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Article/Artigo

In vitro differential activity of phospholipases and acid proteinases of clinical isolates of *Candida*

Atividade diferencial *in vitro* de fosfolipases e proteinases ácidas de isolados clínicos de *Candida*

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ABSTRACT

Introduction: *Candida* yeasts are commensals; however, if the balance of normal flora is disrupted or the immune defenses are compromised, *Candida* species can cause disease manifestations. Several attributes contribute to the virulence and pathogenicity of *Candida*, including the production of extracellular hydrolytic enzymes, particularly phospholipase and proteinase. This study aimed to investigate the *in vitro* activity of phospholipases and acid proteinases in clinical isolates of *Candida* spp. **Methods**: Eighty-two isolates from hospitalized patients collected from various sites of origin were analyzed. Phospholipase production was performed in egg yolk medium and the production of proteinase was verified in a medium containing bovine serum albumin. The study was performed in triplicate. **Results:** Fifty-six (68.3%) of isolates tested were phospholipase positive and 16 (44.4%) were positive for proteinase activity. *C. tropicalis* was the species with the highest number of positive isolates for phospholipase (91.7%). Statistically significant differences were observed in relation to production of phospholipases among species (p<0,0001) and among the strains from different sites of origin (p=0.014). Regarding the production of acid protease, the isolates of *C. parapsilosis* tested presented a larger number of producers (69.2%). Among the species analyzed, the percentage of protease producing isolates did not differ statistically (χ2=1.9 p=0.5901 (χ2=1.9 p=0.5901). **Conclusions**: The majority of *C.* non-*albicans* and all *C. albicans* isolates were great producers of hydrolytic enzymes and, consequently, might be able to cause infection under favorable conditions.

Keywords: *Candida*. Virulence factors. Phospholipases. Proteases.

RESUMO

Introdução: *Candida* são leveduras comensais, porém, se o equilíbrio da flora normal for interrompido ou as defesas imunitárias estiverem comprometidas, espécies de *Candida* podem causar manifestações de doença. Vários atributos contribuem na virulência e patogenicidade de *Candida*, inclusive a produção de enzimas extracelulares hidrolíticas, especialmente fosfolipases e proteinases. O objetivo deste estudo foi verificar a atividade *in vitro* de fosfolipases e proteinases ácidas em isolados clínicos de *Candida* spp. **Métodos**: Oitenta e dois isolados provenientes de pacientes hospitalizados coletados a partir de sítios de origem diversos foram analisados. A produção de fosfolipase foi verificada em meio *egg yolk* e a de proteinase em meio contendo soro albumina bovina. O estudo foi feito em triplicata. **Resultados**: Cinquenta e seis (68,3%) dos isolados testados apresentaram atividade de fosfolipase positiva e 16 (44,4%) foram positivos para atividade de proteinase. *C. tropicalis* foi a espécie que apresentou o maior número de isolados positivos para fosfolipases (91,7%). Diferenças estatisticamente significantes em relação à produção de fosfolipases entre as espécies e entre as cepas provenientes de diferentes sítios de origem foram detectadas. Quanto à produção de proteinases ácidas, os isolados de *C. parapsilosis* testados foram os maiores produtores (69,2%). Entre as espécies analisadas, a porcentagem de produção de proteinase entre os isolados não diferiu estatisticamente (χ2=1.9 p=0.5901 (χ2=1.9 p=0.5901). **Conclusões**: A maioria dos isolados de *C.* não-*albicans*, assim como os de *C. albicans*, foram grandes produtores de enzimas hidrolíticas e, consequentemente, podem ser capazes de causar infecção em condições adequadas.

Palavras-chaves: *Candida*. Fatores de virulência. Fosfolipases. Proteinases.

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INTRODUCTION

The polyphyletic genus *Candida* includes species of pathogenic fungi in humans, including *Candida albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and other species. Together these species are responsible for 34% of opportunistic fungal infections and *C. albicans* is the most common fungal pathogen in humans. Over the past few years, the incidence of fungal infections by non-*albicans* species has increased[1]. The reasons for this change in the species distribution pattern in fungal infections have not been fully elucidated and may be closely related to the virulence potential of these microorganisms. Different species of *Candida* are able to express different gene products to adapt and grow in a variety of extreme physiological conditions, providing infections. These gene products are important for the virulence of *Candida*[2,3].

Several virulence factors of *Candida* have been discovered or proposed, including adhesion, hyphal formation, phenotypic diversity and production of extracellular hydrolytic enzymes, such as phospholipases, lipases and aspartyl proteinases (Saps)[4].

Once they have invaded host tissues, species of *Candida* have the ability to produce, in a constitutive and inducible way, hydrolytic enzymes that destroy or disorganize elements of the host cell membranes, leading to membrane dysfunction and/or physical disruption. These enzymes are proteinases, which hydrolyze peptide bonds, and phospholipases, which hydrolyze phospholipids[5,6]. Phospholipases act by invading and causing tissue damage in host cells, rupturing the epithelial cell membranes and allowing the ends of hyphae penetrate the cytoplasm[5]. Proteinase production is important for increasing the ability of certain organisms to colonize and penetrate the host tissue and deceive the host immune system, breaking a significant number of important proteins for immunity, such as immunoglobulins, complement proteins and cytokines[7].

Due to the increased incidence of invasive infections by *Candida* species[8], interest in the study

of virulence factors of these species has intensified, including the production of hydrolytic enzymes, to establish strategies for the prevention and control of candidiasis and as possible targets for the development of new therapeutic interventions[9]. In contrast to the species *Candida albicans*, only a few somewhat controversial studies exist regarding the virulence and experimental pathogenicity of other species of *Candida*[10-12].

Thus, the objective of this study was to investigate the *in vitro* activity of exoenzymes (phospholipases, acid proteases) in clinical isolates of four species of the genus *Candida*, isolated from several anatomic sites.

METHODS

Strains and culture media

The strains of *Candida* spp. used in this study came from cultures of hospitalized patients (both public and private hospitals in São Luís, State of Maranhão, Brazil) suspected of being infected by microorganisms. The isolates were obtained from 2007 to 2008 and belong to the collection of a private laboratory and were kindly provided for this research. The standard strain *C. albicans* ATCC 18804 (American Type Culture Collection, Rockville, Md) was included in the experiments. The production of aspartyl proteases and phospholipases was studied in 82 clinical isolates of *C. albicans* (47 strains), *C. glabrata* (10 strains), *C. tropicalis* (12 strains), *C. parapsilosis* (13 strains). The isolates were previously identified by the automated system VITEK (BioMériux, Marcy-I'Etoile, France). The yeasts were routinely grown in Sabouraud dextrose agar plates incubated at 37ºC for 24h from stock cultures in BHI-glycerol and stored at 4ºC during the procedures.

Preparation of inoculum

The inoculum of yeast cells was made from stock cultures and incubated for 18 hours at 37ºC in BHI (Brain Heart Infusion – Acumedia Manufectures) or in liquid RPMI-1640 medium and standardized to approximately 10^6 UFC/ml according to the 0.5 McFarland turbidity range (CLSI Clinical and Laboratory Standards Institute - NCCLS, 2007).

Phospholipases production

Production of phospholipases by the isolates was analyzed according the egg yolk agar plate method described by Price et al[13]. The test medium consisted of Sabouraud dextrose agar containing 1M sodium chloride, 0.005M calcium chloride and 2% egg yolk. Each strain was inoculated in triplicate. The Petri plates were incubated at 37ºC and the diameters of the colonies and precipitation area plus the colony were measured 7 days postinoculation. Measurements and calculations of the phospholipase activity zone (Pz) were made according to the method described by Price et al[13]. The Pz of 3 samples of each strain was measured to obtain the average Pz. The Pz coefficients of the *Candida* strains analyzed were grouped into 4 classes: Pz between 0.9 and 1 (+), very low Pz group; 0.89 – 0.80 (+ +), low Pz group; 0.79 – 0.70 (+ + +) high Pz group; and 0.69 (+ + + +), very high Pz group.

Protease production

Determination of the protease production was performed according to Aoki et al[14]. The test medium consisted of plates with agar containing bovine albumin serum (BSA). Sixty milliliters of a solution containing 0.04g $MgSO_4.7H_2O$, 0.5g K_2HPO_4, 1g NaCl, 0.2g yeast extract, 4g glucose and 0.5g BSA (bovine albumin serum. Fraction V, Sigma Chem Co., St. Louis, Mo., USA) was prepared and the pH adjusted to 4.0. The solution was sterilized by filtration and then mixed with 140ml of molten agar. Plates with this medium were incubated at 37ºC for 7 days. Proteinase activity was measured and calculated according to the method described by Price et al[13], in terms of the ratio of the colony diameter and the colony plus the inhibition zone. The study was repeated three times for each strain to calculate the average Pz values. The Pz coefficients were grouped into 4 classes as mentioned above.

Statistical analysis

Data were evaluated by the program BioEstat version 5.0. The association between positivity for hydrolytic enzyme production and *Candida* species and between the same positivity and the origin site was verified by the Chi square test. The Lilliefors normality test was initially performed for Pz numerical variables of proteinases and phospholipases. Since these variables did not show normal distribution ($p < 0.05$), they were analyzed in relation to species and anatomic sites using the Kruskal-Wallis nonparametric variance test. The significance level adopted in all tests was 5%; i.e., values were considered significant when $p < 0.05$.

RESULTS

A total of 82 *Candida* species from different anatomic sites of hospitalized patients were tested for phospholipase and proteinase activities in this study. Analysis of the data obtained verified that *Candida albicans* was the most frequently isolated *Candida* spp. in different origin sites (**Table 1**). Phospholipase activity was detected in 56 (68.3%) of the *Candida* isolates and proteinase activity was detected in 44 (53.6%) of the isolates studied. Of the 47 *C. albicans* isolates studied, 36 (76.6%) showed phospholipase activity and 23 (48.9%) showed positivity for proteinase (**Table 1**).

Among the *Candida* species tested for phospholipase activity, *C. tropicalis* was the species with the highest number of positive isolates (91.7%). *C. parapsilosis* was the species with the lowest number of positive isolates for enzymatic activity (15.4%) (**Table 1**). Observation verified that significant differences ($p < 0.05$) occurred in phospholipase production among the species analyzed.

Regarding acid proteinase production, the *C. parapsilosis* isolates tested showed a higher number of producers (69.2%), followed by *C. glabrata* (60%) and those exceeding the production of *C. albicans*, which was 48.9% (**Table 1**). However, among the species studied, the percentage of isolates producing proteases was not statistically different ($p > 0.05$). All the producer isolates of *C. parapsilosis*, except one, were from blood and catheter specimens.

The distribution of mean values of Pz among the 82 *Candida* isolates analyzed is presented in **Table 2**. The majority of *C. albicans* isolates that showed enzymatic activity (76.6%) were considered to have very strong activity (++++), both regarding phospholipase activity (69.4%) and protease activity (64.2%). The non-*albicans* species mostly showed average phospholipase activity (++) and a very strong proteinase activity (++++) **(Table 3)**. Statistical analysis of the phospholipase activity showed a highly significant difference between the mean Pz values obtained among the *Candida* species ($p < 0.0001$) and in relation to the various anatomic sites ($p = 0.014$). However, the differences between the mean Pz values for proteinase activity among the species ($p = 0.601$) and the anatomic sites ($p = 0.207$) were not statistically significant.

TABLE 1 - Phospholipase and acid proteinase activity of isolated *Candida* spp. according to the site of origin.

		Phospholipase					Proteinase				
Species	Origin site	positive	%	negative	%	total	positive	%	negative	%	total
Candida albicans	urine	17	73.9	6	26.1	23	7	30.4	16	69.6	23
	vaginal secretion	8	72.7	3	27.3	11	6	66.7	3	33.3	9
	tracheal secretion	7	87.5	1	12.5	8	6	75.0	2	25.0	8
	catheter	1	100.0	0	0.0	1	1	100.0	0	0.0	1
	blood	3	100.0	0	0.0	3	3	60.0	2	40.0	5
	peritoneal secretion	0	0.0	1	100.0	1	0	0.0	1	100.0	1
	total	**36**	**76.6**	**11**	**23.4**	**47**	**23**	**48.9**	**24**	**51.1**	**47**
Candida parapsilosis	urine	2	66.7	1	33.3	3	1	33.3	2	66.7	3
	blood	0	0.0	6	100.0	6	5	83.3	1	16.7	6
	catheter	0	0.0	4	100.0	4	3	75.0	1	25.0	4
	total	**2**	**15.4**	**11**	**84.6**	**13**	**9**	**69.2**	**4**	**30.8**	**13**
Candida glabrata	urine	5	71.4	2	28.6	7	5	62.5	3	37.5	8
	tracheal secretion	2	66.7	1	33.3	3	1	50.0	1	50.0	2
	total	**7**	**70.0**	**3**	**30.0**	**10**	**6**	**60.0**	**4**	**40.0**	**10**
Candida tropicalis	urine	6	100.0	0	0.0	6	3	60.0	2	40.0	5
	tracheal secretion	4	100.0	0	0.0	4	2	40.0	3	60.0	5
	blood	1	50.0	1	50.0	2	1	50.0	1	50.0	2
	total	**11**	**91.7**	**1**	**8.3**	**12**	**6**	**50.0**	**6**	**50.0**	**12**
General total		**56**	**68.3**	**26**	**31.7**	**82**	**44**	**53.7**	**38**	**46.3**	**82**

TABLE 2 – Mean Pz values for phospholipase and proteinase for different *Candida* species and sites of origin.

		Phospholipase		Proteinase	
Species	Origin sites	n	$\bar{x} \pm s$	n	$\bar{x} \pm s$
Candida albicans	urine	17	0.62 ± 0.13	7	0.67 ± 0.10
	vaginal secretion	8	0.69 ± 0.10	6	0.67 ± 0.14
	tracheal secretion	7	0.69 ± 0.13	6	0.67 ± 0.16
	catheter	1	0.61 ± 0.00	1	0.52 ± 0.00
	blood	3	0.86 ± 0.15	3	0.71 ± 0.07
Candida parapsilosis	urine	2	0.87 ± 0.02	1	0.73 ± 0.00
	blood			5	0.64 ± 0.05
	catheter			3	0.61 ± 0.07
Candida glabrata	urine	5	0.77 ± 0.11	5	0.62 ± 0.15
	tracheal secretion	2	0.81 ± 0.08	1	0.73 ± 0.00
Candida tropicalis	urine	6	0.8 ± 0.16	3	0.64 ± 0.08
	tracheal secretion	4	0.83 ± 0.04	2	0.65 ± 0.06
	blood	1	0.85 ± 0.00	1	0.86 ± 0.00

TABLE 3 - Distribution of the Pz* value among the isolates of different *Candida* species.

	Candida albicans		*Candida* non-*albicans*	
Pz Values	phospholipase	proteinase	phospholipase	proteinase
≤ 0.69 ++++	24	13	2	16
0.70-0.79 +++	3	8	1	3
0.80-0.89 ++	7	2	15	2
0.90-0.99 +	2	0	2	0
Total (%)	**36 (76.6%)**	**23 (48.9%)**	**20 (57.1%)**	**21 (60.0%)**

*Pz: activity zone for phospholipase and proteinase, ++++: very strong, +++: strong, ++: average, +: weak/poor.

The majority of isolates positive for phospholipases were from tracheal secretion (86.7%) and urine samples (76.9%) (**Table 1**). This difference was statistically significant, with $\chi^2 = 12.6$ and $p = 0.0277$. For proteinase activity, analysis of the isolates revealed no significant differences in enzymatic production between the different anatomic sites ($\chi^2 = 7.2$ and $p = 0.2077$; **Table 1**).

In the present study, 29.3% of the isolates produced both phospholipase and proteinase, including 16 of the 27 strains of *C. albicans*, 5 of the 12 *C. tropicalis*, 1 of the 13 *C. parapsilosis* and 2 of the 10 strains of *C. glabrata*.

DISCUSSION

Phospholipase and proteinase activities are considered to play important roles in the pathogenesis of opportunistic fungi. The roles of these two hydrolytic enzymes in *C. albicans* and other yeast species seem to be related to species virulence[15,16]. The present study aimed to determine *in vitro* phospholipase and proteinase activities in 82 *Candida* isolates, which were collected from several anatomically distinct sites of hospitalized patients. In this work, phospholipase and proteinase activities were observed in both the *C. albicans* and C. non-*albicans* isolates studied.

C. tropicalis proved to be the species with the highest number of positive isolates and *C. parapsilosis* was the species with the lowest number of positive isolates for phospholipase activity, a difference that was statistically significant. These observations are in disagreement with the results obtained by Rorig et al[17], who determined *C. albicans* as the largest enzymatic producer, with neither of the species *C. glabrata* and *C. parapsilosis* revealing phospholipase and proteinase activities. Similarly, a study by Negri et al[18] observed that only one *C. tropicalis* isolate out of seven obtained from a central venous catheter was positive for phospholipases.

Regarding acid proteinase production, the *C. parapsilosis* isolates tested showed a higher number of producers (9 out of 13 strains), followed by *C. glabrata* and those exceeding the production of *C. albicans*; however, this difference was not statistically significant.

De Bernardis et al[10] reported high acid proteinase activity *in vitro* in all *C. parapsilosis* strain isolates from outpatients, regardless of whether they presented candidemia or not or whether they were HIV+ or HIV-. While investigating the presence of virulence factors in 33 *C. parapsilosis* strains, 19 of which were isolated from blood cultures of hospitalized fungemia patients, Dagdeviren et al[19] observed that 42.42% of these strains were acid proteinase producers and 79% showed high activity. Their results are in agreement with the current findings, in which 69.2% of *C. parapsilosis* isolates were acid proteinase producers with high Pz (average Pz = 0.64). However, Kantarcioglu & Yucel[15] showed that the highest producer species of phospholipases was *C. albicans* (93.3%) and only a few strains of *C. kefyr* and *C. glabrata* behaved similarly. In the same study, the authors observed proteinase activity in isolates of *C. albicans* and in a few isolates of *C. kefyr*, *C. lipolytica*, *C. parapsilosis* and *C. tropicalis*, and again *C. albicans* was the largest producer (95%). Both these results differ from those reported here.

In this work, the majority of the *C. albicans* enzyme producers were considered to have very strong activity, both for phospholipase activity and proteinase activity. The non-*albicans* species showed mostly average phospholipase activity and very strong proteinase activity. In contrast to these results, while studying *Candida* samples from healthy people, Oksuz et al[20] verified that the majority of the *C. albicans* isolates showing enzyme activity were ranked as strong. In the same study, most of the *Candida* non-*albicans* isolates that showed phospholipase and proteinase activity were classified as very strong activity for phospholipase and low for proteinase. In this study, the frequencies of phospholipase and proteinase activity in *C. albicans* were 53 and 56.7%, respectively, while for *C.* non-*albicans* isolates, the values obtained were 17 and 43.9%, showing that these frequencies were lower than those obtained in the present study and those reported in other studies.

Different frequencies of enzymatic activity have been reported in *Candida* spp., isolated from different anatomic sites[15,20,21]. The isolates analyzed in this work produced phospholipases independent of the species and this production also varied according to the anatomic site. The majority of the isolates positive for phospholipases were from urine and tracheal secretion samples, although proportionality in the distribution of *Candida* species among the clinical materials is not observed, since higher prevalence of these strains was determined in such sites. Kantarcioglu & Yucel verified that the highest phospholipase activity was determined in respiratory tract isolates[15]. In contrast, Oksuz et al[20] obtained higher extracellular phospholipase activity in isolates from oral (59%) and fecal samples (42.8%), respectively. Regarding proteinase activity among the isolates analyzed, no difference in enzymatic production was observed between the different origin sites, in disagreement with the findings of Kantarcioglu & Yucel regarding proteinase activity, which was higher in isolates from the respiratory tract and urogenital system[15].

Few studies have investigated phospholipase and proteinase production together in the same strains. In this study, 29.3% of the isolates produced both phospholipases and proteinases. Kantarcioglu & Yucel[15] observed that 56 out of 60 strains of *C. albicans* and 2 out of 4 strains of *C. kefyr* tested produced both enzymes, while the other strains studied showed no capacity for producing one of the enzymes analyzed. A study conducted by Shimizu et al[22] investigated the ability of different *Candida* species to simultaneously produce hyaluronidase, chondroitin sulfatase, protease and phospholipase, in order to evaluate whether they were related to *Candida* pathogenicity. They determined that with the exception of the *C. albicans* strains, none of the strains produced all four enzymes simultaneously, in contrast to the results obtained in this study.

Candida tropicalis is a pathogen known to be important in nosocomial infections[12,18]. The majority of the *C. tropicalis* isolates (11 of 12 isolates analyzed) showed phospholipase activity, an enzyme considered to be an important virulence factor and that is probably involved in the pathogenesis of *C. albicans* species[23-25]. However, in the present results, phospholipase production by *C. tropicalis* isolates was considered weak or average (average Pz = 0.83). *C. parapsilosis* has also been indicated as a species capable of causing infection. More than 30% of the notified fungemias during this decade have been associated with *C. parapsilosis*. The rise in fungemia caused by this species is explained by the high capacity of the species to adhere to plastic surfaces, such as catheters, and probably due to the extracellular hydrolytic enzymes they can produce[19]. The *C. parapsilosis* isolates analyzed in this research were large acid protease producers, as were those of *C. tropicalis* and *C. glabrata*.

The results for *C. glabrata* are also important and show that this species can produce acid proteinase *in vitro*, which could be related to species virulence. These data are different from most of those published in the literature, which affirm that secreted proteinase activity has no function in relation to *C. glabrata* virulence[11] and that *C. glabrata* does not seem to produce significant levels of extracellular proteinase activity, at least *in vitro*[15].

In general, the present results showed that *C.* non-*albicans* isolates and those of *C. albicans*, are large producers of hydrolytic enzymes and, consequently, they might be capable of causing infection under favorable conditions. The production levels determined were higher than those reported for *Candida* isolates from healthy individuals. According to these results, proteinase and phospholipase expression can vary according to *Candida* species and strain. Analysis of the results also showed that some species, such as *C. parapisilosis*, which were previously considered SAP-negative, and *C. glabrata*, considered to be a low producer of protease activity, were, in fact, proteolytic.

Analysis of the results obtained indicates differences in phospholipase and acid proteinase production between *Candida* spp. isolates from different sources. This study suggests that the pathogenicity of *Candida* might be related to the site of infection.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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