Egyptian Journal of Medical Microbiology Volume 29 / No.3 / July 2020 37-45 Online ISSN: 2537-0979

ORIGINAL ARTICLE

Molecular diagnosis and antifungal resistance and biofilm production profiles among *Candida* species isolated from immunocompromised patients at Menoufia University Hospitals

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Key words:
Antifungal resistance, biofilm, Candida, chromogenic agar, molecular diagnosis, VITEK-2

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ABSTRACT

Background: *Accurate and rapid identification of Candida species is necessary for proper diagnosis and treatment of candidiasis due to emergences of drug-resistant strains especially among immunocompromised patients.* ***Objectives****: Identification of Candida clinical isolates to the species level using different phenotypic and molecular methods. Biofilm-forming ability and antifungal resistance were also studied.* ***Methodology:*** *Sixty-nine Candida strains were isolated from 220 immunocompromised patients. Identification was performed using chromogenic Candida agar, VITEK 2 system and multiplex polymerase chain reaction (PCR). Biofilm formation was detected by the tube method and antifungal susceptibility was tested using the VITEK2 system.* ***Results:*** *The most common source of Candida isolates was from urine (33.3%) and ICUs (56.6%). VITEK 2 system detected 9 spp.: C. albicans (34.8%), C. tropicalis (21.7%), C. famata (8.7%), C. lusitaniae (7.2%), C. cruzi (7.2%), C. ciferri (5.8%), C. dubliniensis (5.8%), C. parapsilosis (5.8 %) and C. glabrata. Candida isolates showed high resistance to flucytocine (49.3%), and high sensitivity to fluconazole, micafungin, voriconazole and caspofungin (88.4%, 81.2% and 81.2 % respectively). Only 30.4% of all Candida isolates were biofilm producers. There was a positive relationship between antifungal resistance and biofilm formation among Candida isolates.* ***Conclusion:*** *C. albicans was the predominant species. Chromogenic Candida agar and VITEK 2 system were valuable tests compared to PCR in speciation of Candida isolates. Antifungal susceptibility was significantly related to biofilm production and its evaluation is important for proper treatment..*

INTRODUCTION

Opportunistic fungal infections have become one of the major life-threatening nosocomial infections[1]. Switch of *Candida* species from commensals to pathogens is facilitated by virulence factors including adherence to host tissues, biofilm formation and secretion of hydrolytic enzymes[2]. Candidiasis has various clinical types ranging from superficial mucocutaneous to severe life-threatening invasive diseases. Invasive candidiasis is frequent in hospitals especially in neutropenic or immunocompromised patients[1].

Although the most common cause of candidiasis is *Candida albicans*, emergence of other *Candida* species such *as Candida dubliniensis, Candida glabrata, Candida krusei, Candida parapsilosis* and *Candida tropicalis* has increased in the last decades[3,4]. *Candida auris* has also been recognized as an emerging multidrug-resistant species[5,6]. Identification of these fungi at the species level is necessary for selecting the appropriate treatment of serious *Candida* infections[7]. Due to emergence of drug-resistant strains, it is imperative to study these pathogens and to evaluate their susceptibility to the commonly used drugs[8].

This study aimed to isolate *Candida* species from different clinical samples in immunocompromised patients, to identify them to species level. Antifungal susceptibility and their biofilm formation ability were also studied.

METHODOLOGY

Subjects:

This study was done in the Faculty of Medicine, Menoufia University. The involved immunocompromised patients (3-77 years old) were selected from different Departments and Intensive Care Units (ICUs), and were subjected to full history taking and thorough clinical examination. The study was approved by the Local Ethics Committee, Faculty of medicine, Menoufia University.

Isolation of *Candida*:

All samples (urine, blood, sputum, throat and catheter swabs) were immediately sent to the Microbiology laboratory within two hours to be

DOI: https://doi.org/10.51429/EJMM29305

processed and inoculated into Sabouraud Dextrose Broth (SDB) (Difico, USA). Subcultures were done on SDA plates and yeast-like colonies were identified by standard methods. The confirmed *Candida* isolates were preserved in nutrient broth supplemented with 20% glycerol and stored at -80°C[9].

Identification of Candida species:

Chromogenic *Candida* agar (CCA) (Tmmedia, India), a selective and differential medium, facilitates rapid differentiation of *C. albicans*, *C. krusei*, *C. tropicalis* and *C. glabrata* on the basis of colony morphology and colour. Single colonies were streaked on CCA, incubated at 37°C and examined after 24 and 48 hours[10]. *Candida* isolates were also identified by Vitek 2 System (bioMerieux, France). Suspensions were prepared in sterile saline at 2.0 McFarland turbidity standard using a DensiChek instrument (bioMérieux). The individual test cards for species identification were automatically filled with the prepared culture suspension, sealed, and incubated in the VITEK 2 instrument. The cards were incubated at 35.5°C for 18 hours, and optical density was automatically detected every 15 min. The final results were compared with the database to identify the *Candida* spp[11].

Molecular diagnosis of Candida species:

Specific primers for *C. albicans*, *C. famata*, *C. glabrata* and *C. tropicalis* were used. Multiplex PCR was performed in 25 µl reaction mixtures consisting of approximately 5 µl of template DNA, 10 µl of Taq DNA polymerase, 1 µl of each of the forward and reverse primers and 2µl of PCR-grade water. The PCR cycle parameters were: initial denaturation at 95˚C for 5 min; then 35 cycles of denaturation at 95˚C for 30 sec, annealing at 57˚C for 30 sec, and extension at 72˚C for 1 min; and then followed by final extension at 72˚C for 2 min. The amplified products were detected by electrophoresis on 1.5% agarose gel, visualized by ethidium bromide staining, and photographed[12].

The sequences of primers used in the study

***C. albicans*[12]:**

F: (5-TTGAACATCTCCAGTTTCAAAGGT-3).

R: (5- AGCTAAATTCATAGCAGAAAGC-3).

***C. glabrata*[12]:**

F: (5- CCCAAAAATGGCCGTAAGTATG-3).

R: (5- ATAGTCGCTACTAATATCACACC-3).

***C. tropicalis*[12]:**

F: (5-GTTGTACAAGCAGACATGGACTG-3).

R: (5- CAAGGTGCCGTCTTCGGCTAAT-3).

***C. famata*[13]:**

F: (5-TCCTTCTTGGTTGGGTTCCT-3).

R: (5-GGTCCCAACAGCTATGCTCT-3).

Biofilm detection using the tube method:

Isolated *Candida* on SDA plate was inoculated into a tube containing 10 ml of SDB. The tubes were incubated at 35°C for 48 hours. After incubation, the culture supernatants were decanted and the tubes were washed with phosphate buffer saline (pH 7.3) and the dried tubes were stained with 1% crystal violet. Excess stain was removed by washing with de-ionized water and the tubes were dried by inverting them. Biofilm formation was considered positive when a visible film forms lining the wall of the tube[14].

Antifungal susceptibility of Candida isolates: was evaluated using VITEK 2 system[15].

Statistical analysis:

Data were tabulated and statistically analyzed using SPSS program version 20. Chi-square 2**(X^2)** and Fischer Exact tests were performed at 5% level of significance.

RESULTS

Rate of *Candida* isolation among immunocompromised patients:

Out of the collected 220 samples, 69 (31.4%) were positive for *Candida* infections (23 urine, 16 blood,14 sputum,7 ascitic fluid, 6 throat swabs and 3 venous catheter samples). Most positive samples (56.5%) were obtained from patients admitted to ICUs followed by Internal Medicine (14.6%) and Oncology (11.6%) Departments**.** Most *Candida* isolates were found in urine samples (33.3%), blood samples (23.2 %) and sputum (20.3%) (Fig. 1,2).

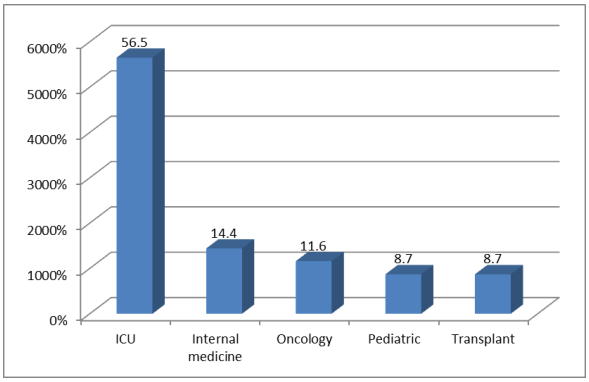

Fig. 1: Distribution of the isolated *Candida* spp. among Hospital Departments. ICU was the most common site.

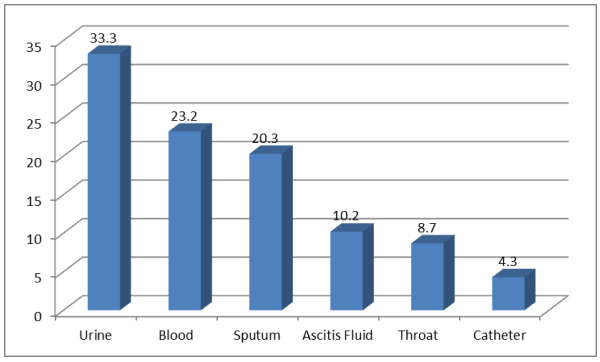

Fig. 2: Distribution of the isolated *Candida* spp. among different samples. Most isolates were from urine, blood and sputum.

Identification of *Candida* species:

Using PCR, *C. albicans* was the most predominant species (34.8%), followed by *C. tropicalis* (21.7%), then *C. famata* (8.7%) and *C. glabrata* (5.8%). However, 20 *Candida* isolates were not identified up to species level by PCR using the available used primers. Specific primers for *C. albicans*, *C. famata*, *C. glabrata* and *C. tropicalis* were only used.

VITEK 2 system was able to identify 9 species; *C. albicans* was the predominant (34.8%) followed by *C. tropicalis* (21.7%). However, CCA identified only 4 *Candida* spp.; *C. albicans*, *C. glabrata*, *C. tropicalis* and *C. cruzi* (42%, 30.5%, 18.8% and 8.7% respectively) (table 1, Fig. 3,4)

Table 1: *Candida* speciation by multiplex PCR, VITEK 2 system and chromogenic *Candida* agar

	Multiplex PCR		VITEK 2		CCA	
***Candida* spp.**	**No**	**%**	**No**	**%**	**No**	**%**
C. albicans	24	34.8	24	34.8	29	42
C. tropicalis	15	21.7	15	21.7	13	18.8
C. famata	6	8.7	6	8.7	-	-
C. cruzi	-	-	5	7.2	6	8.7
C. lusitaniae	-	-	5	7.2	-	-
C. parapsilosis	-	-	4	5.8	-	-
C. dubliniensis	-	-	4	5.8	-	-
C. ciferri	-	-	4	5.8	-	-
C. glabrata	4	5.8	2	2.9	21	30.5

C. albicans and *C. tropicalis* were the most common isolates.

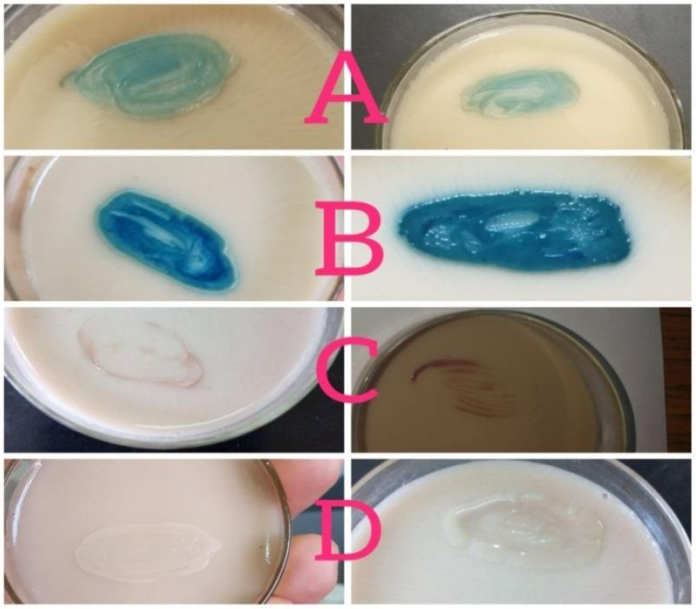

Fig. 3: Different *Candida* spp. on chromogenic *Candida* agar (CCA). A: Light green colonies of *C. albicans.* B: Metallic blue colonies of *C. tropicalis*. C: Purple colonies of *C. cruzi.* D: White to creamy colonies of *C. glabrata*

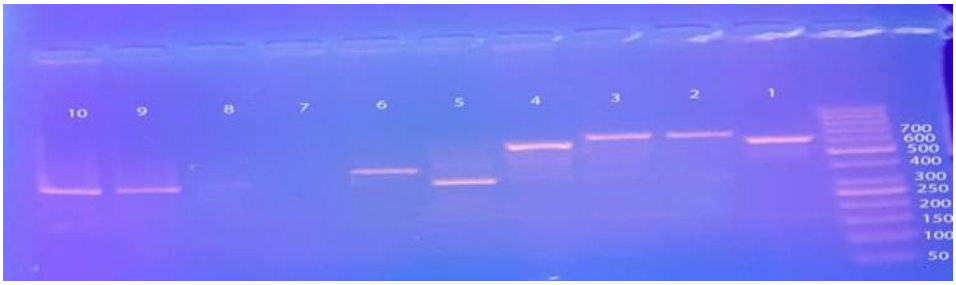

Fig. 4: Multiplex PCR amplified products of *Candida* spp. specific genes. Lanes 1 and 4: *C. albicans* (665 bp) Lanes 2 and 3: *C. glabrata* (674 bp). Lane 6: *C. famata* (460 bp). Lanes 5, 9 and 10: *C. tropicalis* (318 bp)

Antifungal susceptibility of *Candida* species:

There was no significant difference between *C. albicans* and non-albicans *Candida* (NAC) regarding antifungal resistance. There was high resistance to flucytocine (49.3%), fluconazole (33.3%) and amphotericin B (27.5%). On the other hand, high susceptibility rates were detected to micafungin, voriconazole and caspofungin (88.4%, 81.2% and 81.2 % respectively).

C. albicans showed the highest resistance to flucytocine (54.1%), fluconazole (41.6 %) and amphotericin B (41.6 %) and voriconazole (29.1%), while the highest sensitivity was to micafungin (87.5%) and caspofungin (75%). *C. tropicalis* showed the highest resistance to flucytocine (46.7%) while the highest sensitivity (93.3%) was to amphotericin B, voriconazole, caspofungin and micafungin. *C. famata* showed high resistance (66.6%) to flucytocine and fluconazole while the highest sensitivity (83.3%) was to amphotericin B. *C. cruzi* had the highest resistance to amphotericin B (40 %) and the highest sensitivity to caspofungin and micafungin (100%). *C. lusitaniae* showed the high resistance to flucytocine and fluconazole (40%), and highest sensitivity (100%) to voriconazole and micafungin (table 2).

Table 2: Susceptibility patterns to antifungal drugs using VITEK 2 system

Candida spp.	Antifungal drugs					
	Flucytocine No (%)	**Fluconazole No (%)**	**Amphotericin B No (%)**	**Voriconazole No (%)**	**Caspofungin No (%)**	**Micafungin No (%)**
albicans						
R	13 (54.1)	10 (41.6)	10 (41.6)	7 (29.1)	6 (25)	3 (12.5)
S	11 (45.8)	14 (58.3)	14 (58.3)	17 (70.8)	18 (75)	21 (87.5)
tropicalis						
R	7 (46.7)	2 (13.3)	1 (6.7)	1 (6.7)	1 (6.7)	1 (6.7)
S	8 (53.3)	13 (86.7)	14 (93.3)	14 (93.3)	14 (93.3)	14 (93.3)
famata						
R	4 (66.6)	4 (66.6)	1 (16.6)	3 (50)	3 (50)	2 (33.3)
S	2 (33.3)	2 (33.3)	5 (83.3)	3 (50)	3 (50)	4 (66.6)
cruzi						
R	1 (20)	1 (20)	2 (40)	1 (20)	-	-
S	4 (80)	4 (80)	3 (60)	4 (80)	5 (100)	5 (100)
lusitaniae						
R	2 (40)	2 (40)	1 (20)	-	1 (20)	-
S	3 (60)	3 (60)	4 (80)	5 (100)	4 (80)	5 (100)
parapsilosis						
R	2 (50)	1 (25)	1 (25)	-	1 (25)	1 (25)
S	2 (50)	3 (75)	3 (75)	4 (100)	3 (75)	3 (75)
dubliniensis						
R	2 (50)	2 (50)	1 (25)	-	-	-
S	2 (50)	2 (50)	3 (75)	4 (100)	4 (100)	4 (100)
ciferri						
R	2 (50)	-	1 (25)	-	1 (25)	1 (25)
S	2 (50)	4 (100)	3 (75)	4 (100)	3 (75)	3 (75)
glabrata						
R	1 (50)	1 (50)	1 (50)	1 (50)	-	-
S	1 (50)	1 (50)	1 (50)	1 (50)	2 (100)	2 (100)
Total						
R	34 (49.3)	23 (33.3)	19 (27.5)	13 (18.8)	13 (18.8)	8 (11.6)
S	35 (50.7)	46 (66.7)	50 (72.5)	56 (81.2)	56 (81.2)	61 (88.4)

Comparison between the disc diffusion method and VITEK 2 in detection of antifungal resistance:

Using disk diffusion method, *Candida* isolates showed high resistance rate to flucytocine (68.1%), fluconazole (37.7%) and amphotericin B (34.7%).There was a highly significant difference between disk diffusion and VITEK2 in detection of *Candida* spp. susceptibility to flucytocine. The disk diffusion had 57% sensitivity, 94% specificity and 75% accuracy compared to VITEK2 system. On the other hand, there was no significant difference between the two methods regarding fluconazole and amphotericin B **(**table 3).

Table 3: Comparison between disc diffusion method and VITEK 2 in detection of antifungal resistance

Antifungals	Sensitive	Resistant	X^2 (P value)	Sensitivity (%)	Specificity (%)	Accuracy (%)	PPV (%)	NPV (%)
Flucytocine:				57	94	75	91	68
Disk diffusion	22 (31.9)	47 (68.1)	10.91					
VITEK	35 (50.7)	34 (49.3)	**(0.004*)**					
Fluconazole:				91	96	93	98	85
Disk diffusion	43 (62.3)	26 (37.7)	0.34					
VITEK	46 (66.7)	23 (33.3)	(0.844)					
Amphotericin B:								
Disk diffusion	45 (65.3)	24 (34.7)	0.85	84	84	84	93	67
VITEK	50 (72.5)	19 (27.5)	(0.652)					

*PPV: Positive predictive value ** NPV: Negative predictive value

Biofilm formation by *Candida* isolates:

Only 30.4% of the isolated strains were biofilm-producers by the tube method. Biofilm formation was highest among *C. albicans* (58.3%) followed by *C. famata* (33.3%). Both *C. dubliniensis* and *C. ciferri* were biofilm-negative (table 4, Fig. 5).

Table 4: Biofilm formation by various *Candida* spp

Candida spp. (No)	Biofilm formation			
	Positive		Negative	
	No	%	No	%
C. albicans (24)	14	(58.3)	10	(41.7)
C. famata (6)	2	(33.3)	4	(66.7)
C. tropicalis (15)	1	(6.7)	14	(93.3)
C. cruzi (5)	1	(20)	4	(80)
C. lusitaniae (5)	1	(20)	4	(80)
C. parapsilosis (4)	1	(25)	3	(75)
C. dubliniensis (4)		-	4	(100)
C. ciferri (4)		-	4	(100)
C. glabrata (2)	1	(50)	1	(50)
Total (69)	**21**	**30.4**	**48**	**(69.6)**

High rates of biofilm production were found in *C. albicans* and *C. glabrata*.

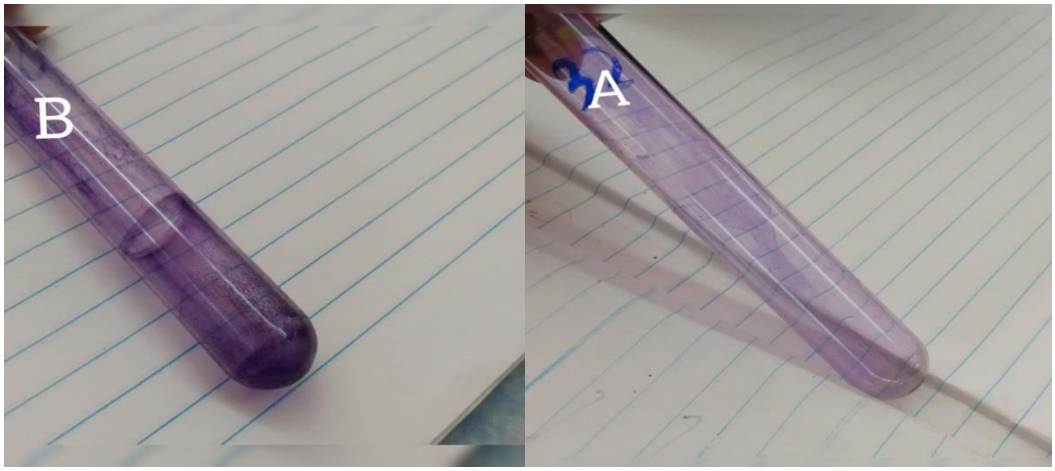

Fig. 5: Tube test for detection of biofilm formation by *Candida* species. **A:** Negative biofilm formation. **B:** Positive biofilm formation

Relation between biofilm formation and antifungal resistance among *Candida* isolates:

There was highly significant difference (p<0.001) between biofilm producers and non-producers regarding resistance to fluconazole, amphotericin B, voriconazole, ketoconazole, caspofungin and nystatin. About 81%, 71.4% and 71.4% of the biofilm-producing *Candida* spp. were resistant to fluconazole, ketoconazole and amphotericin B (table 5).

Table 5: Biofilm formation and antifungal resistance among *Candida* isolates

Resistant *Candida* species (intermediate+ resistant)		Biofilm tube test (n= 69)				Test of significance	
		Positive (n=21)		Negative (n=48)			
	Method	No	%	No	%	Z test	P value
Flucytocine	VITEK	16	76.2	18	37.5	2.7	0.007*
Amphotericin B	VITEK	15	71.4	4	8.3	5.11	<0.001**
Fluconazole	VITEK	17	81	6	12.5	5.27	<0.001**
Voriconazole	VITEK	12	57.1	1	2.1	5.05	<0.001**
Caspofungin	VITEK	10	47.6	3	6.3	3.71	<0.001**
Micafungin	VITEK	6	28.6	2	4.2	2.5	0.012*
Ketoconazole	Disk	15	71.4	7	14.6	4.38	<0.001**
Itraconazole	Disk	12	57.1	12	25	2.3	0.021*
Nystatin	Disk	10	47.6	3	6.3	3.71	<0.001**

***: Significant **Highly significant**

There was a positive relation between anti-fungal resistance and biofilm formation among *Candida* isolates

DISCUSSION

Accurate identification of yeasts is important especially among critically ill patients[16]. In this study, 69 *Candida* strains were isolated from 220 clinical samples (34.5%) obtained from immunocompromised patients. However, lower rates were reported (14.7%[17]and 8.1%[18]). This difference may be due to regional variations in infection control measures and resistance to antimicrobials. In our study, *Candida* species were isolated from ICUs at a high rate (56.6%), a finding which was similarly reported[19]. ICU patients almost have a higher risk of infections compared to non-critical care areas due to increased use of invasive procedures, prolonged extensive antimicrobial regimes, and critically ill status of the patients. On the other hand, *Candida* species were highly (57.0%) isolated from Obstetrics and Gynaecology Department[20]. In our study, most isolates were found in urine (33.3%) and blood (23.2%). Similarly, high isolation rates were from urine (36.3%)[20], (48%)[18]and (43%)[21]. On the other hand, the highest isolation rate (36.2%) was from blood[19].

At least 15 *Candida* species cause human diseases, although most (95%) infections are caused by *C. albicans, C. glabrata, C. tropicalis, C. parapsilosis,* and *C. krusei*[22]. Several techniques have been used to identify *Candida* spp.[16]. In our study, the most frequent were *C. albicans* (34.8%) and *C. tropicalis* (21.7%). Similar results; (56% and 20%)[18], (69.6% and 12.8%)[23] and (80% and 12.5%)[17]were also reported. Therefore, *C. albicans* is the most major pathogenic species. However, the incidence of NAC is generally increasing worldwide[22]. Among our 69 isolates, 65.2% were NAC, and the most common NAC was *C. tropicalis* (21.7%). Similar findings were reported by other investigators[22,24,25]. The distribution of *Candida* species, however, was C. *albicans* (49.3%), *C. stellatoidea* (25.4%) and *C. parapsilosis* (25.3%) in Nigeria[26]. These variations may be attributed to differences in antifungal treatment practices, patient demographic features and chronic underlying diseases in different locations[22].

Antifungal susceptibility testing is essential to monitor resistance and to guide therapy[27].In our study, susceptibility patterns to antifungal drugs were tested by VITEK 2 system. High resistance to flucytocine (49.3%), fluconazole (33.3%) and amphotericin B (27.5%) was found. However, high susceptibility rates were detected to micafungin, voriconazole and caspofungin (88.4%, 81.2% and 81.2 % respectively. Variable susceptibility patterns were reported by different investigators. Voriconazole was the most useful drug against *C. albicans* and NAC while miconazole and ketoconazole were the least[20]. High sensitivity was to amphotericin B (91%), followed by voriconazole (65%) and itraconazole (49%)[28]. High susceptibility was to clotrimazole (82%) followed by fluconazole (64%) whereas 86% of the isolates were resistant to ketoconazole[18]. Resistance to amphotericin B, fluconazole, flucytocine, voriconazole, and itraconazole were 40.63%, 34.38% 46.88%, 18.75%, and 31.25%, respectively[29]. High susceptibility was to amphotericin B (100%), anidulafungin (100%), micafungin (100%), caspofungin (98.4%), flucytosine (98.4%) and voriconazole (84.1%)[22]. However,

susceptibility to itraconazole and fluconazole was comparatively lower (57.9% and 72.2%, respectively). About 72.5%and 81.2% of our strains were sensitive to amphotericin B and voriconazole respectively. Several studies have reported that amphotericin B was the most effective drug[30,31,32,33].

Our results showed that 33.3% of *Candida* isolates were resistant to fluconazole, a finding which is similar to others[21,29]**.** However, lower fluconazole resistance rates were reported in Egypt[34], Taiwan[35]**,** Brazil[30] and Portugal[36]. The lowest fluconazole resistance (0.6%) was reported in Australia and Kuwait[37,38]. These variations may be due to differences in clinical samples, immune system status, azole administration in the different geographical areas[39].

In our study, 18.8% of *Candida* spp were resistant to voriconazole and among these resistant strains, 43.5% were *C. albicans.* Similar findings were reported by other investigators[40]. Our *C. albicans* had high resistance to flucytocine (54.1%), fluconazole and amphotericin B (41.6 %), but had high sensitivity to micafungin (87.5%) and caspofungin (75%). On the other hand, *C. albicans* had high sensitivity to voriconazole (73.1%) followed by fluconazole (63.5%) and amphotericin B (61.5%)[20]. Maximum resistance was seen with miconazole (51.9%) followed by ketoconazole (40.4%) and fluconazole (34.6%). However, all *C. albicans* isolates (100%) were susceptible to amphotericin B and voriconazole[39].

In our study, *C. tropicalis* had high resistance to flucytocine (46.7%) and high sensitivity to amphotericin B, voriconazole, caspofungin and micafungin (93.3%). Similarly, it had 83% sensitivity to fluconazole and 17% resistance to fluconazole, ketoconazole, clotrimazole and amphotericin B[8]. However, *C. tropicalis* had reduced susceptibility to voriconazole (76.7%), fluconazole (76.7%) and itraconazole (39.5)[22]. In our study, no significant difference was found between *C. albicans* and NAC. On the other hand, resistance to fluconazole and itraconazole was more common in NAC (*C. glabrata*, *C. krusei*, and *C. guilliermondii*), and it was suggested that these antifungal agents should not be used for these NAC infections[19].

In our study, a large proportion of *Candida* strains showed no biofilm formation (69.6%) while 30.4 % were biofilm producers. This finding was similarly reported by some investigators[39]. A relationship was suggested between biofilm production and pathogenesis, however, this may vary, depending on the location of infection and number of *Candida* strains[39].

In our study, there was a positive relationship between biofilm formation and antifungal resistance especially to fluconazole, amphotericin B, voriconazole, ketoconazole, caspofungin and nystatin. Similarly, Mohammadi et al[39] showed a correlation between biofilm formation and resistance to fluconazole and itraconazole. On the other hand**,** no significant correlation was detected between biofilm formation and antifungal susceptibility[21]. Biofilm-associated *Candida* may exhibit dramatic resistance (intrinsic or acquired) by multiple mechanisms[14]; as reduction of drug penetration, decreased metabolism of cells, and increased expression of resistance genes in the biofilm[39].

CONCLUSION

Candida speciation can be reliably done by VITEK 2 system and CCA. High and variable antifungal resistance was found among our *Candida* isolates and was correlated to biofilm formation. Therefore, accurate speciation and antifungal susceptibility testing of *Candida* isolates should be done routinely to prevent therapeutic failures.

Acknowledgment:
Safa Saber, Researcher at Central Laboratory, Faculty of Medicine, Menoufia University for cooperation during work.

Conflicts of interest: The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.

- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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