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Investigating virulence factors of clinical *Candida* isolates in relation to atmospheric conditions and genotype

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Aim: To investigate some virulence factors in *Candida* species isolated from patients with suspected invasive fungal infection and to identify their relationship with *Candida* genotypes.

Materials and methods: Overall 45 isolates (20 *Candida albicans* and 25 non-*albicans Candida* spp.) genotyped by rep-PCR were included in this study. Virulence factors were studied in both aerobic and anaerobic conditions. In isolates, egg yolk agar was used for determining phospholipase activity, while bovine serum albumin agar was used for proteinase activity, Tween-80 medium for esterase activity, and Sabouraud dextrose agar with sheep blood for hemolysin activity. Biofilm formation was detected by the microplate method.

Results: In both *Candida* spp., it was found that hemolytic activity and proteinase activity were higher in aerobic conditions, whereas biofilm formation was higher in anaerobic conditions. It was also found that phospholipase and esterase activity were only detected in *C. albicans* isolates, which were found to be higher in aerobic conditions. No difference was found in virulence factors evaluated among the *C. albicans* genotypes.

Conclusion: The amount of oxygen in the atmosphere may affect the virulence of *Candida* spp. Further comprehensive studies are needed in order to identify the relationship between *Candida* genotypes and virulence factors.

Key words: *Candida* spp., virulence factors, atmospheric condition, genotype

Introduction

Candida species are commonly seen yeasts that exist as an element of normal flora in the skin, mucosa, and gastrointestinal tract of humans (1). Fungal infections are generally opportunistic. The extent and severity of *Candida* infections depend on the immune status of the host (2).

Candida spp. have some virulence factors that facilitate proliferation; they may result in adhesion to the epithelium and invasion of the host tissue (3,4). It seems that extracellular hydrolytic enzymes play an important role in candidal overgrowth (5). The extracellular hydrolytic enzymes including secreted aspartyl proteinase and phospholipases degrade immunoglobulins and proteins of the extracellular matrix; they also inhibit the phagocytosis of polymorphonuclear neutrophils and induce inflammatory reactions (6,7). Furthermore, the survival and ability of *Candida albicans* to establish infections within humans are mainly related to its ability to procure elemental iron through hemolysin production (8,9). There is an increasing amount of evidence linking lipases to microbial virulence. It has

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been proposed that microbial extracellular lipases play various roles including the digestion of lipids for nutrient acquisition, adhesion to host cells and tissues, synergistic interactions with other enzymes, unspecific hydrolysis due to additional phospholipolytic activities, and the initiation of inflammatory processes by affecting immune cells and self-defense by lysing the competing microflora (10,11). Moreover, it has been reported that biofilm formation also plays an essential role in the pathogenicity of *Candida* spp. (1,12). It is also known that *Candida* spp. have the ability to grow in both aerobic and anaerobic conditions. Moreover, *Candida* spp. possess some adaptive mechanisms to survive in both situations (4,13). In addition, it has been reported that the virulence of distinct genotypes of *Candida* spp. may be different (4). Although there are many studies about pathogenic fungi, it has been reported that studies on virulence factors are still needed (14).

In the present study, we aimed to investigate some virulence factors in *Candida* spp. isolated from patients with suspected invasive fungal infection and to identify their relationship with *Candida* genotypes.

Materials and methods

Yeast isolates

Forty-five *Candida* isolates, obtained from patients with suspected invasive fungal infection and genotyped by repetitive extragenic palindromic polymerase chain reaction (rep-PCR) at the hospitals of Erciyes University's School of Medicine, were included in this study. All isolates were obtained from different patients. These isolates were identified according to germ tube test, morphology on corn-meal agar with Tween-80, and carbohydrate assimilation test using a commercially available API 20C AUX kit (bioMérieux, France). Of the 45 isolates (isolated from blood, bronchoalveolar lavage, tissue sample, and peritoneal, pleural, and cerebrospinal fluid), 20 isolates (44.4%) were identified as *C. albicans*, while 6 (13.3%) were identified as *C. glabrata*, 5 (11.1%) as *C. kefyr*, 5 (11.1%) as *C. parapsilosis*, 1 (2.2%) as *C. krusei*, 1 (2.2%) as *C. rugosa*, and 7 (15.6%) as *C. tropicalis* by methods based on phenotype (15).

The rep-PCR based fingerprint patterns of all isolates were obtained in gel-like images by using DiversiLab 2.1.66 web-based interpretation software. For analysis, the Pearson correlation coefficient was used in the similarity calculation and UPGMA was used to automatically compare the rep-PCR profiles. Strains with a similarity of 90% or above were accepted as main clones, and those in the main clone category with a having similarity of 95% or above were accepted as subclones. Isolates with a similarity of 90% or below were accepted as different clones. According to this definition of the *C. albicans* isolates, 12 were classified as genotype A1, 7 as genotype A2, and 1 as genotype B, while non-*albicans* strains were classified as C-O genotypes (15). In the present study, *C. albicans* ATCC 90028 was employed as the reference strain.

Preparation of the yeast suspensions: Yeast suspensions were prepared from the yeast isolates included in the study to evaluate phospholipase, proteinase, esterase, and hemolytic activity. First, a small amount of stock culture was inoculated on Sabouraud dextrose agar (SDA) (Oxoid) containing chloramphenicol by using a sterile loop and incubated at 37 °C for 24–48 h. Then the yeasts were harvested and suspended in sterile phosphate buffered solution (PBS) at turbidity equal to optical density (OD) of 0.5 McFarland. The final suspension was adjusted to contain 1×10^7 yeast cells/mL.

Determination of phospholipase activity: To determine phospholipase activity, the egg yolk agar method of Price et al. (16), which was modified by Samaranayake et al. (17), was employed. The culture medium consisted of 1 L of SDA (Oxoid) containing 1 M NaCl, 0.005 M $CaCl_2$, and 10% sterile egg yolk. Ten microliters of previously prepared yeast suspension was inoculated onto plates; these were then incubated at 37 °C for 5 days in both aerobic and anaerobic conditions. Anaerobic jars were used to obtain anaerobic conditions.

The presence of enzyme activity was determined by the formation of a precipitation zone around the yeast colonies. Phospholipase activity (P_z) was calculated by dividing the diameter of the colony by the diameter of the colony plus precipitation zone. The P_z was scored as follows: $P_z = 1$, negative phospholipase activity; $P_z = 0.64–0.99$, positive phospholipase activity; and $P_z \leq 0.63$, very strong phospholipase activity (16).

Determination of proteinase activity: To determine proteinase activity, bovine-serum albumin agar defined by Staib (18) was employed. The agar contained 0.1% KH_2PO_4, 0.05% $MgSO_4$, 2% agar, and 1% bovine serum albumin (Sigma-Aldrich, St Louis, MO, USA). The final pH was adjusted to 4.5. Ten microliters of previously prepared yeast suspension was inoculated onto the plates; these were then incubated at 37 °C for 10 days in both aerobic and anaerobic conditions. The presence of proteinase activity was determined by the formation of a transparent halo around the yeast colonies. Enzyme activity (Pr_z) was calculated according to the method described by Price et al. (16). Pr_z was scored as follows: $Pr_z = 1$, negative proteinase activity, $Pr_z =$ 0.64–0.99, positive proteinase activity; and $Pr_z \leq 0.63$, very strong proteinase activity.

Determination of hemolytic activity: To determine hemolytic activity, SDA (Oxoid) containing 7% sheep blood and 3% glucose with a final pH adjusted to 5.6 ± 0.2 was employed. Ten microliters of previously prepared yeast suspension was inoculated onto plates; these were then incubated at 37 °C for 48 h in both aerobic and anaerobic conditions. After incubation, a transparent/semitransparent zone around the inoculation site was considered as positive hemolytic activity (8).

Esterase activity: To determine esterase activity, Tween-80 opacity test medium was used. The test medium with a pH adjusted to 6.8 consisted of 1% peptone, 0.5% NaCl, 0.01% $CaCl_2$, and 1.5% agar. After cooling the medium (50 °C), 0.5% of Tween-80 was added. Ten microliters of previously prepared suspension from each isolate was carefully deposited on the Tween-80 opacity test medium; this was then incubated at 37 °C for 10 days in both aerobic and anaerobic conditions. Esterase activity was considered as positive in the presence of a halo pervious to light around the inoculation site (19).

All strains were tested 3 times in the enzymatic evaluation and interpreted by 2 independent observers.

Biofilm formation: Sterile 96-well microplates were used to evaluate biofilm formation (20). By using a loop, a spot of each isolate was placed into tubes containing 2 mL of brain heart infusion broth (BHIB) medium with glucose (0.25%) and incubated at 37 °C for 24 h. Then all tubes were diluted at a ratio of 1:20 by using freshly prepared BHIB. From this final solution, 200 µL was placed into the microplates, which were then incubated at 37 °C for 24 h. After incubation, the microplates were rinsed with PBS 3 times and then inverted to blot. Then 200 µL of 1% crystal violet was added to each well, followed by incubation for 15 min. After incubation, the microplate was again rinsed with PBS 3 times. Then 200 µL of ethanol:acetone mixture (80:20 w/v) was added to each well. They were read at 450 nm using an enzyme-linked immunosorbent assay (ELISA) reader (Biotek EL x 808, USA) and the OD was recorded for each well. Three wells were used for each strain and the arithmetical mean of 3 readings was used in analysis. *Enterococcus faecalis* ATCC 29212 was employed as the control strain. Sterile BHIB without microorganism was employed as the negative control. The cutoff value was determined by arithmetically averaging the OD of the wells containing sterile BHIB and by adding +2 standard deviation. Samples with an OD higher than the cutoff value were considered positive, whereas those with lower value than cutoff were considered negative.

Statistical analysis

All statistical analyses were performed using SPSS 15.0. Normal distributions of the variables were analyzed by visual (histogram and o-probability graphics) and analytic methods (Shapiro–Wilk test). Wilcoxon's test and the Mann–Whitney U test were used as the data were ordinal. $P < 0.05$ was considered significant.

Results

Phospholipase activity: Among all the isolates, 17 (37.8%) expressed phospholipase activity in aerobic conditions, whereas 15 (33.3%) did so in anaerobic conditions. The P_z value range was 0.67–0.95 (mean: 0.812 ± 0.095) in aerobic conditions, while it was 0.88–0.95 (mean: 0.924 ± 0.026) in anaerobic conditions. No significant difference was found between the 2 atmospheric conditions ($P > 0.05$). When considered in terms of species, 17 (85%) of the *C. albicans* isolates expressed phospholipase activity in aerobic conditions, whereas 15 (75%) did so in anaerobic conditions. No significant difference

was seen between the 2 atmospheric conditions (P > 0.05). It was seen that *C. albicans* isolates expressed strong phospholipase activity in neither aerobic nor anaerobic conditions. It was also seen that non-*albicans Candida* spp. had phospholipase activity in neither aerobic nor anaerobic conditions.

Proteinase activity: Among all the isolates, 25 (55.6%) expressed proteinase activity in aerobic conditions, whereas 19 (42.2%) did so in anaerobic conditions. The P_z value range was 0.50–0.83 (aerobic, mean: 0.671 ± 0.074; anaerobic, mean: 0.668 ± 0.085) in both atmospheric conditions. It was found that overall 6 isolates (13.3%) had strong positive proteinase activity in both aerobic and anaerobic conditions. Proteinase activity was detected on day 3 in aerobic conditions versus on day 7 in anaerobic conditions. It was seen that there was a significant difference favoring aerobic conditions in terms of proteinase activity among all isolates according to atmospheric condition (P = 0.14). Among *C. albicans* strains, 19 isolates (95%) expressed proteinase activity in aerobic conditions, whereas 18 isolates (90%) did so in anaerobic conditions, suggesting no significant difference between the 2 atmospheric conditions (P < 0.05). Among non-*albicans Candida* spp., 6 isolates (24%) expressed proteinase activity in aerobic conditions and 1 isolate (4%) in anaerobic conditions; there was a significant difference favoring aerobic conditions (P = 0.025).

Esterase activity: Esterase activity was detected in only 1 (2.2%) *C. albicans* strain isolated from blood in aerobic conditions, while no isolate expressed esterase activity in anaerobic conditions. No statistical analysis could be performed for esterase activity, as the number of isolates expressing esterase activity was considerably small.

Hemolytic activity: Among all the isolates, 41 (91.1%) expressed hemolytic activity in aerobic conditions, whereas 18 (40.0%) did so in anaerobic conditions. All cases of hemolysis were beta hemolysis type. The hemolytic index was 0.32–0.86 (mean: 0.730 ± 0.087) in aerobic conditions and 0.54–0.90 (mean: 0.722 ± 0.074) in anaerobic conditions. There was a significant difference favoring aerobic conditions in terms of the hemolytic activity of isolates according to atmospheric conditions (P < 0.001). Among *C. albicans* strains, 19 isolates (95%) expressed hemolytic activity in aerobic conditions, whereas 1 (5%) did so in anaerobic conditions, and there was a significant difference favoring aerobic conditions (P < 0.001). Among non-*albicans Candida* spp., 22 isolates (88%) expressed hemolytic activity in aerobic conditions, whereas 17 (68%) did so in anaerobic conditions, suggesting no significant difference between the 2 atmospheric conditions (P < 0.05).

Biofilm formation: Among all the *Candida* isolates, biofilm formation was detected in 10 isolates (22.2%) in aerobic conditions, whereas it was detected in 23 isolates (51.1%) in anaerobic conditions. There was a significant difference in terms of biofilm formation according to atmospheric conditions among the isolates (P < 0.001). Among the *C. albicans* strains, biofilm formation was detected in 2 isolates (10%) in aerobic conditions and in 11 isolates (55%) in anaerobic conditions. Among non-*albicans Candida* spp., biofilm formation was detected in 8 isolates (32%) in aerobic conditions and in 12 isolates (48%) in anaerobic conditions. A significant difference was detected in both *Candida* spp. in terms of biofilm formation according to atmospheric conditions (P = 0.003 and P = 0.046, respectively). The enzyme activities and characteristics of biofilm formation are shown in Table 1.

Genotype and virulence factors: Among the *C. albicans* isolates stratified as genotype A1, 11 (91.7%) expressed phospholipase activity, while all (100%) expressed proteinase activity in both atmospheric conditions. Hemolytic activity was detected in all (100%) aerobic isolates and in 1 anaerobic isolate (8.3%), while biofilm formation was found in 2 aerobic isolates (16.7%) and 7 anaerobic isolates (58.3%). There was a significant difference favoring aerobic condition in terms of hemolytic activity (P = 0.001), while there was a significant difference favoring anaerobic conditions in terms of biofilm formation (P = 0.014). Esterase activity was only detected in 1 isolate (8.3%) in aerobic conditions. Among the isolates stratified as *C. albicans* genotype A2, phospholipase activity was detected in 5 aerobic isolates (71.4%) and 3 anaerobic isolates (42.9%), while proteinase activity was detected in 6 isolates (85.7%) in both conditions. Hemolytic activity was detected only in aerobic conditions in 6 isolates (85.7%), while biofilm formation was detected only

Table 1. The relationship between *Candida* spp. and virulence factors.

Virulence factors	***C. albicans* n (%)**		**non-*albicans Candida* spp. n (%)**		**Total n (%)**	
	Aerobe	Anaerobe	Aerobe	Anaerobe	Aerobe	Anaerobe
Phospholipase	17 (85)	15 (75)	-	-	17 (37.8)	15 (33.3)
Proteinase	19 (95)	18 (90)	*6 (24)	1 (4)	*25 (55.6)	19 (42.2)
Esterase	1 (0.5)	-	-	-	1 (2.2)	-
Hemolysin	*19 (95)	1 (0.5)	22 (88)	17 (68)	*41 (91.1)	18 (40)
Biofilm	2 (10)	*11 (55)	8 (32)	*12 (48)	10 (22.2)	*23 (51.1)

*(*significantly higher)*

in anaerobic conditions in 3 isolates (42.9%). No esterase activity was detected in any of the isolates. There was a significant difference only in hemolytic activity favoring aerobic conditions in terms of hemolytic activity (P = 0.034). In *C. albicans* genotype B was isolated from blood. Hemolytic and proteinase activity were detected only in aerobic conditions, whereas biofilm formation was detected only in anaerobic conditions. In this isolate, phospholipase activity was expressed in both atmospheric conditions, but no esterase activity was expressed (Table 2). No statistical analysis was performed in *C. albicans* belonging to genotype B and non-*albicans Candida* spp. belonging to other genotypes (C-O), as the number of isolates was small.

Discussion

It has been reported that the enzymatic activity of *Candida* spp. may vary depending on the species and source of isolates (21–25). Birinci et al. (21) reported phospholipase activity in aerobic conditions of 61.3% in *C. albicans* strains isolated from sterile samples. In 2 individual studies on isolates from blood, it was noted that 79% and 100% of *C. albicans* isolates produced phospholipase (9,22). Mohan das and Ballal (23) reported that *C. albicans* species isolated from blood produced more phospholipase than did non-*albicans Candida* spp. In 2 individual studies from Turkey on blood culture isolates, phospholipase activity was 60.3% and 100% in *C. albicans* strains, while no such activity was detected in non-*albicans Candida* spp. (24,25). According to the above-mentioned studies, 85% of the *C. albicans* strains produced phospholipase, while there was no such activity in non-*albicans Candida* spp. Sardi et al. (4) found that 94.5% of the *C. albicans* strains isolated from subgingival biofilms of diabetic patients with chronic periodontitis produced phospholipase in a medium with reduced oxygen content; however, none had phospholipase activity in anaerobic conditions. In contrast to that study, we detected that 75% of the *C. albicans* strains expressed phospholipase activity in anaerobic conditions. We think that these discrepancies in results may be due to variations in the sources of isolates and numbers of isolates.

It has been reported that more than 90% of *Candida* isolates produce proteinase (14). In several studies investigating the proteinase production of *C. albicans* in aerobic conditions, Yenişehirli et al. (9) found that 83% of isolates expressed proteinase activity, while Fotedar and Al-Hedaithy (22) reported this rate as 100%. In another study, proteinase production was detected in 74.56% of *Candida* spp. and it was also reported that non-*albicans Candida* spp. produced more proteinase than *C. albicans* strains (23). Gökce et al. (24) found proteinase production at a level of 89.7% in *C. albicans* isolated from blood, whereas it was 25.8% in non-*albicans Candida* spp. According to the above-mentioned studies, proteinase activity was detected in 95% of the *C. albicans* versus 24% of non-*albicans Candida* spp. in aerobic conditions. Fotedar and Al-Hedaithy (22) reported that 88% of *C.*

Table 2. The relationship between *C. albicans* genotypes and virulence factors.

Genotypes of *C. albicans*	Atmospheric conditions	Phospholipase n (%)	Proteinase n (%)	Esterase n (%)	Hemolysin n (%)	Biofilm n (%)
Genotype A1 (n = 12)	Aerobic	11 (91.7)	12 (100)	1 (8.3)	*12 (100)	2 (16.7)
	Anaerobic	11 (91.7)	12 (100)	-	1 (8.3)	*7 (58.3)
Genotype A2 (n = 7)	Aerobic	5 (71.4)	6 (85.7)	-	*6 (85.7)	-
	Anaerobic	3 (42.9)	6 (85.7)	-	-	3 (42.9)
Genotype B (n = 1)	Aerobic	1 (100)	1 (100)	-	1 (100)	-
	Anaerobic	1 (100)	-	-	-	1 (100)
Total (n = 20)	Aerobic	17 (85)	19 (95)	1 (0.5)	*19 (95)	2 (10)
	Anaerobic	15 (75)	18 (90)	-	1 (0.5)	*11 (55)

*(*significantly higher)*

albicans strains isolated from blood expressed strong proteinase activity, while the rate was 25% in our study. Moreover, isolates expressing strong proteinase activity were not affected by atmospheric conditions. Sardi et al. (4) reported that, although 94.5% of *C. albicans* isolates produced proteinase in a medium with reduced oxygen content, none of the isolates expressed proteinase activity in anaerobic conditions. In contrast to the above-mentioned studies, it was found that both *C. albicans* and non-*albicans Candida* spp. expressed proteinase activity in anaerobic conditions. However, enzyme activity took longer to become apparent in anaerobic conditions. Rosa et al. (13) reported that proteinase activity increased in anaerobic conditions in *C. albicans* strains isolated from a patient with chronic periodontitis. In contrast to that study, no difference was found among *C. albicans* strains in terms of proteinase activity according to atmospheric conditions, while proteinase activity decreased in non-*albicans Candida* spp. in anaerobic conditions in our study. The differences among studies may result from variations in the sources of isolates, incubation periods, and methods used to achieve anaerobic conditions.

It has been reported that both *C. albicans* and non-*albicans Candida* spp. express esterase activity (25,26). Rosa et al. (13) reported that esterase activity substantially decreased in anaerobic conditions. In the present study, esterase activity was detected in only 1 *C. albicans* strain in aerobic conditions, whereas no esterase activity was detected in anaerobic conditions.

Another characteristic regarded as a virulence factor is hemolysin production in *Candida* spp. Yenişehirli et al. (9) demonstrated that all *C. albicans* strains isolated from various clinical samples had hemolytic activity in aerobic conditions, as being beta hemolysis in all *C. albicans* strains. In another study, it was found that 98.5% of all *C. albicans* isolates expressed hemolytic activity including alpha, beta, and gamma hemolysis in both anaerobic conditions and in conditions with reduced oxygen. It was also reported that alterations in atmospheric conditions had no effect on the hemolytic activity of *C. albicans* strains (4). Rosa et al. (13) stated that the hemolytic activity of *C. albicans* strains substantially decreased in anaerobic conditions. According to the study by Yenişehirli et al. (9), all the isolates produced were beta-hemolysis type. Similar to the study by Rosa et al. (13), a significant decrease was detected in the hemolytic activity of *C. albicans* strains in anaerobic conditions.

Biofilm formation is one of the most extensively investigated virulence factors of *Candida* spp.

Gültekin et al. (25) reported that no biofilm formation was detected in any *C. albicans* strains by microplate method, while it was found in 50% of non-*albicans Candida* spp. Demirbilek et al. (27) detected that the biofilm formation rate was higher in non-*albicans Candida* spp. than in *C. albicans* strains by the microplate method. According to the above-mentioned studies, the biofilm formation rate was higher in non-*albicans Candida* spp. compared to *C. albicans* isolates in aerobic conditions. In our study the biofilm formation rates were found to be higher in both species in anaerobic conditions.

Sardi et al. (4) found that the *C. albicans* genotypes A and B had higher phospholipase and proteinase activity in conditions with reduced oxygen content compared to aerobic conditions and that genotype A expressed higher phospholipase activity compared to genotype B. Moreover, the authors emphasized that genotype A was more virulent than genotype B. In our study, neither phospholipase and proteinase activity was affected by atmospheric conditions in *C. albicans* genotype A1 and genotype A2. In addition, hemolytic activity increased in aerobic conditions, while there was increased biofilm formation in anaerobic conditions. Furthermore, no difference was found in virulence factors evaluated between genotypes A1 and A2. In our study, the number of *C. albicans* isolates belonging to genotype B was smaller; thus no comparison was performed between genotypes A and B.

There are some limitations to our study: these include the limited number of isolates and isolates from different species.

In conclusion, it was suggested that the virulence of *Candida* spp. may be affected by type of species and amount of oxygen in the media. It is thought that further comprehensive studies with a larger sample size are needed to identify the relationship between *Candida* genotypes and virulence factors investigated.

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