

# Determination of germ tube, phospholipase, and proteinase production by bloodstream isolates of *Candida albicans*

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## ABSTRACT

**Introduction:** *Candida albicans* is a commensal and opportunistic agent that causes infection in immunocompromised individuals. Several attributes contribute to the virulence and pathogenicity of this yeast, including the production of germ tubes (GTs) and extracellular hydrolytic enzymes, particularly phospholipase and proteinase. This study aimed to investigate GT production and phospholipase and proteinase activities in bloodstream isolates of *C. albicans*. **Methods:** One hundred fifty-three *C. albicans* isolates were obtained from blood samples and analyzed for GT, phospholipase, and proteinase production. The assays were performed in duplicate in egg yolk medium containing bovine serum albumin and human serum. **Results:** Detectable amounts of proteinase were produced by 97% of the isolates, and 78% of the isolates produced phospholipase. GTs were produced by 95% of the isolates. A majority of the isolates exhibited low levels of phospholipase production and high levels of proteinase production. **Conclusions:** Bloodstream isolates of *C. albicans* produce virulence factors such as GT and hydrolytic enzymes that enable them to cause infection under favorable conditions.

**Keywords:** Virulence. Germ tube. Phospholipase. Proteinase. *Candida albicans*. Candidemia.

## INTRODUCTION

*Candida albicans* is a common colonizer of human skin and mucosal surfaces. The balance between colonization and candidiasis depends on changes in the expression of virulence factors in response to environmental changes and the competence of the host immune system<sup>1</sup>.

Several virulence factors of *C. albicans* have been discovered or proposed, including adhesion factors, hyphal formation, germ tubes (GTs), phenotypic diversity, and the production of extracellular hydrolytic enzymes such as phospholipases and proteinases<sup>2-6</sup>.

Hydrolytic enzymes destroy or disorganize elements of host cell membranes, leading to membrane dysfunction and/or physical disruption. Phospholipases act by invading host cells, causing tissue damage, rupturing the epithelial cell membranes, and allowing hyphae to penetrate the cytoplasm. Proteinase production increases the ability of certain organisms to colonize and penetrate host tissue, deceive the host immune

system, and disrupt a significant number of proteins important for immunity such as immunoglobulins, complement proteins, and cytokines<sup>5,7-9</sup>. Yeast can be induced to produce GTs, which develop into mycelia. This morphological transition can be induced by changes in a variety of environmental factors and by blocking the host immune response<sup>10</sup>.

Because of the increasing incidence of invasive *C. albicans* infections, interest in their virulence factors has increased. This avenue of research may help to establish strategies for the prevention and control of candidiasis and identify possible targets for the development of new therapeutic interventions<sup>7,11</sup>. However, studies of the relationship between GT production and hydrolytic enzyme activity in bloodstream isolates are scarce<sup>2,12</sup>.

Therefore, this study aimed to investigate the known virulence factors, including phospholipase and proteinase activity and GT production, in bloodstream isolates of *C. albicans* *in vitro*.

## METHODS

One hundred fifty-three human isolates of *C. albicans* were obtained from blood samples between 1999 and 2009, and the yeast was identified using the ID 32C assay (BioMérieux, Marcy-l'Étoile, France); isolates were stored at -20°C in a freezer at a mycology laboratory (Santa Casa de Porto Alegre, Rio Grande do Sul, Brazil). The isolates were analyzed for GT, phospholipase, and proteinase production. The standard strain of *C. albicans*, ATCC 28367 (American Type Culture Collection, Rockville, MD, Manassas, USA), was included in the experiments as a control.

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TABLE 1 - Hydrolytic enzyme activity for 153 bloodstream *Candida albicans* isolates.

		Phospholipase (n)				total (n)
		high (0.35-0.5)	moderate (0.51-0.74)	low (0.75-0.99)	negative (1)	
Production of extracellular hydrolytic enzymes (Pz)						
Proteinase (n)	High (0.35-0.5)	10	19	34	18	81
	Moderate (0.51-0.74)	4	16	24	12	56
	Low (0.75-0.99)	1	2	5	3	11
	Negative (1)	2	1	2	0	5
Total (n)		17	38	65	33	153

Pz: activity zone for phospholipase and proteinase.

The *Candida albicans* inoculum was prepared from stock cultures, incubated for 24h at 37°C in Sabouraud dextrose agar, and standardized according to the 0.5 McFarland turbidity range using a densitometer (BioMérieux).

The isolates were screened for their extracellular phospholipase activity by growing them on egg yolk medium and measuring the diameter of the zone of precipitation as previously described by Vidotto et al.<sup>8</sup>. The egg yolk medium consisted of 22.5g of malt agar, 29.2g of NaCl, 0.28g of CaCl<sub>2</sub>, and 10% sterile egg yolk. Extracellular phospholipase activity was detected by inoculating 10-µL aliquots of *C. albicans* suspensions (approximately 1 × 10<sup>8</sup> cells/mL) into wells punched through the surface of the egg yolk medium. The diameter of the precipitation zone around the well was measured after incubation at 37°C for 6 days. Phospholipase activity (Pz value) was determined as the ratio of the diameter of the colony to the total diameter of the zone of precipitation.

The determination of protease production was performed in agar containing bovine serum albumin, as previously described by Mohan & Ballal<sup>2</sup>. A solution was prepared by dissolving 11.7g of yeast carbon base, 0.1g of yeast extract, and 2g of bovine serum albumin in 200mL of distilled water. The solution was sterilized by filtration and added to a previously sterilized stock solution of 16g of agar-agar in 800mL of distilled water. After inoculation, plates were incubated at 37°C, and the results were recorded after 6 days of incubation. Enzyme activity was measured as the diameter of the lytic area surrounding the growth area on the serum medium.

The Pz value was determined as the ratio of the diameter of the colony to the total diameter of the colony plus the precipitation zone, and it was scored and categorized as follows: Pz value = 1 (negative); Pz value = 0.75-0.9 (low producers); Pz value = 0.51-0.74 (moderate producers); and Pz value = 0.35-0.5 (high producers). The assay was performed in duplicate, and the enzyme activity values were recorded as the average of the two measurements.

Germ tubes production is defined as a filamentous outgrowth from a blastoconidium at least twice as long as the parent cell without constriction at the junction. Isolated colonies of *C. albicans* were inoculated in glass tubes with 1mL of human serum and incubated at 37°C for 2h. A drop of the inoculum

was then placed on a clean microscope slide, and a coverslip was placed on the slide. Wet mount preparations were examined under the objective lens of a microscope (×40 magnification), and tests were performed in duplicate.

### Ethical considerations

The hospital (Santa Casa de Porto Alegre, Rio Grande do Sul, Brazil) ethics committee approved this study.

## RESULTS

Phospholipase activity was detected for 78% (120/153) of *C. albicans* isolates, and proteinase activity was detected for 97% (148/153) of isolates.

The mean Pz value was 0.79 (range, 0.48-1.00). The average value for proteinase activity was 0.71 (range, 0.49-1.00).

Isolates were categorized by their levels of phospholipase and proteinase production. The activity of these enzymes is shown in **Table 1**. A majority of *C. albicans* isolates with enzymatic activity displayed high proteinase activity and low phospholipase activity. Of the isolates studied, 73% produced both phospholipase and proteinase.

Germ tubes induction was positive in 95% (146/153) of bloodstream *C. albicans* isolates. The associations of GT production with phospholipase and proteinase activity are described in **Table 2**.

TABLE 2 - Virulence factor (GT, phospholipase, and proteinase production) associations in bloodstream isolates of *Candida albicans*.

Virulence factor associations	Number	Percentage
Phospholipase + GT	5	3.0
Proteinase + GT	33	22.0
Phospholipase + proteinase	7	5.0
Phospholipase + proteinase + GT	108	70.0
Total	153	100.0

GT: germ tube.

## DISCUSSION

Yeasts of the genus *Candida* continue to be among the most important etiologic agents of nosocomial infections. Deep-seated candidemia, the occurrence of which has considerably increased, is most commonly observed in ICU patients with indwelling catheters, individuals receiving oncological treatment, organ transplant recipients, or other immunocompromised individuals subjected to heavy therapeutic protocols<sup>2,13</sup>.

Although many studies have investigated the exoenzyme activity of *Candida* spp. isolated from mucosal surfaces<sup>3,7-9,12,14,15</sup>, few have investigated bloodstream isolates<sup>2,12</sup>. The virulence of *Candida* spp. is believed to be caused by the combination of several factors<sup>12</sup>, including its ability to colonize a variety of different anatomic sites<sup>7</sup>.

The phospholipases and proteinases of *C. albicans* are considered important virulence factors, and the absence or decreased expression of these enzymes may indicate reduced virulence for some *Candida* species<sup>2</sup>.

In our study, 78 and 97% of the isolates displayed phospholipase and proteinase activity, respectively. Mohan & Ballal<sup>2</sup> found a similar percentage of phospholipase activity; however, only 44% of the isolates that they studied exhibited proteinase activity, suggesting that the isolates used in this study were comparatively more virulent.

Few studies have investigated phospholipase and proteinase production simultaneously in the same strains. In the present study, 73% of the isolates produced both phospholipase and proteinase, which is in concordance with the results obtained by D'Eça Júnior et al.<sup>7</sup> and Kantarcioglu & Yucel<sup>15</sup>, who observed concurrent production in 60 and 93% of *C. albicans* isolates, respectively. However, the isolates used in those studies were isolated from different anatomic sites.

In another similar study, measures of enzymatic activity indicated that all *C. albicans* isolates were pathogenic<sup>2</sup>. However, the present study identified no hydrolytic enzyme activity in 38% of the isolates.

Germ tubes production was observed for 95% of the isolates. High levels of phospholipase production were observed in 13 of the GT-positive isolates. Vidotto et al.<sup>12</sup> described a correlation between high phospholipase activity and GT production in oral isolates, which suggests that these virulence factors are necessary for *C. albicans* colonization and infection of the oral cavity. It is possible that high levels of phospholipase activity and GT production can facilitate penetration through the mucosa because phospholipase activity is particularly concentrated in hyphal tips.

Ghannoum<sup>4</sup> and D'Eça Júnior et al.<sup>7</sup> suggested that the quantity of phospholipase produced by *C. albicans* varies with each specific isolate, and it is correlated with the site of infection.

In our study, bloodstream isolates of *C. albicans* produced extracellular phospholipase and proteinase. In general,

bloodstream isolates are known to produce much higher levels of phospholipase and proteinase than wound or urine isolates<sup>4,15</sup>.

Bloodstream isolates of *C. albicans* produce virulence factors, including GT and hydrolytic enzymes, making them capable of causing infection under favorable conditions.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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