

## DIFFERENCES IN EXOENZYME PRODUCTION AND ADHERENCE ABILITY OF *Candida* spp. ISOLATES FROM CATHETER, BLOOD AND ORAL CAVITY

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### SUMMARY

Phospholipase and proteinase production and the ability of adhesion to buccal epithelial cells (BEC) of 112 *Candida* isolates originated from oral cavity of HIV infected patients and from blood and catheter of intensive care unit patients were investigated. The proteinase production was detected by inoculation into bovine serum albumin (BSA) agar and the phospholipase activity was performed using egg yolk emulsion. A yeast suspension of each test strain was incubated with buccal epithelial cells and the number of adherence yeast to epithelial cells was counted. A percentage of 88.1% and 55.9% of *Candida albicans* and 69.8% and 37.7% of non-*albicans Candida* isolates produced proteinase and phospholipase, respectively. Non-*albicans Candida* isolated from catheter were more proteolytic than *C. albicans* isolates. Blood isolates were more proteolytic than catheter and oral cavity isolates while oral cavity isolates produced more phospholipase than those from blood and catheter. *C. albicans* isolates from oral cavity and from catheter were more adherent to BEC than non-*albicans Candida* isolates, but the adhesion was not different among the three sources analyzed. The results indicated differences in the production of phospholipase and proteinase and in the ability of adhesion to BEC among *Candida* spp. isolates from different sources. This study suggests that the pathogenicity of *Candida* can be correlated with the infected site.

**KEYWORDS:** Adherence ability; *Candida*; Exoenzyme activity.

### INTRODUCTION

Many *Candida* species can be carried as an oral commensal by healthy individuals but they can cause opportunistic infection, especially in HIV patients, the elderly, neonates and patients undergoing chemotherapy, antibacterial therapy or invasive procedures<sup>22,24,27</sup>. HIV infected patients often develop oropharyngeal candidiasis, while candidemia has been cited as the fourth most frequent bloodstream infection in patients at intensive care units (ICUs) in the USA<sup>5,13,28</sup>.

In addition to predisposing factors related to the host, *Candida* species are able to cause infection depending on intrinsic characteristics related to the microorganism itself. Germ tube production, growth at 37 °C, protease and phospholipase production and adherence ability to BEC are considered important factors in establishing the infection<sup>14,16,29</sup>. Proteinase and phospholipase production can lead to dysfunction or even rupture of cellular membranes, which facilitate adhesion of the microorganism to the host<sup>20,21</sup>. The successful colonization and infection by a microorganism depend upon their initial capacity to adhere to host tissue<sup>3,29</sup>.

In the present study, we investigated the *in vitro* phospholipase and proteinase activity and the ability of the adhesion to BEC of *C. albicans*

and non-*albicans Candida* isolates from oral cavity of HIV infected patients and from blood and catheter of hospitalized patients in ICU.

### MATERIALS AND METHODS

A total of 112 *Candida* strains isolated from three sources [58 obtained from oral cavity of HIV-infected patients and 54 obtained from patients in the intensive care unit (ICU) of a tertiary hospital (33 from catheter and 21 from blood)] were included in this study. The samples from oral cavity were obtained by swabbing the oral mucosa from *Candida* carriage patients undergoing antiretroviral therapy. The samples from the catheter were collected from the tip, and the last 3 to 5 cm of the catheter, and were cultured.

All the patients gave written informed consent and the study was approved by the Bioethics Committee of *Hospital de Doenças Tropicais de Goiás* (protocol 007/2004) and of *Hospital das Clínicas da Universidade Federal de Goiás* (protocol 027/07). Species stratification of the various isolates is as follows: 59 *Candida albicans*, 22 *Candida parapsilosis*, 14 *Candida tropicalis*, seven *Candida guilliermondii*, five *Candida famata*, three *Candida krusei*, one *Candida lusitanae*, and one *Candida kefyr*. *Candida* strains were identified by germ tube test,

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clamidoconidia formation, carbon and nitrogen assimilation and sugar fermentation<sup>18</sup>. In addition, all the isolates were streaked in parallel onto CHROMagar® *Candida* medium plates (CHROMagar, Paris, France) for presumptive identification of *C. albicans*. API 20 *Candida* identification kit (bioMérieux, France) was used when the yeast could not be identified by the methods cited above. Growth at 45 °C on Sabouraud dextrose broth, and assimilation tests by using xylose and  $\alpha$  methyl-D-glucoside were used to help us to discriminate between *C. albicans* and *Candida dubliniensis*<sup>7,8</sup>. All isolates were stored in sterile distilled water at room temperature and subsequently inoculated onto Sabouraud dextrose agar (SDA- DIFCO) for 24 - 48 hours at 35 °C for subsequent analysis. The assays were repeated three times for each isolate studied.

**Detection of secreted aspartyl proteinase:** The proteinase production was detected by the method of RUCHEL *et al.*<sup>25</sup>. Briefly, a single colony (~6mm) from the *Candida* isolates was inoculated into bovine serum albumin (BSA) agar that contained yeast carbon base (1.17%); protovit (0.25%) and BSA (0.2%), adjusted to pH 5.0, sterilized by filtration, and added to autoclaved 2% agar. The plates were incubated at 37 °C for five days. Proteolytic activity (Pz) was measured by ratio of the diameter of colony and total diameter of colony plus precipitation zone as described by PRICE *et al.*<sup>23</sup>.

**Detection of Phospholipase:** The phospholipase production was assayed according to the method of PRICE *et al.*<sup>23</sup>. SDA medium supplemented with 1 mol/L sodium chloride, 0.005 mol/L calcium chloride and 8% sterile egg yolk emulsion (*in natura*) was poured (20 mL) in plates of 90 mm diameter and the single colony (~6 mm) of each strain of the yeast was inoculated on the surface of the medium. The plates were incubated at 37 °C for five days. Phospholipase activity (Pz) was measured according to proteinase testing.

**Adhesion assay:** This test was performed based on BIASOLI *et al.*<sup>3</sup>. The buccal epithelial cells were obtained from the cheeks of healthy individuals. These cells were suspended in phosphate buffered saline (PBS - pH 7.2), washed three times by centrifugation with the same buffer, and adjusted to a final concentration of 2 X 10<sup>5</sup> cells/mL. The yeast inoculum was prepared from cultures 24 h-old on Sabouraud dextrose

agar. A colony originated from this growth was inoculated in Sabouraud glucose broth and incubated at 37 °C for 24 h with agitation. The yeasts were centrifuged, washed twice with PBS and the cell density was adjusted with a spectrophotometer (at 530 nm wavelength) to yield a yeast stock of 1X 10<sup>7</sup> yeast/mL. Five hundred microliters of each yeast suspension was mixed with 500  $\mu$ L of the BEC pool and incubated at 37 °C with agitation for one hour. The mixture was filtered through a 20  $\mu$ m size pore membrane filter. In order to remove non-adhered yeasts the filter was washed with 10 mL PBS and then transferred to a slide by pressing the filter paper against it. The cells fixed with methanol were stained using the Gram-Nicolle technique and the number of adhered cells to 100 BEC was counted. The test was repeated three times and a mean value was defined.

**Statistical analysis:** The resulting data was statistically analyzed by SPSS for Windows version 11.0 using the mean and median of phospholipase, of proteinase and mean number ( $\bar{x}$ ) of yeast adhered to 100 BEC. The virulence differences of *C. albicans* and non *albicans Candida* isolates from blood, catheter and oral cavity were evaluated using Kruskal-Wallis. Mann Whitney test was used to verify the virulence differences between *C. albicans* and non *albicans Candida* isolates. A *p*-value of less than 0.05 was considered to be statistically significant.

## RESULTS

Fifty two (88.1%) *C. albicans* and 37 (69.8%) non-*albicans Candida* isolates were proteinase positive (*p* > 0.05). Among the species of non-*albicans Candida*, *C. parapsilosis* and *C. guilliermondii* showed a high proteinase activity (81.8% and 85.7% respectively). The proteinase activity was observed in all isolates of *C. famata* (05) and of *C. lusitaniae* (01) obtained from catheter. Proteinase was produced by 41 (70.6%) of the isolates from oral cavity, by 21 (100%) of the isolates from blood and by 27 (81.8%) of the isolates from catheter (*p* = 0.016). The comparison among *Candida* isolates from these sites showed that in catheter non-*albicans Candida* were better proteinase producers than *C. albicans* isolates (*p* < 0.001) (Table 1).

Phospholipase production was detected in 33 (55.9%) *C. albicans* isolates, and only in 20 (37.7%) non-*albicans Candida* (*p* > 0.05). *C.*

**Table 1**  
Number of *Candida* spp. isolates from oral cavity (58), blood (21) and catheter (33) positive for proteinase production

Species	Oral cavity		Blood		Catheter		Total	
	n/total	(%)	n/total	(%)	n/total	(%)	n/total	(%)
<i>C. albicans</i>	29/31	93.5	15/15	100	08/13	61.6	52/59	88.1
non- <i>albicans Candida</i>	12/27	44.4	06/06	100	19/20	95.0	37/53	69.8
<i>C. parapsilosis</i>	05/09	55.6	03/03	100	10/10	100	18/22	81.8
<i>C. tropicalis</i>	04/13	30.8	01/01	100	0	0	05/14	35.7
<i>C. guilliermondii</i>	02/03	66.7	02/02	100	02/02	100	06/07	85.7
<i>C. krusei</i>	01/01	100	0	0	01/02	50	02/03	66.7
<i>C. kefyr</i>	0/01	0	0	0	0	0	0/01	0
<i>C. lusitaniae</i>	0	0	0	0	01/01	100	01/01	100
<i>C. famata</i>	0	0	0	0	05/05	100	05/05	100

*kefyr* and *C. lusitaniae* were phospholipase negative. Phospholipase was detected in 37 (64%) of the isolates from oral cavity, in seven (33.3%) from blood and in nine (27.3%) from catheter. Oral cavity isolates were significantly higher producers of this enzyme than blood and catheter isolates ( $p = 0.003$ ), but the comparison between species of *Candida* isolated from these sites did not show a significant difference (Table 2).

The adherence ability to BEC was higher in *C. albicans* ( $\bar{x} = 227.5 \pm 107.1$ ) than in non-*albicans Candida* ( $\bar{x} = 154 \pm 76.5$ ) ( $p < 0.001$ ). *C. guilliermondii* isolates showed a high adherence ability ( $\bar{x} = 196.1 \pm 93.7$ ), but could not be statistically analyzed because the sample size was too small. The adhesion ability of *Candida* spp. from blood, catheter and oral cavity to BEC was similar ( $p = 0.113$ ). The comparison among isolates and the sites showed that *C. albicans* isolates from oral cavity ( $p = 0.002$ ) and from catheter ( $p = 0.032$ ) were more adherent to BEC than non-*albicans Candida* isolates (Table 3).

The phenotypic tests used to discriminate between *C. albicans* and *C. dubliniensis* showed that this last species was not found in our study.

## DISCUSSION

Phospholipase, proteinase activity and the adhesion of microorganisms to host cell surfaces are considered important virulence factors, facilitators of infection establishment, mainly in opportunist fungi<sup>4,29</sup>. There are several studies related to virulence factors of *Candida* species. According to some authors, secreted aspartic proteinases and phospholipase are produced at high rates in *C. albicans*, while non-*albicans Candida* species usually present low rates of these enzymes<sup>10,11,14,17</sup>. KOGA-ITO *et al.*<sup>16</sup> showed an increased proteinase and phospholipase production among the *C. albicans* strains isolated from oral candidiasis patients when compared to those ones isolated from control individuals. There are few reports in the literature investigating the relationship of virulence in yeast species and sites in the host. In our work, proteinase and phospholipase were detected in 88.1% and 55.9% of *C. albicans* isolates and in 69.8% and 37.7% of non-*albicans Candida*, respectively. These results may be attributed to strain characteristics or be related to the site of infection. In blood isolates, independent of species, we found high proteinase activity; in oral cavity the production of this enzyme was higher in *C. albicans* than in non *albicans*, while the non-*albicans Candida* isolates

**Table 2**  
Number of *Candida* spp. isolates from oral cavity (58), blood (21) and catheter (33) positive for phospholipase production

Species	Oral cavity		Blood		Catheter		Total	
	n/total	(%)	n/total	(%)	n/total	(%)	n/total	(%)
<i>C. albicans</i>	24/31	77.4	07/15	46.6	02/13	15.4	33/59	55.9
non- <i>albicans Candida</i>	13/27	48.2	0/06	0	07/20	35.0	20/53	37.7
<i>C. parapsilosis</i>	07/09	77.8	0/03	0	03/10	30.0	10/22	45.4
<i>C. tropicalis</i>	03/13	23.1	0/01	0	0	0	03/14	21.4
<i>C. guilliermondii</i>	02/03	66.6	0/02	0	01/02	50.0	03/07	42.8
<i>C. krusei</i>	01/01	100	0	0	01/02	50.0	02/03	66.7
<i>C. kefyr</i>	0/01	0	0	0	0	0	0/01	0
<i>C. lusitaniae</i>	0	0	0	0	0/01	0	0/01	0
<i>C. famata</i>	0	0	0	0	02/05	40.0	02/05	40.0

**Table 3**  
Results obtained for adherence testing according to the site of isolation

Etiology	Average number of yeasts adhered 100 BEC (SD)			
	Oral cavity $\bar{x} \pm SD$	Blood $\bar{x} \pm SD$	Catheter $\bar{x} \pm SD$	Total $\bar{x} \pm SD$
<i>C. albicans</i>	258.9 ± 119.3	180.7 ± 51.5	207.6 ± 105.6	227.5 ± 107.1
non- <i>albicans Candida</i>	164.9 ± 95.7	130.8 ± 46	146.2 ± 50.6	154 ± 76.5
<i>C. parapsilosis</i>	171.4 ± 83.7	126.0 ± 46.9	149.5 ± 52.5	155.3 ± 65.6
<i>C. tropicalis</i>	125.2 ± 87.6	102 <sup>a</sup>	-	123.5 ± 84.4
<i>C. guilliermondii</i>	258 ± 109.2	152.5 ± 65.8	147 ± 66.5	196.1 ± 93.7
Other species*	254.5 ± 54.5	-	141.9 ± 52.21	164.4 ± 68.6

a = one isolate; \* *C. kefyr*, *C. krusei*, *C. famata*, *C. lusitaniae* - one isolate of each specie;  $\bar{x}$  = mean

from catheter produced higher levels of proteinase. All the isolates of *C. parapsilosis*, *C. guilliermondii*, *C. lusitanae* and *C. famata* were proteinase positive (no statistical analysis was made because of small sample sizes). High activity of this enzyme has been observed among isolates of *C. parapsilosis* in clinical specimens of hospitalized patients<sup>6</sup>.

On the other hand, although no significant differences of phospholipase production among the *Candida* isolates from different sources have been found, we verified that blood and catheter isolates presented lower phospholipase activity than oral cavity isolates (Table 2). PRICE *et al.*<sup>23</sup> observed differences in the phospholipase activity in isolates from blood (55%), wounds (50%) and urine (30%). The high phospholipase production in oral cavity found in our study is similar to some previous studies. One hundred percent phospholipase activity among oral mucosa isolates has been reported by KUMAR *et al.*<sup>17</sup>. Phospholipase production was also increased in the strains isolated from oral cavity of HIV infected patients according to KOGA-ITO *et al.*<sup>16</sup>.

Most of the studies have shown that *C. albicans* is more adherent to BEC than other *Candida* species<sup>3,19</sup>. In our work, a significant difference ( $p < 0.001$ ) was verified in adhesion of *C. albicans* and non-*albicans Candida*. *C. albicans* isolates from oral cavity and from catheter showed higher adhesion than non-*albicans* isolates. The high adhesion ability expressed by *C. albicans* may indicate more virulence when compared with non-*albicans* species. The adherence to host cell is considered an event probably connected to invasion of that cell or tissue<sup>26</sup>. Interestingly, high adhesion ability was verified among *C. guilliermondii* ( $\bar{x} = 196.1 \pm 93.7$ ) isolates. HENRIQUES *et al.*<sup>12</sup> suggested that the factors which affect *Candida* adhesion to epithelial cells can depend on the yeast. According to KENNEDY<sup>15</sup>, the adherence can be considered a specific multifactorial process involving several types of *Candida* cell surface adhesins, as well as a variety of binding sites on host cells.

The non-identification of *C. dubliniensis* after the use of phenotypic tests validated by different researchers to distinguish them from *C. albicans*<sup>1,2,9</sup> shows that *C. dubliniensis* probably has a low incidence in our region.

In conclusion, the results of the present study indicated differences in phospholipase and proteinase activity which are closely related to *Candida* species and to sites where the samples were collected (catheter, blood and oral cavity). This work suggests that the pathogenicity of this yeast can be correlated with the infected site.

## RESUMO

### Diferenças na produção de exoenzimas e habilidade de aderência entre isolados de espécies de *Candida* provenientes do cateter, sangue e cavidade bucal

A produção de proteinase e fosfolipase e habilidade de adesão à célula epitelial bucal de 112 isolados de *Candida* originadas da cavidade bucal de pacientes infectados pelo HIV e de sangue e cateter de pacientes hospitalizados foram investigados. A produção de proteinase foi detectada por inoculação em ágar soro albumina bovina e a atividade de fosfolipase foi realizada usando emulsão de gema de ovo. A suspensão de levedura de cada isolado foi incubada com célula epitelial e o número de leveduras aderidas a célula epitelial foi contada. Uma porcentagem

de 88,1 e 55,9% de *C. albicans* e 69,8 e 37,7% de isolados de *Candida* não *albicans* produziram proteinase e fosfolipase, respectivamente. *Candida* não *albicans* obtidas do cateter foram mais proteolíticas que isolados de *Candida albicans* ( $p < 0,001$ ). Isolados do sangue foram mais proteolíticos do que isolados do cateter e cavidade bucal, enquanto isolados da cavidade bucal produziram mais fosfolipase do que aqueles isolados do sangue e cateter. *C. albicans* isoladas da cavidade bucal e do cateter foram mais aderentes à célula epitelial bucal do que isolados de *Candida* não *albicans*, mas não houve diferença na adesão entre os três locais analisados. Os resultados indicaram diferenças na produção de fosfolipase e proteinase e na habilidade de adesão à célula epitelial bucal entre os isolados de *Candida* das diferentes fontes. Este estudo sugere que a patogenicidade de *Candida* spp pode estar correlacionada ao local infectado.

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