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Original article

Detection of *ALS1* and *HWP1* genes involved in biofilm formation in *Candida albicans* isolated from catheter associated candiduria

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ABSTRACT

Background: *Candida* species are opportunistic fungal pathogens that can affect immune compromised patients and may cause life threatening infections. Biofilm formation is an important virulence factor that enables *Candida* species to invade the host tissues. This study aimed to assess the ability of *Candida albicans* to form biofilm in patients with catheter associated candiduria and to determine the presence of some genes involved in biofilm formation at the molecular level. **Methods:** This cross-sectional descriptive study was carried out from June 2019 to June 2020 on 93 catheterized patients admitted to different wards at Suez Canal University Hospitals (SCUHs), Ismailia. *Candida albicans* strains were identified phenotypically by their growth on sabouraud dextrose agar, Gram staining, growth on hypertonic sabouraud broth, chlamyospore formation and germ tube formation test. Biofilm formation was detected by tissue culture plate method. Detection of the genes involved in biofilm formation was done by PCR. **Results:** Twenty eight *Candida albicans* strains were isolated from 36 *Candida* species. Nine isolates (32.1%) were strong biofilm producers, 6 isolates (21.4%) were moderate biofilm producers and 13 isolates (46.4%) were weak or non-biofilm producers. *ALS1* gene was detected in 13 isolates (46.4%), while *HWP1* gene was detected in 16 isolates (57.1 %). **Conclusion:** This study documented the prevalence of candiduria in catheterized patients and found a statistically significant higher prevalence of the tested biofilm forming genes among biofilm forming isolates as compared to the weak or non-biofilm forming ones; but additional studies should be carried out as the management of catheter associated candiduria is still controversial.

Introduction

Candida species belong to the normal microbiota of an individual's mucosal oral cavity, gastrointestinal tract and vagina. These yeasts are commensal in healthy humans but they are considered as opportunistic fungal pathogens that may cause many infections in immune compromised patients ranging from superficial infections of skin and mucous membranes to systemic life-threatening invasive infections [1].

The presence of *Candida* in urine is known as candiduria. Previous studies have demonstrated *Candida albicans* (*C.albicans*) to be the most frequent etiologic agent detected in more than half of candiduria cases, followed by *C. glabrata* as well as *C. tropicalis* [2].

There are many predisposing factors to the occurrence of candiduria, for example, the presence of patient's immunocompromised conditions,

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immunosuppressive therapy, extremes of age, underlying abnormality of the genitourinary tract, female sex, invasive procedure as prior surgery that causes physical alteration of the normal barrier, indwelling urinary catheters, diabetes mellitus, the use of broad-spectrum antibiotics that leads to the reduction of normal bacterial microbiota with the overgrowth of *Candida* spp., as well as the prolonged hospital stay [3].

The Centers for Disease Control and Prevention (CDC) and the National Healthcare Safety Network (NHSN) have eliminated *Candida* spp. from the Catheter-associated urinary tract infections (CAUTIs) surveillance definition and *Candida* spp. were excluded as the causative agents of CAUTIs in 2015. Candiduria in catheterised individuals mostly represent colonisation events and the incidence of colonisation increases with duration of indwelling catheterisation [4].

The pathogenicity of *Candida* spp. is attributed to specific virulence factors that are encoded by *C. albicans* genes and assist the fungus to invade the host tissues such as the ability of *C. albicans* to change from the budding to filamentous yeast form, the expression of adhesion factors, the ability to form biofilm (on host tissue and on medical devices) and the production of tissue-damaging hydrolytic enzymes such as proteases, phospholipases and haemolysin [5].

The adhesion and biofilm formation in *C. albicans* are mediated through the hyphal wall protein 1 (*Hwp1*) present on the surface of the hyphae. The gene encoding *Hwp1* is part of a core of eight genes induced during filamentation of *C. albicans* [6]. The adhesion of *C. albicans* is also mediated by agglutinin-like sequence 1 gene (*ALS1*). There are two genes with similar sequences and functions, *ALS1* and *ALS3*. These genes mediate attachment to endothelial cells, hyphal formation as well as adherence [7].

Therefore, the aim of this study was to assess the ability of *C. albicans* to form biofilm in patients with catheter associated candiduria and admitted to Suez Canal University Hospitals (SCUHs) as well as to determine the presence of some virulence genes that control biofilm formation at the molecular level.

Materials and Methods

This cross-sectional descriptive study was conducted over a one-year period from June 2019 to June 2020. It was carried out on 93 patients with

indwelling urinary catheters of both sexes and different age groups; and admitted to different wards at SCUHs, Ismailia. Ethical approval was obtained from the Ethical Committee of Faculty of Medicine, Suez Canal University. Informed consent was taken from each patient to participate in this study.

Samples collection and processing

Urine samples were taken from each patient 48 hours after urinary catheter insertion. The catheter was clamped off to allow collection of freshly voided urine. 10 ml of urine was aspirated from each patient by a sterile syringe through the sampling port of the indwelling catheter under complete aseptic precautions. The collected samples were transported rapidly (within two hours) in sterile screw capped tubes to be processed in the microbiology laboratory.

Urine specimens were inoculated on blood agar, MacConkey's agar and Sabouraud dextrose agar (SDA) plates (Oxoid, UK) supplemented with chloramphenicol, and incubated aerobically at 37°C for 24-48 hours.

Phenotypic identification of *Candida albicans*

Identification of *Candida* spp. was based on the macroscopic appearance of the colonies on SDA as white to creamy, round, soft and smooth colonies with characteristic yeast odor. Microscopic examination of Gram-stained samples showed Gram positive large oval budding yeast cells with or without pseudohyphae. *C. albicans* was further identified phenotypically as follows:

- 1-The ability to grow on hypertonic Sabouraud broth. The presence of visible colony growth in the test tube indicated *C. albicans* isolates [8].
- 2-Chlamydospore formation test on corn meal Tween-80 agar. *C. albicans* isolates had feathery or spidery outgrowths with spores [9].
- 3-Positive germ tube formation test. The germ tube appeared as short, slender hyphal (filamentous) extension projecting laterally from a yeast cell surface with no constriction at the point of origin of the germ tube and is classically described as hand mirror appearance [10].

All *C. albicans* isolates were then preserved as frozen stocks in 15% glycerol (Sigma-Aldrich) at –20°C for further processing.

Detection of biofilm formation by tissue culture plate (TCP) method

All *C. albicans* isolates were screened for their ability to form biofilm by the TCP method as follows [11]:

- a) *Candida albicans* isolates from fresh SDA plates were inoculated in Sabouraud dextrose broth (Oxoid) and incubated for 24 hours at 37°C and then diluted (1: 100) with fresh medium.
- b) Individual wells of sterile, polystyrene, flat-bottom TCP were filled with 200 µl of the diluted cultures then the plate was covered and incubated for 48 hours at 37°C.
- c) After that, the content of wells was gently removed by tapping the plate and the wells were washed four times with 200 µl of phosphate buffer saline (pH 7.2) to remove free-floating planktonic bacteria.
- d) Then 110 µl of 0.4% solution of crystal violet was added to each well plate. The plate was covered and incubated at room temperature for 45 minutes, rinse thoroughly and repeatedly with water. Adherent cells, which usually formed biofilm on all side wells, were uniformly stained with crystal violet.
- e) Crystal violet-stained biofilm was solubilized in 200µl of 95 % ethanol (to extract the violet color), of which 100µl was transferred to a new plate, which had been read.
- f) The optical density (OD) at 595nm was recorded and the results were interpreted as follows:

Biofilm formation was categorized into three grades (**Table 1**) according to biofilm density based on the established OD cut-off values (ODc), which were derived from the mean values of negative controls

(mean ODnc) summed with three standard deviations of the negative controls ($3 \times \text{SDnc}$): $\text{ODc} = \text{mean ODnc} + (3 \times \text{SDnc})$ [12].

Detection of virulence genes involved in biofilm formation by polymerase chain reaction (PCR)

The genome of *C. albicans* isolates was extracted using DNA extraction kit (Sigma, USA) according to the manufacturer's instructions. The quantity and quality of the DNA was analyzed using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, USA).

The biofilm formation genes (*ALS1* and *HWPI*) were amplified by PCR using a specific set of primers listed in **table (2)**.

The reaction mixture consisted of 5 µL of 10x reaction buffer (Fermentas, USA), 0.5 µL of each deoxynucleoside triphosphate at 10 mM (Fermentas), 2.5 µL of MgCl₂ at 25 mM, 1 U of Taq polymerase (Fermentas), 1.5 µL of primers, and approximately 4 µL of template DNA at 10 ng in a final volume of 25 µL [13].

DNA was amplified in a thermo cycler using the following conditions: initial denaturation (95 °C for 5 minutes), followed by 30 cycles consisting of denaturation (94 °C for 1 minute), annealing (60 °C for 1 minute) and extension (72 ° for 1 minute), with a single final extension step of 10 minutes at 72 °C [14].

The amplified PCR products were separated in 2% agarose gel, stained with ethidium bromide (0.5g/ml) in TBE buffer, for 50–110 minutes at 120 volts and detected by ultraviolet transillumination (wavelength 312 nm). Amplicon size (bp) of the tested genes was determined by comparison to the 100 bp molecular size standard DNA ladder (Cleaver scientific, UK).

The data was analyzed by SSPS version 22 for windows (SPSS Inc., Chicago, IL, USA). The comparison was performed by Chi-Square test and the statistical significance level was considered at p value ≤ 0.05 .

Table 1. Biofilm formation categories according to biofilm density [12].

Biofilm density categories	How to be calculated?
Weak or non-biofilm forming	$OD \leq 2 \times ODC$
Moderate biofilm forming	$2 \times ODC < OD \leq 4 \times ODC$
Strong biofilm forming	$OD > 4 \times ODC$

Table 2. Primers used for detection of biofilm formation genes in *C. albicans*.

Primer	Sequences (5' to 3')	Amplicon (bp)	References
<i>ALSI-F</i>	5'-GAC TAG TGA ACC AAC AAA TAC CAG A -3'	318	[13]
<i>ALSI-R</i>	5'-CCA GAA GAA ACA GCA GGT GA -3'		
<i>HWPI-F</i>	5'-TCAGTTCCAATCATGCAACCA-3'	99	[14]
<i>HWPI-R</i>	5'-AGCACCGAAAGTCAATCTCATGT-3'		

Results

Thirty six clinical isolates of *Candida* spp. (38.7%) were collected from urine samples of 93 hospitalized patients with indwelling urinary catheters and admitted to SCUHs, Ismailia. These isolates were collected from 15 male patients (41.7 %) and 21 female patients (58.3 %) with a mean age of 58.7 ± 11.93 years. Most of these isolates (61.1%) were from patients admitted in the intensive care unit (ICU). These isolates were further identified as 28 (77.8%) *C. albicans* and 8 (22.2%) non- albicans *Candida* (NAC).

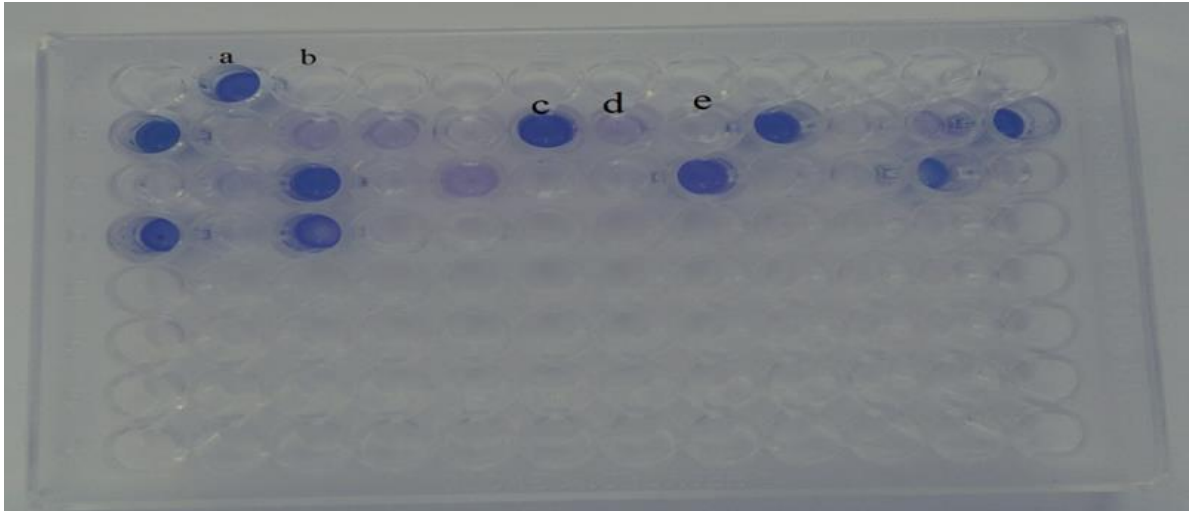
Biofilm formation among *C. albicans* isolates was detected by the TCP method (**Figure 1**). It was found that nine out of 28 isolates (32.1%) were strong biofilm producers, six isolates (21.4%) were moderate biofilm producers and 13 isolates (46.4%) were weak or non-biofilm producers as illustrated in **figure (2)**.

Screening for some virulence genes involved in biofilm formation among *C. albicans*

isolates was carried out using PCR as shown in **figures (3&4)**. It was found that *ALSI* gene was detected in 13 isolates (46.4%), while *HWPI* gene was detected in 16 isolates (57.1 %).

The relation between biofilm formation detected by the TCP method and the presence of biofilm formation genes among *C. albicans* isolates was determined; there was a statistically significant ($p \leq 0.05$) higher prevalence of the tested genes among biofilm forming isolates as compared to the weak or non-biofilm forming ones. As shown in **table (3)**; eight out of 15 biofilm forming isolates (53.3%) harbored *ALSI* gene (7 were strong biofilm forming isolates and one was moderate biofilm forming isolate) while 13 out of 15 biofilm forming isolates (86.7%) harbored *HWPI* gene (9 were strong biofilm forming isolates and 4 were moderate biofilm forming isolates). Combination between the two genes was detected in 7 strong biofilm forming isolates.

Figure 1. Detection of biofilm formation by *C. albicans* by the TCP method.



a: positive control, b: negative control, c: strong biofilm forming isolate, d: moderate biofilm forming isolate, e: weak or non-biofilm forming isolate.

Figure 2. The percentage of biofilm formation among the isolated strains.

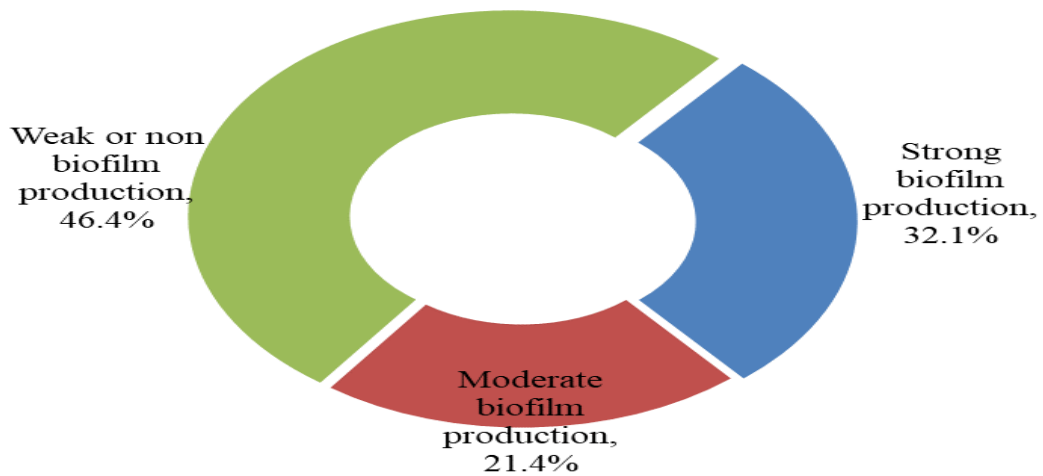


Figure 3. Agarose gel electrophoresis of *ALS1* gene amplicons (318 bp).

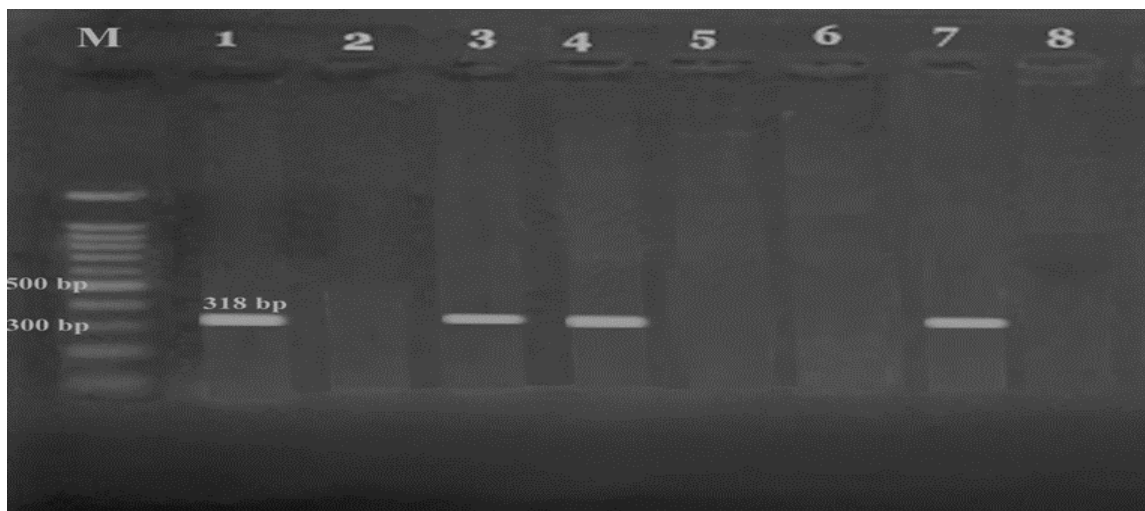
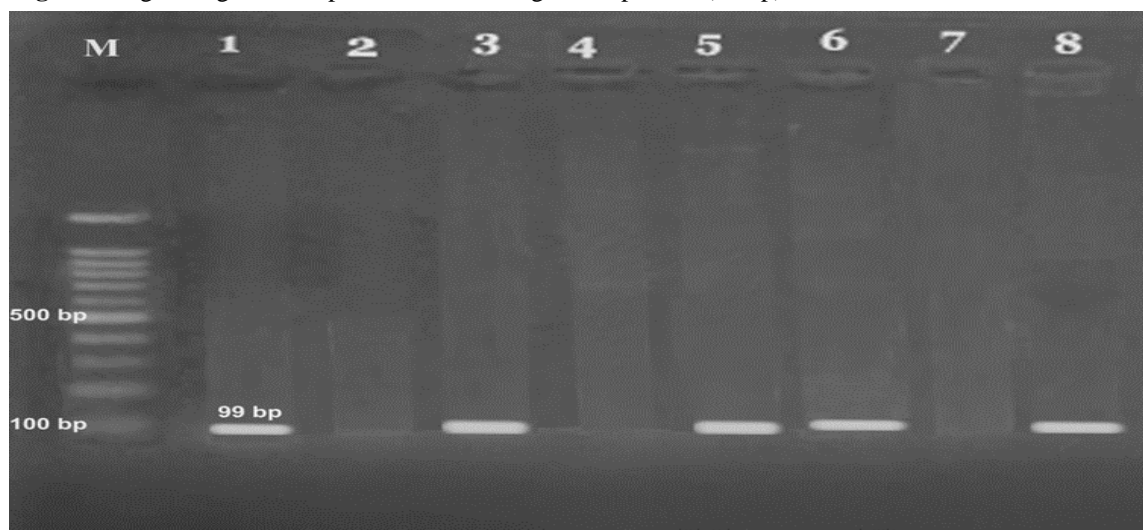


Figure 4. Agarose gel electrophoresis of *HWPI* gene amplicons (99 bp).**Table 3.** The distribution of *ALS1* and *HWPI* genes among biofilm forming and non biofilm forming *C. albicans* isolates.

Genes	Strong biofilm forming isolates	Moderate biofilm forming isolates	Weak or non biofilm forming isolates	<i>p. value</i>
<i>ALS1</i> : positive	7 (77.8%)	1 (16.7%)	5 (38.4%)	0.049*
<i>ALS1</i> : negative	2 (22.2%)	5 (83.3%)	8 (61.5%)	
<i>HWPI</i> : positive	9 (100%)	4 (66.7%)	3 (23%)	0.001*
<i>HWPI</i> : negative	0 (0%)	2 (33.3%)	10 (77%)	
Total No. (%)	9 (32.1%)	6 (21.4%)	13 (46.4%)	

*statistically significant result

Discussion

Candida spp. are categorized as the fourth common cause of UTIs, especially in hospitalized and ICU patients [15]. Indwelling urinary catheter is one of the most important predisposing factor that contributes to the overgrowth of *Candida* spp. on which *Candida* can colonize and form biofilm [16]. In this study, thirty six clinical isolates of *Candida* spp. (38.7%) were collected from urine samples of 93 hospitalized patients with indwelling urinary catheters and admitted to SCUHs. Further identification revealed that among the isolated *Candida* spp., 77.8% were *C. albicans* while only 22.2% were NAC.

A previous study conducted in Menoufia, Egypt revealed that 20% of catheterized patients had candiduria and regarding *Candida* species, the CHROMagar differentiated 37.5 % of them as *C.*

albicans and 62.5 % as NAC [17]. Although NAC spp. are emerging nowadays as potential pathogens responsible for candiduria [18], *C. albicans* is still being considered as the predominant species infecting the urinary tract as reported by **Edward and colleagues** in Alexandria, Egypt who found that NAC spp. were less prevalent (35.7%) than *C. albicans* (64.3%) among the *Candida* spp. isolated from patients with UTIs [19]. Similarly, *C. albicans* was the most prominent species (64.7 %) recovered from urine cultures from candiduric patients in a tertiary care hospital in Kuwait [20]. However, in contrary, **Chandak et al.** in their study found that NAC spp. were predominantly higher 74 %, while *C. albicans* was 26% [21]. Also, **Sahai & Kumar** in India reported that NAC spp. contributed to 83.3% of the isolates and only 16.7% of isolates were *C. albicans* [22].

This study revealed that most of the patients with positive candiduria were with a mean

age of 58.7 ± 11.93 years with predominance of female gender (58.3 %) over male gender (41.7 %). Older age is a classical risk factor for candiduria due to the attenuated host defense mechanisms in this age group. These findings coincide with reports of other studies as **Ismail and colleagues** in Cairo, Egypt confirmed positive candiduria in patients whose ages were between 50-70 years with predominance of female gender 64 % [16]. Also, **Jiménez-Guerra et al** and **Melges et al** found that the average age of patients with candiduria was 65 and 63 years, respectively [23,24]. Although females are more susceptible to higher risk of developing candiduria due to frequent colonization of their vulvo-vestibular area with *Candida* spp. [2], other observers found that it was more common in males [19,25]. This could be attributed to the involvement of other risk factors not approached in the current study.

Regarding other associated predisposing risk factors, antibiotic therapy is one of the most important risk factor exposing the patients to candiduria. This correlates with our results as most of the *Candida* spp. isolates (61.1%) were from patients admitted in ICU with prolonged antibiotic intake. Our results are supported by **Ismail et al.**, **Chandak et al.** and **Melges et al.** who reported that 78 %, 89.2% and 100 %, respectively of all positive candiduria patients were on antibiotic treatment [16, 21, 24].

Biofilm formation among *C. albicans* isolates was detected phenotypically in this study by the TCP method. It was found that 32.1% of the isolates were strong biofilm producers, 21.4% were moderate biofilm producers and 46.4% were weak or non-biofilm producers.

There are various studies that have explored biofilm formation in *C. albicans* isolates phenotypically as **Ismail et al.** in Egypt found that biofilm production was 84.6% (11/13) among *C. albicans* isolates and of them; 7.6% was strong biofilm producers, 7.6% was moderate biofilm producers and 69.2% were weak biofilm producers [16]. Moreover, **Shrief et al.** in Mansoura, Egypt revealed that the biofilm capacity was identified by the microplate method in 58% of *C. albicans* isolated from ICU patients with nosocomial infections. The OD was intense in 20 isolates, moderate in 21 isolates and mild in 17 isolates [26]. Similarly, **İnci et al.** in Turkey reported that the slime factor was found to be positive in 34.5% of *C. albicans* isolates when using the microplate test

[13]. **Kaminska et al.** in Poland demonstrated that 43% of the *C. albicans* isolates from oral mucosa were capable of forming biofilm using the MTT assay [14].

It is known that adhesion is an initial step of biofilm formation. Genes belonging to the ALS gene family and *HWP1* gene encode cell-surface related glycosylphosphatidyl inositol (GPI) that acts as an adhesin that binds to glycoprotein, which then mediates the adherence of *C. albicans* strains to mucosal surfaces [27,28]. It has been reported that these genes play important roles in *C. albicans* biofilm formation, both in vivo and in vitro, and therefore help in colonization and disease [7]. In this study, screening for the *ALS1* and *HWP1* genes among *C. albicans* isolates was carried out using conventional PCR. *ALS1* gene was detected in 46.4% while *HWP1* gene was detected in 57.1 % of the *C. albicans* isolates. In addition, there was a statistically significant higher prevalence of the tested genes among biofilm forming isolates as compared to the weak or non-biofilm forming ones. 53.3 % of the biofilm forming isolates harbored *ALS1* gene while 86.7 % of the biofilm forming isolates harbored *HWP1* gene. The existence of both genes together occurred in 7 strong biofilm forming isolates.

In Egypt, **Shrief et al.** demonstrated a higher prevalence of *HWP1* (77 %) and *ALS1* (65 %) genes in *C. albicans* isolates from nosocomial infections. Additionally, there was statistically significant increase in the prevalence of these virulence genes (96.6% for both) among biofilm forming as compared to non-biofilm forming *C. albicans* isolates (*ALS1* 21.4% and *HWP1* 50%) [26]. A previous study in Iraq revealed higher frequency for *ALS1* (100%) and *HWP1* (90.9%) among *C. albicans* isolates [29]. In addition, **İnci et al.** in Turkey reported that *ALS1* was present in 53.9%, while *HWP1* was present in only 5.3% of *C. albicans* isolates. Moreover, among the strains in which phenotypic slime formation was detected, the *ALS1* and/or *HWP1* genes were found to be positive in 76.6% and negative in 23.3% [13]. The difference in the prevalence rates of virulence genes from this study may be attributed to several factors including the number of isolates studied and the difference of the isolation sites of *C. albicans*.

This study documented the prevalence of candiduria in catheterized patients and highlights a high prevalence of *ALS1* and *HWP1* genes among *C. albicans* isolated from catheter associated

candiduria; but additional studies should be carried out as the management of catheter associated candiduria is still controversial.

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Contributors

Both authors have contributed to performing the practical part, data analysis and writing of this manuscript.

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