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ORIGINAL ARTICLE

Hyphal wall protein 1 gene and Biofilm formation among *Candida albicans* isolates from Ain Shams University Hospitals

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ABSTRACT

Background: *Candida albicans (C. albicans) is considered a common fungal pathogen, many genes like HWP1 regulates formation of biofilm which is considered as an important mediator of pathogenicity of this organism.* ***Objective:*** *to assess the ability of C. albicans to form biofilm and to determine the association of hyphal wall protein 1 (HWP1) gene and biofilm forming ability of isolates.* ***Methodology:*** *This study was conducted on 30 C. albicans isolates. Detection of biofilm formation was performed by using tissue culture plate method then polymerase chain reaction was done to detect HWP1 gene.* ***Results:*** *Biofilm forming isolates were 14 (46.7%) among tested isolates while HWP1 gene was found in 13 isolates (43.3%). Regarding antifungal susceptibility all isolates were susceptible to Fluconazole, voriconazole, micafungin, and flucytosine, while 93.3% of isolates were found susceptible to amphotericin-B and caspofungin.* ***Conclusion:*** *The rate of biofilm formation among C. albicans is high and is highly associated to presence of HWP1 gene.*

INTRODUCTION

C. albicans is considered one of the common fungal flora in humans. Although its generally a harmless commensal in immunocompetent individuals, several factors contribute to its pathogenicity, causing complications ranging from localized superficial infections to systemic life-threatening candidiasis[1].

There are many virulence factors that play a role in mediating the pathogenicity of *C. albicans* facilitating its invasion into the host tissues. Some of these factors are; its ability to produce hyphae, expressing adhesion factors, producing hydrolytic enzymes that cause tissue damage such as proteases, phospholipases and haemolysin . Formation of biofilm is counted as one of the most crucial virulence factors that allows its colonization especially on tissues of the host and on medical devices[2].

Biofilm offers a secure environment which allows its survival in excessively high concentrations of antimicrobials**.** Biofilm is a community of organisms enclosed in a self-produced extracellular matrix formed of filaments and yeasts. This community acts as an endless reservoir in spreading infection. Moreover, it becomes resistant to lots of antifungal agents, when compared to free cells[3]. Biofilm formation starts by the adhesion of yeast cells to a substrate, that allows the attachment of yeast-free cells to each other [4].

The hyphal wall protein 1 (*HWP1)* protein has shown a significant association between the pathogenicity and the virulence ability of *C. albicans* as many studies have proved that the *HWP1*-producing gene is highly expressed in the earlier stages of biofilm formation [5].

The ability of *C. albicans* to form biofilm is exhibited through *(HWP1)* that is present on the hyphae[6]. *HWP1*, a glycosyl-phosphatidyl-inositol linked manno-protein that is resemble agglutinin-like sequence *(ALS)* proteins, is the best characterized hyphal adhesin. It is the substrate of transglutaminase activity derived from a host, and so it allows covalent attachment of *C. albicans* to host cells [7].

Genetic investigations and research into *C. albicans* genome help in clarifying the mechanism of biofilm formation, so in current study we will evaluate the *C. albicans ability* to form biofilm and to determine the association of *HWP1* gene and biofilm forming ability of isolates

METHODOLOGY

This research work was conducted on 30 *C. albicans* isolates identified by VITEK2 (bioMérieux, Inc., Hazelwood, MO) that is a fully automated system. All isolates included in the study were retrieved from different samples from Ain Shams University Hospital, Main Microbiology laboratory, Cairo, Egypt. The study was approved by The Research Ethics Committee, Ain Shams University, Faculty of Medicine (FWA 000017585), and from MASRI (FMASU R153/2022).

Detection of Antifungal susceptibility pattern in *C. albicans* isolates:

All isolates were tested for antifungal susceptibility by using VITEK 2 system for the following drugs (Oxoid, England): Fluconazole, Voriconazole,

Caspofungin, Micafungin, Amphotericin-B and Flucytosine.The minimum inhibition concentration (MIC) results obtained using the VITEK 2 system were interpreted.

Biofilm detection by tissue culture plate (TCP) method:

All *C. albicans* isolates were evaluated for their ability to form biofilm by the TCP method by the following steps[8].

- All isolates were cultured on Saboraud Dextrose agar plates then fresh isolates were sub-cultured on Sabouraud dextrose broth (Oxoid) and were incubated for 24 hours at 37°C and then diluted (1:100) with fresh medium.
- 200 µl of the diluted culture were added to the Individual TCP well then it was covered and incubated for 48 hours at 37°C.
- Plate was tapped to remove the content of the well, wells were washed four times with 200 µl of phosphate buffer saline (pH 7.2) to remove free planktonic fungi.
- 110 µl of crystal violet (0.4%) were added to each well, then the plate was covered and incubated for 45 minutes at room temperature, then rinsed with water. Biofilm forming cells were evenly stained with crystal violet.
- biofilm cells stained with Crystal violet were emulsified in 200µl of 95 % ethanol to remove the dye.
- The optical density (OD) at 595nm was detected and the results were interpreted as follows: Formation of biofilm was classified into three grades (shown in 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 × SDnc): ODc = mean ODnc + (3 × SDnc) [9].

Table 1: Biofilm formation categories according to biofilm optical density[9]

Biofilm density categories	How to be calculated?
Non-biofilm forming	OD ≤ ODC
Weak biofilm forming	ODC < OD ≤ 2 x ODC
Moderate biofilm forming	2 x ODC < OD ≤ 4 x ODC
Strong biofilm forming	OD > 4 x ODC

Detection of *HWP1* by PCR [5].

Then Detection of *HWP1* gene for all isolates were performed by using PCR. The DNA of *C. albicans* isolates was extracted using QIAGEN DNA extraction Kit® (QIAGEN, USA), then amplification of *HWP1* gene was done using primers shown in table (2) by using Go Taq® Green Master Mix (Promega, USA).

Table 2: Primer sequences of *HWP1*[5].

Gene	Primer Sequence	Amplified Product Size
HWP1 –F	(F) ATGACTCCAGCTGGTTC	503bp
HWP1 –R	(R) TAGATCAAGAATGCAGC	

By using a thermal cycler (Applied Biosystems, USA), the amplification conditions were adjusted as shown in table (3). Then the amplified products were visualized by using (2%) agarose gel electrophoresis. DNA in the gel was visualized using UV transilluminator and the product size of the genes were shown in figure (1).

Table 3: PCR conditions

Phases	Number of cycles	Temperature	Time
Initial hold	1	95 ˚C	2 minutes
Amplification	35		
Denaturation		95 ˚C	45 seconds
Annealing		58 ˚C	45 seconds
Extension		72 ˚C	1 minutes
Final extension	1	72 ˚C	10 minutes

Fig 1: Agarose gel electrophoresis of *HWP1* gene amplicons (503 bp).

Statistical analyses and Statistical Package:

All collected data entered in Microsoft Office Excel Worksheet and statistical analysis was done. Data were analysed using the Statistical Package for Social Sciences (SPSS version 25).

RESULTS

Thirty *C.albicans* isolates were retrieved from different clinical samples. 20 (66.6%) isolates were

from urine samples, 8 (26.7%) from blood samples and 2 (6.7%) from pus samples as shown in figure (2). Ain Shams University Hospital, Main Microbiology laboratory, Cairo, Egypt. The isolates were retrieved from 11 male patients (36.6%) and 19 female patients (63.3%) with a mean age of 43 years.

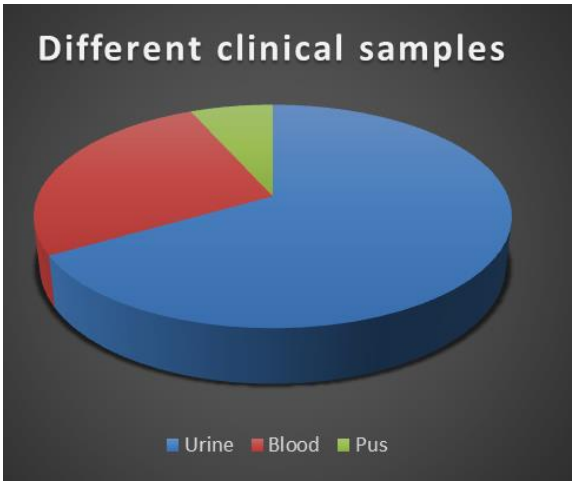

Fig. 2: Distribution of different clinical samples

On performing susceptibility testing to antifungal agents, all isolates were susceptible to Fluconazole, voriconazole, micafungin and flucytosine while susceptibility to amphotericin-B and caspofungin were 93.3% as shown in table (4).

Table 4: Frequency of antifungal susceptibility testing:

		N(30)	%
Fluconazole	Resistance	0	0.0%
	Sensitive	30	100.0%
Voriconazole	Resistance	0	0.0%
	Sensitive	30	100.0%
Caspofungin	Resistance	2	6.7%
	Sensitive	28	93.3%
Micafungin	Resistance	0	0.0%
	Sensitive	30	100.0%
Amphotericin B	Resistance	2	6.7%
	Sensitive	28	93.3%
Flucytosine	Resistance	0	0.0%
	Sensitive	30	100.0%

The biofilm forming ability of *C. albicans* isolates was detected by using TCP method fig (3). The biofilm formation was found in 14 (46.7%) isolates, 8 (26.6%) of these isolates were strong biofilm former and 6 (20 %) isolates were moderate biofilm former while 16 (53.3 %) isolates were non-biofilm former as shown in table (5).

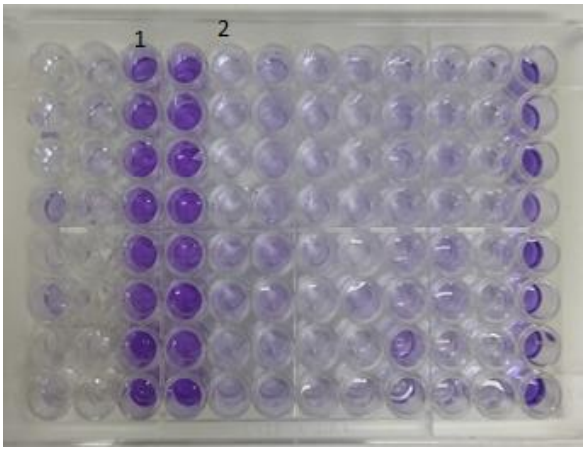

Fig. 3: Detection of biofilm formating *C. albicans* by the TCP method. 1- moderate biofilm former. 2- nonbiofilm former

Table 5: Frequency of biofilm and *HWP1* gene:

		N (30)	%
Biofilm	Non	16	53.3%
	Moderate	6	20%
	Strong	8	26.6%
HWP1	Negative	17	56.7%
	Positive	13	43.3%

The *HWP1* gene was detected among all isolates by using PCR as shown in fig (1). It was found that 13 isolates (43.3%) contained *HWP1* gene.

The association between biofilm forming ability and presence of *HWP1* gene in *C. albicans* isolates was highly significant (P<0.001). The isolates harboring *HWP1* gene distributed as follow 8 isolates were strong biofilm former, 4 isolates were moderate biofilm former and only 1 isolate was non-biofilm former as shown in table (6).

Table 6: The association between biofilm and *HWP1* gene:

		Biofilm Formation						Chi-squared test	P value
		Non		Moderate		Strong			
		N	%	N	%	N	%		
HWP1	Negative	15	93.7%	2	33.3%	0	0.0%	20.752	<0.001*
	Positive	1	6.3%	4	66.7%	8	100.0%		

As shown in table (7), no association was found between biofilm formation among *C. albicans* and susceptibility pattern of tested antifungal as almost all isolates were susceptible to antifungal agents even biofilm forming isolates .

Table 7: association between antifungal susceptibility and biofilm formation

		biofilm						Chi-squared test	P value
		non or weak		Moderate		Strong			
		N	%	N	%	N	%		
Fluconazole	Resistance	0	0.0%	0	0.0%	0	0.0%	0	0
	Sensitive	14	46.7%	6	20.0%	10	33.3%		
voniconazole	Resistance	0	0.0%	0	0.0%	0	0.0%		
	Sensitive	14	46.7%	6	20.0%	10	33.3%	0	0
canpofungin	Resistance	0	0.0%	1	50.0%	1	50.0%	2.51	0.271
	Sensitive	14	50.0%	5	17.9%	9	32.1%		
micafungin	Resistance	0	0.0%	0	0.0%	0	0.0%	0	0
	Sensitive	14	46.7%	6	20.0%	10	33.3%		
amphotenicin B	Resistance	0	0.0%	1	50.0%	1	50.0%	2.114	0.343
	Sensitive	14	50.0%	5	17.9%	9	32.1%		
Flucytonine	Resistance	0	0.0%	0	0.0%	0	0.0%	0	0
	Sensitive	14	46.7%	6	20.0%	10	33.3%		

DISCUSSION

Our study was conducted on 30 isolates of *C. albicans* that retrieved from different clinical samples. Antifungal susceptibility testing was done for all isolates, Isolates showed 100% sensitivity to Fluconazole, voriconazole, micafungin and flucytosine, While sensitivity to Caspofungin and amphotericin-B was 93.3%.

Similarly, Shrief et al.[10], found that the pattern of susceptibility of *C. albicans* isolates to different antifungals was as follow, 92% for fluconazole, caspofungin and itraconazole and was 91% for amphotericin B by microdilution method.

Moreover, Mohammadi et al.[5] tested the susceptibility pattern of 47 isolates of *C. albicans* and stated that 100% of tested isolates were found sensitive to voriconazole and amphotericin B, but they recorded different results for Fluconazole with 76.6% sensitivity.

Ghaddar et al[11], found that the susceptibility pattern of *C. albicans* isolates included in their study to amphotericin B and voriconazole was 97.5%, and to Fluconazole was 90%. Marak and Dhanashree[12], tested 41 *C. albicans* and found that 100%, 83% and 78% of isolates showed susceptibility to amphotericin B, fluconazole and voriconazole respectively.

In this study, 14 (46.7%) out of 30 isolates were biofilm former, 8 (26.6%) of these isolates were strong biofilm formers and 6 isolates (20%) were moderate biofilm former while 16 isolates (53.3 %) were non-biofilm formers.

In Egypt, Hamady and Marei[13], assessed 28 isolates of *C. albicans* for their ability to form biofilm. They obtained analogous results. Nine isolates (32.1%) showed strong biofilm, six isolates (21.4%) showed moderate biofilm, while 13 isolates (46.4%) were found to be weak or non-biofilm producers. Similarity in these results may be attributed to same geographical area and similar method of detection of biofilm formation i.e. by TCP method.

In accordance to the results of this study, a study done in Egypt by Shrief et al.[10] who worked on isolates retrieved from ICU patients using microplate method (MTT). About 58% of *C. albicans* isolates were biofilm formers. Strong, moderate and mild biofilms were detected in 20%, 21% and 17% of isolates respectively.

In a study performed in Iran, Mohammadi et al.[5] concluded that the biofilm production was detected in 54.3% of Candida spp which obtained from vaginal specimen. Biofilm grades of strong, moderate and weak levels was reported in 21.4%, 11.4% and 20% respectively. Another study done by Mohammadi et al.[14] on *C. albicans* isolates which isolated from onychomycosis patients, They stated that only 4.3% of isolates showed strong biofilm forming ability, while 18.6% and 77% showed moderate and weak or no biofilm forming ability respectively. In Poland, *Kaminska et al.,*[15] stated that 43% of the *C. albicans* isolates retrieved from oral cavity were reported as biofilm former by using MTT method.

In the current study almost all biofilm forming isolates were found sensitive to all tested antifungal drugs this may be attributed to the difference that exists between in vitro environment and the in vivo one

It has been reported that *HWP1* gene is crucial in the ability of *C. albicans to form biofilm*. Nobile et al.[16]. In the current study, screening of that gene was done among 30 isolates of *C. albicans* and was found in 13 (43.3%) isolates. 12 (92.3%) out of 13 isolates were biofilm formers. These isolates resemble 8 strong biofilm formers, 4 moderate biofilm formers and only 1 non-biofilm formers. A high statistically significant association was found between the biofilm formation and the presence of *HWP1* gene. It is noteworthy that many factors may affect the ability of microorganism to form biofilm, and this explains that some of biofilm forming isolates lacks the presence of *HWP1* gene.

Similarly, Hamady and Marei[13] detected *HWP1* gene in 57.1% of *C. albicans* isolates. They concluded that there is a significant association between biofilm formation and presence of tested gene as 86.7% of isolates harboring gene were biofilm former.

A higher prevalence was reported by Shrief et al.[10] who reported the presence of *HWP1* gene in 77% of isolates. They also found that 96.6% of isolates harboring the gene were biofilm formers with statistically significant association between the presence of *HWP1* gene and the ability of biofilm formation.

Mohammadi et al.[5] stated that the frequency of *HWP1* gene among 47 *C. albicans* isolates was 89.4% and reported that the gene was found in 89.2% of biofilm-producing Candida species.

On the other hand, Kaminska et al.[15] , reported that the frequency of *HWP1* gene in biofilm forming isolates obtained from oral cavity was 84% (58/ 69 isolates), while the frequency among non-biofilm forming isolates obtained from oral cavity was 63.7% (58/ 91 isolates), with no statistically significant association between the biofilm formation and existence of *HWP1* gene . Moreover İnci et al.[17], concluded that the *HWP1* gene was positive only in 5.3% of *C. albicans* strains. 11.3% of biofilm forming strains that was tested by using the microtiter plate method, contained the gene.

CONCLUSION

This study highlights a high rate of biofilm formation among *C. albicans* isolates and emphasizes the role of presence of *HWP1* gene to help in biofilm formation.

This manuscript has not been previously published and is not under consideration in the same or substantially similar form in any other reviewed media. I have contributed sufficiently to the project to be included as author. To the best of my knowledge, no conflict of interest, financial or others exist. All authors have participated in the concept and design, analysis, and interpretation of data, drafting and revising of the manuscript, and that they have approved the manuscript as submitted.

Ethical considerations:

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest:

All authors declare no conflict of interest in this work.

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