Original Article

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Correlation of phospholipase and proteinase production of *Candida* with *in vivo* pathogenicity in *Galleria mellonella*

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Abstract

An essential factor to the virulence of the genus *Candida* is the ability to produce enzymes and this may be crucial in the establishment of fungal infections. **Aim:** This study investigated *in vitro* enzymatic activities of *Candida* species and their virulence in an *in vivo Galleria mellonella* experimental model. **Methods:** Twenty-four clinical strains of *Candida* spp. isolated from the human oral cavity were evaluated, including the following species: *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. tropicalis*, *C. krusei*, *C. parapsilosis*, *C. norvegensis*, *C. lusitaniae* and *C. guilliermondii*. All *Candida* strains were tested *in vitro* for production of proteinase and phospholipase. The *Candida* strains were also injected into *Galleria mellonella* larvae to induce experimental candidiasis, and after 24 hours, the survival rate was assessed. **Results:** Phospholipase and proteinase activity were observed in 100% of the *C. albicans* strains. In the non-*albicans* species, proteinase and phospholipase activity were observed in 25 and 43% of the studied strains, respectively. The most pathogenic *Candida* species in *G. mellonella* were *C. albicans*, *C. dubliniensis* and *C. lusitaniae*, whereas *C. glabrata* was the least virulent species. Furthermore, a positive significant correlation was found between both enzymatic activities with virulence in *G. mellonella*. **Conclusions:** The virulence of *Candida* strains in *G. mellonella* is related to the quantity of proteinases and phospholipases production of each strain.

Keywords: *Candida*, virulence factors, invertebrates.

Introduction

As the most common yeasts in humans, *Candida* spp. are responsible for most fungal diseases. Therefore, understanding the mechanisms by which these microorganisms colonize and cause disease in humans is a great challenge for planning and establishing treatments[1]. *Candida* species have many virulence factors, which in the presence of local and systemic host failures may result in their transition from commensal to pathogenic organisms.

The virulence factors vary between different *Candida* species. *C. albicans* is the most virulent species, and it has virulence factors that allow it to adhere to oral tissues, invade tissue, escape host defenses, form germ tubes and hyphae, and produce histolytic enzymes such as proteinases and phospholipases[2-3].

There are two major families of histolytic enzymes produced by *Candida* species: the secretory aspartyl proteinases (SAP) and phospholipases (PL). Proteinases are central virulence factors that facilitate the colonization and invasion of host

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tissues via degradation of physiological substrates, such as albumin, immunoglobulins and skin proteins, and inhibition of phagocytosis-inducing inflammatory reactions[4-6]. Phospholipases also play a role in *Candida* pathogenesis, as they may damage the host cell membranes. Various *Candida* species produce proteinases and phospholipases, although in lower amounts than *C. albicans*[6].

The pathogenic mechanisms of *Candida* yeasts and fungus-host interactions have been studied in rat and mouse experimental models of candidiasis[7-11]. Recent *in vivo* studies in invertebrate models of candidiasis investigated the humoral and cellular immune responses of the host. Invertebrates have several advantages over the conventional mammalian models, including lower costs, faster results and fewer ethical issues[12-13].

Galleria mellonella, the greater wax moth larva, is an interesting model for *in vivo* studies of pathogenic yeasts, such as *C. albicans*, *C. parapsilosis*, *C. glabrata*, *C. tropicalis*, and *C. krusei*, and bacteria, including *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Escherichia coli* and *Bacillus cereus*[14]. Aside from its low costs, *G. mellonella* allows systemic inoculation of these pathogens. Moreover, *G. mellonella* is the insect most widely used to study human immune responses[15-16] .

Brennan et al.[17] demonstrated that virulence deviations of hyphal-deficient mutants detected in *Galleria mellonella* are similar to those observed in mice. The response of insects to infection shows strong similarities to that found in mammals and opens the possibility of reducing the need to use mammals for testing the virulence of mutants by employing *Galleria* larvae as a primary screening mechanism. The ability to screen a large number of mutants in a short time using *G. mellonella* would have a number of cost and labor advantages compared to the use of conventional vertebrate models.

Since enzymatic activity may be crucial in the establishment of fungal infections[18-19], the aim of this study was to correlate the production of proteinases and phospholipases by *Candida* species with their virulence in *G. mellonella*.

Materials and methods

Candida isolates

A total of 24 clinical *Candida* strains recovered from the oral cavity of HIV-positive patients seen at the Emílio Ribas Institute of Infectious Diseases (São Paulo, Brazil) were studied. The isolates included the following species: *C. albicans* (n = 8), *C. glabrata* (n = 4), *C. dubliniensis* (n = 3), *C. tropicalis* (n = 2), *C. krusei* (n = 2), *C. parapsilosis* (n = 2), *C. guilliermondii* (n = 1), *C. lusitaniae* (n = 1) and *C. norvegensis* (n = 1).

The identification of *Candida* species was done by growth on Hicrome *Candida* (Himedia, Mumbai, Maharashtra, India), germ tube test, clamydospore formation on corn meal agar, and API20C for sugar assimilation (BioMerieux, Marcy l'Etoile, France). The identity of *C. dubliniensis* was determined by a multiplex polymerase chain reaction (PCR) procedure.

The study was approved by the Ethics Committee of the School of Dentistry of São José dos Campos/UNESP (protocol 21/2010/CEPa), according to the Declaration of Helsinki, and followed the guidelines established by "Ethical Principles for Animal Experimentation of the Brazilian College of Animal Experimentation" (COBEA).

All isolates were revived from stock cultures maintained in YPD broth (Himedia) with 20% glycerol (Amresco, Solon, OH, USA) at -80ºC. The cultures in maintenance medium were transferred onto fresh Sabouraud's dextrose agar (SDA) plates (Himedia) and incubated at 37 ºC for 24-48 h. The fresh cultures were used in enzyme assays and in the *G. mellonella* infection model.

Phospholipase activity

The *Candida* isolates were evaluated for phospholipase production using the egg yolk agar plate method described by Price et al.[20] and Pereira et al.[21]. The SDA plates contained 57.3 g NaCl, 0.55 g $CaCl_2$ and 8% sterile egg yolk emulsion. The test strains were spot inoculated (~6 mm), and the plates were incubated at 37 ºC for up to 5 days. Each isolate was tested in quadruplicate. The diameter of the colony and the surrounding precipitation zone (Pz) were measured, and phospholipase activity was scored as per the described method[20]. The Pz value represented the ratio of the colony diameter to the diameter of the colony plus the precipitation zone. The results were classified as follows: no activity ($Pz = 1$), moderate activity ($0.64 \geq Pz < 1$) and strong activity ($Pz < 0.64$). By this classification, a high Pz value indicates low enzymatic activity.

Proteinase activity

All *Candida* isolates were tested for proteinase secretion in bovine serum albumin (BSA) agar that contained yeast carbon base (1.17%), yeast extract (0.01%) and BSA (0.2%) using the method of Ruchell et al.[22].The medium was adjusted to pH 5.0, sterilized by filtration and added to autoclaved 2% agar. The test strains were spot inoculated (~6 mm) and the plates were incubated at 37 ºC for up to 5 days. Each isolate was tested in quadruplicate. The post-incubation clearance zone around the colony was recorded, and the Pz value for proteinase activity was calculated as described above.

Galleria mellonella infection model

G. mellonella were infected with *Candida* as previously described by Fuchs et al.[23] and Junqueira et al. [24]. In brief, *G. mellonella* caterpillars in the final instar larval stage (Entomology and Plant Pathology Laboratory, State University of North Fluminense, RJ, Brazil) were stored in the dark and used within 7 days from the date of shipment. Sixteen randomly chosen caterpillars (330 ± 25 mg) were infected for each *Candida* isolate for a total of 384 assays.

Candida inocula were prepared by growing 5 mL YNB broth (1% yeast extract, 1% dextrose and 2% peptone) cultures overnight at 37 ºC. The cells were pelleted at $2000 \times g$ for 10 min and washed three times in PBS (Laborclin, Pinhais,

PR, Brazil). The cell densities were determined by hemacytometer counting. The *Candida* inocula were confirmed by determining the colony-forming units per milliliter (CFU/mL) on SDA.

A Hamilton syringe (Hamilton, Reno, NV, USA) was used to deliver *Candida* inocula (10^5 cells in 10 µL) into the hemocoel of each larva via the last left proleg. The larvae were incubated in plastic containers (37 ºC) and the number of dead *G. mellonella* was counted after 24 h of infection.

Statistical analysis

Percent survival and killing curves of *G. mellonella* were plotted and statistical analysis was performed by the Log-rank (Mantel-Cox) test using GraphPad Prism statistical software (GraphPad Software, Inc., California, CA, USA) The Pearson test was used to correlate phospholipase and proteinase activity with virulence in *G. mellonella*. This test was performed in the Minitab Program with a 5% significance level.

Results

All *C. albicans* isolates (100%) were positive for proteinase and phospholipase activity. Only 25% and 43.75% of the non-*albicans* species showed proteinase and phospholipase activity, respectively (Table 1).

The most pathogenic *Candida* species *in vivo* were *C. albicans*, *C. dubliniensis* and *C. lusitaniae*, which caused over 90% mortality at 24 h post-infection. The least virulent species was *C. glabrata* with 9.37% mortality (Log-rank test: $p = 0.0001$) (Table 2).

The results obtained for each *Candida* strain, including the Pz values for enzymatic activity and the *G. mellonella* mortality rates, are shown in Table 3.

Using a Pearson test, a significant correlation between the proteinase Pz values and *G. mellonella* mortality was observed (Pearson correlation = -0.571, $p = 0.004$). There was a similar correlation between phospholipase Pz values and *in vivo* mortality (Pearson correlation = -0.447, $p = 0.029$). According to Pereira et al.[21], low Pz values indicate high enzymatic activity. This study demonstrated that greater production of proteinases and phospholipases by *Candida* strains led to increased virulence in *G. mellonella* (Fig. 1).

Discussion

The identification of virulence factors in *Candida* strains can elucidate their adhesion, invasion and infection process, thus leading to the development of more efficient antifungal therapies[25]. In the present study was evaluated the production of two types of extracellular enzymes, proteinases and phospholipases, which are virulence factors that are crucial to *Candida* pathogenesis. Furthermore, the enzymatic activity

Table 1. Activity of proteinase and phospholipase for the *Candida* species studied according to the classification of Price et al.[20]: strong activity (+ +), moderate activity (+) and no activity (-)

Candida species(number of strains)	Proteinase activity			Phospholipase activity		
	++	+	-	++	+	-
C. albicans (n=8)	3	5	0	5	3	0
C. glabrata (n=4)	0	0	4	0	0	4
C. dubliniensis (n=3)	0	2	1	0	2	1
C. parapsilosis (n=2)	2	0	0	0	0	2
C. tropicalis (n=2)	0	0	2	1	1	0
C. krusei (n=2)	0	0	2	0	2	0
C. norvegensis (n=1)	0	0	1	0	1	0
C. lusitaniae (n=1)	0	0	1	0	0	1
C. guilliermondii (n=1)	0	0	1	0	0	1

Table 2. *G. mellonella* larvae killed 24 h after infection by *Candida* species

Candida species (number of *G. mellonella*)	Number of deaths (24 h post-infection)	Mortality rate (24 h post-infection)
C. albicans (n = 128)	125	97.65%
C. glabrata (n = 64)	6	9.37%
C. dubliniensis (n=48)	47	97.91%
C. parapsilosis (n=32)	18	56.25%
C. tropicalis (n=32)	6	18.75%
C. krusei (n=32)	13	40.62%
C. norvegensis (n=16)	2	12.5%
C. lusitaniae (n=16)	15	93.75%
C. guilliermondii (n=16)	5	31.25%
Total (n=384)	237	61.71%

Table 3. Pz values for enzymatic activity and *G. mellonella* larvae killed 24 h after infection with different *Candida* strains

Candida strain(Identity code)	Proteinase(Pz)	Phospholipase (Pz)	*G. mellonella*(n=16)
C. albicans (15-S)	0.64	0.54	16
C. albicans (7)	0.64	0.54	13
C. albicans (21)	0.80	0.73	16
C. albicans (60)	0.48	0.72	16
C. albicans (10-S)	0.56	0.46	16
C. albicans (24-S)	0,70	0.55	16
C. albicans (31-S)	0.86	0.75	16
C. albicans (39-S)	0.50	0.41	16
C. glabrata (55)	1.00	1.00	0
C. glabrata (217-S)	1.00	1.00	4
C. glabrata (89-S)	1.00	1.00	2
C. glabrata (12-S)	1.00	1.00	0
C. dubliniensis (222-S)	0.75	0.67	16
C. dubliniensis (183-S)	1.00	1.00	16
C. dubliniensis (155-S)	0.75	0.87	15
C. parapsilosis (127-S)	0.50	1.00	13
C. parapsilosis (156-S)	0.43	1.00	5
C. tropicalis (140-S)	1.00	0.70	3
C. tropicalis (212S)	1.00	0.59	3
C. krusei (54-S)	1.00	0.84	10
C. krusei (64-S)	1.00	0.90	3
C. norvegensis (52-S)	1.00	0.67	2
C. lusitaniae (114-S)	1.00	1.00	15
C. guilliermondii (166-S)	1.00	1.00	5

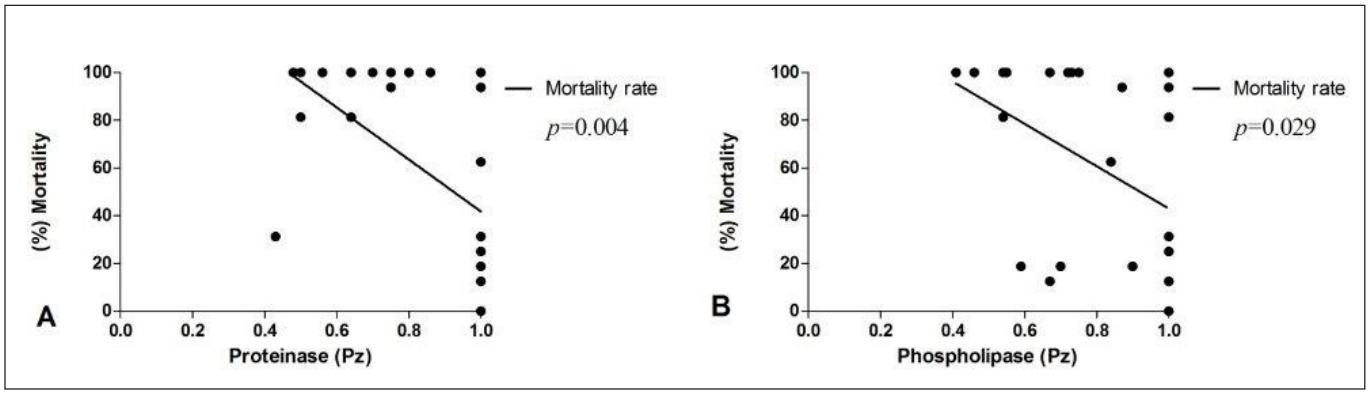

Fig. 1. Scatter plot and respective regression line of mortality rate versus enzymatic activity. A) Proteinase: y = -109.0*X + 150.8; R^2= 0.326. B) Phospholipase: y = -88.84*X + 131.8; R^2 = 0.200. There was significant difference between mortality rate and enzymatic activity ($p<0.05$).

of various *Candida* strains was correlated with *in vivo* virulence in *G. mellonella*.

All *C. albicans* isolates (100%) were positive for proteinase and phospholipase production. This result is in agreement with Pereira et al.[21], who studied the enzymatic activity of 51 strains of *Candida* in oral isolates and found that all *C. albicans* strains were positive for proteinase and phospholipase activity. Martins et al.[26] also observed presence of these enzymes in all tested isolates of *C. albicans*. In non-*albicans* species, the enzymatic activity was lower. Only 25% of the strains produced proteinases and 43% of the strains produced phospholipases.

These data agree with a study by Junqueira et al.[19] that evaluated the enzymatic activity of 64 *Candida* isolates. Among the 31 non-*albicans* species, 21% had proteinase activity and 39% had phospholipase activity. Previous studies also reported that proteinases and phospholipases are produced at high levels in *C. albicans*, whereas non-*albicans* species generally have lower production of these enzymes. In a work by Mane et al. [27], enzymatic activity was evaluated in 65 strains of *Candida* (39 *C. albicans* and 26 non-*albicans*) isolated from HIV-positive, oral candidiasis patients. The

authors verified that most *C. albicans* strains had active proteinases (89% of strains) and phospholipases (59%), while only 13% or 6% of non-*albicans* strains were positive for proteinase or phospholipases, respectively.

In the present study, was used an *in vivo G. mellonella* infection model to evaluate the pathogenicity of oral *Candida* isolates. There are some benefits to using *G. mellonella* larvae as a model host. For example, the larvae can be maintained at a temperature range from 25 ˚C to 37 ˚C, thus facilitating a number of temperature conditions under which fungi exist in either natural environmental niches or mammalian hosts[12,25]. Recently, Mesa-Arango et al.[28] investigated the efficacy of antifungal drugs in this infection model and verified that amphotericin B, caspofungin, fluconazole and voriconazole had a protective effect at concentrations equivalent to therapeutic doses used in human, concluding that *G. mellonella* offers a simple and feasible model to study drug efficacy.

C. albicans, *C. dubliniensis* and *C. lusitaniae* were more pathogenic in the *G. mellonella* experimental model. *C. albicans* is the most pathogenic *Candida* species due to its virulence factors, which include Hwp1, a hyphal cell wall protein responsible for adherence and biofilm formation[29]. Cotter et al.[14] tested the pathogenicity of *Candida* species in *G. mellonella* experimental models. Microbial suspensions were inoculated into hemolymphs of larvae, which were then incubated at 30 ˚C for 72 h. The resulting mortality rates were similar to those in the present study. *C. albicans* killed 90% of the larvae, while the mortality rates for other species were 70%, 45%, 45%, 20% and 0% for *C tropicalis*, *C. parapsilosis*, *C. pseudotropicalis*, *C. krusei* and *C. glabrata*, respectively.

The virulence of *Candida* strains in *G. mellonella* was dependent on the intensity of enzyme production. This study was the first to correlate *in vitro* enzyme production with *in vivo* pathogenicity. In *Candida* strains with higher proteinase and phospholipase activity, *G. mellonella* virulence was greater. This correlation may be due to the high protein and lipid content in *G. mellonella* bodies[30].

Fuchs et al.[23] correlated *C. albicans* filamentation with pathogenicity in *G. mellonella*. Five mutant strains lacking the genes BCR1, FLO8, KEM1, TEC1 and SUV3, which are required for filamentation, were studied. Only the mutant strain FLO8 did not form filaments in *G. mellonella*, and this strain also showed reduced virulence in the larvae. In contrast, the TEC1 mutant strain exhibited reduced pathogenicity and hyphae formation in larvae. The authors concluded that filamentation was not sufficient for a lethal infection in *G. mellonella* and suggested that other *Candida* virulence factors were associated with *in vivo* pathogenicity.

The number of studies using *G. mellonella* as a model host has increased significantly in the last few years. In addition, there has been an improvement in the techniques used with this model, which allows further studies of *Candida* virulence factors and pathogenicity in this experimental model of infection that will elucidate host-pathogen interaction.

In conclusion, the virulence of *Candida* strains in *G. mellonella* is related to the quantity of proteinases and phospholipases production of each strain.

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