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ORIGINAL ARTICLE

Investigation of possible virulence factors in *Candida* strains isolated from blood cultures

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Abstract

Background: The *Candida* species, which are one of the most common causes of nosocomial bloodstream infections, present with high mortality and morbidity rates. This study aims to investigate the production of esterase, phospholipase, proteinase, and biofilm formation ability of the *Candida* strains isolated from the blood cultures.

Materials and Methods: Between June 2011 and July 2012, the *Candida* strains, which were isolated from blood cultures of a total of 50 patients, were studied. The esterase activity was analyzed in the Tween-80 agar, while phospholipase activity was studied in the egg yolk agar. The proteinase activity and biofilm formation were identified by using the petri dish method and microplate method, respectively.

Results: Of 50 specimens obtained from individual patients, 17 (34%) were identified as *C. albicans*, 14 (28%) as *C. glabrata*, 9 (18%) as *C. parapsilosis*, 5 (10%) as *C. krusei*, 4 (8%) as *C. kefyr*, and 1 (2%) as *C. tropicalis*. The rate of proteinase, phospholipase, and esterase positivity was higher in the *C. albicans* isolates. Biofilm formation was the highest in the *C. parapsilosis* strains.

Conclusions: Higher rate of virulence factors in the most commonly isolated *Candida* species than other species indicates that these virulence factors play a crucial role in the pathogenesis.

Key words: Biofilm, blood culture, candidemia, esterase, phospholipase, proteinase

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Introduction

In the recent years, the incidence of serious fungal infections, invasive *Candida* infections particularly, has been increasing due to an increased number of patients receiving immunosuppressive therapy, increased major surgeries and broad-spectrum antibiotherapy, hyperalimentation, prolonged intensive care unit stay for patients with poor health status, and increased use of artificial prostheses.[1,2] The *Candida* species are the fourth most common cause of nosocomial bloodstream infections, accounting for nearly 10% of all. It follows *coagulase-negative staphylococci*, *Staphylococcus aureus*, and enterococci with high morbidity and mortality rate.[2-4] Virulence factors as well as attenuated defense mechanism of the host play a critical role in the development of these infections. Extra-cellular hydrolytic enzymes of *Candida* species facilitate adherence and tissue penetration, and therefore invasion of the host.[5] However, biofilm producing *Candida* species are known to be more resistant to immune response and antimicrobial agents, which lead to treatment failure.[6] Although there is a growing number of studies reporting virulence factors of other strains, *C. albicans* is the most commonly analyzed strain in which several virulence factors including phenotypic changes, morphological dimorphism, adhesion molecules, hydrolytic enzymes (e.g. phospholipase, lipase, and aspartic proteinases), catalase, superoxide dismutase, and heat-shock proteins have been identified.[7,8]

In this study, we aimed to investigate the production of esterase, phospholipase, proteinase, and biofilm formation ability of the *Candida* strains isolated from the blood cultures.

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Materials and Methods

Definition of species

Between June 2011 and July 2012, the *Candida* strains, which were isolated from blood cultures of a total of 50 patients, were studied. The blood samples which were sent from various departments of our hospital to the central laboratory were incubated in the BacT/Alert 3D Automation System (Biomerieux, France). Some preparations were done using the blood samples with a hint for bacterial production, and those with yeast cells as confirmed by Gram-staining were passaged to the CHROMagar *Candida* (BBL, France) and Sabouraud dextrose agar (Oxoid, UK). The isolates were identified by the germ tube test results; morphological images obtained from the Tween-80 corn-meal agar, and API 20C AUX system (Biomeriuex, France).

Investigation of virulence factors

The phospholipase activity was studied in the egg yolk agar. 10 μ yeast suspension was added at 0.5 McFarland turbidity and incubated at 37°C for 4 days. The ratio of colony diameter to the diameter of precipitation zone was calculated (precipitation zone; Pz). With respect to the phospholipase activity, Pz: ++++ or ≤0.69 = very strong, + + + or 0.70–0.79 = strong, ++ or 0.80–0.89 = medium, + or 0.90–0.99 = weak positive. Pz ≥ 1: Negative.[7,9]

The esterase activity was studied using an agar containing 1% peptone, 0.5% NaCl, 0.01% $CaCl_2$, and 1.5%/L at 6.8 pH. When heated at 45–50°C, 5 mL Tween-80 was added. The round-shaped strains with 10-mm in diameter were incubated at 30°C for 10 days. The presence of a transparent halo around the inoculum site indicated positivity.[7,10]

The proteinase activity was analyzed by using an agar containing 0.1% KH_2PO_4, 0.05% $MgSO_4$, 2% agar, and 1% bovine serum albumin per liter at 4.5 pH. 10 μL yeast suspension was added and a transparent zone around the colony was investigated.[9]

The biofilm formation ability of the isolates was identified by using the microplate method. The brain heart infusion broth (BHIB; Oxoid, UK) containing 0.25% glucose was prepared and put into 2-mL tubes. A loop full of each isolate was added and incubated at 37°C for 24 h. Then, each tube was diluted at 1:40 with a fresh BHIB. The final solution was seeded into a 96-well polystyrene microplate (Nunc, Denmark) and incubated at 37°C for 24 h. At the end of the incubation period, it was washed with phosphate buffer solution 3 times, inverted, and dried. 200 μL of crystal violet 1% was added and standard for 15 min. At the end of the incubation period, it was washed 3 times, and 200 μL mixture of ethanol-acetone (80:20 v/v) was added to each well. It was studied by the enzyme-linked immunosorbent assay kit (BioTek, ELX 800, USA) at 450 nm wavelength. The optic density (OD) of the well was recorded. A sterile BHIB, which contained no microorganism, was identified as a negative control. The mean OD of sterile BHIB wells was calculated, and the cut-off value was estimated using + 2 standard deviations. The samples which produced OD above the cut-off value were considered as positive, while samples with OD below the cut-off value were considered as negative.[11]

Candida albicans ATCC 90028 was used as a control stain in the phospholipase, esterase, and proteinase tests. All tests were repeated 3 times for each strain. Statistical analysis was performed using SPSS (SPSS Inc., Chicago, IL, USA) version 11.5 software. The Chi-square test was also done. $P < 0.05$ was considered as statistically significant.

Results

A total of 50 patients were included in the study. Then median age of the patients was 50 years and 32 (64%) were male, and 18 (36%) were female. Most patients were from the intensive care units (27/50; 54%), 18/50 (36%) from medical wards and 5/50 (10%) from surgical wards. At least one underlying medical condition was identified in 47 cases of candidemia (94%). The most frequent underlying disease in patients with candidemia was cancer (38%), especially solid tumors (28%). Lung disease and acute/chronic kidney failure were the 2nd and the 3rd most frequent underlying disease, respectively (18% and 14%, respectively). Only three patients had diabetes (6%).

Of 50 specimens obtained from different patients, 17 (34%) were identified as *C. albicans*, 14 (28%) as *C. glabrata*, 9 (18%) as *C. parapsilosis*, 5 (10%) as *C. krusei*, 4 (8%) as *C. kefyr*, and 1 (2%) as *C. tropicalis*. Eleven (64.7%) of the *C. albicans* strains had positive esterase activity, 14 (82.4%) had positive proteinase activity, 15 (88.2%) had positive phospholipase activity, and 1 (5.9%) had positive biofilm

Table 1: Esterase, phospholipase, and proteinase activity and biofilm formation ability of the *Candida* species

Candida spp. (*n*)	Positivity (%)					
	Esterase	Proteinase	Phospholipase			Biofilm
			VS	S	M/W	
C. albicans (17)	11 (64.7)	14 (82.4)	5 (29.4)	5 (29.4)	5 (29.4)	1 (5.9)
C. glabrata (14)	1 (7.1)	4 (28.6)	0 (0)	4 (28.6)	1 (7.1)	0 (0)
C. parapsilosis (9)	0 (0)	4 (44.4)	0 (0)	0 (0)	1 (11.1)	7 (77.7)
C. krusei (5)	2 (40)	2 (40)	0 (0)	1 (20)	1 (20)	0 (0)
C. keyfr (4)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
C. tropicalis (1)	1 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Total (50)	15 (30)	24 (48)	5 (10)	10 (20)	8 (16)	8 (16)

VS=Very strong; S=Strong; M/W=Medium/weak; *C. albicans=Candida albicans; C. glabrata=Candida glabrata; C. parapsilosis=Candida parapsilosis; C. krusei=Candida krusei; C. keyfr=Candida keyfr; C. tropicalis=Candida tropicalis*

formation. In addition, 1 (7.1%) of the C. *glabrata* strains had positive esterase activity, 4 (28.6%) had positive proteinase activity, and 5 (35.7%) had positive phospholipase activity. However, biofilm formation was not identified in this strain. The rate of proteinase, phospholipase, and esterase positivity was statistically higher in the C. *albicans* isolates than non-*albicans Candida* isolates ($P < 0.001$). Biofilm formation was the highest in the C. *parapsilosis* strains, indicating statistical significance ($P < 0.001$). However, no virulence factor was identified in the C. *kefyr* strains [Table 1].

Discussion

In parallel to the increasing number of high-risk immunocompromised patient population, the *Candida* species has become particularly important as one of the most common causes of nosocomial bloodstream infections.[12] The C. *albicans* is the most isolated pathogen in the *Candida* species-associated bloodstream infections, followed by C. *parapsilosis*, C. *glabrata*, C. *krusei,* C. *tropicalis* with an increasing incidence.[13,14] Despite varying incidence according to the region, C. *glabrata* and C. *parapsilosis* may be a cause of candidemia.[15,16] In our study, C. *albicans* (34%) is the leading cause of candidemia, followed by C. *glabrata* (28%).

Extra-cellular hydrolytic enzymes of *Candida* species acting as virulence factors which contribute to host-tissue invasion by digesting proteins (hemoglobin, keratin, collagen, etc.)[17] formation of *Candida* biofilms occurring on devices such as indwelling intravascular catheters, infections become more refractory to antimicrobial agents.[18] Earlier studies demonstrated that C. *albicans* produced extra-cellular phospholipase alone. However, further studies showed that non-*albicans Candida* produced also phospholipase but at a lesser extent.[19] In the present study, we found that 15 (88.2%) of 17 C. *albicans* strains and only 8 (24.2%) of 33 non-*albicans Candida* species (C. *glabrata* [$n = 5$], C. *krusei* [$n = 2$], and C. *parapsilosis* [$n = 1$]) had positive phospholipase activity. Birinci *et al*.[20] reported that 42 (67.7%) of 62 C. *albicans* strains isolated from the blood samples had phospholipase activity. However, the authors reported no phospholipase activity in the non-*albicans Candida* species. Similarly, De Luca *et al*.[21] showed that 32 (100%) of the C. *albicans* strains and only one of the C. *parapsiolosis* strains had phospholipase activity. Another study revealed that 41 of the C. *glabrata* strains, 50% of the C. *parapsilosis* strains, 70% of the C. *tropicalis*, 80% of the C. *lusitaniae*, and 10% of the C. *krusei* had phospholipase activity. The authors, however, concluded that relatively low rates were associated with small sample size of the *Candida* strains ($n = 51$).[22] In our study, using a similar sample size with the aforementioned study, we found 35.7% of the C. *glabrata* strains, 40% of the C. *krusei* strains, and 11.1% of the C. *parapsilosis* strains with phospholipase activity.

Furthermore, we observed that 11 (64.7%) of 17 C. *albicans* and 4 (12.1%) of 33 non-*albicans Candida* (C. *glabrata* [$n = 1$], C. *krusei* [$n = 2$], and C. *tropicalis* [$n = 1$]) had esterase activity. Review of literature has demonstrated that C. *albicans* and C. *tropicalis* are the most common strains exerting esterase activity.[7,10,23,24] Yücesoy and Marol[23] reported that 95% of the C. *albicans*, 93% of the C. *tropicalis*, 57% of the C. *parapsilosis*, and 10% of the C. *glabrata* had esterase activity. In addition, Aktas *et al*.[24] found that 98.3% of the C. *albicans*, and 100% of the C. *tropicalis* and C. *guilliermondii* had esterase activity. In our study, we observed a lower rate of esterase activity in the C. *albicans* strains along with one of the C. *tropicalis* strains with positive activity. We also found esterase activity in 40% of the C. *krusei* and 7.1% of the C. *glabrata*. However, none of the C. *parapsilosis* and C. *keyfr* had esterase activity.

Moreover, we reported that 82.4% of the C. *albicans* and 30.3% of the non-*albicans Candida* had proteinase activity. Consistent with our findings, Gokce *et al*.[17] showed proteinase activity in 89.7% of the C. *albicans* and 25.2% of the non-*albicans Candida* of the isolated from blood cultures. Similarly, Bramono *et al*.,[25] reported a significantly higher rate of proteinase activity in the C. *albicans* strains, followed by the C. *parapsilosis* C. *tropicalis*. In our study, the C. *albicans* had the highest proteinase activity, followed by C. *parapsilosis* (44.4%), C. *krusei* (40%), and C. *glabrata* (28.6%).

Based on our study results, only 1 (5.9%) of the C. *albicans* and 77.7% (7/9) of the C. *parapsilosis* strains had the biofilm formation ability. On the other hand, Gültekin *et al*.[7] had reported that none of the C. *albicans* isolates had the biofilm formation ability with the highest positivity ratio of the C. *parapsilosis* strains. In addition, Shin *et al*.[26] also showed that the C. *parapsilosis* had the highest ratio for the biofilm formation, followed by the C. *albicans*. The authors concluded that there was a significant difference in the biofilm formation ability between the C. *parapsilosis* strains isolated from the blood samples and other samples.

Conclusion

We suggest that inconsistent results of previous studies in respect of the phospholipase, proteinase, and esterase activity as well as the biofilm formation ability of the *Candida* species can be attributed to the nonstandardized sample size including the number of strains, patient characteristics, regional variations, and methodological differences. Higher rate of virulence factors in the most commonly isolated *Candida* species than other species indicates that these virulence factors play a crucial role in the pathogenesis. However, further studies in a higher number of strains of a limited number of species are required to confirm these findings.

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