Hemolytic activity and production of germ tubes related to pathogenic potential of clinical isolates of Candida albicans

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ABSTRACT

We assessed the virulence factor profile and in vitro antifungal susceptibility of 27 hospital isolates of C. albicans; 19 of these were from infections (16 urinary and three blood), and the other eight were isolated from sites of colonization (two from hands of health professionals, and six from central venous catheters). The virulence factors assayed were germ tube formation and production of extracellular products (hemolysins, proteinases, and phospholipases). Susceptibility to fluconazole, itraconazole, voriconazole and amphotericin B was determined by E-test. Regarding the virulence factors, the infection isolates produced significantly more hemolysin and germ tubes than the colonization isolates (p<0.05). There were no significant differences in the production of other factors between isolates from the two sources (p>0.05). Amphotericin B showed the lowest minimum inhibitory concentrations for all the isolates. The highest resistance was observed for the azoles, especially in the clinical isolates. These results suggest that the capacity of C. albicans to produce hemolysins and germ tubes may be associated with its pathogenic potential. Colonization isolates may pose a high risk of nosocomial infection, especially when the yeasts show resistance to antifungals.


INTRODUCTION

The incidence of hospital fungal infections has increased significantly over the last decade. According to Tortorano et al. (2004) Candida albicans is the most frequently isolated microorganism, and the fourth most important agent responsible for bloodstream infections. Several factors may favor the occurrence of fungal infections in hospitals, including the use of anti-bacterial agents, colonization of various anatomical sites, cross-colonization via the hands of health professionals, and the use of central venous catheters (CVCs) (Trape et al., 2002; Bonassoli et al., 2005; Charles et al., 2005). C. albicans has a number of attributes that may be involved in the invasion process. Adhesins, dimorphism, and the secretion of specific hydrolytic enzymes have all been suggested as possible virulence factors (De Bernardis et al., 2001).

While a number of detailed studies have been published on some hydrolytic enzymes, such as lipases, proteases, and phospholipases (Ghanoun, 2000), little is known of the hemolytic activity of Candida species. It is certain that numerous pathogenic microorganisms grow in the host by using hemin or hemoglobin as a source of iron (Manns et al., 1994; Watanabe et al., 1999; Luo et al., 2001).

The physiopathogenesis of candidiasis is a complex and multifactorial mechanism, which involves features of both the host and the microorganism. Some predisposing characteristics of the host are well known, but it is not yet clear if illnesses can be associated with the virulence or antifungal resistance of the yeast stain. The objective of the present study was to test samples of C. albicans collected from hospital sources for possible virulence factors and their response in vitro to several antifungal drugs.

MATERIAL AND METHODS

Sampling and identification of yeasts

This study was conducted with 27 samples of C. albicans obtained from July 2004 through June 2005 at the University Hospital (UH) in Maringá (PR, Brazil). The samples were classified in two groups: INFEC, 19 isolates from infections (16 from urine cultures and three from blood cultures, all from patients admitted to the Intensive
Antifungal susceptibility test methods

AB BIODISK (Solna, Sweden) provided E-test strips, in which the concentration ranged from 0.002 to 256 µg/mL for fluconazole (FLU); and from 0.002 to 32 µg/mL for itraconazole (ITR) and for amphotericin B (AMB). Each isolate was tested against the three antifungal agents by the E-test method as recommended by the manufacturer.

Quality control was performed by E-test in accordance with Clinical and Laboratory Standards Institute (CLSI) document M27-A2 (2002) by using *Candida krussei* ATCC 6258 and *C. parapsilosis* ATCC 22019 in all runs, and all results were within published limits (Barry et al., 2002). The minimum inhibitory concentrations (MIC) of fluconazole, itraconazole, and amphotericin B were read as the lowest concentration at which the border of the elliptical inhibition zone intercepted the scale on the strip. MIC<sub>50</sub> and MIC<sub>90</sub> were defined as the MIC for 50% and 90% of isolates, respectively. The endpoints for antifungal agents were evaluated by E-test in accordance with Pfaffer et al. (2003) and CLSI document M27-A2. We chose the following criteria for the purposes of comparison in this study: ≤ 1 µg/mL=S; ≥2 µg/mL=R, as used by Pfaffer et al. (2003).

Statistical analysis: Data were analyzed by Student’s t-test. The Mann-Whitney non-parametric method was used when appropriate. Prism 3.00 (Graphpad Software, Inc) was used throughout the analysis. Differences were regarded as significant if *p* < 0.05.

## RESULTS

Table 1 shows the mean of quadruplicate experiments, comparing putative virulence factors and antifungal susceptibility of two groups of *C. albicans* isolates: COL and INFEC.

### Table 1. Comparison of putative virulence factors and *in vitro* susceptibility of *Candida albicans* isolated from infection or colonization sites.

<table>
<thead>
<tr>
<th>Virulence factors <em>a</em></th>
<th>INFEC (n=19)</th>
<th>COL (n=8)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Germination in 2 h</td>
<td>26.47 ± 12.19</td>
<td>12.75 ± 7.66</td>
<td>0.0023</td>
</tr>
<tr>
<td>Germ tubes length (µm)</td>
<td>13.14 ± 8.73</td>
<td>12.83 ± 6.34</td>
<td>0.3279</td>
</tr>
<tr>
<td>Phospholipase produced&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.87 ± 0.15</td>
<td>0.85 ± 0.13</td>
<td>0.3669</td>
</tr>
<tr>
<td>Proteinase&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.87 ± 0.20</td>
<td>0.80 ± 0.13</td>
<td>0.2247</td>
</tr>
<tr>
<td>Hemolysin&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.25 ± 0.01</td>
<td>0.42 ± 0.19</td>
<td>0.0003</td>
</tr>
<tr>
<td>MIC&lt;sub&gt;50&lt;/sub&gt;/MIC&lt;sub&gt;90&lt;/sub&gt; (µg/mL)</td>
<td>0.19/0.38</td>
<td>0.19/0.30</td>
<td>0.4577</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>0.125/0.38</td>
<td>0.19/0.30</td>
<td>0.2031</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>0.25/0.50</td>
<td>0.125/0.50</td>
<td>0.2051</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>0.38/0.32</td>
<td>0.023/0.32</td>
<td>0.1691</td>
</tr>
<tr>
<td>Percent resistance</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amphotericin B</td>
<td>5.2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Fluconazole</td>
<td>42.1</td>
<td>12.5</td>
<td></td>
</tr>
<tr>
<td>Itraconazole</td>
<td>36.8</td>
<td>12.5</td>
<td></td>
</tr>
<tr>
<td>Voriconazole</td>
<td>47.4</td>
<td>25</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> All values are means ± standard deviations.

<sup>b</sup> Ratio of colony diameter/zone diameter, as defined by Price et al (1982): ratio=1.00 means that the test strain is negative for enzyme activity, while a ratio < 1.00 means that the test strain is positive for enzyme activity.

## Virulence factors

Statistical analysis showed that the mean percentage of cells germinating in 2h and amount of hemolysin secreted differed between the isolation site groups. INFEC exhibited more germination and hemolysis than COL (p<0.05). There was no significant difference between the yeasts groups in germ tube length, phospholipase or proteinase.

Thus, in INFEC, proteinase was observed in seven isolates (36.8%) and phospholipase in eight (42.1%), while in COL, six (75%) produced proteinase and five (62.5%) produced phospholipase.
Hemolytic activity, germ tubes in C. albicans

MIC

The MIC\textsubscript{50} and MIC\textsubscript{90} and percent resistance values of the three antifungal agents tested on the C. albicans isolates are shown in Table 1.

Of the drugs tested, amphotericin B showed the lowest MIC for all isolates. Resistance to amphotericin B was observed in one clinical isolate (5.2%). C. albicans isolates in the COL group were more susceptible to fluconazole than those in INFEC \((p<0.05)\). The largest percent resistance was observed for voriconazole in nine (47.4%) INFEC and two (25%) COL strains.

DISCUSSION

In this study, the percentage and length of germ tubes were lower in the COL than in the INFEC yeasts. However, there was no significant difference in germ tube length between the two groups (Table 1). Ibrahim et al. (1995) assessed the virulence of isolates of C. albicans from blood and commensal sources, and reported that the blood isolates were capable of producing longer germ tubes, in greater frequency, than the commensal ones. Those data reinforce the idea that the ability of C. albicans to change its cellular morphology from blastoconidia to hyphae contributes to the pathogenicity of the fungus (Hammer et al., 2000).

This capacity of C. albicans seems to be an important virulence factor, but is not essential in the pathogenesis of disseminated infection. In spite of recent progress, the mechanisms governing these morphogenetic conversions are still not fully understood (Sugita et al., 2002).

Regarding the relationship between the formation of the germ tube and the extracellular enzymes, important results can be found in Ibrahim et al. (1995) study. These authors reported significant differences between strains from colonization and blood infection. The blood isolates produced greater extracellular phospholipase activity, had a higher rate of germination and produced longer germ tubes. Some investigators have observed that tests to determine extracellular compounds (phospholipase, proteinase, and hemolysis) are important to define the microorganism as from infection or colonization (Luo et al., 2001; Matsumoto et al., 2002; Sugita et al., 2002).

Manns et al. (1994) and Watanabe et al. (1999) demonstrated that C. albicans produced hemolytic activity. Luo et al. (2001) observed that species of Candida are capable of producing one or more types of hemolysins \textit{in vitro} and that species differ in the production of these activities.

C. albicans COL showed partial hemolysis or none; in contrast, total hemolysis was observed in all INFEC strains. Luo et al. (2001) reported differences between pathogenic and commensal isolates in relation to hemolytic activity. On the other hand, Bonassoli et al. (2005) observed that 96.1% of C. parapsilosis from colonization were capable of producing hemolysis \textit{in vitro}. Unfortunately, this virulence factor is still little investigated, and further studies are needed to investigate the nature of the hemolytic factor in C. albicans, its usefulness for diagnosis, and mainly its effect on the host cells. However, the hemolytic factors may, like the exoenzymes, aid in the characterization of isolates.

With respect to other secreted enzyme, C. albicans COL produced more proteinase and phospholipase than INFEC; however, this difference was not statistically significant. De Bernardis et al. (1999) also observed that isolates of Candida sp. from hands had greater enzyme activity than blood isolates. These differences may be explainable by the capacity of some drugs to inhibit proteinases and phospholipases (Willis et al., 2001).

Colonization isolates with virulence potential may pose a risk for the development of invasive illnesses, specially if they have been isolated from the hands of health workers or from medical devices surfaces such as catheters (Maki et al., 1977; Traoré et al., 2002; Bonassoli et al., 2005; Tamura et al., 2007).

Among the drugs tested, amphotericin B showed the lowest MICs for all isolates. Resistance to amphotericin B was observed in one clinical isolate (5.2%). Resistance of Candida spp. to amphotericin B is uncommon. The contribution of specific factors, such as previous exposure to polyene or azole, to the development of amphotericin B resistance has yet to be defined epidemiologically (St-Germain et al., 2001).

In this study, the largest percentage of resistant isolates was observed for voriconazole; also, the fluconazole-resistant isolates were also fluconazole- and/or itraconazole-resistant (results not shown). Thus, as has been noted elsewhere, the action of voriconazole was less effective in isolates that were less susceptible to other azoles (Pfaller et al., 2002; Cuenca-Estrella et al., 2005)

This is one of the first studies to demonstrate the high capacity of INFEC strains to form germ tubes and to produce hemolysins. These data suggest that the capacity of C. albicans to produce hemolysins and germ tubes may be a factor in its pathogenic potential. On the other hand, COL may pose a high risk of nosocomial infection, especially when these yeasts show antifungal resistance and the capacity to produce biofilms.

RESUMO

Atividade hemolítica e produção de tubos germinativos relacionados ao potencial patogênico de isolados clínicos de Candida albicans

O perfil de virulência e de susceptibilidade \textit{in vitro} aos antifúngicos de 27 amostras de C. albicans de origem hospitalar foi avaliado, sendo que 19 delas foram isoladas de infeccões (16 urinárias e três sanguíneas) e as outras oito foram isoladas de colonização (duas de mãos de profissionais da saúde e seis de cateter venoso central).

Os seguintes fatores de virulência foram investigados: formação de tubo germinativo e produção de compostos extracelulares (hemolisinas, proteinases e fosfolipases). Suscetibilidade ao fluconazól, itraconazól, voriconazól e anfotericina B foram determinadas por E-test. Em relação aos fatores de virulência, os isolados de infecção produziram significativamente mais hemolisina e tubos germinativos do que os de colonização (\textit{p}<0.05). Não houve diferença significativa na produção das outras enzimas, entre os isolados de duas fontes (\textit{p}>0.05).

Anfotericina B mostrou as menores concentrações inibitórias mínimas para todos os isolados. Maiores...
indices de resistência foram observados aos azólicos, especialmente entre os isolados clínicos. Estes resultados sugerem que a capacidade de C. albicans produzir hemolisinas e tubos germinativos pode estar associada com seu potencial patogênico. Por outro lado, leveduras em colonização podem oferecer alto risco para infecções hospitalar, especialmente quando têm perfil de resistência aos antifúngicos.


REFERENCES


Hemolytic activity, germ tubes in C. albicans


