TÜBİTAK

Turkish Journal of Medical Sciences

http://journals.tubitak.gov.tr/medical/

Research Article

Turk J Med Sci
(2013) 43: 125-130
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doi:10.3906/sag-1205-90

Investigations of *ALS1* and *HWP1* genes in clinical isolates of *Candida albicans*

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Received: 22.05.2012 • **Accepted:** 05.07.2012 • **Published Online:** 18.01.2013 • **Printed:** 18.02.2013

Aim: To explore the presence of *ALS1* and *HWP1* genes by multiplex polymerase chain reaction (PCR) in *Candida albicans* strains.

Materials and methods: By using the multiplex PCR method, the presence of agglutinin-like sequence 1 (*ALS1*) and hyphal wall protein 1 (*HWP1*) genes were investigated in 206 *C. albicans* strains that were isolated from various clinical samples. Phenotypic identification of slime formation by microplate and tube adherence tests was performed.

Results: The presence of the *ALS1* gene was detected in 53.9% of all strains, while the *HWP1* gene was present in 5.3%. Slime formation was phenotypically detected in the 62.2% of the strains in which the *ALS1* and/or the *HWP1* gene was found, using the microplate and/or tube adherence test. The genes evaluated were found to be present in the 76.7% of strains in which slime formation was detected by phenotypic tests. There was a moderate correlation between the presence of the *ALS1* gene and the microplate method, yet there was no correlation when using the tube adherence test.

Conclusion: It was concluded that various genes other than those evaluated could be present in slime formation of *C. albicans*, and the presence of the genes may not always be represented in the phenotype.

Key words: *Candida albicans*, *ALS1*, *HWP1*, slime formation

1. Introduction

Candida species are a commonly seen form of yeast present in the gastrointestinal tract, mucosa, and skin of healthy individuals as a part of normal flora. When the immune defense of a host is deteriorated, *Candida* spp. can cause life-threatening disorders by invading tissues. *Candida albicans* is the most commonly isolated species of nearly all forms of candidiasis (1).

Biofilm formation plays an essential role in the pathogenicity of *C. albicans* (2,3). Biofilm is a cell population that is surrounded by an extracellular matrix consisting of yeast cells and filaments; it has a relationship with the surface and exhibits different phenotypic features than planktonic cells (4). This cell population behaves as a continuous reservoir in the spread of infection. Additionally, it is resistant to many antifungal agents, as compared to planktonic cells (2,3). The initiation of biofilm formation is dependent on the attachment of yeast cells to a substrate, which is followed by the attachment of yeast cells to each other (5). Adherence must extend to the hyphal layers. The agglutinin-like sequence (*ALS*) gene family is the largest family among known adhesins in *C. albicans* (6). It is known that *ALS* family members interact with several substrates, including host cells and proteins (7–9). The finding that expression of *ALS1* and other family members increases during biofilm development in vitro suggested that the *ALS* family plays a role in biofilm formation (10–12). Hyphal wall protein 1 (*HWP1*), another adhesion gene, is also upregulated during biofilm development (13). *HWP1*, a glycosylphosphatidylinositol-linked mannoprotein like the *ALS* proteins, is the best-characterized hyphal adhesin (8,14). It is a substrate for transglutaminase activity derived from a host, and thus it mediates covalent attachment of *C. albicans* to host cells (8,15). Although there are many studies in the literature investigating pathogenic fungi, antifungal resistance, and

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virulence factors of fungi, new studies are still needed on these issues (3,6,11,16–18).

In the present study, our aim was to explore the presence of *ALS1* and *HWP1* genes by multiplex polymerase chain reaction (PCR) in *C. albicans* strains isolated from various clinical samples, and to evaluate slime factor by the microplate and tube adherence test methods in order to determine the phenotypic reflection of these genes.

2. Materials and methods

2.1. Strains

In the present study, 206 *C. albicans* strains isolated from various clinical samples were used. Clinical isolates were provided by the Microbiology Laboratory of Mustafa Kemal University and the School of Medicine and Mycology Laboratory of Erciyes University. All yeast isolates were taken from different patients. Strains were isolated from samples obtained from urine (n = 82), blood (n = 13), vaginal smears (n = 77), wounds (n = 17), cerebrospinal fluid (n = 2), peritoneal fluid (n = 3), and respiratory tracts (n = 12). *C. albicans* identification was performed according to positive germ tube tests, chlamydospore formation in corn-meal Tween-80 agar, and carbohydrate assimilation tests with the commercially available API 20C AUX kit (bioMérieux, France).

2.2. PCR assay

DNA extraction: DNA extraction was achieved by a modified version of the method that de Baere et al. (19) described previously. A suspension with 0.5 McFarland standard was prepared from strains that were grown in Sabouraud dextrose agar (SDA) culture media and loaded into Eppendorf tubes. They were centrifuged at 10,000 rpm for 5 min. After the discharge of supernatant, 500 µL of distilled water was added. The tubes were kept in a heat block at 100 °C for 10 min. After centrifuging at 12,000 rpm for 15 min the supernatant was put into a new tube and kept at –20 °C until PCR tests.

PCR reagents and amplification conditions: PCR amplification was carried out in a total volume of 25 µL of reaction mixture by using *ALS1* and *HWP1* primers (Table 1) (11,20). The reaction mixture consisted of 5 µL of 10X reaction buffer without $MgCl_2$ (Fermentas, USA), 10 mM of each deoxynucleoside triphosphate at 0.5 µL (Fermentas), 25 mM of $MgCl_2$ at 2.5 µL, 1 U of Taq polymerase (Fermentas), 1.5 µL of primers, and approximately 10 ng of template DNA at 4 µL, and was then brought to a final volume of 25 µL with distilled water. Each cycle consisted of 3 steps: denaturation, annealing, and extension. PCR was conducted as follows: 1 cycle of 94 °C for 4 min, followed by 35 cycles of 94 °C for 30 s, 52 °C for 1 min, and 72 °C for 2 min. A final extension cycle was performed at 72 °C for 5 min. The amplified products were analyzed in a 2% (w/v) agarose gel in 1X TAE buffer (40 mmol/L Tris-acetate, 1 mmol/L EDTA). Ethidium bromide (0.5 µg/mL TAE)-stained DNA amplicons were seen using a gel imaging system (Wealtec, Dolphin-View, USA).

2.3. Phenotypic tests

Tube adherence test for slime production in *C. albicans* strains: One colony was taken from *C. albicans* strains that were grown in SDA culture media. It was inoculated into 5 mL of Sabouraud dextrose broth (SDB) culture media containing 2% glucose. It was incubated at 37 °C for 48 h. After incubation the tube contents were evacuated. After adding 1% safranin solution, they were kept at room temperature for 30 min. Tube contents were then poured out. Tubes were washed twice using sterile phosphate buffered saline (PBS) and the tubes were then inverted and placed onto a blotter for drying. After 48 h, strains that formed an adherent biofilm layer were considered positive, while those not forming such a layer were considered negative. Involvement at the air–fluid junction was not considered (21). Each tube was rated by 2 independent observers.

Microplate method for slime production in *C. albicans* strains: *C. albicans* strains were grown in a SDA culture at 37 °C for 48 h. They were diluted at 1/100 with SDB containing 2% glucose, and then 200 µL of this dilution was placed into each well of a sterile, flat-based microplate and incubated at 37 °C for 48 h. The content of each well

Table 1. Primer sequences.

Primer	5'-sequence-3'	Length of PCR product (bp)
C. albicans ALS1	(Forward) GAC TAG TGA ACC AAC AAA TAC CAG A (Reverse) CCA GAA GAA ACA GCA GGT GA	318
C. albicans HWP1	(Forward) ATG ACT CCA GCT GGT TC (Reverse) TAG ATC AAG AAT GCA GC	572

bp: base pairs.

was evacuated and washed with PBS 4 times. Next, 200 µL of methylene blue was placed into each well and stained at room temperature for 1 h. Each well was washed with distilled water 3 times and microplates were inverted and placed onto a blotter for drying. Sterile SDB was used as a negative control in this study. The microplate was read at 492 nm with an ELISA reader (TekTIME, Organon Tecnica, France). These processes were performed 3 times for each sample, and the arithmetic mean of optical densities was taken. The cut-off value was determined by adding +2 standard deviations to the optical density of wells containing sterile SDB. Specimens found to be above the cut-off value for optical density were considered positive (22).

2.4. Statistical analysis

Statistical analyses were performed using SPSS 15.0. While investigating the associations between ordinal variables, the correlation coefficients and their significance were calculated using the Spearman test. A 5% type-I error level was used to infer statistical significance.

3. Results

Among all strains, the *ALS1* gene was positive in 111 strains (53.9%) and negative in 95 strains (46.1%), whereas the *HWP1* gene was positive in 11 strains (5.3%) and negative in 195 strains (94.7%). The presence of the *ALS1* gene was seen in all strains containing the *HWP1* gene. The presence of the *ALS1* and *HWP1* genes are shown in the Figure. In the present study, slime activity was evaluated in order to see the reflection of gene presence to phenotype. The slime factor was found to be positive in 71 strains (34.5%) and negative in 135 strains (65.5%) when using the microplate test, whereas it was positive in 34 strains (16.5%) and negative in 172 strains (83.5%) when using the tube adherence test. However, slime formation was found to be phenotypically positive in 90 strains (43.7%) when using the microplate and/or tube adherence test. Table 2 shows the results of slime formation by phenotypic test, according to the source from which strains were isolated, and the presence of the *ALS1* and *HWP1* genes.

It was observed that, of the strains in which the *ALS1* or/and *HWP1* genes were present, slime formation was found to be phenotypically positive in 59 strains (53.2%) when using the microplate method and in 22 strains (19.8%) when using the tube adherence test. Slime

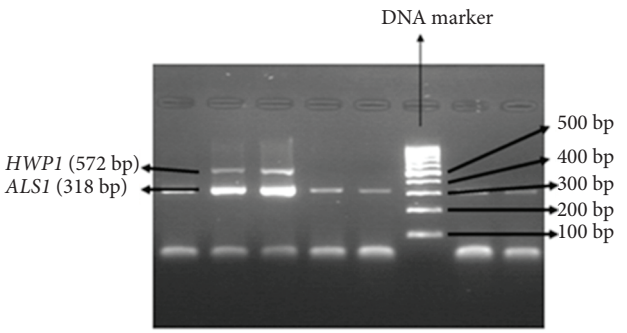

Figure. Multiplex PCR amplification products showing presence of the *HWP1* and *ALS1* genes of *Candida albicans*.

Table 2. Presence of *ALS1* and *HWP1* genes and slime production according to source of strains.

Strains isolated from specimens	Presence of *ALS1* gene	Presence of *HWP1* gene	Positive slime production by MPM*	Positive slime production by TAT**
	n (%)	n (%)	n (%)	n (%)
Urine (n = 82)	43(52.4)	4 (48.8)	26 (31.7)	15 (18.3)
Vaginal swabs (n = 77)	37 (48.0)	3 (3.9)	26 (33.8)	8 (10.4)
Wounds (n = 17)	7 (41.7)	2 (11.8)	6 (35.3)	5 (29.4)
Blood (n = 13)	11 (84.6)	1 (7.7)	7 (53.8)	2 (15.4)
Respiratory specimens (n = 12)	8 (66.7)	1 (8.3)	5 (41.7)	2 (16.7)
Peritoneal fluid (n = 3)	3 (100)	-	-	2 (66.7)
Cerebrospinal fluid (n = 2)	2 (100)	-	1 (50)	-
Total (n = 206)	111 (53.9)	11 (5.3)	71 (34.5)	34 (16.5)

*MPM: microplate method, **TAT: tube adherence test.

formation was found to be positive in 69 strains (66.2%), while phenotypically, no slime formation was detected in 42 strains (37.8%) when using the microplate and/or tube adherence test. A moderate correlation was detected between the *ALS1* gene and the microplate method ($r = 0.425$, $P < 0.001$), while no correlation was found between the *ALS1* gene and the tube adherence test ($r = 0.097$, $P = 0.168$). It was found that there was a moderate correlation between the *ALS1* gene and microplate and/or tube adherence test ($r = 0.403$, $P < 0.001$). However, this correlation coefficient was lower when compared to the microplate test.

Gene presence was found in 59 (83.1%) of the strains in which slime formation was detected by the microplate method, and in 22 (64.7%) of those in which slime formation was detected by the tube adherence test. The *ALS1* and/or *HWP1* genes were found in 69 (76.7%) of the strains in which slime formation was detected by the microplate and/or tube adherence test, while neither was found in 21 strains (23.3%). Comparisons of both genes and phenotypic tests are shown in Table 3.

4. Discussion

Genes belonging to the *ALS* gene family and *HWP1* genes encode cell-surface–related glycosylphosphatidylinositol that binds to glycoprotein, which then mediates the adhesion of *C. albicans* strains to mucosal surfaces (7,15). It has been reported that the *HWP1* gene and the *ALS1* gene of the *ALS* family play important roles in *C. albicans* biofilms, both in vivo and in vitro (8,23,24). Detecting the presence of the *ALS1* and *HWP1* genes in *C. albicans* strains isolated from clinical specimens will help to ascertain the roles of these genes in colonization and disease.

Green et al. (11) evaluated the expression patterns of genes in the *ALS* gene family in a reconstituted human buccal epithelium (RHE) model and clinical oral specimens by reverse transcriptase-PCR (RT-PCR). They showed that the *ALS1* gene was expressed at all time points and inoculation densities. Moreover, they reported that gene expression patterns were found to be similar in the RHE model and the clinical strains. In another study, biofilms were formed on silicone elastomers by using reference *C. albicans* strain and clinical isolates; *ALS* gene expression was explored in these biofilms by the real-time PCR method (12). It was found that the *ALS1* gene was clearly upregulated in biofilms when compared to planktonic cells. García-Sánchez et al. (13) explored specific features of *C. albicans* biofilms by using transcript profile. They found that genes were expressed in 325 different manners in biofilms developed under different environmental conditions, and they reported that the *ALS1* gene was clearly upregulated in cells of *C. albicans* biofilms. Nailis et al. (16) explored *ALS1* and *ALS3* gene expression levels in biofilm cells and free cells of *C. albicans* by 4 different normalization strategies. They found that the *ALS1* gene was overexpressed in biofilm cells when compared to planktonic cells. In another study by Nailis et al. (25), expressions of several adhesin genes (including the *ALS* gene family and *HWP1* gene) were evaluated in *C. albicans* biofilms produced in different model systems by using the real-time PCR technique. They found that the *HWP1* gene and most members of the *ALS* gene family were upregulated in all tested model systems and at all time points of biofilm formation. Expression of *ALS* genes was evaluated in both clinical samples and vaginal candidiasis models by using the RT-PCR method in another study (26). *ALS1*, *ALS2*, *ALS3*, and *ALS9* were reported as the most commonly expressed genes of the *ALS* gene family and, in the same study, similar expression frequencies and patterns were reported on clinical samples and model systems. Nas et al. (20) investigated the expression of *ALS1* and *HWP1* genes in vaginal swab

Table 3. Comparison of the *ALS1* and *HWP1* genes by phenotypic tests.

Presence of genes		Microplate method		Tube adherence test		Total n (%)
		Pos** (n)	Neg* (n)	Pos (n)	Neg (n)	
ALS1	Pos (n)	59	52	22	89	111 (53.9)
	Neg (n)	12	83	12	83	95 (46.1)
HWP1	Pos (n)	8	3	2	9	11 (5.3)
	Neg (n)	63	132	32	163	195 (94.7)
Total n (%)		71 (34.5)	135 (65.5)	34 (16.5)	172 (83.5)	206 (100)

*Neg: negative, **Pos: positive.

samples taken from pregnant, postmenopausal, and reproductive-aged women with vulvovaginal candidiasis by using the RT-PCR method. They found that the *ALS1* gene was expressed in 70%, 75%, and 67% of the strains isolated from these groups, respectively, while the *HWP1* gene was expressed in 60%, 25%, and 73% of the same strains, respectively. *HWP1* is suggested as a substrate for host transglutaminases, which allow *C. albicans* to bind covalently to epithelial cells (8,15). It has been suggested that *HWP1* surface protein requires *ALS1/3* association to initiate in vivo biofilm formation (23). A complementary role was suggested for *ALS1/3* and *HWP1* genes in in vivo and in vitro biofilm formation (24). Ene and Bennett (27) reported that *HWP1*, *HWP2*, and *Rbt1* genes played an important role in biofilm formation and emphasized that, among these, *HWP1* was the most important factor. In our study, it was found that *ALS1* was present in 53.9% of *C. albicans* isolates, while *HWP1* was present in 5.3%. We thought the difference in the results of the *ALS1* and *HWP1* genes might have resulted from the sample group and the distinct methods used.

There are various studies that have explored biofilm formation in *C. albicans* isolates phenotypically (28–30). By using the tube adherence test, Dolapci and Tekeli (28) found that 15.1% of 350 clinical *Candida* strains produced slime factor. Demirbilek et al. (29) reported slime factor positivity as 23% in 79 clinical *C. albicans* isolates by using the microplate method. Yücesoy and Karaman (30) evaluated biofilm formation in *Candida* species isolated from different clinical samples by the tube adherence and microplate methods. When using the microplate method, they found that 16.7% of strains produced slime factor, whereas when using tube adherence test, they found that 27.6% produced slime factor. They found a rate of biofilm formation of 17%–55% by the tube adherence test and of 0%–48% by the microplate method. They reported that no significant difference was found between *C. albicans* and non-*albicans Candida* species in terms of biofilm formation by the tube adherence test, whereas a significant difference was found by the microplate method. In our study, slime positivity was found to be 34.5% by the microplate method, whereas it was found to be 16.5% by the tube adherence test. The discrepancy of our study from other studies may be due to the difference in sources from which samples were isolated and sample number.

In the present study, slime formation was phenotypically found in 62.2% of the strains in which *ALS1* and/or *HWP1* genes were detected. Slime positivity was found to be higher by the microplate than by the tube adherence test. Phenotypically, no slime formation was seen in 37.8% of the strains in which the gene was detected. This could result from inadequacy of phenotypic tests or the presence of various other genes. Of 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%. This can be explained by the presence of different genes involved in slime formation.

There are some limitations of the present study. First, this study is oriented toward demonstrating the *ALS1* and *HWP1* genes in DNA. This is not a gene expression study. Second, only 2 of many genes implicated in slime production in clinical strains of *C. albicans* are focused on. Other genes should be evaluated in further comprehensive studies. Third, the strains included in the study were obtained from varying sources.

In conclusion, the presence of the *ALS1* gene was found in about half of the clinical isolates of *C. albicans*, whereas *HWP1* was present in a minority of the strains. A significant relationship was found between the *ALS1* gene and slime production by the microplate method, whereas no relation was found by the tube adherence test. There is a need for further comprehensive studies that include more clinical samples isolated from the same source and also various other genes in order to assess the slime formation of *C. albicans* strains by genotypic and phenotypic aspects.

Acknowledgments

This study was conducted with the financial support of the Mustafa Kemal University Scientific Research Council, Project Number: 1003 M 0102.

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