

Virulence factors of *Candida* species from the oral mucosa and prostheses of elderly people from a riverside community in the Amazon state, Brazil

Fatores de virulência de espécies de *Candida* provenientes da mucosa oral e das próteses dos idosos de uma comunidade ribeirinha no Amazonas – Brasil

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How to cite: Silva DM, Souza TC, Alencar CFC, Souza IS, Bandeira MFCL, Fernandes OCC. Virulence factors of *Candida* species from the oral mucosa and prostheses of elderly people from a riverside community in the Amazon state, Brazil. Rev Odontol UNESP. 2019;48:e20190094. <https://doi.org/10.1590/1807-2577.09419>

Resumo

Introdução: A *Candida albicans* é a levedura que mais acomete a cavidade oral, podendo causar infecção. Porém diversos fatores podem estar associados ao aparecimento da candidíase, que podem estar relacionados com a higiene e saúde dos indivíduos, mas também com a patogenicidade destes microrganismos. **Objetivo:** Avaliar os fatores de virulência de leveduras do gênero *Candida* isoladas da mucosa oral dos idosos residentes na Comunidade Lago do Limão - Iranduba - Amazonas – Brasil. **Material e método:** Foram realizados os testes de urease, proteinase, fosfolipase, e avaliação da produção de hemólise. Na análise estatística utilizou-se teste Exato de Fisher e Quiquadrado. **Resultado:** Obteve-se a prevalência de espécies não-*albicans*, quanto aos fatores de virulência, todos os isolados foram ureases negativos, houve prevalência de produção muito forte de proteinase, enquanto que no teste da fosfolipase, a maioria dos isolados não apresentou produção desta enzima; todas as leveduras analisadas apresentaram produção de hemolisina, sendo mais prevalente a hemólise grau IV. Não houve diferença estatisticamente significativa entre a virulência dos isolados oriundos da cavidade oral e da prótese dos idosos analisados. **Conclusão:** Diversos fatores de virulência podem apresentar-se com alta intensidade na presença de alterações da microbiota oral. Além disso, as espécies não-*albicans* apresentam fatores de virulência tanto quanto a *C. albicans*, com graus de patogenicidade elevados. Este estudo permite a análise de estratégias de prevenção da candidíase, com intuito de promover melhor saúde e qualidade de vida para os idosos.

Descritores: *Candida*; mucosa oral; prótese dentária; virulência.

Abstract

Introduction: *Candida albicans* is the yeast most commonly affecting the oral cavity, sometimes causing infection. However, several factors may be associated with the onset of candidiasis, which may be related not only to the hygiene and health of individuals, but also to the pathogenicity of these microorganisms. **Objective:** To evaluate the virulence factors of *Candida* yeasts isolated from the oral mucosa of elderly people living in the “Comunidade Lago do Limão”, municipality of Iranduba, Amazonas state, Brazil. **Material and method:** Testes were performed to assess the production of urease, proteinase, phospholipase and hemolysin. Statistical analysis used the Fisher's exact test and the Chi-squared test. **Result:** Prevalence of non-*albicans* species was observed. As for virulence factors, all isolates were negative ureases, and there was prevalence of very strong proteinase production, whereas most isolates did not produce this enzyme in the phospholipase test. All yeasts analyzed presented hemolysin production, with grade IV hemolysis as the most prevalent. There was no statistically significant difference between the virulence of isolates from



the oral cavity and the prostheses of the elderly analyzed. **Conclusion:** Several virulence factors may present with high intensity in the presence of oral microbiota changes. In addition, non-*albicans* species present number of virulence factors similar to that of *C. albicans*, with high pathogenicity. This study allows a better analysis of candidiasis prevention strategies aiming to promote improvement in the health and quality of life for the elderly.

Descriptors: *Candida*; oral mucosa; dental prosthesis; virulence.

INTRODUCTION

Several physiological and metabolic changes of systemic order and in the oral cavity occur during aging. The use of dental prostheses that favor the colonization of *C. albicans* by altering the oral microbiota lead to the emergence of candidiasis¹⁻⁴.

Candidiasis is an infectious process caused by fungi of genus *Candida*. This microorganism has a commensal relationship with the host, but when there is an imbalance in this flora, it can become pathogenic, affecting approximately 10% of elderly people with poor health. *Candida albicans* is the species most commonly associated with infection, accounting for over 80% of the fungi isolated from the oral cavity^{5,6}.

Manifestations of oral candidiasis are classified as acute or chronic, with the latter being found more frequently in the elderly. The acute manifestation is pseudomembranous and erythematous, whereas the chronic manifestation presents lesions such as chronic hyperplasia, prosthetic stomatitis, medial rhomboid glossitis, angular cheilitis, and hairy tongue^{5,6}.

Prosthetic stomatitis is an infectious process that affects the palatal mucosa in close contact with the prosthesis. It has a multifactorial etiology and may also be associated with poor hygiene of the prostheses with consequent biofilm accumulation⁷.

However, not only host-related factors can cause candidiasis, but also the virulence factors of these microorganisms, including secretion of hydrolytic enzymes (phospholipases and proteinases), toxins, hemolysins, and adhesion and biofilm formation capacity⁸⁻¹⁰.

Colonization by fungi of the genus *Candida* in the human oral microbiota may cause some diseases when the patient immune system is weakened, resulting in local or even systemic impairment.

These facts demonstrate the clinical relevance of the analysis of the main virulence factors of *Candida* species, highlighting the knowledge of these mechanisms involved in the pathophysiology of candidiasis, which may provide prevention and treatment strategies.

MATERIAL AND METHOD

Forty isolates of *Candida* yeast from the palate and prostheses of elderly people residing in the "Comunidade Lago do Limão", municipality of Iranduba, Amazonas state, Brazil, were used in this study. The samples were collected for a master's thesis entitled Oral health conditions of the elderly population in a rural community of Amazonas state (process no. CEP 61618016.0.0000.5020) and transferred to the Amazon Fungi Collection of "Instituto Leônidas e Maria Deane" (CFAM-ILMD-FIOCRUZ-Amazonia). The isolates were previously identified by CHROMagar *Candida*® chromogenic medium (BioMerieux, France), a presumptive method for identifying *Candida*, and stored under refrigeration at 20 °C in Sabouraud Agar (K25-610103-Kasvi, Italy) with glycerol as a wall protector.

The virulence factors of these yeasts were evaluated through production of urease, proteinase, phospholipase and hemolysin tests. For the urease test, isolates were reactivated and seeded in

Christensen¹¹ agar medium (Urea Agar) and soon after incubated at 37 °C with daily observation for seven days. A change in medium color to fuchsine-red is indicative of a positive result, otherwise the culture is negative. After seven days of incubation, the result was read.

The study of proteinase production was performed according to the methodology described by Teixeira et al.¹², in which the test medium consisted of sterile plates containing agar, gelatin and skimmed milk, diluted in Citrate-Phosphate Buffer solution (pH 5.0).

Phospholipase production was analyzed according to the egg yolk plate method described by Price et al.¹³ with modifications, where the medium consisted of Sabouraud Dextrose Agar plus sodium chloride, calcium chloride, and yeast extract. After sterilization of the medium, 30% egg yolk emulsion was aseptically added.

For enzymatic analysis, yeast fragments with 24 h of growth were inoculated on the surface of the specific medium for each enzyme investigated, and incubated for seven days at 37 °C. Enzyme production was observed by the formation of transparent and opaque halos around the yeast colony for proteinase and phospholipase, respectively.

The tests were performed in triplicate and the precipitation zone value (Pz) was given as the mean of the evaluated diameters (colony/halo+colony). Production was classified according to the Pz value as very strong ++++ (Pz ≤ 0.69), strong +++ (Pz between 0.70 and 0.79), average ++ (Pz between 0.80 and 0.89), or weak + (Pz between 0.90 and 0.99).

The hemolysin production test was performed according to the method by Malpezzi et al.¹⁴; Berlinck et al.¹⁵; Costa-Lotufo et al.¹⁶ with modifications as follows: sheep blood was diluted in saline (0.85% NaCl + 10mM CaCl₂), washed three to four times in saline, and then centrifuged (1500 rpm/10 min). An erythrocyte pellet was obtained and resuspended in saline to prepare the 2% erythrocyte solution (ES). The test was performed using 96-well microplates containing 150 µL of yeast solution and an additional 150 µL of ES. The negative control was obtained with 0.2% DMSO-Dimethyl sulfoxide, whereas the positive control was obtained with 0.2% Triton X-100.

After this process, the samples were incubated for 1 hour under constant agitation at room temperature (26 °C) and then centrifuged at 1500 rpm/10 min. Reading of the results was classified according to the hemolysis intensity compared with the control as grade I (without hemolysis), II (weak), III (strong), and IV (very strong) hemolysis.

Data were processed using R 3.6.0 and Rstudio 1.1.4, with various packages (tidyverse, epiDisplay, gridExtra, and sjPlot)¹⁷. A significance level of 0.05 was adopted in all statistical analyses.

The Fisher's Exact and Chi-squared tests were used to analyze the relationship between the "Experiment Location" and the qualitative (categorical) variables.

As test hypotheses, we used H₀: the variables are independent; H₁: the variables are dependent. Decision Rule: Reject H₀ if *p*-value is <0.05.

RESULT

The 40 yeasts analyzed (21 from samples collected from the mouth (palate) and 19 from the prostheses) in the elderly from "Comunidade Lago do Limão", municipality of Iranduba, showed prevalence of 80% *Candida non-albicans* species, and 21% of isolates originating from the palate of the elderly analyzed (Table 1).

Table 1. Correlation between *Candida* species and pathogenicity tests with the origin of the samples collected from elderly people in the “Comunidade do Lago do Limão”, municipality of Iranduba, Amazonas state, Brazil

| Variables | Experiment Location | | p-value |
|------------------------|---------------------|------------------|---------|
| | MT n = 21 (%) | PT n = 19 (%) | |
| PROTEINASE | | | 0.342 |
| Very Strong | 12 (57.1) | 8 (42.1) | |
| NEG | 9 (42.9) | 11 (57.9) | |
| PHOSPHOLIPASE | | | 0.873 |
| Very Strong | 6 (28.6) | 5 (26.3) | |
| NEG | 15 (71.4) | 14 (73.7) | |
| HEMOLISIN | | | 0.698 |
| GRADE III | 5 (23.8) | 3 (15.8) | |
| GRADE IV | 16 (76.2) | 16 (84.2) | |
| SPECIES | | | 0.28 |
| <i>C. albicans</i> | 3 (14.3) | 3 (15.8) | |
| <i>C. glabrata</i> | 7 (33.3) | 4 (21.1) | |
| <i>C. krusei</i> | 7 (33.3) | 3 (15.8) | |
| <i>C. parapsilosis</i> | 2 (9.5) | 7 (36.8) | |
| <i>C. tropicalis</i> | 2 (9.5) | 2 (10.5) | |

Captions: MT - mouth, PT - prosthesis. p-value of Chi-squared test or Fisher's Exact test. Percentage given by column. Significance level of 0.05.

The results presented in Table 1 showed no evidence according to the *p*-values (none <0.05) found to reject the independence hypothesis.

In the virulence tests, the isolates did not present urease production. However, analysis of the production of hydrolytic enzymes showed prevalence of isolates collected from the mouth of the elderly with positive results for proteinase (57.1%), being classified as very strong, whereas prevalence of negative results (71.4%) was observed in the phospholipase test.

In this study, all isolates of *Candida* sp. found hemolysin production, with prevalence of grade IV hemolysis in samples from the prostheses of the elderly people (84.2%).

The species with the highest production of proteinases was *C. glabrata*, followed by *C. krusei*. In the phospholipase test, of the isolates that tested positive, *C. parapsilosis* showed the highest production. However, in the hemolysin test, all species analyzed had hemolytic potential, especially *C. parapsilosis* and *C. krusei* as major causes of grade IV hemolysis. Comparisons between the virulence tests and the *Candida* species found in the study are depicted in Figures 1, 2 and 3, respectively.

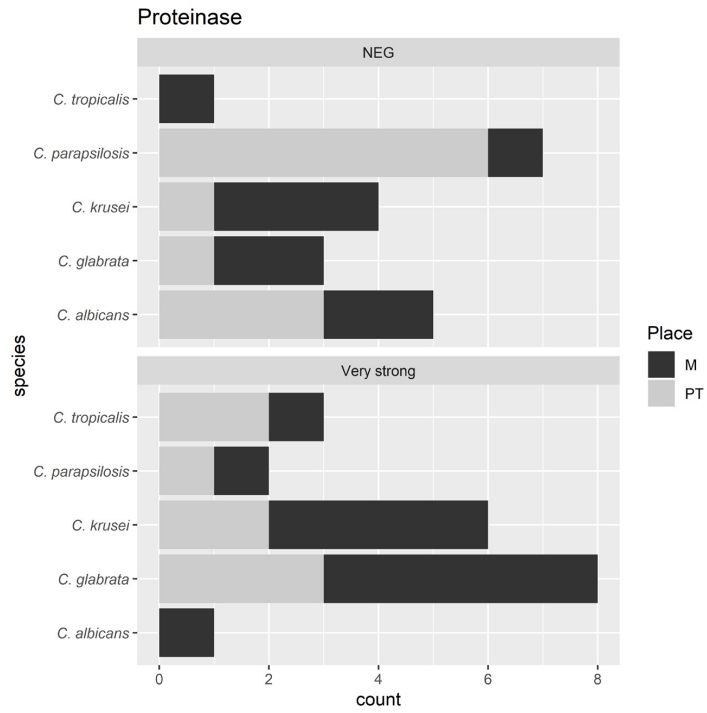


Figure 1. Proteinase-producing *Candida* species as virulence factor derived from isolates of the mouth and prostheses of the elderly from “Comunidade Lago do Limão”, municipality of Iranduba, Amazonas state, Brazil.

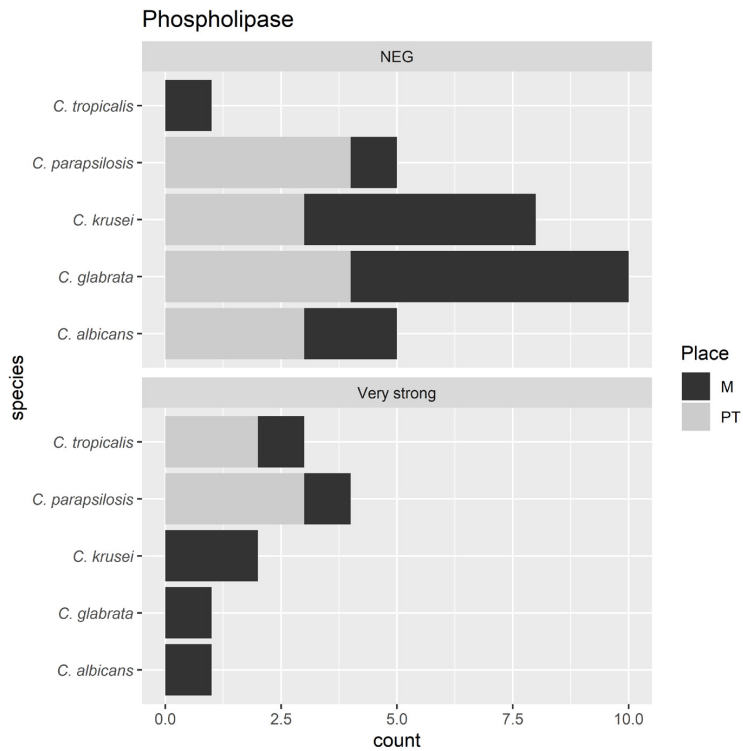


Figure 2. Phospholipase-producing *Candida* species as virulence factor derived from isolates of the mouth and prostheses of the elderly from “Comunidade Lago do Limão”, municipality of Iranduba, Amazonas state, Brazil.

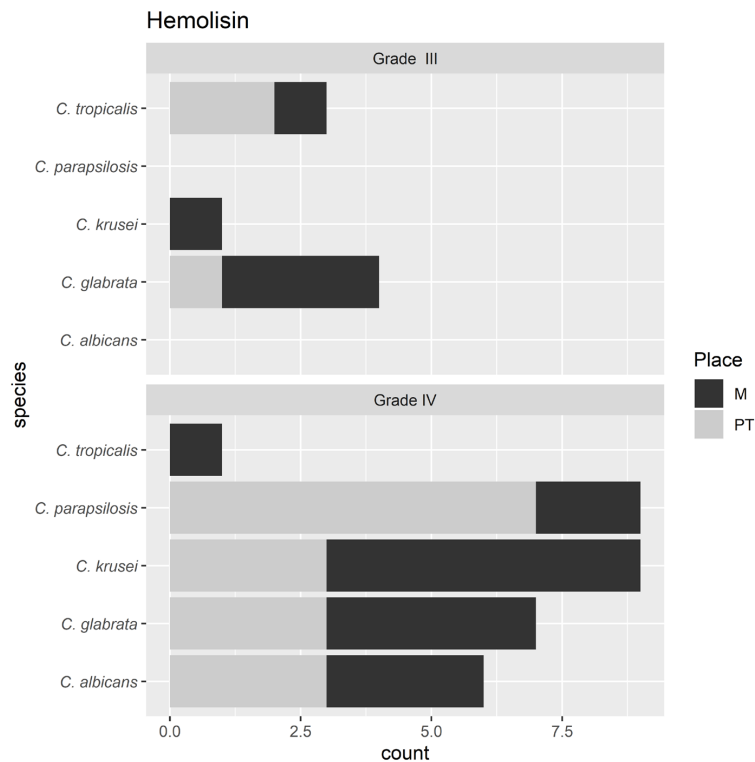


Figure 3. Hemolysin-producing *Candida* species as virulence factor derived from isolates of the mouth and prostheses of the elderly from “Comunidade Lago do Limão”, municipality of Iranduba, Amazonas state, Brazil.

DISCUSSION

Despite the fact that *Candida albicans* is the yeast species that most affects the oral cavity, thus being the most pathogenic, the present study revealed a predominance of pathogenicity among non-*albicans* species, in line with the study conducted by Mohandas, Ballal¹⁸ with hospitalized patients in southern India.

Some pathogenic fungi are able to produce urease to use urea as a source of nitrogen, but as a result of the breakdown reaction of urea, ammonia, a toxic substance to host cells, can cause the immune system to evade and, consequently, promote infection. A study by Seeliger¹⁹ tested the 14 *Candida* species to identify urease production, but only one *Candida humicola* culture showed a positive result. However, studies have stated that most yeast species responsible for causing pathogens in humans are negative urease²⁰.

Other virulence factors that *Candida* species may exhibit are the production of proteinases and phospholipases that facilitate the invasion of hyphae into the epithelium. In the case of proteinase, host protein degradation occurs by hydrolysis of peptide bonds, both those located on the mucosal surface and those related to the immune system (immunoglobulin, complement system proteins, and cytokines); and in the presence of phospholipases, phospholipid hydrolysis occurs resulting in the disintegration of cell membranes^{8,9,21}.

Results of this study corroborate those by D'Eça et al.²¹, who found that 50% of the yeasts showed proteinase activity. However, in the study by Andreola et al.⁹, approximately 97% of *Candida* spp. were positive for proteinase. Regarding *Candida* species, studies²¹ have shown prevalence of 64.2% of very strong proteinase activity by *Candida albicans* isolates, and even proteinase production by 100% of *C. albicans* isolates, whereas only 25% of isolates from non-*albicans* species obtained positive results²².

In the phospholipase test, there were difference between the results obtained in the study and those by D'Eça et al.²¹, who reported activity in 68.3% of *Candida* spp. and 76.6% prevalence of *C. albicans*, as well as those by Martins et al.²³, who verified that all isolates were phospholipase positive; however, only 43% of the non-*albicans* species presented phospholipase production. In contrast, the results of this study were similar to those observed by Mohandas, Ballal¹⁸, who found higher phospholipase production in non-*C. albicans* species, differing only in their prevalence - 44.1% of the isolates.

Nevertheless, although *C. albicans* is the most pathogenic species, studies have shown a considerable increase of non-*albicans* species involved in infectious processes, which may lead to systemic infection in debilitated patients⁸.

Candida fungi may also present hemolytic capacity, aiming to obtain iron from the host tissues for their metabolism through erythrocyte lysis, with hemoglobin being of great importance in this process. The oral cavity is rich in iron from both lactoferrin (a protein present in saliva) and ferritin (intracellular), facilitating the invasion of hyphae and possibly leading to systemic candidiasis²².

Regarding the hemolysin production test, the study by Rossoni et al.²² indicated predominance of 100% *Candida albicans* in the production of this protein, thus being an important virulence factor responsible for causing systemic infections. However, the comparison between the hemolytic activity developed by non-*albicans* species and *C. albicans* showed no statistically significant difference, being characterized with the same hemolytic potential. Prevalence of hemolysin-producing non-*albicans* species was observed by Pakshir et al.²⁴ in a study with clinical analyses of *Candida* species.

In all associations tested in Table 1, no statistical dependence was found between the virulence factors and the fungi collected from the oral cavity and prostheses of elderly people from the “Comunidade Lago do Limão”, municipality of Iranduba, Amazonas state, Brazil, thus accepting the H_0 hypothesis, that is, the variables are not dependent.

CONCLUSION

Based on the methodology used, we conclude that *Candida* yeasts have several virulence factors that may present with high intensity in the presence of oral microbiota changes. Regarding the comparison between the *Candida* species analyzed, it was found that non-*albicans* species present number of virulence factors similar to that of *C. albicans*, with high pathogenicity, and may even resemble them with respect to infectious potential.

The study of host-related factors, as well as virulence factors in *Candida* species, allows a better analysis of candidiasis prevention strategies in order to promote improvement in the health and quality of life of these elderly people.

ACKNOWLEDGEMENTS

The author is sincerely grateful to everyone who contributed to the execution of this study, especially to Dr. Ormezinda Fernandes (advisor) and Professor Maria Fulgência Bandeira for all their support, as well as to Carol Falcão for kindly providing me with her master's thesis collection samples, and to Ingrid Souza and Thayana Souza for assisting with the pathogenicity tests. The author also thanks Fiocruz Amazônia/ILMD for investing in health research and providing opportunities for enhancing knowledge and “Fundação de Amparo à Pesquisa do Estado do Amazonas” (FAPEAM) for the funding received.

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CONFLICTS OF INTERESTS

The authors declare no conflicts of interest.

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Received: September 29, 2019

Accepted: October 9, 2019