin B and flucytosine [16].

Detection of virulence factors

Proteinase activity was detected using bovine serum albumin (BSA) agar assay, by inoculating the medium with 10 µL of 0.5 McFarland turbidity cell suspensions, after incubation at 37°C for six days, the positive protease activity and the cleavage of BSA were indicated by the presence of white precipitation zone around the colonies. The diameters of the yeast colonies and the white precipitation zone were measured [17].

Phospholipase activity: It was assessed using egg yolks agar plate. Egg yolk agar was inoculated aseptically with 10 µl of of 0.5 McFarland turbidity cell suspensions. Plates were incubated at 37 °C for 48 h. The positive phospholipase activity was indicated by the presence of white precipitation zone around the colonies. The diameters of the yeast colonies and the white precipitation zone were measured [18].

Haemolysin activity: In order to assess the hemolytic activity of candida, an inoculum size of 1 × 106 cells/mL of yeast cells were inoculated in sabouraud dextrose broth (SDB) medium. SDB medium with 7% fresh blood was infected in duplicate with 5 µL of the cell suspension and incubated at 37 °C for 48 hours. Positive hemolysis was indicated by the presence of a clear translucent halo zone, the yeast colony and the zone were measured [19].

Esterase activity: The tween medium was prepared by addition of 15.0 g agar, 10.0 g peptone, 5.0 g NaCl, 0.1 g CaCl2 to 1 L distilled water, sterilized by autoclaving and cooled to around 50°C, 5 ml of autoclaved tween 80 was added. The plates were examined for three days, after being inoculated and incubated at 30°C. A positive test result and the production of an esterase by the candida isolate were shown by the formation of a halo surrounding an inoculum on the medium [20].

The enzymatic activity of all enzymes was calculated by dividing the colony diameter by the precipitation or clear zone plus colony diameter (Pz value). Enzymatic activity was considered high with values <0.399, moderate with values 0.699 and 0.400, weak with values between 0.999 - 0.700, negative result was considered with values equal to 1 [21].

Coagulase activity: The assessment of coagulase activity (Cgl) was conducted by inoculation of 100 µL of 0.5 mcfarland suspensions of c. albicans to tubes with 500 µL of human serum. Clot formation indicating the enzymatic activity of Cgl was observed at 2, 4, 6, and 24 hours post incubation at 37°C. The Cgl activity was interpreted as follows: high (+++), moderate (++), weak (+), and negative (−). For quality control *Staphylococcus aureus* (ATCC 25923) and *Staphylococcus epidermidis* (ATCC 14990) were used as positive and negative control respectively [22].

Determination of biofilm formation:

Using the colorimetric microtiter method. 0.5 McFarland standard of c. albicans strains were cultivated in SDB medium overnight at 37°C, after which the suspension was transferred into a sterile 96-well micro-titer plate in an amount of 100 µL per well, wells with SDB medium without *C. albicans* were considered as a negative control for each isolate, the plates were incubated at for 48 hours at 37°C. After which, the suspension was removed, followed by washing each well with 200 µL PBS for removal of non-adherent cells, and left to air dry for 30 min, 200 µL of 0.4% crystal violet was added per well for 40 min at room temperature, the plates are washed twice with distilled water, followed by de-staining the biofilm by addition of 200 µL of absolute ethanol to each well at room temperature for 40 min.

The Biotek elx800 ELISA reader was used to measure the amount of biofilm at a wavelength of 590 nm. The optical density cut-off values (ODc) was calculated by addition of the mean values of optical density of the negative controls (mean ODnc) to three standard deviations of the negative controls (3 × SDnc), so ODc = mean ODnc + (3 × SDnc). Density of the biofilm was graded as: mild= ODC < OD ≤ 2 x ODC, moderate= 2 x ODC < OD ≤ 4 x ODC, high =OD > 4 x ODC, biofilm negative=OD ≤ ODC [23].

Genotyping of *C. albicans*

All germ tube and hypertonic Sabouraud broth positive candida isolates underwent ABC genotyping PCR and were identified as candida albicans by the Vitek 2 compact system (BioMérieux, France). The previously established isolation procedure was used to extract DNA [24]. The primers CA–INT–L (5′-ATA AGG GAA GTC

GGC AAA ATA GAT CCG TAA-3′), and CA–INT–R (5′-CCT TGG CTG TGG TTT CGC TAG ATA GTA GAT-3′) were used, and the ABC genotyping was carried out in accordance with the 25S rDNA type. Using the previously mentioned protocol, in applied biosystems thermal cycler [7].

PCR products were sorted by the size using 2% agarose gel electrophoresis, visualized by UV and compared with DNA ladder after staining with ethidium bromide. Candida isolates were classified according to size of the amplified DNA, depending on the existence or absence of an intron in the ITS1 region of candida albicans into three genotypes, group A (450 bp), group B (840 bp), and group C (450 and 840 bp).

Statistical Analysis

The data was analyzed using SSPS 26. The categorical variables (qualitative data) were expressed as percentage and counts. The comparison was done by Kruskal- Wallis and Chi-Square test and P was significant if its value was less than 0.05.

Results:

In table (1): ABC genotyping of the 55 isolated candida albicans strains revealed three genotypes A, B and C. However, genotype D or E hadn't been identified in our study. Genotype A, B and C reported as 28 (50.9%), 17 (30.9%) and 10 (18.2%) respectively. AFST was performed on c. albicans genotypes against the antifungals drugs and revealed that caspofungin, micafungin and miconazole were found to be sensitive to all studied isolates. Amphotericin B and Voriconazole were with least resistance of 1.8%. While flucytosine and fluconazole showed a higher resistant (3.6%) for each. All the resistant antifungal drugs were in genotype A (5/28) except one drug (Amphotericin B) in genotype B (1/17) with a frequencies of 17.9% and 5.9% respectively. In table (2): Regarding to the secretion of hydrolytic enzymes, 85.4%, 96.4% and 83.6% of c. albicans isolates were respectively positive for the synthesis of proteinase (Prt), phospholipase (Pls) and esterase (Est) activities respectively. Similarly, hemolysin (Hz) and coagulase activities (Cgl) were observed in 74.5% and 60.0% of isolates, respectively. Regarding to the Pz values obtained, the activities of these enzymes differ from one strain to another as described in table 2, with the highest activity was observed for Pls in 76.4% and the lowest for Cgl in 23.6% of the isolated strains. Also, this table shows that, according to the results of the biofilm method, 83.6% of c. albicans isolates were biofilm forming with varying levels with 52.7% of them were highly biofilm-forming. In table (3): Among different extracellular enzymes of c. albicans genotype A, Pls showed the highest enzymatic activity in 43.4%, while the least one with high enzymatic activity was Cgl in 15.2%. Regarding to BF, all isolates had the ability to form biofilm with variable degrees, with 34.8% isolates showed highest ability to form biofilm. Among isolates of Genotype B; Hz shows the highest enzymatic activity in 31.7%. Regarding to BF, 14 isolates had the ability to form biofilm with variable degrees with 17.4% had had highest ability to form strong biofilm. Regarding to Genotype C; Prt shows the highest enzymatic activity in 17%. Regarding to BF, 10.9% isolates had highest ability to form biofilm.

Table 1. Frequency of *C. albicans* genotypes and resistant pattern of antifungal agent among different genotypes

***C. albicans* genotypes**	**Genotype frequency**		**Resistant pattern of Antifungal agent (n, %)**							**total**
	N	%	AMB	5FC	FLC	MCF	VRC	CSF	MIC	
Genotype A	28	50.9 %	0	2	2	0	1	0	0	5 (11.4%)
Genotype B	17	30.9 %	1	0	0	0	0	0	0	1 (12.5%)
Genotype C	10	18.2 %	0	0	0	0	0	0	0	0
Total	55	100%	1/55	2/55	2/55	0	1/55	0	0	6 (10.9%)

AMB, Amphotericin B; 5FC,5-fluorocytosine; FLC, fluconazole; MCF, micafungin; VRC, voriconazole; CSF, caspofungin; MIC, Miconazole.

Table 2. Distribution of enzymatic activities and biofilm-formation among *C. albicans* isolates.

Candida spp.	Enzymes	Enzymatic activity and Biofilm formation						
		Total -ve (N, %)	Weak	Moderate	High	Range (Pz values)	Mean	Total +ve (N, %)
C. albicans (55)	Proteinase (Prt)	8 (14.6%)	2 (3.6%)	8 (14.6%)	37 (67.3%)	0.15 - 1	0.38	47 (85.4%)
	Phospholipase(Pls)	2 (3.60%)	–	11 (20.0%)	42 (76.4%)	0.27 - 1	0.36	53 (96.4%)
	Esterase (Est)	9 (16.4%)	6 (10.9%)	10 (18.2%)	30 (54.5%)	0.10 - 1	0.43	46 (83.6%)
	Haemolysin (Hz)	14 (25.5%)	–	7 (12.7%)	34 (61.8%)	0.33 - 1	0.58	41 (74.5%)
	Coagulase (Cgl)	22 (40.0%)	8 (14.6%)	12 (21.8%)	13 (23.6%)	–	–	33 (60.0%)
	Biofilm (BF)	9 (16.4%)	5 (9.1%)	12 (21.8%)	29 (52.7%)	–	–	46 (83.6%)

Enzymatic activity had four categories (Pz value): Negative (-ve)= 1, Low = 0.999 - 0.700, Moderate = 0.699 - 0.400, High= <0.399

Table3. Frequency and distribution of enzymatic activities and biofilm-formation between different *C. albicans* genotypes.

Virulence Factors	PZ value	Genotype A 28 (50.9%)	Genotype B 17 (30.9%)	Genotype C 10 (18.2%)	Total		p- value
Proteinase (Prt)	**Weak**	-	-	2 (4.3%)	2 (4.3%)	**47**	0.31
	Moderate	6 (12.8%)	2 (4.3%)	-	8 (17.0%)		
	High	16 (34.0%)	13 (27.7%)	8 (17.0%)	37 (78.7%)		
Phospholipase(Pls)	**Weak**	-	-	-	-	**53**	0.33
	Moderate	5 (9.4%)	3 (5.7%)	3 (5.7%)	11 (20.8%)		
	High	23 (43.4%)	13 (24.5%)	6 (11.3%)	42 (79.2%)		
Esterase (Est)	**Weak**	2 (4.4%)	2 (4.4%)	2 (4.4%)	6 (13.0%)	**46**	0.51
	Moderate	7 (15.2%)	3 (6.5%)	-	10 (21.7%)		
	High	14 (30.4%)	11 (23.9%)	5 (10.9%)	30 (65.2%)		
Haemolysin (Hs)	**Weak**	-	-	-	-	**41**	0.11
	Moderate	2 (4.9%)	4 (9.8%)	1 (2.4%)	7 (17.1%)		
	High	15 (36.6%)	13 (31.7%)	6 (14.6%)	34 (82.9%)		
Coagulase (Cgl)	**Weak**	4 (12.1%)	3 (9.1%)	1 (3.0%)	8 (24.2%)	**33**	0.34
	Moderate	4 (12.1%)	6 (18.2%)	2 (6.1%)	12 (36.4%)		
	High	5 (15.2%)	5 (15.2%)	3 (9.1%)	13 (39.4%)		
Biofilm formation (BF)	**Weak**	2 (4.5%)	3 (6.5%)	-	5 (10.9%)	**46**	0.63
	Moderate	6 (13.0%)	3 (6.5%)	3 (6.5%)	12 (26.1%)		
	High	16 (34.8%)	8 (17.4%)	5 (10.9%)	29 (63.0%)		

Pz value: Values = 1 were considered Negative, 0.999 - 0.700 = Weak, 0.699 - 0.400 = Moderate, values <0.399 = high enzymatic activity

Discussion

Candida albicans is the third most common pathogen isolated from all ICU infections globally [4] and represents a leading cause of healthcare-acquired candidemia and invasive infections with a mortality rate of about 40% [5].

ABC genotyping is a simple, reproducible PCR method for c. albicans typing according to the band size into four subgroups: genotypes A, B, C and E while genotype D is c. dubliniensis [10]. This study aimed to investigate the rate of different c. albicans genotypes isolated from immunocompromised patients with candidemia admitted in critical care units of ZUHs and to study some virulence factors production that facilitate its pathogenicity and antifungal resistant pattern among the c. albicans isolates.

In the current study, the majority of Candida albicans isolated from candidemic patients were genotype A (50.9 %, 28/55) followed by genotype B (30.9%, 17/55) and genotype C (18.2%, 10/55) while, genotype D and E bands pattern weren't observed in any of the samples. These results were consistent with several studies reported that genotype A is the most predominate followed by genotype B and the least common was genotype C [17, 24-26]. However, other reported genotype A was the most frequent and genotypes B and C were equal, another study reported genotype C was the most common genotype followed by genotype B and genotype A [27]**.** While others detected genotypes C was more frequent than genotypes B [28]**.** Moreover, in another study the most frequent genotype was B, followed by A and genotype C was not found [29]**.** From the majority of previous studies, we can conclude that genotype A of c. albicans is the most frequent genotype. This variability of genotype distribution from individual studies may be related to the geographical location.

Disseminated invasive infections of c. albicans may be contributed to potential virulence factors, several virulence factors, including the secretion of several extracellular enzymes (such as proteinase (Prt), phospholipase (Pls), esterase (Est), hemolysin (Hs), and coagulase (Cgl), as well as the formation of biofilms, contribute to candida pathogenicity by promoting adherence, invasion, and evasion of immune cells [30]. Virulence factors types and activities are based on the infecting species, the type and location of infection, the host's response and the origin of the illness [31].

In the present study, 96.4% and 85.4% of c. albicans isolates had Pls and Prt activity and were the most active enzymes regarding to the Pz value 76.4% and 67.3% respectively (table 2).This result was matched with Pinto et al [32], who reported 90% and 99.4% of isolates had Prt and Pls activity and Ramos et al [21], who reported that 100% of isolates had Prt and Pls activity. However, Rosca et al. reported that 38.3 % and 92.2 % of isolates had no Prt and Pls activity respectively [28].

Regarding Est and Hs activity, this study showed that 83.6%, 74.5% of isolates had Est and Hs activity but the highest activity was in Hs 61.8% regarding to Pz value (table2). Our result matched with other study reported 94.6%, 82.7% of c. albicans isolates had EST and Hs activity but the highest activity was in Hs 55.4% versus 20.9% in Est [33]. However, in another study, 100% of isolates had Est activity with 94.7% showed very strong activity, while Hs showed activity in 94.7% of isolates with 68.4% had very strong activity [26].

Regarding the coagulase (Cgl) activity, only 60.0% of isolates had Cgl activity with high activity in 23.6%. Other previous study detected similar result of 64.7% for coagulase activity [23]. However our result was lower than Tefiani et al [**22**] who reported 80% activity for Cgl but higher than other studies that detected Cgl activity in only 3.0% and 45.3% of isolates respectively [34,35]. Moreover, other study reported that all tested isolates were negative for coagulase activity [6]. Our study exhibited no significant difference between ABC genotyping and all studied virulence factors ($P > 0.05$) table (3). These results were in line with other reports except for esterase activity with $p \leq 0.04$ [17, 26]. However, others reported a significant difference between each of Hs, Pls and Est production and genotyping (except for proteinase ($P > 0.05$) [33].

Biofilm, a structured microbial communities, is one of an essential virulence factors for the development of clinical infections [36]. The current study detected biofilm formation in 83.6%, with variable degrees of ability to form biofilm where, 52.7% and 21.8% had high and moderate biofilm-forming activity respectively (table2). This result agrees with other studies detected high percentage of biofilm formation (76.1%, 100%) respectively [17, 22]. However, lower result was detected by others, who showed biofilm-producing activity in only 58.1% [36]**.**

C. albicans has developed resistance to common antifungal drugs such as polyenes, azoles, and echinocandins, due to overuse of these drugs as they are involved in empirical therapy [37].

In our study, the overall antifungal drug resistant was 10.9% (6/55) among c.albicans isolates with 1.8% for each of amphotericin B and voriconazole and 3.6% for each of fluconazole and flucytosine. However all c.albicans isolates were sensitive to micafungin, miconazole and caspofungin (table1). Other studies showed different results; one study reported that out of the 153 c. albicans isolates, 24.0%, 22.2%, 1.96%, showed resistance to fluconazole, voriconazole and amphotericin B respectively [38], Jafarian et al showed that among c. albicans isolates, 15.8% showed intermediate susceptibility to caspofungin, all were wild type for amphotericin B, and all were susceptible to voriconazole and fluconazole[26]. In a study, the resistance rate for fluconazole (MIC: ≥8 μg/mL) was 43.8%, while, others reported resistance rate of 6% to fluconazole, but they did not report any resistance to amphotericin B [39]. Moreover, others reported similar resistant pattern to our study for voriconazole (3.1%), flucytosine (2.8%) and amphotericin B (1.4%), similar sensitivity for miconazole, but had higher resistance for fluconazole (6%), caspofungin (3.5%) and micafungin (2.8%) [25].

In present study, most of the resistant isolates were among *C. albicans* genotype A (5/28) with a rate of 17.9%, while only one strain was found resistant to amphotericin B of wild type belonged to genotype B with frequency of 5.9%. All isolates of genotype C were susceptible to all antifungal drugs used in the study. Fluconazole and flucytosine resistant strains detected in this study had genotype A, while genotypes B and C did not have. This might show an association between the genotype A and the resistance to these drugs, that in accordance with previous studies that revealed a direct causal link between an increase in flucytosine susceptibility and the group 1 intron in the 25S rDNA (genotype A did not have this group) [7]**.** Also, a previous study reported less susceptibility of c. albicans to fluconazole in antifungal susceptibility testing [7]. However, several previous studies found no association between c. albicans genotypes and resistant antifungal drugs [8, 40]**.**

In conclusion, ABC genotyping can classify *C. albicans* species well into subgroups. All *Candida albicans* isolates were sensitive to caspofungin, micafungin and miconazole. The association of genotype A and resistance to fluconazole and flucytosine need further assessment. Routine AFST to guide empiric to targeted therapy should be considered.

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