Adherence to buccal epithelial cells, enzymatic and hemolytic activities of Candida isolates from HIV-infected individuals

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Candida spp., along with other microorganisms are usually found in the normal flora of the human oral mucosa. However in HIV-infected individuals these yeasts can become opportunistic pathogens and lead to mucosal infections. The transformation from a harmless commensal to a virulent pathogen under conditions of dysfunctional host defense system is attributable to an extensive repertoire of selectively expressed virulence determinants. The present study was undertaken to analyze the production of four important virulence factors viz. adherence to buccal epithelial cells (BEC), proteinase activity, phospholipase activity and hemolysis, and to evaluate the correlation between these virulence factors in 65 Candida isolates from HIV-infected individuals with oral candidiasis. A total of 95.3%, 67.7%, 41.5% and 100% of the Candida isolates showed adherence to BEC, and proteinase, phospholipase and hemolytic activities, respectively. Production of proteinase and phospholipase enzymes was seen in 89.7% and 59.0% of C. albicans isolates and 34.6% and 15.4% of non-Candida albicans Candida (NAC) isolates, respectively. C. albicans showed significantly greater level of virulence factor expression with regards to adherence to BEC (P < 0.001), phospholipase production (P < 0.044) and hemolysis (P = 0.037) as compared to NAC. A correlative relationship between proteinase activity and adherence to BEC, as well as phospholipase production was noted.

Keywords Candida species, virulence factors, HIV, oral candidiasis

Introduction

Candida spp., along with other microorganisms are usually found in the normal flora of the human oral mucosa [1]. However, in HIV-infected subjects these yeasts can become opportunistic pathogens and lead to mucosal infections, particularly oropharyngeal candidiasis [2]. The transformation from a harmless commensal to a virulent pathogen under conditions of dysfunctional host defense system is attributable to an extensive repertoire of selectively expressed virulence determinants [3]. These include the ability to adhere to host tissue, to produce extracellular hydrolytic enzymes and to undergo dimorphic transition [4]. Adherence to the host tissue required for colonization and subsequent infection is cited as the first stage of the infection process for the members of the genus Candida [5]. Extracellular hydrolytic enzymes, especially proteinases and phospholipases, are seen to play an important role in Candida overgrowth as these facilitate adherence, tissue penetration and subsequent invasion of the host. Furthermore, the ability of Candida to acquire elemental iron through hemolysin production is pivotal in its survival and ability to establish infections within humans, in particular in disseminated candidiasis [6].

The identification of these virulence factors unique to a particular Candida spp. could provide powerful insights...
into the pathogenic process and will hopefully reveal novel antifungal drug targets that can be exploited in the search for improved therapies [5]. With very limited data on *Candida* spp. virulence in India, the present study was undertaken to analyze the expression of four virulence factors and evaluate the association between them in *Candida* spp. isolated from HIV-infected individuals with oral candidiasis.

**Material and methods**

Sixty-five *Candida* isolates obtained from 65 different HIV-positive patients with oral candidiasis attending the National AIDS Research Institute clinic (NARI) in Pune, India, from January 2008 to December 2009 were maintained in our laboratory stock collection and were included in the study. Species distribution of the isolates was as follows; *C. albicans* (39), *C. tropicalis* (9), *C. glabrata* (8), *C. krusei* (6), and *C. parapsilosis* (3). All isolates were revived from the stock cultures maintained on Sabouraud Dextrose Agar (SDA) plates and incubated at 37 °C for 24 – 48 h.

The fresh cultures were used in the virulence assays as follows:

1. Adherence assay was performed as described by Al-Abeid *et al.* [7] with slight modifications. Briefly, cultures of *Candida* spp. were incubated overnight at 37 °C in 0.67% (wt/vol) yeast nitrogen base broth (YNB) supplemented with 2.5% (wt/vol) glucose. Flasks containing 25 ml of the same medium were inoculated with 0.5 ml of the overnight culture and grown for 24 h in a shaking water bath at 37 °C. The cells were harvested by centrifugation (1200 g for 10 min) and washed twice with sterile phosphate buffered saline (PBS). BEC were collected from healthy human volunteers by gently rubbing the buccal mucosa from the cheeks with a sterile cotton swab, which were then washed twice with PBS and cells collected by centrifugation. Yeast cells and BEC were suspended in PBS at 1 × 10^7 cells\(^{-1}\) and 2 ml of yeast suspension was mixed with 2 ml BEC suspension. The mixture was shaken at 37 °C for 2 h and then filtered through a 20 μm filter to remove non-adherent yeast cells. The BECs on the filter were washed in 5 ml of PBS and finally suspended in 5 ml of PBS. A drop of this suspension was spotted on a glass slide and Gram stained. The adherence was determined microscopically by counting the mean number of yeast cells adhering to 100 BECs. *C. albicans* ATCC 90028 was used as a control strain.

2. Protease production was performed according to Aoki *et al.* [8] using bovine serum albumin agar plates. Inoculated plates were incubated at 37 °C for 7 days followed by staining with Coomassie brilliant blue R250 [9]. The diameter of the colonies was measured before staining and the diameter of the clear zones was measured after staining. Protease activity (Prz) was expressed as the ratio of the colony diameter to the clear zone. *C. albicans* ATCC 10231 and *C. kefyr* ATCC 2512 were included as positive and negative controls, respectively.

3. Phospholipase production was assayed according to Kumar *et al.* [3] by using the egg yolk agar plate method. Inoculated plates were incubated at 37 °C for 5 days after which the diameter of the zone of precipitation around the colony was determined. Phospholipase activity (Pz) was expressed as the ratio of the colony diameter to the colony diameter plus zone of precipitation. *C. albicans* ATCC 10231 and *C. kefyr* ATCC 2512 were used as positive and negative controls, respectively.

4. Hemolytic activity was determined by using the blood agar plate assay described by Luo *et al.* [10]. Plates were incubated at 37 °C in 5% CO\(_2\) for 48 h. The presence of a distinct translucent halo around the inoculum site, viewed with transmitted light, indicated positive hemolytic activity. Hemolytic activity (Hz) was expressed as the ratio of the colony diameter to the diameter of the translucent zone of hemolysis. *C. albicans* ATCC 90028 and *C. parapsilosis* ATCC 2201 were employed as positive and negative controls, respectively.

All isolates were tested for all four virulence factors in duplicate on two separate occasions.

Statistical analysis was performed using the SPSS software version 15.0 (SPSS Inc, USA). The Chi square test was used to test the association between *Candida* spp. and virulence factor activity. The t-test was used to check the difference in mean virulence factor production between *C. albicans* and NAC.

**Results**

We investigated in the present study the virulence factors of 65 *Candida* isolates. Table 1 shows the production and quantitative expression of these factors in different *Candida* species.

Overall, 95.3% of *Candida* isolates showed adherence to BECs, i.e., adherence was seen in all *C. albicans* and 88.5% NAC isolates. Among the strains, the number of yeast cells adherent per 100 BEC ranged from 8–989. Maximum adherence was seen in *C. albicans* isolates (mean ± SD, range; 261.59 ± 143.28, 28–989) followed by *C. tropicalis* (170.89 ± 90.70, 24–299), *C. glabrata* (131.5 ± 104.28, 20–315), *C. parapsilosis* (123.33 ± 3.51, 120–127) and *C. krusei* (68.4 ± 64.78, 8–229).
### Table 1: Production and expression of virulence factors in *Candida* species.

<table>
<thead>
<tr>
<th>Species (n)</th>
<th>Adherence to BEC</th>
<th>Proteinase</th>
<th>Phospholipase</th>
<th>Hemolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No (%)</td>
<td>BEC Mean (± SD)</td>
<td>n (%)</td>
<td>PR Mean (± SD)</td>
</tr>
<tr>
<td><strong>Candida albicans (39)</strong></td>
<td>39 (100)</td>
<td>261.59 ± 143.28</td>
<td>35 (89.7)</td>
<td>0.765 ± 0.107</td>
</tr>
<tr>
<td>Non-albicans Candida (26)</td>
<td>23 (88.5)</td>
<td>132.13 ± 88.18</td>
<td>9 (34.6)</td>
<td>0.839 ± 0.06</td>
</tr>
<tr>
<td>Total (65)</td>
<td>62 (95.3)</td>
<td>213.56 ± 139.88</td>
<td>44 (67.7)</td>
<td>0.78 ± 0.10</td>
</tr>
<tr>
<td>P value</td>
<td>ND &lt; 0.001</td>
<td>&lt; 0.001</td>
<td>0.056</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

| Non-albicans Candida         |                  |            |             |           |
| Candida tropicalis (9)       | 9 (100)          | 170.89 ± 90.70  | 4 (44.4)  | 0.89 ± 0.02   | 3 (33.3) | 0.84 ± 0.06   | 9 (100) | 0.60 ± 0.11    |
| Candida glabrata (8)         | 6 (75.0)         | 131.5 ± 104.28 | 1 (12.5)  | 0.87 ± 0.01*  | 1 (12.5) | 0.80 ± 0.01*  | 8 (100) | 0.51 ± 0.09    |
| Candida krusei (6)           | 5 (83.3)         | 68.4 ± 64.78   | 3 (50.0)  | 0.77 ± 0.05   | –        | –            | 6 (100) | 0.66 ± 0.11    |
| Candida parapsilosis (3)     | 3 (100)          | 123.33 ± 3.5   | 1 (33.3)  | 0.8 ± 0.02*   | –        | –            | 3 (100) | 0.66 ± 0.06    |

*SD from different experiments performed on the same isolate included.*

Statistical analysis is not done (ND) as all *Candida albicans* isolates showed adherence to BEC and all *Candida* spp. exhibited hemolytic activity.

Proteinase production was found in 89.7% of *C. albicans* isolates and 34.6% of the NAC isolates, respectively. Proteinase activity (PR) values ranged from 0.53–0.93, with the PR mean ± SD (range) for *C. albicans* was 0.765 ± 0.107 (0.53–0.93), 0.89 ± 0.02 (0.87–0.9) for *C. tropicalis*, 0.87 ± 0.01 for the single positive isolate of *C. glabrata*, 0.77 ± 0.05 (0.72–0.82) for *C. krusei* and 0.8 ± 0.02 for the single positive isolate of *C. parapsilosis*.

Phospholipase production was observed in 59% of *C. albicans* isolates and 15.4% of NAC strains, respectively. None of the *C. krusei* or *C. parapsilosis* isolates exhibited phospholipase activity, but among positive isolates, phospholipase activity (Pz) values ranged from 0.52–0.88. The Pz mean ± SD (range) for *C. albicans* was 0.710 ± 0.109 (0.52–0.86), 0.84 ± 0.069 (0.76–0.88) for the single positive *C. tropicalis* isolate and 0.80 ± 0.01 for the single positive isolate of *C. glabrata*.

All *Candida* isolates were found to have hemolytic activity, with values that ranged from 0.4–0.8. The Hz mean ± SD (range) for *C. albicans* was 0.59 ± 0.11 (0.47–0.77), 0.60 ± 0.11 (0.5–0.8) for *C. tropicalis*, 0.51 ± 0.09 (0.4–0.66) for *C. glabrata*, 0.66 ± 0.11 (0.52–0.77) for *C. krusei* and 0.66 ± 0.06 (0.61–0.72) for *C. parapsilosis*.

The level of virulence factor expression was significantly greater in *C. albicans* isolates compared to NAC isolates with regards to adherence to BEC (P value < 0.001), phospholipase production (P = 0.044) and hemolytic activity (P = 0.037). A positive association between proteinase production and both adherence to BEC (P = 0.010) or phospholipase production was seen (P = 0.002).

### Discussion

The adhesion of *Candida* cells to host mucosal surfaces is a vital prerequisite for successful colonization and infection.

In the present study, all *C. albicans* isolates and 88.5% of NAC were found to adhere to BEC. Maximum adherence was seen in *C. albicans* followed by *C. tropicalis*, *C. glabrata*, *C. parapsilosis* and *C. krusei*. Similar results have been reported in previous studies [11,12]. However, Ellepola et al. [13] found no difference in the adherence abilities of different *Candida* species.

The extracellular hydrolases like proteinases and phospholipases are major facilitators of host tissue invasion and of the disease process that ensues. A total of 89.7% of *C. albicans* isolates were proteinase producers, a finding that compares favorably with previous studies [3,12,14–16]. The phospholipase production in these isolates was also in agreement with other reports in the literature [4,16,17]. Our data shows proteinase and phospholipase production in 34.6% and 15.4% of NAC isolates, respectively. Different studies have reported a wide variation in the production of these enzymes in NAC [3,4,12,17,18]. The expression of virulence factors by *Candida* spp. may vary depending on the type of infection, the site and stage of infection, and the host response. This could explain the variability observed among studies [11].

Hemolytic activity was detected in all *Candida* spp. including *C. parapsilosis*, which is in accordance with other studies [4,6,10]. However none of the five *C. parapsilosis* discussed by Luo et al. [10] showed hemolysis. Since none of the *C. parapsilosis* and *C. krusei* isolates produced phospholipase, it is unlikely that the hemolytic activity observed in our study was due to a bias from extracellular phospholipases.

Although several studies have indicated that the production of virulence factors is greater in *C. albicans* compared to NAC, very few have quantitatively compared the expression of virulence factors [3,17,19,20]. In the present study *C. albicans* isolates showed significantly greater levels of...
virulence factor expression with regards to adherence to BEC, phospholipase production and hemolysis compared to NAC. It may be possible that the higher expression of virulence factors by C. albicans strains can explain that these isolates are found more frequently associated with severe and disseminated infections.

A positive correlation was seen between proteinase activity and adherence to BEC, as well as proteinase activity or phospholipase production. While Lyon et al. [21] only found a correlation between proteinase activity and adherence to BEC, Ghannoum et al. [22] demonstrated a correlative relationship between proteinase production, adherence and pathogenicity.

To conclude, we describe the expression of four important virulence factors in Candida isolates. To our knowledge this is the first report on adherence to BEC and hemolytic activity in Indian Candida isolates from HIV-infected individuals with oral candidiasis. Our findings indicate that the level of virulence factor expression is greater in C. albicans isolates compared to NAC and a correlative relationship exists between proteinase activity and adherence to BEC as well as phospholipase production. It would be interesting to further study the contribution of these virulence factors in disease establishment and progression.

**Declaration of interest:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

**References**


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