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Acid proteinase, phospholipase, and biofilm production of *Candida* species isolated from blood cultures

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Abstract Three virulence factors comprising proteinase, phospholipase, and biofilm among 68 *Candida albicans* and 31 non-albicans *Candida* strains (11 *C. tropicalis*, 8 *C. parapsilosis*, 6 *C. glabrata*, 4 *C. guillermondii*, 2 *C. krusei*) isolated from blood cultures were analyzed. In total, 61 (89.7%) *C. albicans* strains were detected as proteinase positive whereas eight (25.8%) non-albicans *Candida* strains were proteinase positive ($P < 0.05$). Phospholipase production was detected in 41 (60.3%) *C. albicans* strains. All non-albicans *Candida* strains were phospholipase negative. Biofilm production was determined by both visual and spectrophotometric methods. Eight (11.8%) of *C. albicans* strains and 13 (41.93%) of 31 non-albicans *Candida* strains were biofilm positive with two of the methods ($P < 0.05$). According to our results, we may suggest that detection of hydrolytic enzyme and biofilm production abilities of the *Candida* isolates in clinical mycology laboratories may warn the clinican for a possible hematogenous infection.

Keywords Virulence · *Candida* · Blood

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Introduction

Since the early 1980s the frequency of nosocomial yeast infections has increased dramatically, with a consequent rise in related mortality and prolonged hospitalizations [1]. According to the results of the National Nosocomial Infections Surveillance System surveys, *Candida* species have become the fourth most common isolate recovered from blood cultures in the US and rates of candidemia have increased substantially as well in Europe [2]. Although *C. albicans* is the most commonly isolated organism from blood cultures, other *Candida* species have emerged as clinically important pathogens [3].

Several *Candida* species are normal inhabitants of human skin or mucosal surfaces and changes in the host are generally required before diseases can be established and progress. *Candida* species can respond rapidly to environmental changes, and this flexibility could allow these organisms to take advantage of impaired immunity and facilitate establishment of disease. Multiple characteristics of *Candida* species have been proposed to be virulence factors that enable the organism to cause disseminated infections in susceptible hosts. The ability to recognize and adhere to host tissues, to respond rapidly to changes in the external environment and to secrete enzymes are all thought to be important in virulence [4]. There are few reports comparing virulence factors of *Candida* species isolated from blood [5–7]. To our knowledge, this is the first study

analyzing three virulence factor including proteinase, phospholipase, and biofilm production among *Candida* strains isolated from blood samples.

Materials and methods

Identification of isolates

Candida species isolated from blood cultures of patients admitted to Marmara University Hospital were included in the study. The Center for Disease Control (CDC) definition for blood stream infection (BSI) was used and detection of *Candida* in at least one blood culture specimen was considered to represent candidemia [8]. For each patient only one strain was analyzed. Yeast growth was confirmed by observation of typical cells on Gram stained preparates and all isolates were named with germ tube test and carbohydrate assimilation reactions (ID32C, Biomerioux, France).

Testing protease activity

A yeast suspension of an each test strain was made in a broth of yeast extract, peptone, and glucose (YEPD). From this suspension, a 10 µl sample was put on a sterile paper disk placed on the surface of bovine serum albumin agar medium (pH 5.0) as described previously [9]. The inoculated plates were incubated at 30°C for 6 days. The plates were observed each day for an increasing opacity around the disks caused by growing fungi. Subsequently, clearing of the opacity by hydrolysis of precipitated albumin was affected by acid protease of the fungi; this was recorded. The millimetric zone measurements were evaluated as negative (–) for no clearance, (+) for mild activity (a lysis zone 1–2 mm around the zone), and (++) for strong activity (a lysis zone of 3–5 mm around the disk). The *C. albicans* CBS 2730 strain was used as positive control and the experiment was carried on three different occasions.

Testing phospholipase activity

About 10 µl of droplets of yeast suspension in sterile saline were placed on the surface of agar medium (pH 4.3) containing egg yolk at 37°C for 4 days [10]. Phospholipase activity was measured by dividing colony diameter by the diameter of the precipitation zone (pz) around the colony formed on the plate. A pz of 1.0 was evaluated as negative (–), 0.99–0.9 as weak (+), 0.89–0.8 as mild (++), 0.79–0.7 as relatively strong (+++) and <0.69 (++++) as very strong positive. The *C. albicans* SC 5314 strain was used as positive control and the experiment was carried on three different occasions.

Determination of biofilm formation

Visual detection

A loopful of organisms from the surface of Sabouraud dextrose agar (SDA) plate was inoculated into a polystyrene tube (Falcon conical tube with a screw cap [Becton Dickinson]) containing 10 ml of Sabouraud dextrose broth (SDB) supplemented with glucose (final concentration, 8%). After incubation at 35°C for 48 h, the broth in the tubes were aspirated gently, and tubes were washed with distilled water twice and than stained with 1% safranin for 10 min, after which they were examined for the presence of an adherent layer. Biofilm production was scored as negative, weak (+), moderate (++), or strong (+++) positive [6, 11]. Biofilm producer *Staphylococcus epidermidis* ATCC 35984 was used as positive control and each isolate was tested at least three times and read independently by two different observers.

Spectrophotometric detection

Organisms were grown at 35°C for 24 h on SDA plates and saline washed suspensions of each strains were prepared. The turbidity of each suspension was adjusted to 3×10^7 cfu/ml with SDB supplemented with glucose (final concentration, 8%). Next, 1 ml of suspension was inoculated into a polystyrene tube containing 9 ml of SDB and each well of microtitration plates was inoculated with aliquots of 200 µl of yeast cell suspension, plates were then incubated at 35°C for 24 h without agitation. The microtiter plate was then washed for four times with phosphate buffered saline, stained with 1% safranin, aspirated,

and spectrophotometer readings were performed at 490 nm with a micro titer plate reader [6]. The percent transmittance (%*T* value) for each test sample was subtracted from the %*T* value for the reagent blank to obtain a measure of the amount of light blocked when passing through the wells ($\%T_{bloc}$). Biofilm production by each isolate was scored as either negative ($\%T_{bloc}$, <5), + ($\%T_{bloc}$, 5–20), ++ ($\%T_{bloc,}$ 20–50), +++ ($\%T_{bloc}$, ≥50). Each isolate was tested at least three times.

Statistics

The differences for acid proteinase, phospholipase, and biofilm production capabilities of *C. albicans* and non-albicans *Candida* strains were determined by chi-square and Fisher's exact tests. *P* value <0.05 was considered to be significant.

Results and discussion

Bloodstream infections due to *Candida* spp. have become an important cause of morbidity and mortality among patients and a number of studies have documented the increased incidence of systemic candidiasis in recent decades. Although *C. albicans* remains as the most common cause of fungemia, there has been an increase in infections due to other *Candida* species [12, 13].

Candida species have the ability to produce virulence factors that enhance their capacity to colonize mucosal or synthetic surfaces and to invade host tissues by disrupting host-cell membranes. Proteinases and phospholipases acting as virulence factors which contribute to host-tissue invasion by digesting proteins such as hemoglobin, keratin, collagen, and by degrading cell membranes, respectively. There are several reports indicating invasive strains of *C. albicans* produced significantly more extracellular enzyme activity than the commensal strains did [5, 9, 10, 14, 15]. Since the number of studies in other *Candida* species is limited we compared the positivity of these enzymes in different *Candida* species. *Candida albicans* (*n*: 68) and non-albicans *Candida* strains (*n*: 31) isolated from blood cultures of adults (*n*: 66) and pediatric (*n*: 33) patients were analyzed. Non-albicans *Candida* strains were distributed as follows: 11 (35.4%) *C. tropicalis*, eight (25.8%) *C. parapsilosis*, six (19.3%) *C. glabrata*, four (12.9%) *C. guillermondii* and two (6.4%) *C krusei*. Virulence factor positivity of the strains was given in Table 1. In total, 61 (89.7%) *C. albicans* strains and only eight (25.8%) non-albicans *Candida* strains were proteinase positive ($P < 0.05$) (Table 2). Phospholipase production was detected in 41 (60.3%) *C. albicans* strains with strong positivity in 28 (68.29%) of them whereas all non-albicans *Candida* strains were phospholipase negative. We detected higher levels of proteinase activity in *C. albicans* strains than non-albicans *Candida* strains (89.7% vs. 25.8%, $P < 0.05$) which is consistent with the

Table 2 Comparison of virulence factors among blood *Candida* isolates

Virulence factor	*C. albicans* (*n*: 68) *n* (%)	Non-albicans *Candida* spp (*n*: 31) *n* (%)	*P*
Proteinase	61 (89.7)	8 (27.6)	<0.05
Phospholipase	41(60.3)	0 (100)	*
Biofilm[a]	8 (11.8)	13 (41.93)	<0.05
Biofilm[b]	8 (11.8)	12 (38.71)	<0.05

[a] Tube adherence method

[b] Microtiter plate method

* Since all non-albicans isolates were negative statistical analysis could not be performed

Table 1 Virulence factor positivity among blood *Candida* isolates

Species	Proteinase (*n*)			Phospholipase (*n*)					Biofilm[a] (*n*)				Biofilm[b] (*n*)			
	(–)	(+)	(++)	(–)	(+)	(++)	(+++)	(++++)	(–)	(+)	(++)	(+++)	(–)	(+)	(++)	(+++)
C. albicans (*n*: 68)	7	18	43	27	3	10	3	25	60	1	1	6	60	2	1	5
Non-albicans *Candida* spp (*n*: 31)	23	4	4	31	0	0	0	0	18	1	2	10	19	0	2	10

[a] Tube adherence method

[b] Microtiter plate method

findings of Ozkan et al. (40% vs. 10%, $P < 0.05$) [16]. Phospholipase positivity among non-albicans *Candida* strains were reported in few studies [17–19]. However, none of non-albicans *Candida* strains were phospholipase producers in our study, while 60.3% of *C. albicans* were found to be positive.

Although bacterial biofilms and their role in diseases have been investigated in details over a number of years much less are known about fungal biofilms. Recent studies indicate that with the formation of *Candida* biofilms occurring on devices such as indwelling intravascular catheters, infections become more refractory to conventional therapy because of resistance to antimicrobials [20]. We determined biofilm production by tube adherence and microtiter plate methods (Table 1). Eight (11.8%) of *C. albicans* strains were detected as biofilm positive with either methods. About 13 (41.93%) of non-albicans *Candida* strains by tube adherence and 12 (38.71%) by microtiter plate method were found to be positive. The differences between biofilm positivity among *C. albicans* and non-albicans *Candida* strains were significant by two of the methods ($P = 0.006$, $P = 0.011$), Table 2. All *C. krusei* and *C. guillermondii*, 45% of *C. tropicalis*, 16% of *C. glabrata*, 12.5% of *C. parapsilosis* strains were biofilm positive by tube adherence method. No major discrepancy was observed between methods (except 1 *C. guillermondii* which was biofilm positive by tube adherence but not by microtiter plate method). Branchini et al. [11] showed that 80% of 31 *C. parapsilosis* blood isolates produced slime in vitro and suggested that capacity of producing large amounts of viscid slime material in glucose containing solutions may contribute to the ability of this organism to adhere plastic catheters and cause infections in individuals receiving total parenteral nutrition where the glucose concentration of the solution being administered is usually high. Authors investigating biofilm production among *Candida* species detected higher levels of positivity among non-albicans *Candida* strains than that of *C. albicans* strains (51–61% vs. 8–12%) [6, 7].

We may conclude that in the pathogenesis of hematogenous candida infections, proteinase and phospholipase activity for *C. albicans* strains and biofilm production for non-albicans *Candida* strains appear to be important virulence factors. The role of these factors in morbidity and mortality of the patients remains unclear. Cerikcioglu et al. suggested exhibition of strong hydrolytic enzyme activities for long duration of colonization might contribute to the development of candidemia in preterm infants [21]. We believe that determination of these factors might be a helpful tool to inform the clinicians about the possible virulence of the strain.

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