ORIGINAL ARTICLE

Study of Virulence Genes, Biofilm Formation and Antifungal Susceptibility of Medical Device Associated Candida Infection in ICU Hospitalized Patients

Reem M. Elkholy, Amira A. El-hendy, Hagar S.M. Nassar*, Eman M. Zaher
Department of Clinical Pathology Faculty of Medicine -Menoufia University

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Corresponding Author:
Hagar Said Mousa Nassar, Clinical Pathology, Faculty of Medicine, Menoufia University
hagarsaid76@gmail.com
Tel: 01062850668

ABSTRACT

Background: *Candida species are increasingly reported as a cause of hospital acquired infection. Biofilms play a role in Candida adherence to medical devices. Candida within biofilms is less susceptible to antifungal agents. HWP1, ALS1, ALS3, SAP1, PLB1 and INT1S are biofilm regulators genes.* ***Objectives****: the aim of the present work is to study medical device associated candida infection in ICU patients regarding virulence genes, biofilm formation and antifungal susceptibility.* ***Methodology****: Fifty candida isolates were identified by chromogenic media and VITEK 2 compact system and subjected to antifungal susceptibility test by modified disc diffusion method and VITEK -2. Biofilm study was carried out by microtiter plate method (MTP). Molecular study for virulence genes was done by conventional PCR.* ***Results****: Non albicans candida (NAC) were isolated at higher rate (64%) than candida albicans (36%). C. tropicalis was the commonest among non albicans. There was high agreement between Chromogenic media and VITEK -2 regarding identification of C.albicans and C.tropicalis. Kappa agreement was 0.87, 0.95 respectively. The resistance rates for candida isolates were as the followings: Fluconazole (28%), Amphotericin B (26%), Caspofungin (18%), Micafungin (16%), Voriconazole (14%) and (8%) for Flucytocine. HWP1, ALS1, ALS3 genes correlated with biofilm formation (p -values: 0.021, 0.001 and 0.014) respectively.* ***Conclusion****: Both NAC and Candida albicans are involved in device associated infection in ICU with higher resistance rate for Fluconazole and Amphotericin B. Drug resistance increases in isolates with biofilm forming capacity. Moreover, there was association between Virulence genes (HWP1, ALS 1 and ALS 3) and biofilm formation.*

INTRODUCTION

Healthcare-associated infections (HAIs) are defined as infections related to care delivery that are not incubated or present at the time of admission[1]. Being global public health problem, significant adverse effects represented in increased mortality and prolonged hospital stay with huge economic burden are pronounced [2]. Intensive care units (ICUs) patients have significant higher risk of acquiring HAI due to immunodeficiency and exposure to invasive procedures and medical devices. In addition, ICU is often a focus of multi-drug resistance (MDR) microorganisms [3].

The centers for disease control and prevention (CDC) broadly categorizes the types device-associated HAIs (DA-HAIs) as central line-associated bloodstream infection (CLABSI), catheter-associated urinary tract infection (CAUTI), ventilator-associated pneumonia (VAP), and surgical-site infection (SSI) [4].

DA-HAIs are frequently associated with the colonization of medical devices with microorganisms organized in biofilms[5]. From the group of biofilms forming organisms, *Candida spp.*, are one of the major causative agents of HAIs [6] and ranked the third among various leading cause of catheter associated infections [7].

Candida albicans was the most common clinically isolated *Candida spp.*, However, there has been a recent trend toward the isolation of other *non-albicans candida (NAC)* species such as: C. *glabrata, C. tropicalis, C. parapsilosis, C. dubliniensis, C. guilliermondii and C. krusei*. These NAC are known as a major cause of serious infections that resist antifungal treatment [8].

Biofilm forming capacity in medically important Candida species is a putative virulence factor as it leads to resistance to antifungal therapy, limiting the penetration of antifungal drugs through the extracellular matrix and protects cells from the host's immune responses allowing spread of infection. Biofilm formation is under the influence of several mechanisms related to the yeast adhesion, morphogenesis and formation of extracellular matrix. These mechanisms are regulated by different genes [9].

Hyphal wall protein (HWP1) presents on the surface of the hyphae and mediate adhesion and filamentation. The gene that encode for HWP1 is part of a core of

eight genes induced during filamentation of *C. albicans.* Adhesion is also mediated by Agglutinin-like sequence (ALS). ALS1 and ALS3 are two genes with similar sequences and functions. These genes encode large glycosyl- phosphatidyl inositol (GPI)-linked cell surface glycoproteins and mediate attachment to endothelial cells, hyphal formation as well as adherence [10].

Secretion of secreted aspartic proteinases (SAPs) like SAP1 and phospholipases proteins (PLBs) like PLB1 is another virulence factors that contribute to colonization by enhancing the degrading of components of host cell membranes and cell wall proteins and phospholipids. These proteins by consequence mediate adherence to host tissues and invasion. Secretion of SAP1 and PLB1 is mediated by SAP1 and PLB1 genes respectively [11]. The integrin 1 protein (INT1) is another virulence factor that facilitate the capacity *of Candida albicans* to adhere to epithelial cells and form hyphae [12].

The aim of this work was to study medical device associated candida infection in ICU and to investigate the rate of antifungal resistance, biofilm formation and their relation to some virulence genes, such as HWP1, ALS1, ALS3, PLB1, SAP1 and INT1among candida isolates.

METHODOLOGY

Ethical approval statement

The study was approved by the Menoufia Faculty of Medicine Ethical Committee (5/22CPATH 42).

Study design:

This prospective study was carried out during the period extended from January 2023 to January 2024.It was conducted on fifty *candida* isolates from medical devices. They were collected from patients with HAIs who admitted at different ICUs units at Menoufia University Hospitals. The workup was held in Microbiology Unit of Clinical Pathology Department, Faculty of Medicine, Menoufia University.

Samples collection and Processing:

In cases of CA-UTI, Urine samples were collected from sampling port of the indwelling urinary catheters. In cases of suspected CLABSI, two blood cultures ***(BD BACTEC TM)*** were collected (one from the catheter lumen and the other from accessible peripheral vein). In suspected VAP, morning endotracheal aspirates were collected. All samples were collected using standard microbiological practice and transported to microbiology laboratory as soon as possible.

Samples were cultivated on blood agar, MacConkey's agar, CLED agar and SDA. The plates were incubated at 37°C for a period of 24 to 48 hours for further identification.

Phenotypic Identification of Candida species:

Candida spp., were identified by Gram Stain, Germ tube test [13], growth on Chromogenic Candida agar (CCA) *(HiCrome™ Candida, India)*[14,15] and automated identification by VITEK 2 Compact system *(BioMérieux, France*) using YST-ID Cards [16]

Antifungal Susceptibility Testing:

It was evaluated by Modified Disc Diffusion Method on Muller-Hinton agar **(Himedia, India)** supplemented with 2% glucose and 0.5 µg/mL of methylene blue according to CLSI [17, 18] and by VITEK 2 Compact system using YST-AST -YS08 cards [16].

Candida albicans ***(ATCC10231) (BD)*** was used as a positive control strain. Isolates of *Candida* were stored in tryptic soy broth (TSB) ***(Oxoid, UK)*** containing 20% glycerol at - 80°C until further steps.

Detection of virulence

Biofilm formation study by microtiter plate method (MTP):

One fresh *Candida* colony was dissolved in 5ml trypticase soya broth (TSB) and incubated at 37°C for 24 hours then diluted 1:100 in TSB containing1% glucose with turbidity adjusted to 0.5 McFarland. Two hundred microns of the dilution were inoculated into sterile flat bottomed 96 well of polystyrene tissue culture plates. The plates were incubated at 37°C for 24h then washed, stained and resolubilized with 95% ethanol. The optical density (OD) of stained wells was measured at 595 nm using ELISA reader. [19].

Molecular detection of virulence genes by conventional PCR

Using ***(DNeasy Plant Mini Kit, QAIAGENE*)**, DNA was extracted and purified from fresh colonies sub-cultured on SDA according to the manufacturer's instructions. after DNA extraction, its concentration and purity were evaluated using the Nanodrop ***(Implen, Germany).***

PCR assay was done by Thermal Cycler ***(Biometra, Germany)*.** The PCR reaction mixture was done in a total volume of 25 µl as follows: 12.5µl Taq Green PCR Master Mix **(*Thermo Fisher Scientific, UK),*** 5µl of template DNA, 0.5µl from each primer, and 6.5µl of nuclease free water.

The PCR conditions were as follows: initial denaturation step, cycles of: denaturation, annealing a, extension, followed by a final extension step. a Primer sequence and PCR condition of tested virulence genes were stated in table (1)[20,21]. Amplified products were examined using electrophoresis on ethidium bromide-stained 2% agarose gel. UV transilluminator was used to visualize DNA bands. Based on the size of the fragments in contrast to a 100 bp DNA ladder, genes were detected [22].

Table 1: Primer sequence (5'-3') and PCR condition of the tested virulence genes [20,21].

Genes	Primer sequence (5'-3')	Product Size (bp)	PCR condition
HWP1-F *HWP1-* R	*ATGACTCCAGCTGGTTC* *TAGATCAAGAATGCAGC*	503	94ºC4 min 1x 94 ºC30 sec 52 ºC 1 min 35x 72 ºC2 min 72ºC5 min 1x
ALS1- F *ALS1* -R	*GACTAGTGAACCAACAAATACCAG* *CCAGAAGAAACAGCGGTGA*	318	94ºC 2min 1x 94ºC 4 min 52ºC 1min 35x 72ºC 2 min 72ºC 5 min 1x
AINT1-F *AINT1-R*	*AAGCTCTGATACCTACACTAGCGA* *GTTAGGTCTAAAGTCGAAGTCATC*	239	92ºC 5min 1x 92ºC 1 min 65ºC 1min 30x 72ºC 1min 72ºC 5 min 1x
SAP1-F *SAP1-R*	*GCTCTTGCTATTGCTTTATTA* *CAT CAG GAA CCC ATA AAT CAG*	253	95ºC 5min 1x 95ºC 4 min 49ºC 1min 30x 72ºC 1min 72ºC 5min 1x
ALS3-F *ALS3-R*	*CCAAGTGTTCCAACAACTGAA* *GAACCGGTTGTTGCTATGGT*	185	95ºC 5min 1x 95ºC 1min 52 ºC 1 min 32x 72 ºC 1 min 72ºC 5min 1x
PLB1-F *PLB1*-R	*ATGATTTTGCATCATTTG* *AGTATCTGGAGCTCTAC*	751	95ºC 5min 1x 95ºC 1min 49ºC 1 min 32x 72 ºC 1 min 72ºC 5min 1x

Statistical Analysis

Data was analyzed using IBM SPSS (version 26.0, Armonk, NY: IBM Corp). Data were expressed as Number (N), percentage (%). Chi-square test ($\chi 2$) and Fisher's Exact test were used. The significance of the obtained results was judged at the 5% level. In all tests, P value of less than 0.05 was considered statistically significant. Kappa agreement interpretation was as follow: Kappa = 0: no agreement, 0.01– 0.20: none to slight agreement, 0.21–0.40: fair agreement, 0.41– 0.60: moderate agreement, 0.61–0.80: strong-good agreement, 0.81–0.99: very strong-excellent agreement and1.00: perfect agreement.

RESULTS

This study included 50 candida isolates from ICU patient with DA-HAIs. The frequency of CA-UTI, CLABSI and VAP was 54%, 38% and 8% respectively. *Candida spp.*, were identified by subculture on CCA confirmed by VITEK 2 compact system. Using CCA, *C. albicans*, *C. tropicalis* and *C. glabrata represented* 42%, 32% and 26% respectively. **Figure (1)**. VITEK 2 compact system identified 7 subspecies; *C. albicans* (36%) *C. tropicalis* (34%), *C. parapsilosis (*10%), *C. auris* (6%), *C Ciferri (*6%), *C. Glabrata* (4%) and *C. guilliermondi*(4%) as illustrated by **Figure(2).** Chromogenic Candida Agar was excellent in identification of *C. albicans* and *C. tropicalis* with strong agreement to VITEK 2 compact system (kappa=0.0.87,0.95 respectively) **(Table 2).**

Table 2: Comparison between CCA and VITEK 2 compact system regarding identification of Candida Spp.

CCA	VITEK 2											
	C.albicans N=18		NAC N=32		C.tropicalis N=17		Non C.tropicalis N=33		C.glabrata N=2		Non-C.glabrata N=48	
	No	%	No	%	No	%	No	%	No	%	No	%
Positive Negative	18 -	100 -	3 29	9.4 90.6	16 1	94.1 5.9	- 33	- 100	2 -	100 -	11 37	22.9 77.1
Kappa agreement	0.874				0.955				0.212			
P-value	<0.001 (HS)				<0.001 (HS)				0.015 (S)			
Sensitivity	100%				94.1%				100%			
Specificity	90.6%				100%				77.1%			
Accuracy	94%				98%				78%			

NAC: non-albicans candida HS: highly significant S: significant

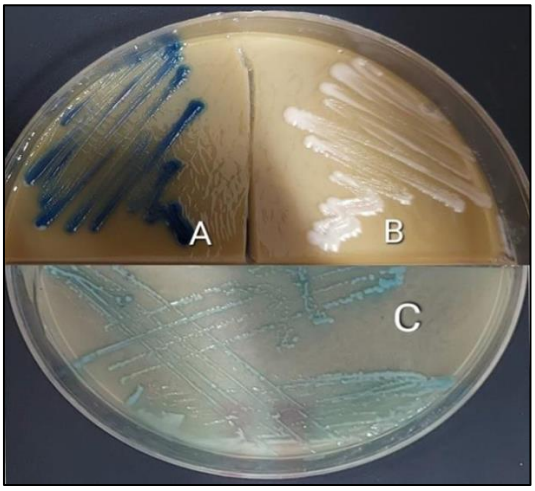

Fig. 1: *Candida spp.*, on Chromogenic Candida Agar (CCA). A: Metallic blue colonies of *C. tropicalis*- B: White to creamy colonies of *C. glabrata*- C: Light green colonies of *C. albicans.*

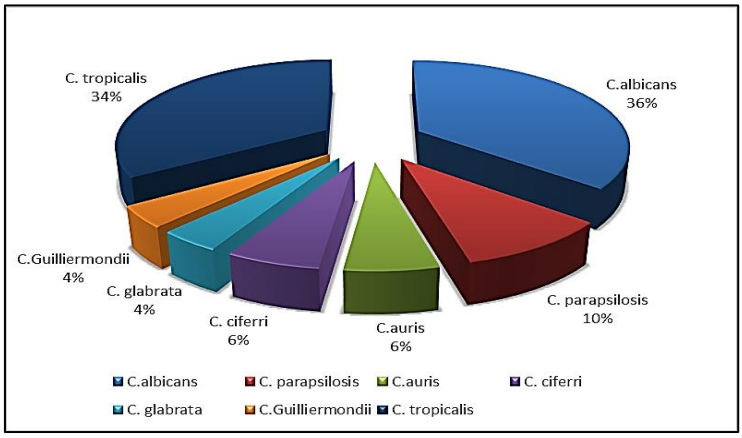

Fig. 2: *Candida Spps*., distribution by VITEK 2 compact system.

Regarding antifungal Susceptibility testing, Fluconazole, Voriconazole, Amphotericin B and Caspofungin were tested by modified disc diffusion method and confirmed with VITEK 2 compact system in addition to Micafungin and Flucytocine. The highest resistance rate was for fluconazole (28%) followed by Amphotericin- B (26%), Caspofungin (18%) and Micafungin (16%). Flucytocine and Voriconazole had better antifungal activity with resistance rate (8% and 14%) respectively **(Table 3).**

Table 3: Detection of antifungal resistance rates by modified disc diffusion method and VITEK 2 compact system

Antifungal resistance	Modified disc diffusion method		VITEK 2	
	No	%	No	%
Fluconazole	15	30	14	28
voriconazole	11	22	7	14
Amphotericin B	6	12	13	26
Caspofungin	8	16	9	18
Micafungin	Not tested		8	16
Flucytocine.			4	8

There was good agreement between modified disc diffusion method and VITEK 2 compact system in antifungal susceptibility testing to Fluconazole (kappa=0.76), voriconazole (kappa=0.73), Caspofungin (kappa=0.65) and Amphotericin B (0.56) **(Table 4).**

Table 4: Comparison between Modified Disc Diffusion method and VITEK 2 compact system regarding detection of antifungal Susceptibility among Candida isolates

		VITEK 2									
		Fluconazole									
Modified disc diffusion method		**Resistant**		**Sensitive**		**Kappa (P-value)**	**Sensitivity (%)**	**Specificity (%)**	**PPV (%)**	**NPV (%)**	**Accuracy (%)**
		N	**%**	**N**	**%**						
	Resistant	12	85.7	3	8.3	0.757					
	Sensitive	2	14.3	33	91.7	**(<0.001) HS**	**85.7**	**91.7**	**80**	**94.3**	**90**
		voriconazole									
		Resistant		**Sensitive**		**Kappa (P-value)**					
		N	**%**	**N**	**%**						
	Resistant	7	100	4	9.3	0.732	**100**	**90.7**	**63.6**	**100**	**92**
	Sensitive	-	-	39	90.7	**(<0.001) HS**					
		Amphotericin B									
		Resistant		**Sensitive**		**Kappa (P-value)**					
		N	**%**	**N**	**%**						
	Resistant	6	46.2	-	-	0.559	**46.2**	**100**	**100**	**84.1**	**86**
	Sensitive	7	53.8	37	100	**(<0.001) HS**					
		caspofungin									
		Resistant		**Sensitive**		**Kappa (P-value)**					
		N	**%**	**N**	**%**						
	Resistant	6	66.7	2	4.9	0.646	**66.7**	**95.1**	**75**	**92.9**	**90**
	Sensitive	3	33.3	39	95.1	**(<0.001) HS**					

PPV: positive predictive value, NPV: negative predictive value HS: highly significant (P<0.001)

The biofilm formation capacity was evaluated by microtiter plate method Figure (3). Thirty-eight isolates (76%) were biofilm forming; thirteen isolates (72%) of *C. albicans*, eleven isolates *(65%) of C. tropicalis*, four isolates (80%) of *C. parapsilosis*, three isolates (100%) of *C. auris*, three isolates (100%) of *C. Ciferri*, two isolates (100%) of *C. glabrata* and two isolates (100%) of *C.guilliermondii.* No statistically significant difference between *candida albicans* and *NAC* as regard capacity to form biofilm.

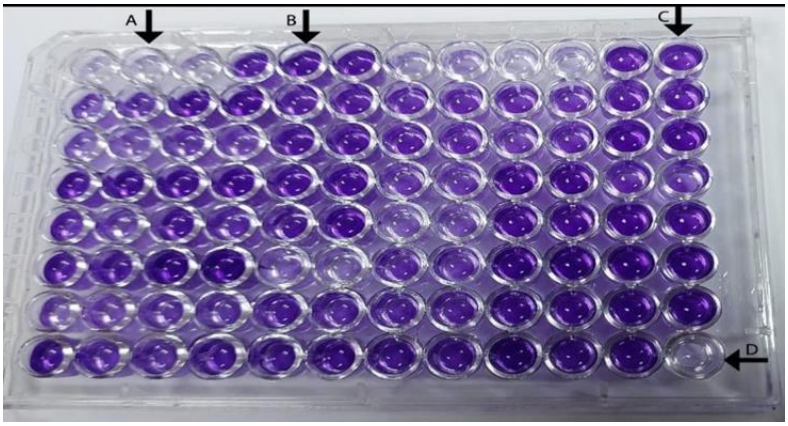

Fig. 3: Biofilm study on microtiter plate: A: negative control - B: positive control – C: biofilm forming candida -D: non biofilm forming candida.

Antifungal resistance was increased in biofilm forming *Candida* isolates versus non biofilm forming ones. For fluconazole, the resistance rate was; (34.2%) in biofilm forming versus (8.3%) in non-biofilm forming isolates. For Amphotericin B, it was (26.3%) in biofilm forming versus (25%) in non-biofilm forming isolates. For Caspofungin, it was (21.1%) in biofilm forming versus (8.3%) in non-biofilm forming isolates. For Micafungin, it was (18.4%) in biofilm forming versus (8.3%) in non- biofilm forming *Candida isolates*. All Flucytosine and Voriconazole resistant *Candida* isolates were biofilm forming **(Table5)**.

Table 5: Distribution of antifungal resistance among biofilm forming and non-biofilm forming Candida isolates

Antifungal resistance	Biofilm formation				Total (n= 50)	
	Negative (N=12)		Positive (N=38)			
	N	%	N	%	N	%
Fluconazole	1	8.3	13	34.2	14	28
Voriconazole	0	0	7	18.4	7	14
Amphotericin B	3	25	10	26.3	13	26
Caspofungin	1	8.3	8	21.1	9	18
Micafungin	1	8.3	7	18.4	8	16
Flucytocine	0	0	4	10.5	4	8

In molecular studying of the virulence genes among the isolated *candida* species, the most prevalent gene was ALS3 (84%) followed by ALS1 (68.0%), SAP1(60%), HWP1(54%) and PLB1(46%). The least prevalent one was INT1 (34%) as illustrated in **Table (6) & Figure 4.**

Table 6: Frequency of virulence genes among candida isolates

Virulence genes	Candida isolates (N= 50)			
	Positive		Negative	
	N	%	N	%
ALS3	42	84	8	16
ALS1	34	68	16	32
SAP1	30	60	20	40
HWP1	27	54	23	46
PLB1	23	46	27	54
INT1	17	34	33	66

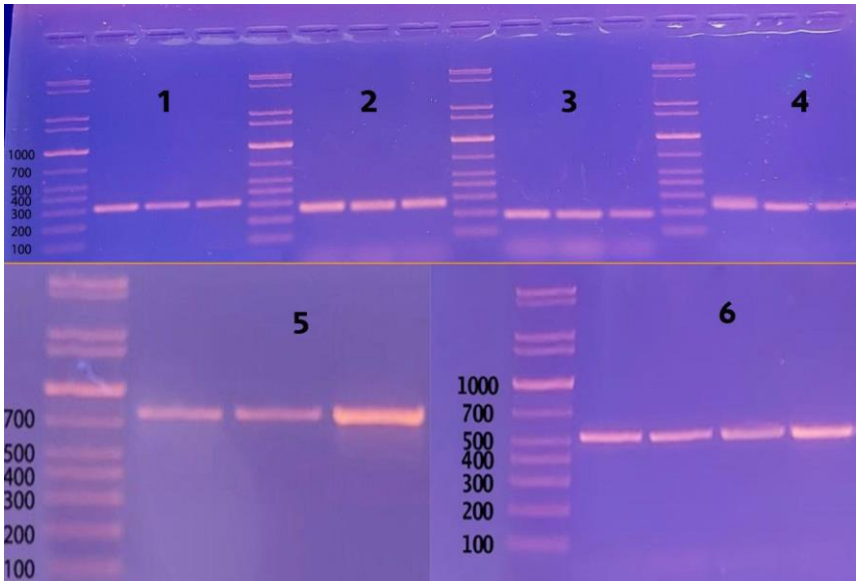

Figure (4): Molecular study of virulence genes: 1-ALS1 gene (318 bp), 2-SAP1 gene (253 bp), 3-ALS3 (185 bp), 4-INT1 gene (239 bp), 5-PLB1 gene (751 bp) and 6-HWP1 (503 bp).

The studied virulence genes were increased in *Candida* isolates with capacity to form biofilm as compared to non-biofilm forming *Candida* isolates. There was statistically positive relation between capacity of candida to form biofilm and the presence of the following virulence genes HWP1, ALS3 and ALS1 with p value: 0.021, 0.014 and 0.001 respectively**. (Table 7)**

Table 7: Relation between biofilm formation and virulence genes

	Biofilm formation				**Total (n= 50)**		**Test of significance**	**P-value**
	Negative (N=12)		**Positive (N=38)**					
	N	**%**	**N**	**%**	**N**	**%**		
HWP1:								
Positive	3	25	24	63.2	27	54	χ^2 5.35	**0.021 (S)**
Negative	9	75	14	36.8	23	46		
ALS1:								
Positive	3	25	31	81.6	34	68	***FE*** 13.42	**0.001 (S)**
Negative	9	75.0	7	18.4	16	32		
SAP1:								
Positive	5	41.7	25	65.8	30	60	***FE*** 2.21	0.182
Negative	7	58.3	13	34.2	20	40		
PLB1:								
Positive	3	25	20	52.6	23	46	χ^2 2.80	0.094
Negative	9	75	18	47.4	27	54		
INT1:								
Positive	4	33.3	13	34.2	17	34	***FE*** 0.003	1
Negative	8	66.7	25	65.8	33	66		
ALS3:								
Positive	7	58.3	35	92.1	42	84	***FE*** 7.74	**0.014 (S)**
Negative	5	41.7	3	7.9	8	16		

S: significant (P<0.05)- FE: Fisher Exact test- χ^2: chi-square test.

DISCUSSION

DA-HAIs are now a challenging clinical issue in health care system and are progressively changing in relation to shift of microbial spectrum and ongoing emergence of extensively-drug resistant strains [23]. *Candida species* have emerged as an important nosocomial pathogen implicated in DA-HAIs. Species identification and antifungal suitability testing is mandatory for proper management [24].

Nosocomial infections are categorized as CLABSI, CAUTI, VAP, and SSI [4]. In this study more than 50% (27 isolates) of candida were isolated from urinary catheters, 38% (19 isolates) from vascular access devices and 8% (4 isolates) from endotracheal tube. CA-UTI was the most common type of DA-HAIs.

In agreement with our finding, *Fateh Allah et al.* [25] studied candida DA-HAIs and revealed that most of candida isolates were isolated from urine and CA-UTI was more frequent than CLABSI and VAP. Also, A meta-analysis study conducted on DA- HAIs point prevalence and stated that CA-UTI was the most prevalent [26].

On the contrary, *Ali et al.* [27] conducted prospective surveillance at Ain Shams burn ICUs and found that CLBSI had the highest incidence.

Identification of *candida* as new emerging critical pathogen up to species level is vital in diagnosis and treatment of fungal infections.

In our study, Identification was reached by germ tube test, chromogenic candida agar (Hi-chrome candida agar) and VITEK 2 compact system. VITEK 2 compact system identified 36% of isolates as *candida albicans* and 64% as *NAC.C. tropicalis (34%)* was the commonest among *NAC* followed by *C. parapsilosis (10%*). This obviously showed shift of infection by *candida* toward *NAC* however, *Candida albicans* continue to be the most frequently isolated species individually. This in accordance with *El-Shabrawy et al.* [28] who reported that NAC was isolated at frequency (64.4%) higher than *C. albicans* (35.6%) and *C. tropicalis* was the predominant isolated species.

Unlike our results, *Fateh Allah et al.* [25] found that most of DA-HAIs are caused by *C.albicans* (66%). *Dahroug et al.* [29] reported that: The NAC frequency in their study was (75.2%) but *C. glabrata* was ranked first before *C. albicans.* The variability in *spp.*, distribution may be attributed to regional distribution, hospital factors and antifungal therapy.

This study found high agreement between Hi-chrome candida agar and VITEK 2 compact system in identification of *C.albicans* and *C.tropicalis* (kappa agreement=0.87, 0.95) with accuracy (94% and 98%), sensitivity (100% and 94%) and specificity (91%and 100%) respectively. There were three *C. Ciferri identified* by VITEK2 compact system but misidentified by chromogenic media as *candida albicans* owing to green color produced by them. but unlikely, the sensitivity for *C.glabrata* was100% but with lower specificity and accuracy and no to slight agreement with VITEK 2 (kappa agreement=0.21). This discrepancy is because other *NAC* (except *candida tropicalis*) give the same beige or creamy white color of *C.glabrata on* chromogenic media.

These results matched *El-Shabrawy et al* [28] who declared perfect agreement (KAPPA= 0.9) between VITEK-2 compact system and chromogenic media (Brilliance Candida Agar) in identification of *candida spp., C.cieferri* was also non distinguishable from *C.albicans* with difficulty in identification of *C. guilliermondii* and *C. parapsilosis* .

Dahroug et al [29] also compared HiCrome chromogenic agar to VITEK 2 compact system and found perfect agreement between them in identification of *C.tropicalis* (100%).On the contrary to our result ,they found good agreement in identification of *C. glabrata* (95.5%). Although all *C. glabrata* and *C. parapsilosis* isolates included in our study produced creamy white color, 4.4% of *C. glabrata* and 33.3% of *C. parapsilosis* isolates in their study produced unexpected pale purple color.

Antifungal Susceptibility testing is essential in controlling antifungal resistance. We tested it by both modified disc diffusion method and VITEK 2 compact system. The resistance rate was higher for fluconazole (28%) and Amphotericin- B (26%) than voriconazole (14%), Caspofungin (18%), Micafungin (16%) and Flucytosine (8%).

The highest resistance rate was for fluconazole as it is the most widely used, cheap and available one. Some strains also like C. glabrata carry intrinsic resistance to fluconazole. Studies showed wide range of resistance rate to fluconazole ranging from (80%) to low resistance rate (8%) [29,30]. Voriconazole is another azole but with lower resistance rate and this was in agreement with two studies posted excellent sensitivity to voriconazole [28,30] and on the opposite side, study in India showed high voriconazole resistance [32].

Amphotericin-B resistance rate was (26%). This finding was in agreement with *Ghonaim et al.*[33] who found resistance rate (27.5%) for Amphotericin-B. This was unlike other studies that reported no amphotericin B-resistant strain in *Candida spp.*, [29,34].

Micafungin and Caspofungin had better antifungal activity with susceptibility rates (84% and82% respectively) and this was in agreement with *Ghonaim et al.* [33] who detected high susceptibility rates to Micafungin and Caspofungin (88.4%, and 81.2% respectively), also *Dahroug et al.* [29] found that Micafungin sensitivity rate for *Non albicans* strains was 100%.

Modified disc diffusion method is simple and cheap method in testing antifungal susceptibility compared to VITEK 2 compact system. We found excellent good agreement between the two methods for Voriconazole, Fluconazole and Caspofunfin and moderate agreement for Amphotericin B. Sensitivity was higher for voriconazole and fluconazole (100%) and (86%) than for Caspofungin and Amphotericin B (66.7%) and (46.2%) respectively. The accuracy for voriconazole, Fluconazole, Caspofunfin and Amphotericin B were (92%, 90%, 90%and 86%respectively).

Dahroug et al. [29] found strong agreement between modified disc diffusion method and the VITEK 2 compact system. The sensitivity of the modified disc diffusion method for voriconazole was (100%), (95%) for fluconazole and (93%) for Caspofungin. These results were in accordance with our results. On the opposite side, the sensitivity of disc diffusion method for Amphotericin-B was (100%) which was higher than our result.

In accordance with our results, *Ghoneim et al.* [33] found that the sensitivity of modified disc diffusion method for fluconazole was (91%) and (84%) for Amphotericin B.

Biofilm formation is important in pathogenesis of DA-HAIs. This study showed that most of tested isolates were biofilm forming (76%); (72% of *C. albicans, 65% of C.Tropicalis* and 80% of *C. parapsilosis).* All isolated species of *C. auris, C. Ciferri, C.Glabrata* and *C .Guilliermondii were* biofilm-former. No difference between C. *albicans* and *Candida non albicans* in their capacity of biofilm formation was found.

In accordance with these results, *Awad et al.* [35] found that high percentage of C. *albicans* and *NAC* isolates were biofilm forming without any significant difference between them as well.

Unlike these findings, the study of Marak and Dhanashree [36] revealed that higher percentage of *NAC* (mainly *C.Tropicalis* and *C. parapsilosis*) were capable of biofilm formation than *C. albicans* isolates. Although all *C.Glabrate* isolates included in our study *were* biofilm-former, their *C.Glabrata* isolates didn`t form biofilm.

On the other hand, *Fateh Allah et al.* [25] stated that the majority (83.3%) of *candida* species isolated from medical devices were negative for biofilm formation. Biofilm was formed mostly by *C.albicans*.

Discrepancy in biofilm formation results is attributable to different methods of biofilm study, type and number of candida species and type of samples.

There was a high prevalence of antifungal resistance to Fluconazole, Voriconazole, Amphotericin- B, Micafungin, Caspofungin and flycytosine in biofilm forming Candida isolates. This is due to biofilm which act as a permeability barrier diminishing penetration of drug through biofilm matrix, decrease need for nutrition and metabolism and increase expression of resistance genes [37]. In accordance with these results studies that showed a positive relationship between biofilm formation and antifungal resistance [30,33,38].

On the other hand, some studies revealed that there was no obvious correlation between biofilm formation and antifungal resistance among *candida spp*., [25,36].

In this study, the most prevalent virulence gene was ALS3(84%) followed by ALS1(68.0%), SAP1(60%), HWP1(54%) and PLB1(46%). The least prevalent gene was INT1(34%).

Dikmen et al. [39] investigated some virulence genes of *Candida spp.,* isolated from patients on inhaled steroid. They also revealed that ALS1(64.0%), SAP1(57%) and PLB1(54%) were detected with frequency rates convergent to our results but, HWP1(72%) and INT1(69%) were detected at higher frequency rates.

Unlike the results of this study, *Mohammadi et al.*[40] revealed that HWP1 had the highest frequency (81.4%) among their isolated Candida species. *Shrief et al.* [30] also found that among candida albicans, HWP1 was the most prevalent virulence gene (77%) and the least prevalent one was PLB1 (52%). The frequency of ALS1(65%) and SAP1(65%) was similar to our results.

Variability in the frequency rates of studied genes is related to number and types of samples, distribution of *candida spp.,* and the used molecular methods.

Positive relation between capacity of *candida* to form biofilm and the presence of the following virulence genes (HWP1, ALS3 and ALS1) was found. Also, other studies found HWP1 and ALS1 were strongly detected in biofilm-forming *candida* [35,11]. A study in Iran also found that ALS3 is a mostly expressed in all biofilm forming *candida* isolates [39]. On the other hand, *Shrief et al.* [29] showed a statistically significant increase in INT1, ALS1, HWP1, SAP1 and PLB1 among biofilm forming *C. albicans* as compared to non-biofilm forming isolates.

CONCLUSION

The present study highlights that both *NAC* and *candida albicans* are involved in DA-HAIs in ICU patients through biofilm formation with shift toward *NAC*. Chromogenic media is simple, rapid and accurate method in *candida spp.,* identification when resources are limited. Fluconazole and Amphotericin -B are less effective antifungal drugs with higher resistance rate. Most of *candida spp.,* isolated from patients with DA-HAIs are biofilm forming with no significant difference between *candida albicans* and *NAC* in their capacity to form biofilm. Drug resistance increases in biofilm forming isolates. The most prevalent virulence gene is ALS3 followed by ALS1 and the least prevalent one is INT1. HWP1, ALS1, ALS3 genes correlate with biofilm formation.

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