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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.

Keywords: Nosocomial infection. Virulence. *Candida albicans*. Germ tube. Hemolysins.

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) (Traoré 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 (Ghannoum, 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

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Care Unit); and COL, eight isolates from colonization (two from the hands of UH staff members, and six from CVCs).

The INFEC yeasts were isolated by routine methods, and the COL yeasts, from hands and CVCs, by the methods used by Bonassoli et al. (2005) and Maki et al. (1977), respectively. After the yeast colonies developed, they were subcultured on CHROMagar *Candida*® (CHROMagar BioMerisc, Paris, France) to assess the purity of the culture and the colour of the colonies. From this selective and differential medium, the yeasts were identified by classical methods (Kurtzman & Fell, 1998).

Virulence factors

Germ tubes

These were assayed as described by Vilela et al. (2002). A germ tube was defined as a rounded outgrowth on a cell, whose length is greater than or equal to the diameter of the parent cell, not constricted at the base (Hammer et al., 2000). After 2 hours of incubation, cells were examined with an Olympus CBB microscope, at 100 cells per field. The percentage of cells that formed germ tubes was counted and the germ tube lengths were measured with the aid of a calibrated micrometer disc in the objetive lens. Each experiment was conducted in triplicate.

Proteinase and Phospholipase

Secretion of these enzymes was assayed by the methods of Ruchel et al. (1982) and Price et al. (1982), respectively. The presence of the enzymes was demonstrated by the formation of an opaque halo of substrate degradation around the colony. Phospholipase (Pz) activity was measured as described by Price et al. (1982).

Hemolytic activity

Hemolysin production was evaluated by a modification of the plate assay described by Luo et al. (2001). Freshly cultured colonies of *C. albicans* were produced from inoculum spread on Sabouraud Dextrose Agar plates (Difco, Detroit, Michigan, USA) and incubated for 18-24 hours. For each isolate, a suspension of 10^8 cells/mL was prepared in saline and counted in a Neubauer chamber under a microscope. Aliquots of 10 µL of this suspension were spot-inoculated on sheep-blood agar supplemented with glucose. The plates were incubated at 37°C for 48 hours. Colony and halo diameters were measured with a ruler and haemolytic activity was expressed in terms of the ratio of the diameter of the colony to the outer diameter of the zone of degradation. The assay was conducted in quadruplicate on two separate occasions for each yeast isolate tested. A standard strain, *C. albicans* ATCC 90028, was used as a control in each experiment.

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 krusei* ATTC 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_{50} and MIC_{90} 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 Pfaller 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 Pfaller 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.

	INFEC (n=19)	COL (n=8)	*p* value
Virulence factors [a]			
% Germination in 2 h	26.47 ± 12.19	12.75 ± 2.76	0.0023
Germ tubes length (µm)	13.14 ± 8.73	12.83 ± 6.34	0.3279
Phospholipase produced [b]	0.87 ± 0.15	0.85 ± 0.13	0.3697
Proteinase[b]	0.87 ± 0.20	0.80± 0.13	0.2247
Hemolysis[b]	0.25 ± 0.01	0.42 ± 0.19	0.0003
MIC50/MIC90 (µg/mL)			
Amphotericin B	0.125/0.38	0.19/0.38	0.4577
Fluconazole	8/≥256	0.19/0.50	0.0231
Itraconazole	0.25/≥32	0.125/0.50	0,2051
Voriconazole	0.38/≥32	0.023/≥32	0.1691
Percent resistance			
Amphotericin B	5.2	0	
Fluconazole	42.1	12.5	
Itraconazole	36.8	12.5	
Voriconazole	47.4	25	

[a] All values are means ± standard deviations.

[b] 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.

MIC

The MIC_{50} and MIC_{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 *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 *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 voriconazole-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 o de susceptibilidade *in vitro* aos antifúngicos de 27 amostras de *C. albicans* de origem hospitalar foi avaliado, sendo que 19 delas foram isoladas de infecções (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 fluconazol, itraconazol, voriconazol 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 ($p<0.05$). Não houve diferença significativa na produção das outras enzimas, entre os isolados das duas fontes ($p>0.05$). Anfotericina B mostrou as menores concentrações inibitórias mínimas para todos os isolados. Maiores

índices 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 infecção hospitalar, especialmente quando têm perfil de resistência aos antifúngicos.
Palavras-chave: Infecção hospitalar. Virulência. *Candida albicans*. Tubo germinativo. Hemolisinas.

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