



Acta Scientiarum. Health Sciences

ISSN: 1679-9291

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Universidade Estadual de Maringá  
Brasil

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Acta Scientiarum. Health Sciences, vol. 35, núm. 1, enero-junio, 2013, pp. 1-8  
Universidade Estadual de Maringá  
Maringá, Brasil

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## Characterization of *Candida* spp. isolated from vaginal fluid: identification, antifungal susceptibility, and virulence profile

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**ABSTRACT.** A total of 101 (20.0%) yeast samples were isolated from vaginal fluids of 504 non-hospitalized patients in Maringá, Paraná State, Brazil and *Candida albicans* was more frequent specie (93.1%) identified by seminested PCR method. All the isolates were susceptible to amphotericin B and nystatin, and 93.1% of them were susceptible to fluconazole. The acid proteinase, hemolytic and phospholipase activities were observed in 99.0, 90.0, and 88.0% of *Candida* spp., respectively. Around 67.0% of the strains had adherence indexes of 0.5 to 1.5 yeasts by Vero cell, and most of them showed a hydrophilic profile. Correlation studies indicated hydrophilic yeasts presented higher adherence index, proteinase, and phospholipase activities; and a positive correlation between all enzymes was also observed. In addition, the isolates with high hemolytic activity were less susceptible to fluconazole and amphotericin B. These results of *Candida* prevalence and antifungal susceptibility corroborate with literature's datas and correlation between virulence factors and MIC values suggest *Candida* isolates from vaginal fluid less susceptible to antifungal and with higher extracellular enzymes production can be more virulent to cause tissue damage.

**Keywords:** antifungal susceptibility, *Candida*, identification, virulence.

## Caracterização de *Candida* spp. isoladas de fluido vaginal: identificação, susceptibilidade antifúngica e perfil de virulência

**RESUMO.** Cento e uma leveduras foram isoladas de fluido vaginal de 504 pacientes não hospitalizados em Maringá, Estado do Paraná, Brasil e *Candida albicans* foi a espécie mais freqüente (93,1%) identificada pelo método seminested PCR. Todos os isolados foram susceptíveis a anfotericina B e a nistatina, e 93,1 % susceptíveis ao fluconazol. Atividades da proteinase ácida, hemolisina, e fosfolipase foram observadas em 99,0, 90,0 e 88,0% dos isolados de *Candida* spp., respectivamente. Aproximadamente 67,0% das cepas apresentaram índices de aderência de 0,5 a 1,5 leveduras por célula Vero, sendo que a maioria dos isolados mostrou um perfil hidrofílico. Estudos de correlação indicaram que leveduras hidrofílicas apresentaram maiores índices de aderência e atividade da proteinase e fosfolipase; e uma correlação positiva entre todas as enzimas, também, foi observada. Adicionalmente, os isolados com alta atividade hemolítica foram menos susceptíveis aos fluconazol e a anfotericina B. Esses resultados de prevalência de leveduras do gênero *Candida* e de susceptibilidade aos antifúngicos corroboram com dados da literatura; e as correlações observadas entre os fatores de virulência e valores de CIM sugerem que *Candida* spp. isoladas de fluido vaginal menos susceptíveis aos antifúngicos e com alta produção de enzimas extracelulares podem ser mais virulentas para causar dano tecidual.

**Palavras-chave:** susceptibilidade antifúngica, *Candida*, identificação, virulência.

### Introduction

Vulvovaginal candidiasis (VVC), a *Candida* infection, is a common disease of the lower genital tract in childbearing women. Despite therapeutic advances, VVC remains a common problem worldwide, occurring in all strata of society (SOBEL, 2007). It is estimated that 70.0-75.0% of all women will experience at least one symptomatic VVC episode during their lifetime.

A significant percentage of these women will experience subsequent episodes, and 5.0% will develop frequent recurrent vulvovaginal candidiasis (RVVC), defined as four or more episodes in one year. *Candida* spp. can be isolated from the vagina of about 20.0% of asymptomatic healthy women (SOBEL, 2007). Although *Candida albicans* is the predominant species isolated from vaginal samples (85-95%), *Candida non-albicans* (CNA) species have

been reported with increasing frequency, mainly *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis* and *Candida krusei* (BAUTERS et al., 2002; LOPES-CONSOLARO et al., 2004). Interestingly, *C. glabrata* often is related with RCVV (SOBEL, 2007).

Several factors of the host can be related with VVC, such as genetic susceptibility to colonization by *Candida* species, pregnancy, diabetes mellitus, use of oral contraceptives with high estrogen content and use of broad spectrum antibiotics. Besides, immunological factors, as low innate and adaptative immunity of the host can be also related with *Candida* infection (SOBEL, 2007). In addition, many virulence factors of *Candida* spp. are involved in pathogenic process of VVC. The adherence on host cell and/or medical-devices surfaces is essential for microorganisms to successfully colonize a human host, and is an important step in the infection process. The ability of *Candida* to persist within the host and to cause infection has also been attributed to its capacity to grow in a range of physiological extremes, to produce hydrolytic enzymes (proteinases such as aspartyl proteinases, phospholipases, and hemolysins), the reversible transition between unicellular yeast and filamentous growth, and phenotypic switching (CALDERONE; FONZI, 2001; SILVA et al., 2011).

A limited number of antifungal agents including azoles (imidazoles and triazoles) and polyenes (amphotericin B and nystatin) are used for the treatment of *Candida* vaginitis, and strategies and drug combinations may be useful to VVC therapy (SOBEL, 2007). In addition, oral azoles have a potential side-effect of systemic toxicity, which has dramatically restricted the use of ketoconazole (SOBEL, 2007). Some CNA species, such as *C. krusei* and *C. glabrata*, tend to be less susceptible to azoles, and the prolonged and repeated use of these antifungal can contribute to the emergence of resistant *Candida* strains (COLLIN et al., 1999). Besides the innate or acquired resistance of *Candida* spp., reduced mucosal immunity of host and inappropriate treatment can contribute to treatment failure (CANUTO; RODERO, 2002).

Diagnosis of VVC is frequently based on the history and physical examination, without corroborative evidence from laboratory tests (SOBEL, 2007) and often the VVC therapy is started without even specie identification, may due to conventional methods for the identification of *Candida* species are based on micromorphological and physiological traits, but these methods are laborious and time-consuming (KURTZMAN;

FELL, 1998). In view of this, the aim of the present study was to identify isolates from vaginal fluid by molecular methods based on the polymerase chain reaction, and to determine their profile of susceptibility to amphotericin B, nystatin, and fluconazole. In addition, virulence factors of the *Candida* spp. isolates, such as the activity of extracellular enzymes (proteinases, phospholipases, and hemolysins), adherence on mammalian cells (Vero), and cell-surface hydrophobicity were investigated.

## Material and methods

### Patients and specimen collection

This study was carried out at the Laboratory of Applied Microbiology at Natural and Synthetic Products of the State University of Maringá, Paraná State, Brazil. The study was approved by the local Ethics Committee on Research involving Humans (Reg. no. 026/2002). The specimens were collected from women who visited the gynecology health service during from September 2002 through March 2003, without previous underlying disease associated with immunodeficiency, including AIDS. These patients were sent to the Clinical Analysis Laboratory of Maringá, where samples of vaginal fluids were obtained by scraping the vaginal walls with a sterile cotton swab. Vaginal swabs were used for microscopy analysis to determine the presence of yeasts through Gram staining and culture on Sabouraud dextrose agar medium (Difco, Becton Dickinson Co., Sparks MD, USA) supplemented with 50 mg mL<sup>-1</sup> of chloramphenicol (Sigma Chemical Co., Missouri, USA). The cultures were incubated at 37°C, for 24-48h, under aerobic conditions. Each isolate was kept in sterile distilled water at 4-8°C.

### Yeasts identification

In order to perform a preliminary species identification, all isolates were cultured in CHROMagar *Candida* medium® (CHROMagar Company, Paris, France), for 48h, at 37°C. In addition, PCR-based methods for species identification were performed, using universal and species-specific primers for detection of *Candida* species as follow below.

**DNA extraction:** Genomic DNA extraction was performed according Jain et al. (2001) with some modifications. A single colony on SDA was subcultured in the broth Sabouraud dextrose at 37°C for 48 h. The cells were treated with lysis's buffer TENTS (10 mM Tris HCl pH 8.0 with 2% v v<sup>-1</sup> Triton X-100, 1% SDS, 100 mM NaCl and 1 mM

EDTA) and DNA purification was performed by extraction in phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v).

**Seminested polymerase chain reaction (snPCR):** The snPCR for *Candida* species identification was performed using oligonucleotide primers directed against 3' end of 5.8S and 5' end of 28S rDNA region, including the intervening internally transcribed spacer (ITS2), as described by Ahmad et al. (2002). The first step, PCR is carried out with universal oligonucleotide primers for detection of *Candida* genus (forward primer CTSF 5'-TCGCATCGAT GAAGAACGCAGC-3' and reverse primer CTSR 5'-TCTTTTCCTCCGCTTAT TGATATGC-3'). The product of this amplification was submitted to a second PCR using CTSR and species-specific oligonucleotide primers of species more frequently isolated from clinical specimen: *Candida albicans* (CADET, 5'-ATTGCTTGCGGCGGTAACGTC-3'), *Candida glabrata* (CGDET, 5'-TAGGTTTACCAAC TCGGTGTT-3'), *Candida parapsilosis* (CPDET, 5'-TCTTTTCCTCCGCTTAT TGATATGC-3'), and *Candida tropicalis* (CTDET, 5'-ATTTTGCTAGTGGCC-3'). PCR cycling was carried out in thermocycler under the following conditions: denaturation at 94°C for 60 s, annealing at 60°C for 30 s, and extension at 72°C for 60 s. An initial denaturation step at 94 °C for 180 s and a final extension step at 72°C for 600 s were also included. Besides, the chitin synthase 1 gene amplification were realized according Milde et al. (2000).

#### Antifungal susceptibility testing

The susceptibility of *Candida* spp. isolates to amphotericin B, nystatin (Sigma Chemical Co., Missouri, USA), and fluconazole (Pfizer, São Paulo, Brazil) were determined by broth microdilution method as described in document M27-A3 published by Clinical Laboratory Standards Institute (CLSI, 2008) with some modifications. *Candida albicans* (ATCC 10231) and *Candida parapsilosis* (ATCC 22019) were included in each experiment as standard strains. Briefly, serial two-fold dilution of the antifungal (amphotericin B, nystatin, and fluconazole) were performed in RPMI 1640 medium (Sigma Chemical Co., Missouri, USA), buffered with MOPS 0.16 M pH 7.0, into 96-well microtitre trays. After, the yeast inoculum was adjusted to  $1-5 \times 10^6$  CFU mL<sup>-1</sup> and an aliquot was dispensed into each well to obtain a final concentration of  $0.5-2.5 \times 10^3$  CFU mL<sup>-1</sup>. The susceptibility endpoints for polyenes were defined as the lowest concentrations of antifungal which resulted in total inhibition of growth. For

fluconazole, minimum inhibitory concentrations of 50% (IC<sub>50</sub>) and 90% (IC<sub>90</sub>) were determined by reading the plates in a spectrophotometer (Bio-Tek® PowerWave XS) at 492 nm (CLSI, 2008).

Fluconazole breakpoints were used as describe in document M27-A3 (CLSI, 2008): susceptible (S), MIC ≤ 8 µg mL<sup>-1</sup>; susceptible-dose dependent (SDD), MIC = 16 to 32 µg mL<sup>-1</sup>; resistant (R), MIC ≥ 64 µg mL<sup>-1</sup>. For amphotericin B, resistant strain was considered in concentrations above of 1 µg mL<sup>-1</sup> (NGUYEN et al., 1998).

#### Cellular surface hydrophobicity (CSH)

Yeasts of *Candida* spp. grown in Sabouraud dextrose broth for 24-48 h were harvested, washed with phosphate-buffered saline (PBS) at pH 7.4, resuspended in the same solution, and then the turbidity was read at 660 nm. An aliquot of 2.5 mL of this cell suspension was added to a sterile glass tube containing 1 mL of xylene. The tube was vigorously mixed for 2 min., and then left for 20 min at room temperature to allow separation of the two phases. The aqueous phase was collected and the turbidity was read at 660 nm.

The hydrophobicity index (HI) was calculated using the following equation:  $HI = (A_{660nm\ control} - A_{660nm\ test}) \times 100 / A_{660nm\ control}$ , where  $A_{660nm\ control}$  = optical density of the samples before xylene treatment, and  $A_{660nm\ test}$  = optical density of the samples after xylene treatment (TEIXEIRA et al., 1993).

Cellular surface hydrophobicity of *Candida* isolates was classified as low CSH (HI < 30.0%) and high CSH (HI > 70.0%), which determined them as highly hydrophilic and hydrophobic cells, respectively.

#### Adherence on Vero cells

Green monkey kidney cells (Vero cells) were maintained in Dulbecco's modified Eagle's Medium (DMEM, Gibco Invitrogen Corporation, New York, USA) with 10% fetal bovine serum, 50 UI mL<sup>-1</sup> penicillin, and 50 µg mL<sup>-1</sup> streptomycin. Monolayers of Vero cells ( $5 \times 10^5$  cells mL<sup>-1</sup>) prepared on round coverglasses were incubated with 0.5 mL of *Candida* spp. suspension ( $1 \times 10^6$  CFU mL<sup>-1</sup>) in DMEM at 37°C, 5.0% CO<sub>2</sub>, for 1h. The monolayers were washed with PBS to remove unadhered yeasts, fixed in Bouin's solution overnight, and Giemsa-stained for 30 min. The number of attached yeasts per cell was estimated in at least 200 Vero cells (DOROCKA-BOBKOWSKA et al., 2003).

#### Hydrolytic enzymes production

All analyses were carried out in minimum medium agar (6 g NaNO<sub>3</sub>, 1.5 g KH<sub>2</sub>PO<sub>4</sub>, 0.5 g KCl, 0.5 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.01 g FeSO<sub>4</sub>, 0.01 g ZnSO<sub>4</sub>, 10 g

glucose, and 20 g agar-agar per 1,000 mL), supplemented with: 0.6% skim milk, pH 4.0 for acid proteinase activity; 4.0% egg yolk, 350 mM NaCl and 6.5 mM  $\text{CaCl}_2$ , pH 4.5 for phospholipase activity; and 7.0% sheep blood for hemolytic activity. *Candida* spp. cultures 24-48 h old in Sabouraud dextrose broth were used to determine the enzymatic activities. Yeasts were centrifuged and washed with PBS, counted in an improved Neubauer chamber to obtain a yeast suspension of  $1 \times 10^6$  CFU  $\text{mL}^{-1}$ . An aliquot of 10  $\mu\text{L}$  of this fungal suspension was placed on the surface of the minimum medium and the cultures were incubated at 37°C for 96h for acid proteinase and phospholipase activities. To determine hemolytic activity, the plates were incubated in atmosphere at 5%  $\text{CO}_2$ , at 37°C, for 96h (LUO et al., 2001; RUCHEL et al., 1982; SAMARANAYAKE et al., 1984).

*Candida* isolates that showed degradation or a precipitation halo around the colony were considered positive for enzyme production. The enzymes activities were expressed as Pz values (a/b), where meaning the ratio of the colony diameter (a) to the degradation halo diameter around the colony (b). According this definition, low Pz values mean high enzyme production and, inversely, high Pz values indicate low enzymatic production. The Pz value was scored into four categories: 0.00 – 0.39: high activity, 0.4 – 0.69: moderate activity, 0.70 – 0.99: low activity and 1.00: without activity (PRICE et al., 1982).

*Candida albicans* (ATCC 10231) was used as a positive control for the determination of acid proteinase and phospholipase activity, and the bacteria *Aeromonas hydrophila* (ATCC 7966) for hemolytic activity.

### Statistical analysis

Statistical analyses were performed with the program Statistic for Windows, version 6.0 (Statsoft, Inc., Oklahoma City, Oklahoma, USA) and a p-value less than 0.05 was regarded as significant. The Pearson's test was performed to correlate enzymes activities and MIC values.

## Results

### Species distribution

One hundred and one yeasts (20.0%) were isolated from vaginal fluids of the 504 women between 13 and 80 years old. In microscopic analyses by Gram's stain, the presence of yeasts was detected in only 81 (16.0%) samples of the vaginal fluid. There was an agreement of 78.6% in detecting yeasts by culturing on Sabouraud dextrose agar and by microscopic analysis.

In the identification by the snPCR method, *C. albicans* occurred in 93.1% of samples (94/101), and CNA in 5.7% (6/101), consisting of one *C. parapsilosis*, one *C. tropicalis*, and 4 *Candida* sp. (non-*albicans*, non-*parapsilosis*, non-*tropicalis*, and non-*glabrata*). One isolate was not identified as a member of the *Candida* genus.

### Antifungal susceptibility

Susceptibility testing results to polyenes and fluconazole against the clinical isolates yeasts are shown in the Table 1. All isolates were susceptible to amphotericin B in MICs below 0.4  $\mu\text{g mL}^{-1}$ , and 44.5% (45/101) showed a MIC value of 0.2  $\mu\text{g mL}^{-1}$ . Nystatin presented antifungal action between 3.1 to 12.5  $\mu\text{g mL}^{-1}$ , and 73.2% (74/101) of the isolates were susceptible in concentration of 6.2  $\mu\text{g mL}^{-1}$ . The  $\text{IC}_{50}$  and  $\text{IC}_{90}$  value of fluconazole more frequent was 0.2  $\mu\text{g mL}^{-1}$ , in 89 and 63.0% of isolates, respectively. In addition, 93.1% (94/101) of the yeasts were characterized as susceptible, 1.0% susceptible dose-dependent (one *C. albicans*), and 5.9 % as resistant to fluconazole (six *C. albicans*).

**Table 1.** Minimum inhibitory concentrations (MICs) of antifungal agents and susceptibility profiles of yeasts isolated from vaginal fluid.

Antifungal agents	Isolates (no.)	Range MIC ( $\mu\text{g mL}^{-1}$ )	Mode ( $\mu\text{g mL}^{-1}$ )
Amphotericin B*	All isolates (101)	0.04 - 0.4	0.2
	<i>Candida albicans</i> (94)	0.04 - 0.4	0.2
	<i>Candida parapsilosis</i> (1)	0.1	0.1
	<i>Candida tropicalis</i> (1)	0.4	0.4
	<i>Candida</i> sp. (4)	0.2 - 0.4	0.2
	non- <i>Candida</i> (1)	0.2	0.2
Nystatin*	All isolates (101)	3.1 - 12.5	6.2
	<i>Candida albicans</i> (94)	3.1 - 12.5	6.2
	<i>Candida parapsilosis</i> (1)	3.1	3.1
	<i>Candida tropicalis</i> (1)	6.2	6.2
	<i>Candida</i> sp (4)	3.1-6.2	3.1
Fluconazole*	non- <i>Candida</i> (1)	3.1	3.1
	All isolates (101)	$\leq 0.2$ -128	0.2
	<i>Candida albicans</i> (94)	$\leq 0.2$ -128	0.2
	<i>Candida parapsilosis</i> (1)	$\leq 0.2$	0.2
	<i>Candida tropicalis</i> (1)	$\leq 0.2$	0.2
	<i>Candida</i> sp (4)	0.2-8	0.2
	non- <i>Candida</i> (1)	$\leq 0.2$	0.2

\*MIC results are  $\text{IC}_{50}$  values for fluconazole and  $\text{IC}_{90}$  values for amphotericin B and nystatin; S: susceptible; SDD: susceptible-dose-dependent; R: resistant; -: not classified.

### Adherence on mammalian cells and cellular surface hydrophobicity

The adherent capacity of the *Candida* isolates on Vero cells was variable (0.04 to 3.9 yeasts Vero cell $^{-1}$ ), and 67.0% presented moderate adherence between 0.5 and 1.5 yeasts cell $^{-1}$  (Table 2). Adherence of *C. albicans* (n = 94) and CNA (n=6) were of  $1.1 \pm 0.7$  and  $1.6 \pm 0.3$  yeasts Vero cell $^{-1}$ , respectively.

Around 68.0% of the *Candida* isolates had HI < 30.0%, and only 6 strains presented higher HI: *C. parapsilosis* (85.3%), *C. tropicalis* (72.3%), and 4 *C. albicans* (81.0-95.0%) (Table 2). Although there was no significant difference in cellular surface hydrophobicity between *C. albicans* and CNA, CNA showed be more hydrophobic.

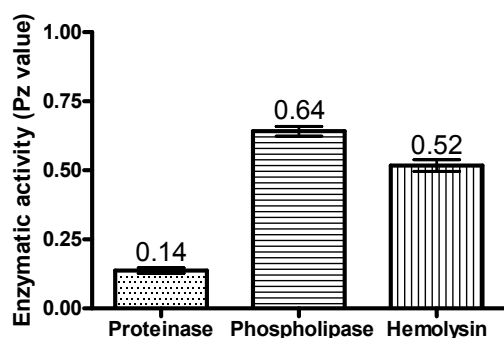
### Extracellular Enzymes Production

After 72h of *Candida* growth on supplemented minimum medium at 37°C, we observed almost all the isolates (99.0%) produced acid protease with Pz values below 0.3 characterizing high enzymatic activity. The hemolysis was observed in 90.0% of isolates being 38.0% with high production and 39.0% with moderate activity, whilst the phospholipase production occurred in 88.0% of *Candida* spp., where the most of them (74.0%) presented moderate activity ( $0.4 < Pz < 0.69$ ) (Table 2). Among enzymes production, the acid protease presented the best enzymatic activity in comparison with hemolysin and phospholipase ( $p < 0.0001$ ) followed by hemolytic activity when compared with phospholipase ( $p < 0.0001$ ) (Figure 1).

**Table 2.** Virulence profiles of *Candida* spp. isolated from vaginal fluid: adherence on mammalian cells, cellular surface hydrophobicity, and hydrolytic enzymes activity (acid proteinase, phospholipase and hemolytic activity).

Virulence factors	Range (classification)	(%)
Adherence (yeasts Vero cell <sup>-1</sup> )	0 – 0.49 (low)	15
	0.5 – 1.5 (moderate)	67
	1.51 – 4 (high)	18
Hydrophobicity index (%)	0 – 30 (hydrophilic)	68
	31 – 69 (-)	26
	70– 100 (hydrophobic)	6
Acid proteinase activity (Pz value)	0.00 – 0.39 (high)	99
	0.4 – 0.69 (moderate)	0
	0.70 – 0.99 (low)	0
Phospholipase activity (Pz value)	0.00 – 0.39 (high)	9
	0.4 – 0.69 (moderate)	74
	0.70 – 0.99 (low)	1
Hemolytic activity (Pz value)	0.00 – 0.39 (high)	14
	0.4 – 0.69 (moderate)	39
	0.70 – 0.99 (low)	38

(-) not classified.



**Figure 1.** Enzymatic activity of *Candida* spp. isolated from vaginal fluid: proteinase > hemolysin > phospholipase ( $p < 0.0001$ ; test  $t'$  student).

### Correlations

The hydrophobicity index showed a negative correlation with adherence on Vero cells ( $r = -0.3$ ) and positive correlation with proteinase and phospholipase Pz values ( $r = 0.3$  and  $r = 0.4$ , respectively). The hemolytic activity had positive correlations with acid proteinase and phospholipase ( $r = 0.4$  and  $r = 0.3$ , respectively), and proteinase and phospholipase also presented positive correlation between them ( $r = 0.3$ ). In addition, the hemolytic activity correlated negatively with fluconazole and amphotericin B MIC values ( $r = -0.2$  for both antifungal), suggesting that isolates with hemolysin high production were less susceptible to antifungal agents.

### Discussion

In this study, 101 yeasts were isolated from 504 fluid vaginal samples (20.0%) of symptomatic and asymptomatic patients and this frequency was consistent with previously published data (ECKERT et al., 1998; LOPES-CONSOLARO et al., 2004). *C. albicans* was more frequent specie in vaginal fluid samples; it was identified in 93.1% of isolates by snPCR method. Although research had shown *C. albicans* is responsible for around 90.0% of symptomatic VVC (SOBEL, 2007), CNA species isolation had increased (RUHNKE, 2006). Different morphological and physiological properties of *C. albicans* strains have been reported by other investigators (KENNEDY et al., 1988; LAN et al., 2002) and these different properties can make species identification difficult. Yeasts have traditionally been classified on the basis of their morphological, physiological and biochemical properties (KURTZMAN; FELL, 1998), however, this is complex and time consuming, and can lead to incorrect classification. Phenotypic identification methods are broadly supplemented by new genotypic methods, which allow a more refined and detailed differentiation of related microorganisms (BUSCH; NITSCHKO, 1999). The gene amplification technology by snPCR used in this study, can detect members of the genus *Candida* by means of a specific and conserved nucleotide sequence, in region 5.8S and 28S rDNA. This methodology has shown specificity and sensitivity for *Candida* species (AHMAD et al., 2002).

In this study, all vaginal isolates were susceptible to amphotericin B and nystatin, in agreement with previously reported data (GUALCO et al., 2007). Even though polyenes were widely used for more than 40 years in many fungal infections, the incidence of resistance in *Candida* spp. is rare

(ELLIS, 2002). On the other hand, resistance of *Candida* spp. to azoles continues to increase in response to the widespread application of triazole therapeutics, although few fluconazole-resistant strains have been isolated from VVC cases (SOBEL, 2007). In agreement with previous studies (GROSS et al., 2007; SOBEL, 2007), we demonstrated resistance to fluconazole in 5.9% of the isolates from vaginal fluid.

For some human pathogenic fungi the virulence factors to have been selected for environmental survival and function in mammalian virulence more by accident or happenstance (CASADEVALL, 2007). These events may be regulated by different pathways, but MAP kinases are key elements that control of essential virulence factors such as invasion and oxidative stress in *C. albicans*. MAP kinases sense different activating signals, which enable them to integrate, amplify and modulate the appropriate protective and adaptive response (ROMÁN et al., 2007). Several virulence factors of *Candida* species can be related to candidiasis pathogenesis, such as CSH, adhesion, hydrolytic enzymes production, and morphogenesis (CALDERONE; FONZI, 2001; SILVA et al., 2011).

The adhesion is an extremely important step for establishing of this infection, colonization and tissue invasion by digesting and destroying cell membranes and/or extracellular matrix components; and the extent of adhesion is dependent on microbial, host and abiotic surfaces properties, such as CSH and fungal cell wall composition (SILVA et al., 2011). In this work, 68% of vaginal isolates showed hydrophilic characteristics and adherence capacity occurred of 0.04 to 3.86 yeasts/Vero cells. Several reports have shown that CSH can be involved in factors related to pathogenesis, such as the cell wall, germ-tube formation, adhesion to cells and matrix proteins, and phagocytosis (HAZEN, 1989). Reports have indicated that CSH is an important factor for adhesion mechanisms of yeasts in cells (ENER; DOUGLAS, 1992), showing hydrophobic yeasts present more adherence than hydrophilic yeasts. However, we show a negative correlation between CSH and adherence on Vero cells. Kennedy et al. (1988) had similar results, which demonstrated hydrophobic cells presented fewer adherences in buccal epithelial cells. This may suggest many factors can be involved in the adhesion process of *Candida* spp. on cells or tissues, in that CSH may not be predominant, and that effect of CSH on adherence depends on the yeast isolated (HAZEN, 1989; KENNEDY et al., 1988). On the other hand, other authors did not find a correlation between CSH and adhesion of *Candida* spp. isolates on

urinary catheters, demonstrating that CSH alone was not a predictor of adhesion (CAMACHO et al., 2007). An important factor that has correlated with adhesion ability of *Candida* spp. and biofilm formation is the presence of specific cell-wall proteins, often referred to as adhesins, being agglutinin-like sequence (ALS) a major *Candida* adhesins group (SILVA et al., 2011).

Many microorganisms produce hydrolytic enzymes to obtain nutrients and assure their survival. However, the hydrolytic enzymes activities of several bacteria and fungi, including proteases, phospholipases and hemolysins are associated with adhesion, cell damage, tissue invasion and blood dissemination. Most of the strains from vaginal fluid isolated in this work presented proteinase, phospholipase, and hemolysis production. Interestingly, we observed positive correlations between all enzymes analyzed; meaning *Candida* isolates that present high acid proteinase production also are characterized by be hemolysins and phospholipases good producers.

The secretion of aspartyl proteinases (Sap1-Sap10) by *C. albicans* is recognized as an important virulence determinant for this specie. Saps facilitate colonization and invasion of host tissue through the disruption of host mucosal membranes and by degrading important immunological and structural defenses proteins (PICHOVÁ et al., 2001; SILVA et al., 2011). Beyond Saps, phospholipases had been described for *Candida* species and are enzymes that hydrolyze phospholipids to fatty acids contributing to host cell-membrane damage, which promote cell damage and/or expose receptors to facilitate adherence of *Candida* (GHANNOUM, 2000). Several studies have suggested that *Candida* isolates that present increased production of proteinases and phospholipases, may have more ability to cause infection, invasion, and tissue damage (GHANNOUM, 2000; SUGITA et al., 2002).

Pathogenic microorganisms can grow in the host by using hemin or hemoglobin as a source of iron by hemolysins action. These enzymes are likely to be key virulence factors as might promote pathogen survival, persistence in the host and can facilitate the microorganism's dissemination (BONASSOLI et al., 2005; MANNS et al., 1994; SILVA et al., 2011; TAKADA et al., 2003). Few studies on the hemolytic activity of yeasts of the *Candida* genus have been reported. Luo et al. (2001) and Bonassoli et al. (2005) showed that some *Candida* species produced hemolysin *in vitro*. In the isolates examined in this study, high hemolytic activity was correlated with lower susceptibility to amphotericin B and fluconazole. Previous study demonstrated

resistance to fluconazole with increased expression of virulence factors, such as proteinases and phospholipases production, adherence, and germ-tube (LYON; RESENDE, 2006; SAMARANAYAKE et al., 1984). These enzymes are also correlated with increase of pathogenesis and overexpression of a gene encoding a multidrug resistance (MDR1) efflux pump (WU et al., 2000).

## Conclusion

We observed 20.0% of the vaginal fluid samples in symptomatic and asymptomatic patients showed a positive culture on Sabouraud dextrose agar, and around 90.0% of the isolates were identified as *C. albicans* by snPCR method. All isolates were susceptible to polyene agents and resistance to fluconazole was rare (5.9%). Moreover, we showed virulence factors of *Candida* isolates (such as CSH, adherence and hydrolytic enzymes activity) had significant correlations between them, which may be related with *Candida* pathogenicity and lower susceptibility to amphotericin B and fluconazole.

## Acknowledgements

This study was supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Financiadora de Estudos e Projetos (FINEP), and Programa de Pós-graduação de Ciências Farmacêuticas da Universidade Estadual de Maringá.

K.I. was supported by a fellowship from CAPES, Brazil.

## References

- AHMAD, S.; KHAN, Z.; MUSTAFA, A. S.; KHAN, Z. U. Seminested PCR for diagnosis of candidemia: comparison with culture, antigen detection, and biochemical methods for species identification. **Journal of Clinical Microbiology**, v. 40, n. 7, p. 2483-2489, 2002.
- BAUTERS, T. G. M.; DHONT, M. A.; TEMMERMAN, M. I. L.; NELIS, H. J. Prevalence of vulvovaginal candidiasis and susceptibility to fluconazole in women. **American Journal of Obstetrics and Gynecology**, v. 187, n. 3, p. 569-574, 2002.
- BONASSOLI, L. A.; BERTOLI, M.; SVIDZINSKI, T. I. S. High frequency of *Candida parapsilosis* on the hands of healthy hosts. **Journal of Hospital Infection**, v. 59, n. 2, p. 159-162, 2005.
- BUSCH, U.; NITSCHKO, H. Methods for the differentiation of microorganisms. **Journal of Chromatography**, v. 722, n. 1-2, p. 263-278, 1999.
- CALDERONE, R. A.; FONZI, W. A. Virulence factors of *Candida albicans*. **Trends in Microbiology**, v. 9, n. 7, p. 327-335, 2001.
- CAMACHO, D.; GASPARETTO, A.; SVIDZINSKI, T. I. The effect of chlorhexidine and gentian violet on the adherence of *Candida* spp. to urinary catheters. **Mycopathologia**, v. 163, n. 5, p. 261-266, 2007.
- CANUTO, M. M.; RODERO, F. G. Antifungal drug resistance to azoles and polyenes. **Lancet Infectious Disease**, v. 2, n. 9, p. 550-563, 2002.
- CASADEVALL, A. Determinants of virulence in the pathogenic fungi. **Fungal Biology Reviews**, v. 21, n. 4, p. 130-132, 2007.
- CLSI-Clinical Laboratory Standard Institute. **Reference Method for Broth dilution antifungal susceptibility testing of yeasts**. Approved standard M27-A3. Villanova: National Committee for Clinical Laboratory Standards, 2008.
- COLLIN, B.; CLANCY, C. J.; NGUYEN, M. H. Antifungal resistance in non-*albicans* *Candida* species. **Drug Resistance Updates**, v. 2, n. 1, p. 9-14, 1999.
- DOROCKA-BOBKOWSKA, B.; KONOPKA, K.; DÜZGUINES, N. Influence of antifungal polyenes on the adhesion of *Candida albicans* and *Candida glabrata* to human epithelial cells *in vitro*. **Archives of Oral Biology**, v. 48, n. 12, p. 805-814, 2003.
- ECKERT, L. O.; HAWES, S. E.; STEVENS, C. E.; KOUTSKY, L. A.; ESCHENBACH, D. A.; HOLMES, K. K. Vulvovaginal candidiasis: clinical, manifestations, risk factors, management algorithm. **Obstetrics and Gynecology**, v. 92, n. 5, p. 757-765, 1998.
- ELLIS, D. Amphotericin B: spectrum and resistance. **Journal of Antimicrobial Chemotherapy**, v. 49, suppl. 1, p. 7-10, 2002.
- ENER, B.; DOUGLAS, L. J. Correlation between cell-surface hydrophobicity of *Candida albicans* and adhesion to buccal epithelial cells. **FEMS Microbiology**, v. 99, n. 1, p. 37-42, 1992.
- GHANNOUM, M. A. Potential role of phospholipases in virulence and fungal pathogenesis. **Clinical Microbiology Reviews**, v. 13, n. 1, p. 122-143, 2000.
- GROSS, N. T.; ARIAS, M. L.; MORAGA, M.; BADDASAROW, Y.; JARSTRAND, C. Species distribution and susceptibility to azoles of vaginal yeasts isolated prostitutes. **Infectious Diseases in Obstetrics and Gynecology**, v. 2007, n. 1, p. 1-5, 2007.
- GUALCO, L.; DEBBIA, E. A.; BANDETTINI, R.; PESCIOTTO, L.; CAVALLERO, A.; OSSI, M. C.; SCHITO, A. M.; MARCHESE, A. Antifungal resistance in *Candida* spp. isolated in Italy between 2002 and 2005 from children and adults. **International Journal of Antimicrobial Agents**, v. 29, n. 2, p. 1979-1984, 2007.
- HAZEN, K. C. Participation of yeast cell surface hydrophobicity in adherence of *Candida albicans* to human epithelial cells. **Infection and Immunity**, v. 57, n. 7, p. 1894-1900, 1989.
- JAIN, P.; KHAN, Z. K.; BHATTACHARYA, E.; RANADE, S. A. Variation in random amplified polymorphic DNA (RAPD) profiles specific to fluconazole-resistant and sensitive strains of *Candida albicans*. **Diagnostic Microbiology and Infectious Disease**, v. 41, n. 3, p. 113-119, 2001.



- KENNEDY, M. J.; ROGERS, A. L.; HANSELMEN, L. R.; SOLL, D. R.; YANCEY, R. J. Variation in adhesion and cell surface hydrophobicity in *Candida albicans* white and opaque phenotypes. **Mycopathology**, v. 102, n. 3, p. 149-156, 1988.
- KURTZMAN, C. P.; FELL, J. W. **The yeasts, a taxonomic study**. 4th ed. Amsterdam: Elsevier Science B.V., 1998.
- LAN, C. Y.; NEWPORT, G.; MURILLO, L. A.; JONES, T.; SCHERER, S.; DAVIS, R. W.; AGABIAN, N. Metabolic specialization associated with phenotypic switching in *Candida albicans*. **Proceedings of the National Academy of Sciences**, v. 99, n. 23, p. 14907-14912, 2002.
- LOPES-CONSOLARO, M. E.; ALBERTONI, T. A.; YOSHIDA, C. S.; MAZUCHELI, J.; PERALTA, R. M.; SVIDZINSKI, T. I. E. Correlation of *Candida* species and symptoms among patients with vulvovaginal candidiasis in Maringá, Paraná, Brazil. **Revista Iberoamericana de Micología**, v. 21, n. 4, p. 202-205, 2004.
- LUO, G.; SARAMANAYAKE, L. P.; YAU, J. Y. *Candida* species exhibit differential *in vitro* hemolytic activities. **Journal of Clinical Microbiology**, v. 39, n. 8, p. 2971-2974, 2001.
- LYON, J. P.; RESENDE, M. A. Correlation between adhesion, enzyme production, and susceptibility to fluconazole in *Candida albicans* obtained from denture wearers. **Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontics**, v. 102, n. 5, p. 632-638, 2006.
- MANNIS, J. M.; MOSSER, D. M.; BUCKLEY, H. R. Production of a hemolytic factor by *Candida albicans*. **Infection and Immunity**, v. 62, n. 11, p. 5154-5156, 1994.
- MILDE, K.; KOSTKA, V.; KALETA, E. F.; WILLEMS, H.; JÁGER, C. Multiplex-PCR-based differentiation and characterization of *Candida*-isolates derived from tortoises (*Testudinidae*). **Veterinary Microbiology**, v. 76, n. 4, p. 395-402, 2000.
- NGUYEN, M. H.; CLANCY, C. L.; YU, V. L.; YU, Y. C.; MORRIS, A. J.; SNYDMAN, D. R.; SUTTON, D. A.; RINALDI, M. G. Do *in vitro* susceptibility data predict the microbiologic response to amphotericin B? Results of a prospective study of patients with *Candida* Fungemia. **Journal of Infectious Diseases**, v. 177, n. 2, p. 425-430, 1998.
- PICHOVÁ, I.; PAVLÍCKOVÁ, L.; DOSTÁL, J.; DOLEJSÍ, E.; HRUSKOVÁ-HEIDINGSFELDOVÁ, O.; WEBER, J.; RUML, T.; SOUCEK, M. Secreted aspartic proteases of *Candida albicans*, *Candida tropicalis*, *Candida parapsilosis* and *Candida lusitanae*. Inhibition with peptidomimetic inhibitors. **European Journal of Biochemistry**, v. 268, n. 9, p. 2669-2677, 2001.
- PRICE, M. F.; WILKINSON, I. D.; GENTRY, L. O. Plate method for detection of phospholipase activity in *Candida albicans*. **Sabouraudia**, v. 20, n. 1, p. 7-14, 1982.
- ROMÁN, E.; ARANA, D. M.; NOMBELA, C.; ALONSO-MONGE, R.; PLA, J. MAP kinase pathways as regulators of fungal virulence. **Trends in Microbiology**, v. 15, n. 4, p. 181-190, 2007.
- RUCHEL, R.; TEGELER, R.; TROST, M. A comparison of secretory proteinase from different strains of *Candida albicans*. **Saboraudia**, v. 20, n. 3, p. 233-244, 1982.
- RUHNKE, M. Epidemiology of *Candida albicans* infections and role of non-*Candida albicans* yeasts. **Current Drug Targets**, v. 7, n. 4, p. 495-504, 2006.
- SAMARANAYAKE, L. P.; RAESIDE, J. M.; MACFARLANE, T. W. Factors affecting the phospholipase activity of *Candida* species *in vitro*. **Sabouraudia**, v. 22, n. 3, p. 201-207, 1984.
- SILVA, S.; NEGRI, M.; HENRIQUES, M.; OLIVEIRA, R.; WILLIAMS, D. W.; AZEREDO, J. Adherence and biofilm formation of non-*Candida albicans* *Candida* species. **Trends in Microbiology**, v. 19, n. 5, p. 241-247, 2011.
- SOBEL, J. D. Vulvovaginal Candidosis. **The Lancet**, v. 369, n. 9577, p. 1961-1971, 2007.
- SUGITA, T.; KUROSAKA, S.; YAJITATE, M.; SATO, H.; NISHIKAWA, A. Extracellular proteinase and phospholipase activity of three genotypic strains of human pathogenic yeast, *Candida albicans*. **Microbiology and Immunology**, v. 46, n. 12, p. 881-883, 2002.
- TAKADA, K.; FUKATSU, A.; OTAKE, S.; HIRASAWA, M. Isolation and characterization of hemolysin activated by reductant from *Prevotella intermedia*. **FEMS Immunology and Medical Microbiology**, v. 35, n. 1, p. 43-47, 2003.
- TEIXEIRA, L. A.; FIGUEIREDO, A. M. S.; FERREIRA, B. T.; ALVES, V. M. L.; NAGAO, P. E.; ALVIANO, C. S.; ANGLUSTER, J.; SILVA-FILHO, F. C.; BENCHETRIT, L. C. Sialic acid content and surface hydrophobicity of group B streptococci. **Epidemiology and Infection**, v. 110, n. 1, p. 87-94, 1993.
- WU, T.; WRIGHT, K.; HURST, S. F.; MORRISON, C. J. Enhanced extracellular production of aspartyl proteinase, a virulence factor, by *Candida albicans* isolates following growth in subinhibitory concentrations of fluconazole. **Antimicrobial Agents Chemotherapy**, v. 44, n. 5, p. 1200-1208, 2000.

Received on May 30, 2011.

Accepted on July 22, 2011.

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