Original

Proteinase and phospholipase activity as virulence factors in *Candida* species isolated from blood

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Summary

The number of nosocomial blood stream infections due to *Candida* species has increased over the past few decades. In order to establish an infection, opportunistic pathogens have to evade the immune system, survive, divide in the host environment, and spread to other tissues. Proteinase and phospholipase secretion has been implicated as potential virulence factors for some *Candida* species responsible for catheter related candidemia in intensive care unit (ICU) patients with indwelling devices. We therefore have aimed at demonstrating the secretion of proteinase and phospholipase enzymes as virulent factors by *Candida* species isolated from blood samples collected from ICUs, dialysis units and oncology units. One hundred and fourteen isolates of *Candida* species were obtained from the blood samples and the isolates include 37 *Candida albicans*, 7 *Candida glabrata,* 5 *Candida guilliermondii*, 3 *Candida kefyr*, 45 *Candida krusei*, 5 *Candida parapsilosis*, and 12 *Candida tropicalis.* Proteinase assay was performed by using the Staib method. Phospholipase assay was performed by using the method of Samaranayake et al. Precipitation zone (Pz value) was determined. The percentage of isolates which produced detectable amounts of proteinase is 74.56% and 44.73% of isolates produced detectable amounts of phospholipase. We believe that production of both phospholipase and proteinase enzimes could be an important virulence factor for several *Candida* species.

Key words *Candida* spp., Virulence factor, Proteinase, Phospholipase

Actividad proteinasa y fosfolipasa como factores de virulencia en especies de *Candida* aisladas de sangre

Resumen

El número de fungemias nosocomiales debidas a especies de *Candida* ha aumentado en las últimas décadas. Para producir una infección, los patógenos oportunistas tienen que evadir el sistema inmunológico, sobrevivir y dividirse en los tejidos del huésped y diseminarse a otros órganos. La secreción de proteinasa y fosfolipasa ha sido descrita como factor potencial de virulencia de algunas especies de *Candida* responsables de candidemias en pacientes con catéteres ingresados en las unidades de cuidados intensivos. En este trabajo se ha tratado de demostrar que la secreción de los enzimas proteinasa y fosfolipasa son factores de virulencia de especies de *Candida* aisladas de hemocultivos de pacientes admitidos en las unidades de cuidados intensivos, unidades de diálisis y unidades de oncología. Ciento catorce aislamientos de *Candida* fueron obtenidos de muestras de sangre; se identificaron las siguientes especies: *Candida albicans* (37), *Candida glabrata* (7), *Candida guilliermondii* (5), *Candida kefyr* (3), *Candida krusei* (45), *Candida parapsilosis* (5) y *Candida tropicales* (12). La detección de proteinasa fue realizada utilizando el método de Staib, y la de fosfolipasa utilizando el método de Samaranayake et al.
Se determinó la zona de precipitación (valor de Pz). El porcentaje de aislamientos que produjo cantidades perceptibles de proteinasa fue 74,56%; los aislamientos productores de fosfolipasa fueron el 44,73%. En el presente estudio se pudo observar que la producción de proteinasa y fosfolipasa puede ser un factor importante de virulencia en las fungemias causadas por *Candida* independientemente de la especie que haya sido aislada.

Palabras clave Especies de *Candida,* Factor de virulencia, Fosfolipasa, Proteinasa

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Aceptado para publicación el 23 de julio de 2008

1130-1406/01/10.00 €

Candida is the fourth most common cause of blood stream infection in hospital patients [3,10,12,21]. In order to colonize, infect and evade host defense mechanisms, *Candida* possesses a repertoir of virulence attributes which includes cell morphology, adhesion factors, phenotypic switching and extra cellular lipolytic and proteolytic activity [6,11,20]. The virulence factors expressed or required by *Candida* to cause infections may vary, depending on the type of infection, stage of infection, the site of infection, and the nature of the host response [9].

The secreted aspartic proteinases (Saps) are responsible for the adhesion, tissue damage and invasion of host immune responses. Their proteolytic activity has been associated with tissue invasion [7]. The secretion of extra cellular phospholipases is considered a key attribute that aids invasion of the host mucosal epithelia. The phospholipases, in general, catalyse the hydrolysis of phospholipids, which are major components of all cell membranes [8].

Materials and methods

Blood samples were collected from the intensive care units (ICUs), dialysis units, and oncology units in the hospitals and nursing homes in and around Bangalore. The samples were collected from patients who have had had no antifungal drug exposure during hospitalization. A total of 114 isolates of *Candida* was recovered from the blood samples. Isolates were identified by germ tube formation, chlamydospore formation, sugar fermentation, and assimilation patterns. Comparison study was done using standard strains provided by P.G.I.M.E.R. (Chandigarh, India). Proteinase assay and phospholipase estimation was determined for all the isolates.

Proteinase detection. *Candida* proteinase was detected by the slightly modified Staib method [17] using bovine serum albumin medium (dextrose 2%, KH_2PO_4 0.1%, $MgSO_4$ 0.05%, agar 2% mixed after cooling to 50 ºC with 1% bovine serum albumin solution). Proteinase activity was detected by inoculating 10 µl aliquots of the yeast suspension (approximately 10^8 yeast cells/ml) into the wells punched onto the surface of the medium. The plates were incubated at 37 ºC for two days. After incubation, the plates were fixed with 20% trichloracetic acid and stained with 1.25% amidoblack. Decolourization was performed with 15% acetic acid. Opaqueness of the agar, corresponding to a zone of proteolysis around the wells that could not be stained with amidoblack, indicated degradation of the protein. The diameter of unstained zones around the well was considered as a measure of proteinase production. The proteinase activity (Pz) was determined in terms of the ratio of the diameter of the well to the diameter of the proteolytic unstained zone [18]. When the Pz equaled 1, no proteinase activity was detected in the strain. Thus, a low Pz indicated high production of the enzyme.

Phospholipase estimation. The isolates were screened for their extracellular phospholipase activity by growing them on egg-yolk agar and measuring the size of the zone of precipitation by the slightly modified method of Samaranayake et al. [15]. Briefly, the egg-yolk medium consisted of 13 g Sabouraud dextrose agar (SDA), 11.7g NaCl, 0.111g $CaCl_2$ and 10% sterile egg yolk. The egg yolk was centrifuged at 500 *g* for 10 min at room temperature, and 20 ml of the supernatant was added to the sterilized medium. Extracellular phospholipase activity was detected by inoculating 10 µl aliquots of the yeast suspension (approximately 10^8 yeast cells/ml) into the wells punched onto the surface of the egg-yolk medium. The diameter of the precipitation zone around the well was measured after incubation at 37 ºC for 48 h. Phospholipase activity (Pz value) was determined by the ratio of the diameter of the colony to the total diameter of the zone of precipitation [18]. When the Pz equaled 1, no phospholipase activity was detected in the strain. Thus, again, a low Pz indicated high production of the enzyme.

Results

Included in the one hundred and fourteen *Candida* species isolated from the blood samples were *C. albicans* (37), *C. glabrata* (7), *C. guilliermondii* (5), *C. kefyr* (3), *C. krusei* (45), *C. parapsilosis* (5) and *C. tropicalis* (12). All the isolates were tested for proteinase and phospholipase production. Proteinase activity was detected in 85 (74.56%) isolates and phospholipase activity was detected in 51 (44.73%) isolates (Tables 1 and 2). The isolates tested demonstrated varying degrees of phospholipase activity (Pz value: 0.23-1.0), with most significant phospholipase activity. The Pz value for proteinase activity ranged from 0.17 and 1.0. The highest level of enzymatic activity was for Pz values near zero. ANOVA was used to compare the production of proteinase and phospholipase in different organisms. There was no significant difference between the organisms with respect to mean proteinase and phospholipase production ($p > 0.05$).

Discussion

For the past several years yeasts in the genus *Candida* continue to be amongst the most important etiologic agents of nosocomial infection [13]. The considerable increase in deep-seated candidosis is most commonly observed in patients in ICUs, those with indwelling catheters or receiving oncological treatment, organ transplant recipients, and other immunocompromised individuals subjected to heavy therapeutic protocols. The isolation of non-*C. albicans* has been frequently cited in past few decades [16] and our results agree with this finding. Of the

Table 1. Proteinase activity (mm) exhibed by *Candida* spp. isolated from blood.

Candida spp.	N	Mean	Std. deviation	Minimum	Maximum
C. albicans	37	0.55	0.36	0.20	1.00
C. glabrata	7	0.43	0.26	0.24	1.00
C. guilliermondii	5	0.56	0.40	0.19	1.00
C. kefyr	3	0.59	0.37	0.27	1.00
C. krusei	45	0.42	0.25	0.20	1.00
C. parapsilosis	5	0.38	0.35	0.20	1.00
C. tropicalis	12	0.52	0.37	0.17	1.00

Table 2. Phospholipase activity (mm) exhibed by *Candida* spp. isolated from blood.

Candida spp.	N	Mean	Std. deviation	Minimum	Maximum
C. albicans	37	0.66	0.34	0.23	1.00
C. glabrata	7	0.73	0.35	0.24	1.00
C. guilliermondii	5	0.86	0.31	0.30	1.00
C. kefyr	3	0.59	0.37	0.27	1.00
C. krusei	45	0.73	0.33	0.24	1.00
C. parapsilosis	5	0.88	0.27	0.40	1.00
C. tropicalis	12	0.68	0.35	0.23	1.00

114 isolates 37 were *C. albicans* and 77 were non *C. albicans*. As phospholipases and aspartyl proteinases of *C. albicans* are considered important virulence factors [1], the absence or lowered expression of these enzymes may indicate the less virulent nature of *Candida* species, when compared with *Candida* species with higher expression of these enzymes [2,5]. Eighteen of *C. albicans* tested were phospholipase producers. However, few strains of *C. krusei*, *C. tropicalis* and *C. glabrata* behaved in the same way. *Candida albicans, C. kefyr, C. parapsilosis* and *C. tropicalis* showed almost similar proteinase activity. Our result also indicate that even though all the isolated strains were pathogenic, not all strains of *Candida* produced proteinase and phospholipase as virulent factors. The virulence of *Candida* species is attributed not to a single factor but to a combination of several factors [4], like proteinase, phospholipase [8], biofilm production [19], etc.

The results of our study agrees with those of Ibrahim et al. [8] by demonstrating that *C. albicans* strain isolated from the blood samples shows significant extracellular phospholipase activity. The percentage of non-*C. albicans* isolates producing proteinase is higher than for *C. albicans* strains, whereas *C. albicans* are higher producers of phospholipase than non-*C. albicans.*

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