



Revista Iberoamericana de Micología

www.elsevier.es/reviberoammicol



Note

Protease and phospholipase activities of *Candida* spp. isolated from cutaneous candidiasis



Lívia de Souza Ramos^{a,c}, Leonardo Silva Barbedo^b, Lys Adriana Braga-Silva^c,
André Luis Souza dos Santos^c, Marcia Ribeiro Pinto^a, Diana Bridon da Graça Sgarbi^{a,*}

^a Instituto Biomédico, Universidade Federal Fluminense, Niterói, Rio de Janeiro, Brazil

^b Instituto de Pesquisa Clínica Evandro Chagas, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil

^c Instituto de Microbiologia Paulo de Góes and Programa de Pós-Graduação em Bioquímica – Instituto de Química, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

ARTICLE INFO

Article history:

Received 30 May 2013

Accepted 21 January 2014

Available online 20 May 2014

Keywords:

Candida spp.
Clinical isolates
Cutaneous candidiasis
Protease
Phospholipase
Virulence factors

ABSTRACT

Background: Cases of superficial and invasive mycoses caused by emerging species of *Candida* have been increasingly reported over the last thirty years. The production of hydrolytic enzymes plays a central role in the fungal infective process. In *Candida* infections the secretion of both proteases and phospholipases are well-known virulence attributes.

Aims: To determine the protease and phospholipase production from 58 human clinical isolates of *Candida* obtained from individuals with cutaneous candidiasis seen in the Human and Veterinary Diagnostic Mycology Sector from Universidade Federal Fluminense (UFF), Brazil, from November 2008 to August 2009.

Methods: Fungal identification was performed using biochemical tests. Proteolytic activity was detected on agar plates containing bovine serum albumin, and phospholipase production was determined on egg-yolk plates.

Results: The *Candida* species isolated were *Candida parapsilosis* (27.59%), *Candida famata* (18.96%), *Candida albicans* (15.52%), *Candida haemulonii* (12.06%), *Candida ciferri* (8.62%), *Candida guilliermondii* (6.90%), *Candida tropicalis* (5.17%) and *Candida lipolytica* (5.17%). All isolates of *C. albicans* produced both protease and phospholipase. As regards the isolates of non-*C. albicans* *Candida* species, 53.06% and 4.08% were able to produce protease and phospholipase, respectively. For example, the majority of isolates of *C. parapsilosis* (15/16) produced protease, while 40% of *C. ciferri* isolates (2/5) were phospholipase producers. This study shows, for the first time, that *C. ciferri* and *C. haemulonii* strains were able to produce protease.

Conclusions: Collectively, our results showed that different species of *Candida* isolated from cutaneous lesions were able to produce proteases and/or phospholipases, which are multifunctional molecules directly involved in the infectious process of these fungi.

© 2013 Revista Iberoamericana de Micología. Published by Elsevier España, S.L.U. All rights reserved.

Actividades proteasa y fosfolipasa en aislamientos de *Candida* procedentes de candidiasis cutánea

RESUMEN

Antecedentes: Los casos de micosis superficiales e invasoras relacionados con las especies emergentes de *Candida* se han reportado progresivamente durante las últimas tres décadas. La producción de enzimas hidrolíticas juega un papel central en varios contextos de la patogenicidad fúngica. Con respecto a la infección por *Candida*, la secreción de proteasas y fosfolipasas son atributos de virulencia bien conocidos.

Objetivos: Determinar y comparar la producción de proteasa y fosfolipasa de 58 aislamientos clínicos humanos de diferentes especies de *Candida* obtenidos de pacientes con candidiasis cutánea, atendidos en el Sector de Diagnóstico Micológico Humano y Veterinario de la Universidad Federal Fluminense (UFF), durante el período de noviembre de 2008 a agosto de 2009.

Palabras clave:

Candida spp.
Aislamientos clínicos
Candidiasis cutánea
Proteasa
Fosfolipasa
Factores de virulencia

* Corresponding author.

E-mail addresses: dbridon@id.uff.br, dsgarbi@globo.com (D.B.G. Sgarbi).

Métodos: La identificación de las especies de *Candida* se realizó mediante pruebas bioquímicas, la actividad proteolítica se detectó en placas de agar que contenían albúmina de suero bovino y la actividad fosfolipasa se determinó utilizando el método de la placa de yema de huevo semi-cuantitativa.

Resultados: Las especies aisladas fueron *Candida parapsilosis* (27,59%), *Candida famata* (18,96%), *Candida albicans* (15,52%), *Candida haemulonii* (12,06%), *Candida ciferri* (8,62%), *Candida guilliermondii* (6,90%), *Candida tropicalis* (5,17%) y *Candida lipolytica* (5,17%). Todos los aislamientos de *C. albicans* produjeron tanto proteasa como fosfolipasa. El 53,06% de los aislamientos de *Candida* no-*C. albicans* fueron capaces de producir proteasa y el 4,08% fosfolipasa. La mayoría de los aislamientos de *C. parapsilosis* (15/16) produjo proteasa, mientras que el 40% de los aislamientos de *C. ciferri* (2/5) fueron productores de fosfolipasa. Se describe por primera vez en la literatura científica la producción de proteasas por cepas de *C. haemulonii* y *C. ciferri*.

Conclusiones: Nuestros resultados muestran el potencial que tienen los aislamientos de *Candida* provenientes de lesiones cutáneas para producir proteasas y fosfolipasas.

© 2013 Revista Iberoamericana de Micología. Publicado por Elsevier España, S.L.U. Todos los derechos reservados.

Superficial mycoses of the skin and its appendages are amongst the most prevalent human infectious diseases observed in clinical practice. The etiological agents comprise the dermatophytes and yeasts responsible for infections including dermatophytosis, pityriasis versicolor and candidiasis. The prevalence of these fungi tends to follow geographic variations, cultural habits and migration that can change with time.¹¹ Cases of superficial and invasive diseases related to species belonging to the *Candida* genus have been progressively reported in the last years, involving isolates of *Candida albicans*, *Candida ciferri*, *Candida dubliniensis*, *Candida famata*, *Candida glabrata*, *Candida guilliermondii*, *Candida haemulonii*, *Candida kefyr*, *Candida krusei*, *Candida lipolytica*, *Candida lusitanae*, *Candida parapsilosis*, and *Candida tropicalis*, among others.¹⁹

Crucial steps during pathogenesis of superficial candidiasis comprise fungal adhesion, colonization and subsequent penetration of tissues. In all these phases, the production of hydrolytic enzymes is a fundamental event.^{13,14} In this sense, two major classes of enzymes have prominent and central roles: proteases and phospholipases, since proteins and phospholipids represent the major chemical constituents of the host cell membrane, which must be destroyed in order to obtain nutrients for fungal growth/development as well as to facilitate the dissemination inside the host.^{10,20}

The aim of the present study was to investigate the in vitro production of phospholipase and protease activities in different *Candida* species isolated from human cases of cutaneous candidiasis.

Materials and methods

Candida isolates

The clinical isolates ($n=58$) were obtained from patients presenting cutaneous candidiasis, who were attended during the period of November 2008 until August 2009 at the Sector of Human and Veterinary Diagnostic Mycology, Microbiology and Parasitology Department of Universidade Federal Fluminense, Niterói, Rio de Janeiro, Brazil. The fungal isolates were recovered from the following body location: toe nail ($n=24$), finger nail ($n=20$), sole of the foot ($n=4$) and skin ($n=10$). The study was approved by the Bioethics Committee of Hospital Universitário Antônio Pedro (CEP CMM/HUAP number 188/08 and CAAE number 0147.0.258.000-08).

Identification tests

Clinical isolates were initially tested on CHROMagar *Candida* medium (CHROMagar *Candida* Company, Paris, France). Plates were incubated for 24–72 h at 37 °C under aerobic conditions.

Metabolic properties, such as assimilation of carbon compounds and enzymatic reactions, were analyzed by VITEK 2 yeast identification system (bioMérieux, Marcy l'Etoile, France). In addition, germ-tube tests as well as the capacity of yeasts to grow at different temperatures and salt concentrations were performed.³⁰ Growth was estimated by counting the fungal cells in a Neubauer chamber.

Protease assay

Isolates were tested for protease production by using yeast carbon base (YCB) medium (1.17%) supplemented with 0.2% bovine serum albumin (BSA). The YCB-BSA medium was adjusted to pH 5.0, sterilized by filtration and added to previously autoclaved agar (2%).²³ Aliquots (10 μ l) of 48 h-old cultured fungal cells (10^6 yeasts) were spotted onto the surface of the YCB-BSA medium and incubated at 37 °C for seven days. The proteolytic activity results in a clear zone around the colony, which corresponds to the hydrolysis of the BSA present in the medium. The colony diameter (a) and the diameter of colony plus the precipitation zone (b) were measured by a digital paquimeter.

Phospholipase assay

Phospholipase production was assayed using the egg-yolk agar plate containing 1 M NaCl, 5 mM CaCl₂ and 8% sterile egg-yolk emulsion.²¹ Suspensions of approximately 10^6 yeasts (10 μ l) of each isolate, previously cultivated on Sabouraud during two days, were inoculated onto the surface of the egg-yolk agar plate medium and incubated at 37 °C for seven days. Hydrolysis of lipid substrates present in the egg-yolk results in the formation of a calcium complex with fatty acids released by the action of the secreted enzymes, resulting in a precipitation zone around the colony. The colony diameter (a) and the diameter of the colony plus the precipitation zone (b) were measured by a digital paquimeter.

Enzymatic score and statistical analysis

The enzyme activities were expressed as Pz value (a/b) as described by Price and co-workers.²¹ According to this definition, low Pz values mean high protease or phospholipase production and, inversely, high Pz values indicate low enzymatic production. The enzymatic activity was scored into four categories: a Pz of 1.0 indicated no enzymatic activity; a Pz between 0.999 and 0.700 indicated low enzymatic activity; Pz between 0.699 and 0.400 corresponded to moderate activity; and low Pz values between 0.399 and 0.100 meant high enzymatic activity. All the experiments were repeated at least three times and all assays were carried out in triplicate. The

data were analyzed statistically using Student's *t* test. *P* values of 0.05 or less were considered statistically significant.

Results and discussion

The prevalence of superficial mycoses has tremendously risen in the last years, affecting around 20–25% of the world's population. In this scenario, skin mycoses are the most frequent forms of infection and, consequently, constitute a major public health problem worldwide.^{15,25} Our results revealed that from the 58 *Candida* isolates recovered from cutaneous infections, *C. parapsilosis* was the most frequently isolated species (*n* = 16; 27.6%), followed by *C. famata* (*n* = 11; 19%), *C. albicans* (*n* = 9; 15.5%), *C. haemulonii* (*n* = 7; 12%), *C. ciferri* (*n* = 5; 8.6%), *C. guilliermondii* (*n* = 4; 6.9%), *C. tropicalis* (*n* = 3; 5.2%) and *C. lipolytica* (*n* = 3; 5.2%). Considering the site of the cutaneous infections, *C. parapsilosis* (20.5%) was the most prevalent *Candida* species isolated from finger followed by *C. albicans* (18.2%), *C. famata* (18.2%), *C. haemulonii* (13.6%), *C. ciferri* (11.4%), *C. guilliermondii* (9.1%), *C. tropicalis* (4.5%) and *C. lipolytica* (4.5%), while *C. parapsilosis* (70%) was again by far the most isolated species from skin followed by *C. albicans* (10%), *C. famata* (10%) and *C. tropicalis* (10%).

Epidemiological and etiological studies of superficial mycoses of the foot revealed that the major isolated causal agents were *C. parapsilosis* (25%) and *Trichophyton rubrum* (18.7%).^{5,6} *C. parapsilosis* was the leading cause of onychomycosis in a group of patients from Malta followed by *C. tropicalis* and *C. guilliermondii*.²⁷ Similarly, a study performed in Brazil reported that the leading pathogen detected in samples from infected nails (*n* = 200) was *C. parapsilosis* (40.5%) followed by *C. albicans* (31.5%), *C. tropicalis* (26.0%) and *C. guilliermondii* (2.0%).⁷ Contrarily, *C. albicans* was the most common *Candida* species able to cause cutaneous/nail and vaginal candidiasis in Singapore,¹⁸ Jordan,¹ Slovakia¹² and the United States.⁸ Importantly, the increased tendency for non-*C. albicans* *Candida* infections poses a challenge in the disease management, since these species are often notorious to develop antifungal resistance that is correlated with routine fluconazole prophylaxis adopted in some patients and the intrinsic/acquired azole resistance of *Candida* spp.³

The production of proteases, especially secreted aspartic proteases (Saps), has been reported to be one of the most important determinants for pathogenicity of *Candida*.⁹ The culture of *Candida* yeasts in YCB-BSA at acidic pH is a recognized condition that induces the secretion of Saps.²⁸ Under these conditions, we showed that protease activity was detected in 35 strains of all *Candida* species tested, among which all strains of *C. albicans* and almost all strains of *C. parapsilosis* were protease producers (Table 1). All *C. albicans* isolates exhibited high Pz values, which indicate low enzymatic production, whereas the majority (66.7%) of *C. parapsilosis* isolates displayed moderate Pz and the minority (33.3%) high Pz values. However, strains of *C. famata* showed lower Pz values when compared to the other *Candida* species (Table 1). In addition, the protease activity of *C. famata* was significantly higher than that of *C. albicans* (*P* < 0.001) or *C. parapsilosis* (*P* < 0.03). None of the

C. lipolytica or *C. guilliermondii* isolates showed proteolytic activity (Table 1), under the employed experimental conditions. The analysis of the body location also revealed a significant difference (*P* < 0.05) in the protease production: there was a 90% of producers between the skin isolates, 55% between the finger nails' isolates, 54.2% between the toe nails' isolates, and 50% in the case of the sole of feet isolates.

Kantarcioglu and Yücel showed that 78.9% of all examined *Candida* strains (*n* = 95) were protease-positive.¹⁶ Those authors also interpreted their data by different viewpoints. For example, 95% of *C. albicans* produced proteases. However, considering only the site of fungal isolation, a new profile of protease producers could be seen, as follows: oral cavity (17/22 = 77.3%), respiratory tract (37/46 = 80.4%), urogenital system (21/23 = 91.3%), and blood (0/4 = 0%).¹⁶ These discrepancies in percentages of positivity may be considered as being relevant due to the number of the different species tested as well as the site of isolation. Corroborating these findings, De Bernardis and co-workers reported that all cutaneous isolates of *C. parapsilosis* had uniformly elevated secreted protease activity, more than four times higher than the enzyme activity of the blood isolates.⁴ Additionally, the cutaneous isolates of *C. parapsilosis* were highly vaginopathic in a rat vaginitis model when compared to the blood isolates.⁴ Cassone also detected a higher proteolytic activity in vaginal *C. parapsilosis* isolates when compared with blood isolates.² Different published works revealed that 65–75% of clinical strains of *C. tropicalis* were able to yield aspartic proteases,^{17,26,29} which are in accordance with our findings (≈67%). We reported herein that cutaneous clinical isolates of *C. famata* were the most potent protease producers. For the first time, we emphasize that some clinical strains of *C. ciferri* and *C. haemulonii* are able to secrete aspartic proteases.

Phospholipases play an active role in the invasion of host tissue by *Candida* by disrupting the epithelial cell membranes and allowing the hyphal tip to enter the cytoplasm.¹⁰ Regarding phospholipase activity, all strains of *C. albicans* and only few strains (2/5) of *C. ciferri* were capable of producing phospholipase, while the great majority (81%) of the *Candida* strains isolated from cutaneous lesions was unable to yield it (Table 1). The 2 strains of *C. ciferri* and 5 strains of *C. albicans* able to produce phospholipase activity exhibited high Pz values, while the other 4 remaining strains of *C. albicans* showed moderate Pz values. Samaranayake and co-workers screened 41 *Candida* isolates for phospholipase activity and found no strains of *C. tropicalis*, *C. glabrata* and *C. parapsilosis* producing extracellular phospholipases, whereas 73% of the *C. albicans* isolates screened were found to be positive.²⁴ Kumar and co-workers showed that 100% of clinical isolates of *C. albicans* isolated from HIV positive and cancer patients produced a pronounced phospholipase activity.¹⁷ The absence of protease and/or phospholipase in clinical isolates of *Candida* must be interpreted with caution. In this context, hydrolytic enzymes with activities against other substrates of relevance to human cutaneous infections, distinct to those used in the present study, can help in the detection of these enzymatic classes.

Table 1

Distribution of protease and phospholipase activities from *Candida* spp. isolated from cutaneous infections.

Species	Protease activity (%)	Range (Pz)	Mean (Pz)	Phospholipase activity (%)	Range (Pz)	Mean (Pz)
<i>C. albicans</i>	100 (9/9)	0.73–0.83	0.78 ± 0.04	100 (9/9)	0.53–0.93	0.73 ± 0.13
<i>C. ciferri</i>	20 (1/5)	0.78	0.78 ± 0.02	40 (2/5)	0.73–0.83	0.78 ± 0.07
<i>C. famata</i>	63.6 (7/11)	0.45–0.85	0.57 ± 0.15	0 (0/11)	0	0
<i>C. guilliermondii</i>	0 (0/4)	0	0	0 (0/4)	0	0
<i>C. haemulonii</i>	14.3 (1/7)	0.60	0.60 ± 0.05	0 (0/7)	0	0
<i>C. lipolytica</i>	0 (0/3)	0	0	0 (0/3)	0	0
<i>C. parapsilosis</i>	93.8 (15/16)	0.57–0.90	0.69 ± 0.09	0 (0/16)	0	0
<i>C. tropicalis</i>	66.7 (2/3)	0.71–0.86	0.79 ± 0.11	0 (0/3)	0	0
Total	60.34% (35/58)			18.97% (11/58)		

The comparison between *C. albicans* and non-*C. albicans* *Candida* species revealed that the frequency of isolation (15.5% versus 82.5%), as well as the production of both protease (100% versus 53.1%) and phospholipase (100% versus 4.1%) activities, was significantly different ($P < 0.05$). Only 17.2% (10/58) of the *Candida* isolates produced both enzyme classes (all strains of *C. albicans* and one strain of *C. ciferri*). Previous studies also reported that proteases and phospholipases are produced at high rates in *C. albicans*, whilst non-*C. albicans* *Candida* species usually present low rates of these enzymes.^{16,22} In this sense, the hydrolytic enzyme profiles provide some data about the potential virulence factors produced by *Candida* strains isolated from cutaneous infection, resulting in a great variability production in non-*C. albicans* *Candida* species (probably strain specific), in contrast to a homogeneous production of both protease and phospholipase by clinical isolates of *C. albicans*.

Conflict of interest

The authors report no conflict of interest.

Acknowledgements

This study was supported by grants from the following Brazilian Agencies: Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa no Estado do Rio de Janeiro (FAPERJ). The authors thank Dr. Marta Helena Branquinho (UFRJ) for critical suggestions on the manuscript.

References

1. Abu-Elteen KH. Incidence and distribution of *Candida* species isolated from human skin in Jordan. *Mycoses*. 1999;42:311–7.
2. Cassone A, De Bernardis F, Pontieri E, Carruba G, Girmenia C, Martino P, et al. Biotyping diversity of *Candida parapsilosis* and its relationship to the clinical source and experimental pathogenicity. *J Infect Dis*. 1995;171:967–75.
3. Concia E, Azzini AM, Conti M. Epidemiology, incidence and risk factors for invasive candidiasis in high-risk patients. *Drugs*. 2009;69:5–14.
4. De Bernardis F, Mondello F, San Millán R, Pontón J, Cassone A. Biotyping and virulence properties of skin isolates of *Candida parapsilosis*. *J Clin Microbiol*. 1999;37:3481–6.
5. Djeridane A, Djeridane Y, Ammar-Khodja A. A clinicomycological study of fungal foot infections among Algerian military personnel. *Clin Exp Dermatol*. 2007;32:60–3.
6. El Fekih N, Belghith I, Trabelsi S, Skhiri-Aounallah H, Khaled S, Fazaa B. Epidemiological and etiologic study of foot mycosis in Tunisia. *Actas Dermosifiliogr*. 2012;103:520–4.
7. Figueiredo VT, de Assis Santos D, Resende MA, Hamdan JS. Identification and in vitro antifungal susceptibility testing of 200 clinical isolates of *Candida* spp. responsible for fingernail infections. *Mycopathologia*. 2007;164:27–33.
8. Foster KW, Ghannoum MA, Elewski BE. Epidemiologic surveillance of cutaneous fungal infection in the United States from 1999 to 2002. *J Am Acad Dermatol*. 2004;50:748–52.
9. Fotedar R, Al-Hedaithy SSA. Comparison of phospholipase and proteinase activity in *Candida albicans* and *C. dubliniensis*. *Mycoses*. 2005;48:62–7.
10. Ghannoum MA. Potential role of phospholipases in virulence and fungal pathogenesis. *Clin Microbiol Rev*. 2000;13:122–43.
11. Havlickova B, Czaika VA, Friedrich M. Epidemiological trends in skin mycoses worldwide. *Mycoses*. 2008;51:2–15.
12. Jautová J, Virágová S, Ondrasovic M, Holoda E. Incidence of *Candida* species isolated from human skin and nails: a survey. *Folia Microbiol (Praha)*. 2001;46:333–7.
13. Jayatilake JA. A review of the ultrastructural features of superficial candidiasis. *Mycopathologia*. 2011;171:235–50.
14. Jayatilake JA, Samaranayake LP. Experimental superficial candidiasis on tissue models. *Mycoses*. 2010;53:285–95.
15. Jayatilake JA, Tilakaratne WM, Panagoda GJ. Candidal onychomycosis: a mini-review. *Mycopathologia*. 2009;168:165–73.
16. Kantarcioglu AS, Yücel A. Phospholipase and protease activities in clinical *Candida* isolates with reference to the sources of strains. *Mycoses*. 2002;45:160–5.
17. Kumar CPG, Kumar SSJ, Menon T. Phospholipase and proteinase activities of clinical isolates of *Candida* from immunocompromised patients. *Mycopathologia*. 2006;161:213–8.
18. Kwok YK, Tay YK, Goh CL, Kamarudin A, Koh MT, Seow CS. Epidemiology and in vitro activity of antimycotics against candidal vaginal/skin/nail infections in Singapore. *Int J Dermatol*. 1998;37:145–9.
19. López-Martínez R. Candidosis, a new challenge. *Clin Dermatol*. 2010;28:178–84.
20. Naglik JR, Challacombe SJ, Hube B. *Candida albicans* secreted aspartyl proteinases in virulence and pathogenesis. *Microbiol Mol Biol Rev*. 2003;67:400–28.
21. Price MF, Wilkinson ID, Gentry LO. Plate methods for detection of phospholipase activity in *Candida albicans*. *Sabouraudia*. 1982;20:7–14.
22. Ribeiro MA, Miranda AE, Gambale W, Paula CR. Prevalence and exoenzyme secretion by *Candida albicans* isolates from oral and vaginal mucosae of HIV-infected women. *Mycopathologia*. 2004;157:255–61.
23. Rüchel J, Tegeler R, Trost MA. Comparison of secretory proteinases from different strains of *Candida albicans*. *Sabouraudia*. 1982;20:233–44.
24. Samaranayake LP, Raeside JM, MaxFarlane TW. Factors affecting the phospholipase activity of *Candida* species in vitro. *Sabouraudia*. 1984;22:201–7.
25. Segal E. *Candida*, still number one – what do we know and where are we going from there? *Mycoses*. 2005;48:3–11.
26. Shehaby AA, Nazzal SA, Dajani N. Putative virulence factors of *Candida* species colonizing respiratory tracts of patients. *Microb Ecol Health Dis*. 2004;16:214–7.
27. Vella Zahra L, Gatt P, Boffa MJ, Borg E, Mifsud E, Scerri L, et al. Characteristics of superficial mycoses in Malta. *Int J Dermatol*. 2003;42:265–71.
28. White TC, Agabian N. *Candida albicans* secreted aspartyl proteinases: isoenzyme pattern is determined by cell type, and levels are determined by environmental factors. *J Bacteriol*. 1995;177:5215–21.
29. Yamamoto T, Nohara K, Uchida K, Yamaguchi K. Purification and characterization of secretory proteinase of *Candida albicans*. *Microbiol Immunol*. 1992;36:637–41.
30. Yarrow D. Methods for the isolation maintenance and identification of yeasts. In: Kurtzman CP, Fell JW, editors. *The yeasts, a taxonomic study*. 4th ed. Amsterdam, The Netherlands: Elsevier; 1998. p. 77–100.