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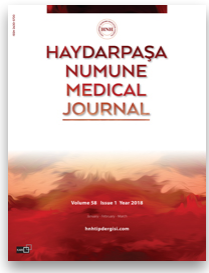

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ORIGINAL ARTICLE

Investigation of Virulence Factors of *Candida albicans* Species Isolated from Clinical Specimens Phenotypically and Genotypically

Deniz Turan[1], Ayşe Barış[2], Nuri Kiraz[3]

[1]Department of Medical Microbiology, University of Health Sciences Türkiye, Haydarpaşa Numune Training and Research Hospital, Istanbul, Türkiye
[2]Department of Medical Microbiology, University of Health Sciences Türkiye, Sisli Hamidiye Etfal Training and Research Hospital, Istanbul, Türkiye
[3]Department of Medical Microbiology, Istanbul University-Cerrahpaşa Cerrahpaşa Faculty of Medicine, Istanbul, Türkiye

Abstract

Introduction: Candida species are commensals in the normal flora of healthy humans but can become opportunistic pathogens, causing significant morbidity and mortality when predisposing factors are present. Various local or systemic factors, particularly their virulence factors, disrupt the normal homeostasis of Candida, leading to the transition from normal flora to pathogenic and opportunistic infections.

Methods: In this study, the acid proteinase, phospholipase, and biofilm formation properties of 100 *Candida albicans* strains isolated from various clinical specimens were investigated phenotypically. Additionally, the presence of agglutinin-like sequences (ALS1) and hyphal wall protein (HWP1) genes, which are thought to be effective in adhesion during biofilm formation, was investigated genotypically.

Results: Proteolytic activity was detected in 86% of the strains, phospholipase activity in 73%, and biofilm formation by the modified tube adherence method in 9%. Genotypically, the presence of ALS1 and HWP1 genes was detected in 29% and 91% of the strains, respectively.

Discussion and Conclusion: The lack of a significant relationship between the presence of ALS1 and HWP1 genes and biofilm formation suggests that different genes may also be effective in this process.

Keywords: Candida albicans; ALS1; biofilm formation; HWP1; hydrolytic enzymes; virulence factors.

Candida species, which are common in nature, are fungal pathogens that are commensal in the normal microbiota of humans and animals. In recent years, they have become increasingly important in parallel with developments in the field of diagnosis and treatment[1]. Today, *Candida* species have become opportunistic pathogens that can cause significant morbidity and mortality due to facilitating factors such as the increase in the number of surgical interventions like tissue and organ transplantation, long-term and excessive use of antibiotics,

Correspondence: Deniz Turan, M.D. Department of Medical Microbiology, University of Health Sciences Türkiye, Haydarpaşa Numune Training and Research Hospital, Istanbul, Türkiye
Phone: +90 535 233 36 86 **E-mail:** dennizturan@hotmail.com
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use of immunosuppressive treatments due to malignant diseases for various conditions, central venous catheter applications, and total parenteral nutrition[2].

Despite the increase in the number of antifungal drug options in recent years, the limited spectrum of action or severe toxic side effects of these drugs cause difficulties in the treatment of invasive fungal infections, and therefore, the expected reduction in high mortality rates cannot be achieved with the use of these drugs[3].

Recent studies conducted to elucidate the pathogenesis of *Candida* infections and to develop new drugs against *Candida* species emphasize the importance of virulence factors pertaining to *Candida* species and the host defense systems[4]. In other words, many virulence factors likely play a role in the pathogenesis of *Candida* infections. These virulence factors include hyphae formation, dimorphism, adhesins, some hydrolytic enzymes, ALS and HWP genes, and biofilm-forming ability[5,6].

Knowledge of the exact mechanisms and signal transduction pathways of the transcriptional factors that carry out the morphological transformation of *Candida albicans* (C. albicans), and the inhibition of these transcriptional factors based on this knowledge, may hinder yeast-hyphae transformation. Detection of these transformation mechanisms and virulence factors may contribute to preventing the formation and development of C. albicans-induced infections[7].

In light of the foregoing, this study was carried out to investigate in vitro the presence of proteinase, phospholipase, and biofilm formation, which are virulence factors of C. albicans species isolated from various clinical specimens, by phenotypic methods, and the presence of ALS1 and HWP1 genes, which are surface adhesins, by genotypic methods.

Materials and Methods

The study material consisted of a total of 100 C. albicans strains (43 blood, 50 urine, seven biopsy) isolated from various clinical specimens sent to İstanbul University-Cerrahpaşa Medical Faculty Hospital, Medical Microbiology Laboratory. The strains were identified based on their colony morphology, germ-tube formation, morphological appearance on cornmeal-Tween 80 agar medium, growth at 45°C, and the results of assimilation reactions assessed using the API 20C AUX (bioMérieux, France) kit.

Medium containing 1% bovine serum albumin and egg yolk medium were used to assess acid proteinase and phospholipase activities, two of the virulence factors, respectively[8]. Evaluation of proteolytic activity was done by measuring the widths of the melting zones of the protein in the medium. Strains without a fusion zone were found to be (-) in terms of acid proteinase activity; strains whose melting zone spread to the area 1-2 mm away from the colony were considered moderate (+); strains whose melting zone spread to the area 3-5 mm away from the colony were evaluated as strong (++)[8].

When evaluating the phospholipase activity (Pz) of strains at the end of incubation, the distinct ring-shaped precipitation zone formed around the colony was measured, and the Pz value was found by calculating the ratio of the colony diameter (a) to the total diameter of the precipitation zone (b) together with the colony diameter. Measurement of colony and zone diameters was made along both axes and average values were taken. According to these results, a value of Pz=1 is considered negative; those with Pz values between 0.9-1.00 are (+); those between 0.89-0.8 (++); those between 0.79-0.7 (+++); those <0.69 were evaluated as (++++)[8].

The modified tube adherence (MTA) method and two different modified microplate methods (Sabouraud Dextrose Broth with 8% Glucose [MMP-SDB][9], Brain Heart Infusion Broth with 0.25% Glucose [MMP-BHIB][10]) were used to assess biofilm formation, another virulence factor. The presence of the ALS1 and HWP1 genes was investigated by multiplex polymerase chain reaction (PCR) (Qiagen, Germany) using site-specific ALS1 and HWP1 primers after DNA extraction (İontek, Türkiye), and the sequences of the primers are listed in Table 1[9-13].

This study was conducted in accordance with the Declaration of Helsinki and approved by the İstanbul University-Cerrahpaşa Faculty of Medicine Clinical Research Ethics Committee with decision no. 83045809/4875 of June 11, 2013.

Statistical Analysis

NCSS (Number Cruncher Statistical System, Utah, USA) and PASS (Power Analysis and Sample Size, Utah, USA) statistical software were used for statistical analyses. The descriptive statistics obtained from the collected data were expressed using mean±standard deviation with minimum and maximum values in the case of continuous variables and as frequency (n) and percentage (%) values in the case of categorical variables. In the comparison of qualitative data, McNemar's test, Fisher's exact test, Fisher-Freeman-Halton exact test, and Yates's correction for

Table 1. *ALS1* and *HWP1* primers of *C. albicans* used in the study

Target Gene	Primers Used	PCR Product (bp)
ALS1	(forward) 5′- GAC TAG TGA ACC AAC AAA TAC CAG A - 3′ (reverse) 5′- CCA GAA GAA ACA GCA GGT GA - 3′	318
HWP1	(forward) 5′- ATG ACT CCA GCT GGT TC - 3′ (reverse) 5′- TAG ATC AAG AAT GCA GC - 3′	572

ALS1: Agglutinin-like sequence protein 1; *HWP1*: Hyphal wall protein 1.

Table 2. The distribution of the virulence factors by the sample type

	Sample Type			p
	Blood Samples (n=43) n (%)	Urine Samples (n=50) n (%)	Biopsy Samples (n=7) n (%)	
Phospholipase (+)	33 (76.7)	34 (68)	6 (85.7)	[c]0.469
Proteinase (+)	39 (90.7)	41 (82)	6 (85.7)	[c]0.484
MTA (+)	7 (16.3)	2 (4)	0 (0)	[b]0.103
ALS1 (+)	10 (23.3)	15 (30)	4 (57.1)	[b]0.190
HWP1 (+)	36 (83.7)	48 (96)	7 (100)	[b]0.103

[b]Fisher-Freeman-Halton exact test; [c]Pearson's chi-square test; MTA: Modified tube adherence method; ALS1: Agglutinin-like sequence protein 1; HWP1: Hyphal wall protein 1.

Table 3. The rates of isolated *C. albicans* strains in terms of *ALS1* and *HWP1* positivity

	ALS1		p
	Negative (n=71) n (%)	Positive (n=29) n (%)	
HWP1			
Negative	9 (9)	0 (0)	0.001**
Positive	62 (62)	29 (29)	

McNemar's test; **p<0.01, ALS1: agglutinin-like sequence protein 1; HWP1: hyphal wall protein 1.

Table 4. The results of the phenotypic and genotypic analyses of virulence factors

	n	%
Phospholipase		
-	27	27
++	12	12
+++	19	19
++++	42	42
Proteinase		
-	14	14
+	69	69
++	17	17
MTA		
-	91	91
+	6	6
++	3	3
MMP -SDB		
-	100	100
MMP-BHIB		
-	100	100
ALS1		
-	71	71
+	29	29
HWP1		
-	9	9
+	91	91

MTA: Modified tube adherence method; MMP-SDB: Modified Microplate Sabouraud Dextrose Broth Method; MMP-BHIB: Modified Microplate Brain Heart Infusion Broth Method; ALS1: Agglutinin-like sequence protein 1; HWP1: Hyphal wall protein 1.

continuity (Yates's chi-squared test) were used. Probability (p) statistics of <0.01 and <0.05 were deemed to indicate statistical significance levels.

Results

The mean age of patients with C. albicans growth was 42.40±28.77 (min. 1, max. 102 years). Of the C. albicans strains, 44% were isolated from male patients and 56% from female patients. Only one sample per patient was analyzed within the scope of the study. Of the strains, 43 were isolated from blood, 50 from urine, and seven from biopsy samples. The distribution of the virulence factors by sample type is shown in Table 2.

There was no statistically significant difference between sample types in terms of phospholipase, proteinase, MTA,

ALS1, and HWP1 results (p>0.05).

The phenotypic analysis of acid proteinase, one of the virulence factors, revealed that 86% of C. albicans strains were positive for acid proteinase production. Of the C. albicans strains found positive for acid proteinase production, 17% and 69% were strongly (++) and moderately proteolytic, respectively. On the other hand, proteinase activity was not detected in 14% of the C. albicans strains.

The phenotypic analysis of phospholipase, another virulence factor, revealed that 73% of C. albicans strains were positive for phospholipase production. The activity level of these strains in terms of phospholipase production was moderate (++) in 12% of the strains, strong (+++) in 19%, and very strong (++++) in 42%. On the other hand, phospholipase activity was not detected in 27% of the C. albicans strains.

The analysis of C. albicans strains for biofilm formation, another virulence factor, by the MTA method, revealed that 9% of the strains were positive for biofilm formation. The biofilm-forming activities were weak (+) in 6% of the strains and moderately strong (++) in 3%. On the other hand, the analysis of C. albicans strains for biofilm formation by the MMP-SDB and MMP-BHIB methods did not reveal any strain positive for biofilm formation. All three methods were repeated twice.

The genotypic analysis of the virulence factors revealed that 71% of the strains were negative for ALS1 and 29% were positive for ALS1, and 9% of the strains were negative for HWP1, while 91% were positive for HWP1. The rates of isolated C. albicans strains in terms of ALS1 and HWP1 positivity are shown in Table 3.

There was a significant difference between ALS1 and HWP1 positivity rates (p=0.001; p<0.01). All 29 C. albicans strains with ALS1 positivity were positive for HWP1, compared to 62 of the 71 C. albicans strains with ALS1 negativity. The results of the phenotypic and genotypic analyses of virulence factors collectively are shown in Table 4.

Discussion

In our study, the virulence factors proteinase, phospholipase, and biofilm formation were investigated phenotypically, and ALS1 and HWP1 genes were investigated genotypically in C. albicans isolates from various clinical samples. Proteolytic activity was detected in 86% of the strains, phospholipase activity in 73%, and biofilm formation with the modified tube adhesion method in 9%. Genotypically, ALS1 and HWP1 genes were detected in 29% and 91% of the strains, respectively.

Proteinases are hydrolytic enzymes that play a role in the early stages of colonization on mucosal surfaces, localize around *Candida* during invasion, and facilitate adherence to the epithelium[14,15]. Phospholipases are another type of hydrolytic enzyme that acts by breaking the epithelial cell membrane, allowing the hyphal structure to enter the cytoplasm and play a role in the pathogenesis of C. albicans[14,15].

In a study featuring the analysis of 153 C. albicans strains isolated from blood samples, Mattei et al.[16] found proteinase and phospholipase activities of the strains to be 97% and 78%, respectively. They also found the isolated strains to have high proteolytic and low phospholytic activities. Similarly, in this study, proteinase and phospholipase activities were found as 86% and 73%, respectively. However, contrary to the said study, the isolated strains were found to have low proteolytic activities and high phospholytic activities. In line with this study, two other studies evaluating phospholipase and proteinase activities in *Candida* species isolated from candidemia also reported the highest activity in C. albicans strains[17,18].

Biofilm formation was another virulence factor that was analyzed using phenotypic analysis in this study. Turan et al.[19] detected weak, moderate, and strong biofilm formation in 29%, 38%, and 23% of the C. albicans isolated from various clinical specimens, respectively. Unlike this study, they reported 90% positivity in their study, in contrast to 9% positivity in the current study. Gültekin et al.[20] assessed 17 out of 46 isolates as C. albicans and found all C. albicans isolates negative for biofilm activity by the MMP-SDB method. Similarly, in this study, the results of all strains were found to be negative with the same method.

Saiprom et al.[15] investigated the virulence factors in *Candida* species that cause candidemia. Consequently, they found that proteinase activity was 57.9% in all strains and that 50% of these strains positive for proteinase activity were C. albicans. Additionally, they found that the phospholipase activity was 26.3% in all strains, and all strains positive for phospholipase activity were C. albicans. Sriphannam et al.[21] determined that C. albicans, among the *Candida* species they isolated from candidemia, had the highest phospholipase and proteinase activity but not the highest biofilm formation activity.

Adhesins, which are involved in adhering to organic and inorganic surfaces, extracellular matrix proteins, and endothelial and epithelial cells, are encoded by the ALS1-7 and HWP1 genes[6,22]. Various studies have reported that ALS and HWP1 gene families play a role in the adhesion

of C. albicans to mucosal surfaces and that HWP1 and ALS1 are effective in biofilm formation both in vivo and in vitro[6,22]. It has been speculated that detecting these genes in C. albicans strains isolated from various clinical samples may give an idea about their role in colonization and disease formation. The HWP1p gene, which uses tissue transglutaminase in the adherence of C. albicans to epithelial cells, provides a complementary effect in in vivo biofilm formation by functioning together with Als1p and Als3p genes[7,23].

In a study investigating the frequency and expression of ALS1 and HWP1 genes in vaginal samples of patients followed for vulvovaginal candidiasis, Monroy-Pérez et al.[24] isolated C. albicans in 50 patients. They assessed the presence of ALS and HWP1 genes in the strains by conventional PCR and their expression levels by reverse transcription polymerase chain reaction (RT-PCR) after the incubation of C. albicans strains in reconstituted vaginal epithelial cells. They concluded that Alsp and Hwp1p proteins play an important role in the pathogenesis of the infection.

In another study, Ardehali et al.[6] determined that ALS1 and HWP1 genes were most commonly found in C. albicans among *Candida* species and that the frequencies of these genes in C. albicans were 92% and 95%, respectively. Similarly, in this study, there was a higher rate of HWP1 positivity compared to ALS1 positivity.

İnci et al.[11] detected ALS1 and HWP1 genes in 53.9% and 5.3% of the strains, respectively. Additionally, they determined biofilm formation in the presence of ALS1 and/or HWP1 in 62.2% of the strains with both methods. They detected a moderate correlation between the presence of ALS1 and positive biofilm activity with the MMP-SDB method but could not detect any correlation with the MTA method. In contrast, in this study, the rates of C. albicans strains with ALS1 positivity, HWP1 positivity, biofilm formation based on the MTA method, and biofilm formation based on the other two MMP methods were determined as 29%, 91%, 9%, and 0%, respectively. There was also no significant correlation between the mentioned genes and biofilm formation.

Due to increasing antifungal resistance, studies for the development of new antifungal drugs have accelerated in addition to the antifungal drugs currently in use. Immunotherapy is one of these methods. As a result, vaccines that provide lifetime immunity may help protect against multidrug-resistant yeasts, including C. auris, and studies are progressing in this direction[25]. In future studies, using fungal cell wall components as adjuvants and utilizing protected fungal epitopes may contribute to vaccine development efforts[26].

The finding that ALS1 and HWP1 genes were detected in high amounts in this study suggests that they may be targeted in studies conducted to develop vaccines or other antifungal agents targeting these adhesins in the cell wall.

Although C. albicans is the most frequently isolated from clinical samples, the exclusion of other *Candida* species, which are increasingly important, is a limitation of our study.

Conclusion

In conclusion, giving an impetus to the analyses of genes and gene products related to virulence factors, which have an important place in the pathogenesis of C. albicans, will likely contribute to the development of new treatment options and, as a result, to finding alternative treatment approaches that can be used in the treatment of infections caused by the genus *Candida*, especially in patients with a severe course.

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