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Enzymatic and hemolytic activity in different *Candida* species

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ABSTRACT

Background: *Candida* species, in conditions of microbiota imbalance or decreased immune defenses, may be one of the main human fungal pathogens. Virulence factors constitute the mechanisms used by the fungus to avoid host defenses.
Aims: This study aimed to investigate the *in vitro* production of virulence factors, such as hemolytic activity, and deoxyribonuclease (DNase), proteinase, and phospholipase activities in *Candida* spp.
Methods: Fifty clinical isolates were analyzed for virulence factors: *Candida albicans* (15), *Candida tropicalis* (15), *Candida parapsilosis* (10), *Candida glabrata* (5), and *Candida krusei* (5). Hemolytic activity was determined in Sabouraud dextrose agar plates containing 3% glucose and 7% sheep red cells. Culture media containing, respectively, agar-base DNA, egg yolk, and bovine albumin were used to determine DNase, phospholipase and proteinase activities, respectively.
Results: Forty-eight (96%) of 50 isolates showed hemolytic activity, with 10 (20%) positive for DNase, 19 (38%) for proteinase, and 16 (32%) for phospholipase. Statistically significant differences were observed between species for phospholipase ($p < 0.0001$) and proteinase ($p < 0.05$) production.
Conclusions: It is concluded that all species had hemolytic activity. DNase activity was detected in all species except in *C. glabrata*; proteinase activity was detected in *C. albicans*, *C. tropicalis*, and *C. parapsilosis*; and phospholipase activity was observed in *C. albicans* and *C. tropicalis*.

La actividad enzimática y hemolítica en diferentes especies de *Candida*

Palabras clave:
Desoxirribonucleasa
Aspartil proteinasa
Factores de virulencia
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RESUMEN

Antecedentes: Las levaduras del género *Candida*, en condiciones de desequilibrio de la microbiota o de disminución de las defensas inmunológicas, pueden ser uno de los principales patógenos fúngicos del hombre. Los factores de virulencia constituyen los mecanismos utilizados por el hongo para evadir las defensas del huésped.
Objetivos: Este estudio tiene como objetivo investigar la producción *in vitro* de algunos factores de virulencia, como la actividad hemolítica, y las actividades desoxirribonucleasa (DNasa), proteinasa y fosfolipasa en *Candida* spp.
Métodos: Se analizaron 50 aislamientos clínicos: *Candida albicans* (15), *Candida tropicalis* (15), *Candida parapsilosis* (10), *Candida glabrata* (5), y *Candida krusei* (5). La actividad hemolítica fue determinada en placas de agar glucosado de Sabouraud, con glucosa al 3% y un 7% de hematíes de oveja. Los medios de cultivo de agar-ADN, yema de huevo y albúmina bovina fueron utilizados para determinar las actividades DNasa, fosfolipasa y proteinasa, respectivamente.
Resultados: De los 50 aislamientos, 48 (96%) presentaron actividad hemolítica, 10 (20%) fueron positivos para DNasa, 19 (38%) para proteinasa y 16 (32%) para fosfolipasa. Se observaron diferencias estadísticamente significativas entre las especies para las actividades fosfolipasa ($p < 0{,}0001$) y proteasa ($p < 0{,}05$).

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Conclusiones: Se concluye que todas las especies estudiadas poseen actividad hemolítica. La actividad DNasa fue detectada en todas las especies, excepto en *Candida glabrata*; la actividad proteinasa fue detectada en *C. albicans*, *C. tropicalis* y *C. parapsilosis*, y la actividad fosfolipasa se observó en *C. albicans* y *C. tropicalis*.

Recent increases in the incidence of fungal infections worldwide are a matter of great concern in public health. Among yeasts, *Candida* species remain the main cause of hospital infections, particularly in adult or pediatric intensive care units.[10,23] The fungal infection is debilitating and recurrent in immunocompromised patients and the co-existence of other disorders or diseases may retard or aggravate diagnosis and inclusively cause death.[1,2,18]

Commensal *Candida* species may become pathogenic for humans if microbiota balance is disturbed. Pathogenicity will depend on factors such as host predisposition (immunodepression), parasitic load and fungal virulence that facilitate tissue invasion and evasion of host defense mechanisms.[2,4,23]

The pathogenicity of fungi has been investigated in an attempt to identify the differences in clinical presentation and severity of the infection. Among the properties of *Candida* species that may explain pathogenicity, different virulence factors are present, which include adhesion to substrates, formation of the germ tube, phenotypic and genotypic variability, toxin production and extracellular enzymes. These constitute important factors in the launching and maintenance of infections. Proteinases and phospholipases are among the hydrolytic enzymes produced. Hemolytic activity and DNase are considered by some authors to be important factors in virulence, as well as the capacity to multiply at high temperatures, for example 39 °C and 42 °C.[6,8,12,15]

Characterization of virulence factors contributes to the understanding of *Candida* infections, including the differences between different species. The aim of this study was to investigate the *in vitro* hemolytic activity, and DNase, phospholipase and proteinases activities in five species of *Candida*: *Candida albicans, Candida tropicalis, Candida parapsilosis, Candida glabrata* and *Candida krusei.*

Materials and methods

Fifty *Candida* spp. strains distributed as *C. albicans* (15), *C. tropicalis* (15), *C. parapsilosis* (10), *C. glabrata* (5) and *C. krusei* (5) were isolated from different clinical materials (blood, synovial and peritoneal liquids), and stored at −20 °C in BHI-glycerol at the Laboratory of the Technical Course in Clinical Analysis, Technical Health School, Federal University of Uberlandia, Minas Gerais, Brazil. Subcultivation for tests was carried out in Sabouraud dextrose (ASD)-chloramphenicol media by incubation at 30 °C for 24 h.

Experimental samples of each isolate were prepared by suspension in saline solution to a turbidity corresponding to tube 2 of the McFarland scale (1×10^8 to 5×10^8 cells/ml). Petri dishes (90 mm × 15 mm) containing, respectively, ASD-blood,[14] DNase agar,[19] phospholipase agar[22] and proteinase agar[16] were each inoculated with 5 μL aliquots of five different isolates at equidistant points.

Results were described and interpreted according to the characteristics of each test. Hemolytic activity was evidenced by the translucent halo with defined borders around colonies, and expressed by an index, hemolytic index (Hi), calculated by dividing the colony diameter by the diameter of colony plus the hemolysis zone. DNase activity was expressed as positive or negative by the detection of a clearing halo without defined borders around the colonies. The activities of phospholipase and proteinase were identified by an opaque zone (precipitation of a calcium complex) and a clear halo around the yeast colonies, respectively, and were expressed by using the Pz value (phospholipase zone) for phospholipase and Prz (proteinase zone) for proteinase. Both Pz and Prz values were calculated by dividing the colony diameter by the colony diameter plus the precipitation or clearing, respectively, according to Price et al.[12] Values of 1.0 for Hi, Pz and Prz mean the isolate did not exhibit the tested activity (score 1); values between 0.64 and 0.99 (score 2) indicate moderate activity, and those values equal to or below 0.63 indicate strong enzymatic activity (score 3).

The Mann–Whitney test was used to calculate statistic differences between enzymatic activities in isolates of *C. albicans, C. parapsilosis, C. krusei, C. glabrata* and *C. tropicalis.* In all tests, $p < 0.05$ was considered significant.[21]

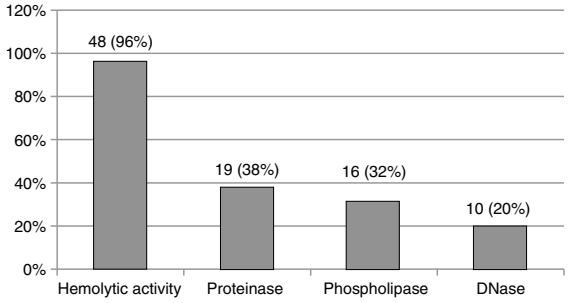

Fig. 1. Percentage of *Candida* spp. ($n = 50$) isolates showing hemolytic activity, and activity of enzymes proteinase, phospholipase and DNase.

Results

Forty-eight (96%) of the fifty isolates included in the study had hemolytic activity, 19 (38%) proteinase activity, 16 (32%) phospholipase activity and 10 (20%) DNase activity (Fig. 1).

Hemolytic activity was characterized as moderate in 45 isolates (90%), with indexes varying between 0.64 and 0.99 and without statistically significant differences between the species. The moderate hemolytic index was shown by all isolates of *C. albicans, C. glabrata*, and *C. krusei*, and by 74% of *C. tropicalis* and 90% of *C. parapsilosis*.

Twenty percent of the isolates had DNase activity distributed as follows: 26% in *C. albicans*, 20% in *C. tropicalis*, 10% in *C. parapsilosis* and 40% in *C. krusei* (Table 1). Statistical differences among species were not significant.

Proteolytic activity was observed in 19 (38%) isolates, distributed in eight (80%) *C. parapsilosis*, seven (46%) *C. tropicalis* and four (26%) *C. albicans*. Statistically significant differences were found between *C. albicans* and *C. parapsilosis* ($p = 0.004$), and *C. tropicalis* and *C. parapsilosis* ($p = 0.003$).

Phospholipase activity was present in all *C. albicans* isolates and one *C. tropicalis*. Statistical differences were significant ($p < 0.0001$) for *C. albicans* and each of the other species. No significant differences were observed between the other species when compared in groups of two.

Table 1
Frequency of *Candida* spp. isolates showing hemolytic activity and activities of phospholipase, proteinase and DNase enzymes according to the respective scores.

Species	Number of isolates (*n*)	Scores of enzymatic activities[a]								Score of hemolytic activity[a]		
		Phospholipase			Proteinase			DNase				
		1	2	3	1	2	3	Positive	Negative	1	2	3
C. albicans	15	0	2	13	11	3	1	4	11	0	15	0
C. tropicalis	15	14	0	1	9	6	0	3	12	1	11	3
C. parapsilosis	10	10	0	0	2	2	6	1	9	1	9	0
C. glabrata	05	5	0	0	5	0	0	0	5	0	5	0
C. krusei	05	5	0	0	5	0	0	2	3	0	5	0

[a] Scores for phospholipase, proteinase and hemolytic activities: 1 – does not show enzymatic activity (Hi, *Prz, Pz* = 1.00); 2 – moderate enzymatic activity (0.63 < Hi, Prz, Pz < 0.99); 3 – strong enzymatic activity (Hi, Prz, Pz ≤ 0.63).

Discussion

Infective processes are dependent on host defense mechanisms, general and local conditions and upon virulence factors characterizing the causative microorganisms. Virulence factors are widely investigated in an attempt to explain differences in pathogenicity of several fungal isolates including *Candida* spp., as well as to understand the parasite–host relationship.

Candida isolates included in this study showed hemolytic activity with the exception of only two strains: one *C. tropicalis* and one *C. parapsilosis* that did not show hemolysis in blood agar in the test conditions. Rorig et al.[14] reported hemolytic activity only in *C. parapsilosis* and *C. albicans* and negative results for *C. glabrata, C. krusei, Candida guilliermondii* and *Candida dubliniensis*. However, other authors observed *in vitro* hemolytic activity in isolates of *C. albicans, C. glabrata* and *C. tropicalis* from blood or other sources, as central venous catheters and urine.[5,11,20]

Some of the discrepancies in the results may be due to difficulties in the quantification of hemolytic activity conducted in different test conditions, definitions and interpretations of results. Thus, the use of suggested models to characterize activities, although empirical, will permit comparison of results from different laboratories. The model proposed by Price et al.,[13] which utilizes a practical, rapid and easy-to-determine score, was used in indexes expressing activity of phospholipases.

Results concerning DNase activity in *Candida* species were not found in the literature. Some strains of all the species, except *C. glabrata*, were able to produce DNase. This lack of information may be due again to experimental problems of the *in vitro* detection of the activity. The method requires prolonged incubation periods, and halos surrounding colonies do not have well-defined borders, suggesting the use of qualitative interpretations as positive or negative. DNase in fungi was reported by Cazin et al.[3] and Sanchez et al.[17] The last authors demonstrated that all clinical and environmental isolates of *Cryptococcus neoformans* and *Cryptococcus gattii* had DNase activity, but it is more frequent in clinical isolates of *C. neoformans*.

The implication of extracellular DNase as a virulence factor for fungi has not yet been defined. Hypotheses are that DNase contributes to the evasion of the immune system, preventing the death of the yeast that can be caused by neutrophils.[17] DNase may be important in combination with other exoenzymes to degrade DNA of other microorganisms in microenvironment, especially bacteria, facilitating the colonization site by reduction of microbial competition. However recent studies have shown the role of extracellular DNase produced by *C. albicans* in maintaining the integrity of the biofilm and in decreasing susceptibility to antifungal drugs[9]. However, future studies should be conducted in order to verify the importance of DNase for *Candida* spp., its association with other virulence factors, especially with adhesins, and its implication in virulence, pathogenicity and antifungal resistance.

Phospholipase activity was detected in all *C. albicans* isolates, confirming the results by Mahmoudabadi et al.,[8] who found this activity in all urogenital isolates of this species. Among the other species, only one isolate of *C. tropicalis* was positive, while Junqueira et al.[7] observed that isolates of *C. albicans, C. dubliniensis, C. tropicalis* and *C. krusei* from oral cavity were phospholipase producers. However D'Eça Junior et al.[4] analyzed clinical isolates from several sources and concluded that among the different species in the study, *C. tropicalis* was the most frequent producer of phospholipase, while the activity was not present in *C. parapsilosis, C. glabrata* and *C. krusei*.

Proteinase activity was present in *C. tropicalis, C. parapsilosis* and *C. albicans*, confirming observations by Junqueira et al.,[7] who detected the activity in isolates of *C. albicans, C. parapsilosis* and *C. dubliniensis* from oral cavity and by Seneviratine et al.[20] in blood isolates of *C. albicans, C. parapsilosis, C. tropicalis* and *C. dubliniensis*.

It is concluded that all the species tested had hemolytic activity; DNase activity was detected in all the species except in *C. glabrata*. Proteinase was detected in *C. albicans, C. tropicalis* and *C. parapsilosis* and phospholipase only in *C. albicans* and *C. tropicalis*. It is suggested that DNase activity observed in clinical isolates of *Candida* spp. may become an interesting attribute to be considered in pathogenicity studies in this genus.

Conflict of interest

The authors declare no conflict of interest.

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