Comparison of the hemolytic activity between *C. albicans* and non-*albicans Candida* species

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Abstract: The ability to produce enzymes, such as hemolysins, is an important virulence factor for the genus *Candida*. The objective of this study was to compare the hemolytic activity between *C. albicans* and non-*albicans Candida* species. Fifty strains of *Candida* species, isolated from the oral cavity of patients infected with HIV were studied. The isolates included the following species: *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. tropicalis*, *C. krusei*, *C. parapsilosis*, *C. dubliniensis*, *C. norvegensis*, *C. lusitaniae*, and C. *guilliermondii*. Hemolysin production was evaluated on Sabouraud dextrose agar containing chloramphenicol, blood, and glucose. A loop-full of pure *Candida* culture was spot-inoculated onto plates and incubated at 37°C for 24 h in a 5% CO_2 atmosphere. Hemolytic activity was defined as the formation of a translucent halo around the colonies. All *C. albicans* strains that were studied produced hemolysins. Among the non-*albicans Candida* species, 86% exhibited hemolytic activity. Only *C. guilliermondii* and some *C. parapsilosis* isolates were negative for this enzyme. In conclusion, most non-*albicans Candida* species had a similar ability to produce hemolysins when compared to *C. albicans*.

Descriptors: *Candida*; Virulence Factors; Acquired Immunodeficiency Syndrome.

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Introduction

The frequency of *Candida* infection has been gradually increasing over the last several years, accompanied by a significant increase in morbidity and mortality. *Candida albicans* is the most pathogenic *Candida* species and is frequently identified in candidiasis lesions in humans.[1] Twenty years ago, *C. albicans* represented 80% of the *Candida* species recovered from patients with oral and systemic candidiasis. Although *C. albicans* continues to be the most frequently isolated species, the number of infections caused by non-*albicans* species has increased significantly over the last two decades.[2]

The increased prevalence of non-*albicans Candida* species found in human candidiasis can be partially attributed to advanced diagnostic methods, such as the use of primary culture media, which are able to differentiate between *Candida* species, and the introduction of molecular techniques for routine diagnosis. Other factors responsible for the increased prevalence of *Candida* species include the introduction and

widespread use of better medical practices (such as immunosuppressive therapy), the administration of broad-spectrum antibiotics, and an increase in the number of invasive surgical procedures. Furthermore, the growing number of *Candida* species causing candidiasis may be a consequence of species selection in the presence of certain antifungal agents, resulting in the high level of antibiotic resistance found in non-*albicans* species.[2]

Candida species produce different virulence factors that contribute to colonization, pathogenicity and infection of tissues, including the adhesion to host epithelial cells and biomaterials, the formation of germ tubes and hyphae, the production of hydrolytic enzymes such as proteinases and phospholipases, and hemolytic capacity.[3,4] Hemolytic capacity is an important virulence factor, that allows fungi of the genus *Candida* to acquire iron from host tissues, which then is used by the fungus for metabolism, growth and invasion during host infection.[5]

Iron is an essential element for almost all organisms, both unicellular and multicellular.[6] In humans, iron is found in some proteins, including hemoglobin (a component of erythrocytes). The ability of *C. albicans* to utilize hemoglobin as an iron source was first described by Moors *et al.*[7] According to their study, the first step of *C. albicans* infection *in vivo* involves binding to erythrocytes through receptors of the complement system. Next, *C. albicans* produces a hemolysis factor that induces lysis of the erythrocyte. This factor most likely corresponds to a mannoprotein bound to the cell surface of the fungus.[5,8] However, the mechanism and molecular basis of hemolysis caused by *C. albicans* remain unknown.[5]

In the oral cavity, extracellular iron is bound mainly to lactoferrin, a protein present in saliva, while intracellular iron is stored as ferritin. Although this element is bound to proteins and/or is present in the cytoplasm of cells, oral infections with *C. albicans* are frequent, suggesting that this yeast is able to take up different forms of iron from the oral cavity.[9] In support of this hypothesis, Almeida *et al.*[9] observed that *C. albicans* caused greater damage to oral epithelial cells containing elevated concentrations of ferritin compared to cells with lower iron levels. In addition, the secretion of hemolysins, followed by the acquisition of iron, facilitates the invasion of hyphae in cases of systemic candidiasis,[10] and *Candida* hyphae possess a higher number of hemoglobin receptors than what the yeast form.[11]

Because the number of fungal infections caused by non-*albicans Candida* species has increased significantly over the last few years and the development of treatment alternatives for fungal infections depends on the study and understanding of virulence factors of these microorganisms, the objective of the present study was to compare the hemolytic capacity of non-*albicans Candida* species and *C. albicans*.

Methodology

Candida strains

We studied fifty *Candida* strains isolated from the oral cavity of HIV-positive patients seen at the Emílio Ribas Institute of Infectious Diseases (*Instituto de Infectologia Emílio Ribas* - IIER, São Paulo, Brazil). The strains were isolated and identified as previously described by Junqueira *et al.*[12] The isolates tested included the following species:

- *C. albicans* (n = 20),
- *C. glabrata* (n = 13),
- *C. parapsilosis* (n = 5),
- *C. dubliniensis* (n = 4),
- *C. tropicalis* (n = 4),
- *C. krusei* (n = 1),
- *C. guilliermondii* (n = 1),
- *C. lusitaniae* (n = 1), and
- *C. norvegensis* (n = 1).

The study was approved by the Ethics Committee of the São José dos Campos Dental School, UNESP (Protocol no. 051/2009/CEP).

All strains were kept in YPD broth (Himedia, Mumbai, India) containing 20% glycerol (Amresco, Solon, USA) at −80°C. The strains were replated on Sabouraud dextrose agar (Himedia, Mumbai, India) and incubated at 37°C for 48 h to analyse hemolysin production.

Hemolysin production

Hemolysin production was evaluated according

to methods by Manns *et al.*,[13] with some modifications. A loop-full of pure *Candida* culture was inoculated into Sabouraud dextrose agar containing chloramphenicol (Inlab, Diadema, Brazil) and incubated at 37°C for 24 h. This growth was used to prepare a suspension of 10^8 cells/mL in sterile phosphate-buffered (0.1 M, pH 7.2) saline (Laborclin, Pinhais, Brazil) using a spectrophotometer (B582, Micronal, São Paulo, Brazil). An aliquot (10 µL) of the standardized suspension was seeded on to blood agar enriched with glucose (Vetec, Duque de Caxias, Brazil). This medium was prepared with 7 mL fresh blood per 100 mL Sabouraud dextrose agar supplemented with chloramphenicol and 3% glucose. The final pH was adjusted to 5.6 ± 0.2. Plates were incubated at 37°C for 48 h in a 5% CO_2 atmosphere.

Hemolytic activity was measured using the method described by Price *et al.*,[14] where Pz corresponds to the ratio of the diameter of the colony alone to the diameter of the colony plus the precipitation zone, this is obtained by dividing the colony diameter in mm by the diameter of the colony plus the halo formed due to enzymatic activity. According to this system, Pz = 1.00 indicates that no halo was produced, i.e., there was a lack of enzymatic activity. The lower the value of Pz, the higher the enzymatic activity of the strain. The results were converted into scores (Table 1).

Statistical analysis

The scores attributed to Pz values were statistically analyzed by the Mann-Whitney test, using GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, USA). A level of significance of 5% was used.

Results

The results of the hemolytic activity assays obtained for all *Candida* species are shown in Table 2. Forty-six (92%) of the 50 *Candida* strains tested produced hemolysin, with 29 (58%) exhibiting strong hemolytic activity. *C. guilliermondii* and three *C. parapsilosis* strains were the only isolates that did not produce hemolysin.

All *C. albicans* species (100%) produced a hemolysis halo, while 86% of the non-*albicans* species exhibited hemolysis (Figure 1). Statistical analysis revealed no significant difference between the hemolysis produced by *C. albicans* and non-*albicans* species ($p = 0.6590$).

As various *C. albicans* and *C. glabrata* isolates were analyzed, statistical comparison between these two species was possible. Hemolytic activity was significantly higher in *C. glabrata* compared to *C.*

Table 1 - Enzymatic activity according to Pz value and score attributed (Price *et al.*,[14] with modifications).

Pz	Enzymatic activity	Score
1.00	Negative	0
≥ 0.64 < 1.00	Positive	1
< 0.64	Strongly positive	2

Table 2 - Distribution of *Candida* species according to hemolytic activity score.

Species (number of isolates)	Hemolytic activity score		
	Score 2	Score 1	Score 0
C. albicans (20)	10	10	0
C. glabrata (13)	12	1	0
C. parapsilosis (5)	1	1	3
C. dubliniensis (4)	3	1	0
C. tropicalis (4)	1	3	0
C. krusei (1)	0	1	0
C. guilliermondii (1)	0	0	1
C. lusitaniae (1)	1	0	0
C. norvegensis (1)	1	0	0

Score 0: no hemolytic activity; score 1: positive hemolytic activity; score 2: strongly positive hemolytic activity.

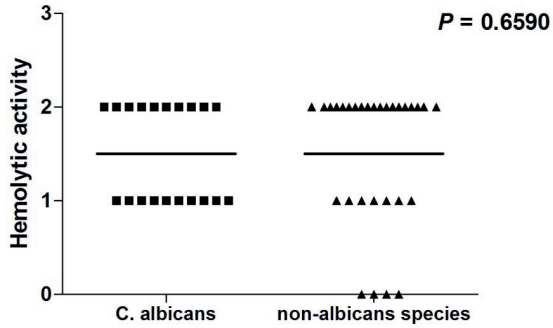

Figure 1 - Values and medians of hemolytic activity observed for *C. albicans* and non-*albicans* species ($p = 0.6590$).

albicans (p = 0.0140). As shown in Table 2, strong positive activity was observed for 50% of the *C. albicans* strains and 92% of the *C. glabrata* isolates.

Discussion

In fungi of the genus *Candida*, the transition from commensalism to pathogenicity can be attributed to the selective expression of different virulence factors that act synergistically under favourable conditions. The type, stage and infection site, in addition to the nature of the immune response, determine which virulence factors the yeast expresses. Among these virulence factors, proteolytic, lipolytic or hemolytic activity seem to play a major role in the pathogenicity of these microorganisms.[4,15]

In the present study, we evaluated hemolysin production, an important virulence factor for yeast from the genus *Candida*. This enzyme degrades host erythrocytes to release iron for use in growth and metabolism of these fungi in cases of systemic infections.[16] We also compared the ability of *C. albicans* and non-*albicans Candida* species isolated from the oral cavity of HIV-positive patients to produce hemolysin. *C. albicans* is the predominant species associated with mucosal and systemic fungal infections. However, the epidemiology of yeast infection is rapidly evolving, and non-*albicans Candida* species have emerged as major opportunistic pathogens primarily in the specific conditions of immunodeficiency, such as acquired immunodeficiency syndrome.[12]

The present results showed that most *Candida* species (92%) produced hemolysins; of these species, 58% had strongly positive hemolytic activity. These findings agree with those of Ramesh *et al.*[17] who compared the hemolytic activity of 50 *Candida* strains isolated from patients with HIV and 10 *Candida* strains isolated from immunocompetent patients. All strains produced hemolysis, but hemolytic activity was significantly higher for *C. albicans* strains isolated from HIV patients when compared to those isolated from immunocompetent patients. Mane *et al.*[18] also studied the hemolytic ability of *Candida* isolates from a cohort of 335 patients, composed of 210 HIV-positive and 135 HIV-negative individuals. The authors verified that isolates from HIV-positive patients had significantly increased production of hemolysin when compared to isolates from HIV-negative individuals. The strongly positive hemolytic activity found in this study (58%) suggests that *Candida* strains in HIV-infected individuals have increased expression of virulence attributes and emphasizes the need for further studies on the development of new approaches for therapeutic intervention.

All *C. albicans* strains analyzed in this study produced a hemolysis halo (100%). Similar results have been reported by Tsang *et al.*,[19] who evaluated the enzymatic activity of proteinase, phospholipase and hemolysin in 126 oral *C. albicans* isolates. All isolates produced the three enzymes. Shreaz *et al.*[20] found 100% hemolysis in 26 *C. albicans* strains isolated from the oral cavity. Among the non-*albicans* species tested, 86% were positive for hemolysin. As *C. albicans* is the most virulent species of the genus *Candida*,[21] the hemolytic activity of non-*albicans* species was compared to that of *C. albicans* and no significant difference was observed, indicating that non-*albicans* species possess the same hemolytic capacity as *C. albicans*.

In the present study, 92% of the *C. glabrata* isolates were strongly positive for hemolysin, and the hemolytic activity of this species was significantly higher than *C. albicans*. However, Mane *et al.*[22] analyzed the hemolytic production of 65 *Candida* isolates from HIV-infected individuals and verified that *C. albicans* (n = 39) produced more hemolysin compared to all other species, including *C. glabrata* (n = 8). Ramesh *et al.*[17] also evaluated the hemolytic capacity of 50 *Candida* strains from HIV patients, and verified that *C. albicans* (n = 45) produced a significantly higher amount of hemolysin than *C. glabrata* (n = 5).

The difference between our results and those from previous studies may be due to the number of strains in each species studied. In the present study, we evaluated a number of *C. glabrata* (n = 13) similar to the number of *C. albicans* (n = 20) strains, while the number of samples of *C. glabrata* was much lower compared to *C. albicans* in previously cited studies.[17,22] These data show the need for more studies with *C. glabrata*, which recently was shown to have the ability to produce α or β hemolysis.[4,23]

This species has emerged as a potential pathogen in the oral cavity of immunocompromised patients and little is known about its role in infection.[24] In an *in vivo* study, Jawhara *et al.*[25] demonstrated the high pathogenic potential of *C. glabrata* in a murine model of colitis characterized by weight loss, colon inflammation, and a high mortality rate of the animals.

The only species that did not produce hemolysin in this study were *C. guilliermondii* and three *C. parapsilosis* strains. Similar results have been reported in the literature. Seneviratne *et al.*[26] analyzed the production of proteinase, phospholipase and hemolysin in 49 bloodstream isolates of *Candida* obtained from patients in Hong Kong and Finland. In that study, *C. albicans*, *C. glabrata* and *C. tropicalis* exhibited high hemolytic activity, whereas *C. guilliermondii* and *C. parapsilosis* did not produce this enzyme. Luo *et al.*[23] also studied the hemolytic activity of different *Candida* species and found that only *C. parapsilosis* did not produce any type of hemolysis.

The present study is the first investigation of hemolytic activity in *C. norvegensis*, a species responsible for 7% of candidemia cases.[27] This fungus was strongly positive for hemolysin, a finding that suggests the capacity of this emerging species to invade and infect an immunocompromised organism.

The four *C. tropicalis* isolates tested produced hemolysin. França *et al.*[28] investigated the hemolytic activity of *C. parapsilosis* and *C. tropicalis* isolated from different human anatomical sites. According to their data, enzymatic activity varies widely from species to species and is associated with the site of isolation. Bloodstream *C. tropicalis* isolates produced a larger hemolysis halo compared to isolates from the trachea and skin, whereas *C. parapsilosis* isolated from tracheal secretion exhibited higher enzymatic activity compared to bloodstream isolates. Overall, these results show that yeast of the genus *Candida* express greater or lesser amounts of hemolysin depending on the anatomical site of isolation and consequent immunological and tissue particularities are required for it to colonize and survive in a host.

The scarcity of studies on the hemolytic activity of emerging *Candida* species and the absence of differential hemolytic activity between non-*albicans Candida* species and *C. albicans* observed in the present study indicate that further investigation is needed to elucidate the exact role of the hemolytic capacity of *Candida* species in fungal infections.

Conclusion

Non-*albicans Candida* species exhibited similar hemolytic capacity as *C. albicans*. The highest hemolytic activity was observed in *C. glabrata*, followed by *C. albicans*. *C. guilliermondii* and some *C. parapsilosis* strains were the only isolates that did not produce hemolysins.

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