MARCIA EDILAINE LOPES CONSOLARO

Aspectos fenotípicos, susceptibilidade a antifúngicos e potencial de virulência de isolados vaginais de *Candida albicans* e sua relação com quadros clínicos

Tese apresentada ao Programa de Pós-graduação em Ciências Biológicas (área de concentração – Biologia Celular) da Universidade Estadual de Maringá para obtenção do grau de doutor em Ciências Biológicas.

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Orientadora: Dr[a]. Rosane Marina Peralta
Co-orientadora: Dr[a] Terezinha Inez Estivalet Svidzinski

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Ao querido **Otávio**
Meu companheiro
de todas as horas

Aos meus filhos **Otávio e Gustavo**
Crianças maravilhosas que
compreenderam e aceitaram
minha ausência em decorrência
desta tese

Dedico

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SUMÁRIO

As leveduras do gênero *Candida* constituem aproximadamente 200 diferentes espécies, que vivem normalmente nos mais diversos habitats corporais, como orofaringe, cavidade bucal, dobras da pele, secreções brônquicas, vagina, urina e fezes. Dentre as espécies que compõem este gênero, *Candida albicans* apresenta maior relevância em função de sua taxa de prevalência em condições de normalidade e de doença. Esta espécie é muito bem adaptada ao corpo humano, sendo capaz de colonizá-lo sem produzir sinais de doença em condições de normalidade fisiológica, existindo delicado balanço entre hospedeiro e este fungo comensal. Alterações neste balanço pode levar a uma relação parasitária, resultando no desenvolvimento da infecção denominada candidíase, que varia desde lesões superficiais em pessoas sadias até infecções disseminadas em pacientes neutropênicos.

C. albicans é um fungo dimórfico que se apresenta sob formas leveduriformes (blastoconídios) no estado saprofítico, associado à colonização assintomática ou como formas filamentosas (pseudo-hifas e hifas verdadeiras), observadas em processos patogênicos. Além disso, sob condições de crescimento sub-óptimas, neste fungo pode ocorrer à formação de clamidósporos, que são esporos arredondados que possuem uma espessa parede celular. Dessa forma, o fungo tem a capacidade de se adaptar a diferentes nichos biológicos, podendo ser considerado, a rigor, um organismo "polimórfico".

Candidíase vulvovaginal (CVV) é uma patologia ocasionada pelo crescimento anormal de fungos do tipo leveduras na mucosa do trato genital feminino. Trata-se de uma infecção de vulva e vagina, causada por leveduras comensais que habitam a mucosa vaginal bem como a mucosa digestiva e respiratória. Aproximadamente 80 a 90% dos casos são devidos à *C. albicans,* e 10 a 20% à outras espécies chamadas não-*C. albicans (C. tropicalis, C. glabrata, C. krusei, C. parapsilosis*), cuja freqüência parece estar elevando-se em algumas populações. Foi observado também um sensível aumento nos casos de CVV nos últimos anos, estando sua incidência próxima a 25% e ocupando o segundo lugar entre as infecções cérvico-vaginais, precedida apenas pela vaginose bacteriana. Estima-se que cerca de 75% das mulheres adultas apresentem pelo menos um episódio de vulvovaginite fúngica em sua vida, sendo que destas, 40 a 50% vivenciarão novos surtos e 5% atingirão o caráter recorrente (CVVR), a qual é definida usualmente como a ocorrência de 3 a 4 episódios de CVV no período de 12 meses. A patogênese da

CVVR entre mulheres que não tem condições predisponentes aparentes está sob investigação.

A principal fonte de leveduras vaginais é o trato gastrointestinal, através de um processo chamado transmissão endógena. As mesmas são veiculadas para a vagina por auto-inoculação, onde se adaptam e se desenvolvem, podendo causar distúrbios imediatos ou constituir-se em reservatório para infecções posteriores. O desenvolvimento da infecção é favorecido por uma série de fatores predisponentes do hospedeiro, como: colonização prévia pela levedura e posterior diminuição da capacidade de resposta imunológica observada em doenças imunossupressoras, diabetes mellitus, gestantes, usuárias crônicas de corticóides. Ainda parecem contribuir o uso de antibióticos, estrogenioterapia, pequenos traumas como o ato sexual, hábito de usar roupas muito justas ou de fibras sintéticas, além da dieta alimentar.

Além de fatores inerentes ao hospedeiro, foi postulado que o fungo não é um mero participante passivo no processo infeccioso. Com isso, uma série de fatores de virulência tem sido propostos, que nada mais são do que propriedades ligadas às células de *C. albicans* que lhes conferem a capacidade de produzir doença. São descritos como fatores de virulência: adesão a substratos inertes e biológicos, formação de tubo germinativo com conseqüente desenvolvimento da forma filamentosa, variabilidade fenotípica ("switching"), variabilidade genotípica, produção de toxinas e enzimas extracelulares hidrolíticas, variabilidade antigênica, imunomodulação dos mecanismos de defesa do hospedeiro e hidrofobicidade de superfície celular.

A habilidade de transição morfológica de *C. albicans* tem sido sugerida como um importante fator de virulência. As hifas têm maior capacidade de aderir e penetrar nas células epiteliais humanas do que os blastoconídios, sendo que mutantes incapazes de produzir hifas perdem a virulência de seus parentais. Essas transições representam uma resposta do fungo à alterações nas condições ambientais e possibilitam a sua adaptação a diferentes nichos biológicos, e conseqüente disseminação fúngica nas células humanas. Porém, poucos estudos foram realizados objetivando verificar se há relação entre a capacidade de formar tubos germinativos e o desenvolvimento da infecção.

Apesar da alta prevalência de CVV e do uso extensivo de antifúngicos tópicos e orais, pouco é conhecido sobre a resistência antifúngica de *C. albicans* isoladas de secreção vaginal de mulheres imunocompetentes. Também são raros os relatos associando

os efeitos da pressão seletiva dos antifúngicos sobre a fisopatogenia da CVV e aumento de microrganismos resistentes.

As infecções por *C. albicans* estão relacionadas diretamente com a produção de exoenzimas. Atualmente, estas foram colocadas entre os mais importantes fatores de virulência deste fungo. Muito foi estudado sobre as proteinases extracelulares, que são implicadas diretamente na virulência da *C. albicans*, sendo que espécies mutantes com capacidade de secreção de proteinases deficiente ou diminuída, são menos virulentas que as demais. As enzimas do tipo fosfolipase também estão associadas à sua virulência.

Nos últimos anos, reconheceu-se a importância da compreensão do processo de adesão microbiana, particularmente em nível bacteriano, estando os estudos em leveduras, em particular a *C. albicans* isoladas de secreção vaginal, com menor densidade de conhecimentos. Existem evidências de que *C. albicans* possa produzir mais de uma estrutura adesiva, sendo que a manoproteína está primariamente atribuída à função adesiva nas reações de adesão. Alguns estudos *in vivo* e *in vitro* foram desenvolvidos para quantificar e caracterizar a aderência de *C. albicans* à superfícies celulares e inanimadas. A ligação de *C. albicans* à superfícies mucosas é descrita como um importante passo no processo infeccioso, particularmente na cavidade oral e mucosa vaginal. Já é conhecido que células leveduriformes hidrofóbicas são mais virulentas em ratos do que células leveduriformes hidrofílicas. A expressão da hidrofobicidade de superfície celular (CSH) por *C. albicans* é desta forma correlacionada com virulência aumentada, provavelmente por estimular fenômenos de aderência, resistência à fagocitose e germinação.

No primeiro trabalho, o objetivo foi correlacionar a freqüência de leveduras e suas respectivas espécies em mulheres assintomáticas e com diferentes manifestações clínicas da CVV; avaliar possíveis relações entre número de colônias de fungos e sintomas desta patologia. Todas as mulheres que foram atendidas no Laboratório de Ensino e Pesquisa em Análises Clínicas da Universidade Estadual de Maringá – Paraná – Brasil, num período de cinco meses, para realização de exames rotineiros de secreção vaginal, independente da presença ou ausência de sintomas da CVV, foram incluídas neste estudo. Destas, foram excluídas as mulheres com imunodeficiência ou infecção no trato genital por outro agente. As leveduras foram identificadas por métodos clássicos e as pacientes foram divididas nos seguintes grupos: assintomáticas, CVV e CVVR. *C. albicans* foi a levedura mais isolada (60,0%) e entre as leveduras não-*C. albicans*, 61,5% foram isoladas de mulheres assintomáticas, 38,7% de pacientes com CVV e 11,1% daquelas com CVVR. *C. albicans*

foi associada com sintomas da CVV, enquanto que a presença de leveduras não-*C. albicans* em mulheres assintomáticas foi evidente. Entretanto, não houve associação entre número de colônias fúngicas e sintomas.

No segundo trabalho, a identificação de vinte *C. albicans* isoladas foi confirmada molecularmente usando análise da seqüência do rDNA. As leveduras foram incubadas em RPMI acrescido de soro fetal bovino para analisar tubos germinativos de 2 até 10 horas. Foi também analisada a sensibilidade *in vitro* para fluconazol, itraconazol, cetoconazol, anfotericina B e nistatina com ensaio de microdiluição, de acordo com o NCCLS-M27-A. Leveduras isoladas de mulheres sintomáticas produziram significativamente mais tubos germinativos do que as de mulheres assintomáticas ($p<0,05$). Entretanto, não houve diferença significativa entre leveduras de CVV e CVVR ($p>0,05$). A variação entre MIC (concentração inibitória mínima)$_{50}$ and MIC_{90} dos antifúngicos testados foi muito discreta entre as leveduras isoladas e não foram detectadas leveduras resistentes. Entretanto, leveduras de CVV foram mais DDS (susceptibilidade reduzida dose dependente) para nistatina e as de CVVR foram mais DDS para cetoconazol. Os resultados sugerem que colonização por leveduras na vagina e ausência de sintomas pode ser parcialmente explicada pela menor capacidade das leveduras para formar tubos germinativos.

O objetivo do terceiro trabalho foi avaliar uma possível correlação entre a atividade proteolítica e os outros fatores de virulência (formação de biofilme, hidrofobicidade, formação de hifa e adesão cellular) em diferentes isolados de *C. albicans* obtidos de pacientes assintomáticas e com quadros de CVV e CVVR. Os fatores de virulência foram estimados nas condições padrão e comparados com os resultados obtidos na presença do inibidor das proteinases aspárticas, pepstatina A. Pepstatina A não causou alteração significativa na formação de biofilme, formação de hifas e hidrofobicidade dos isolados obtidos nas três condições clínicas. Entretanto, pepstatina A reduziu a aderência de *C. albicans* à superfícies humanas. Este resultado sugere que as proteinases de *C. albicans* possam ter um papel auxiliar na adesão celular.

SUMMARY

The yeasts of the *Candida* genus constitute approximately 200 different species, which live normally in the most diverse bodily niches, like the oral-pharynx, buccal cavity, skin folds, bronchial secretions, vagina, urine, and faeces. Among the species that compose this genus, *Candida albicans* shows the greatest relevance in terms of prevalence rate in normal conditions and disease. This species is very well adapted to the human body, being capable of colonising it without production of disease symptoms in normal physiological conditions. A delicate balance exists between the host and this commensal fungus. Alterations in this balance may give rise to a parasitic relation, resulting in the development of the infection called candidiasis, which varies from superficial lesions in healthy people to diffused infections in neutropenic patients.

C. albicans is a dimorphic fungus that presents itself in leveduriform forms (blastoconidia) in the saprophytic state, associated with asymptomatic colonisation, or as filamentous forms (pseudo-hyphae and true hyphae), observed in pathogenic processes. Furthermore, under conditions of sub-optimal growth, the clamydospore formation may occur in this fungus, which are rounded spores that can thicken the cellular wall. In this form, the fungus has the capacity to adapt itself to different biological niches, and may be considered, strictly, a “polimorphic” organism.

Vulvovaginal candidiasis (VVC) is a pathology caused by the abnormal growth of yeast-type fungi in the mucosa of the female genital tract. It is an infection of the vulva and vagina, caused by commensal yeasts that inhabit the vaginal mucosa as well as digestive and respiratory mucosa. Approximately 80 to 90% of the cases are caused by *C. albicans*, and 10 to 20% to other species called non-*C. albicans* (*C. tropicalis, C. glabrata, C. krusei, C. parapsilosis*), with frequency seems to be increasing in some populations. A noticeable increase in the frequency of VVC in the last few years has also been observed, its incidence rate being close to 25% and occupying second place among cervical-vaginal infections, preceded only by bacterial vaginosis. It is estimated that around 75% of adult women have at least one episode of fungal vulvovaginitis in their life, and among them 40 to 50% will experience new outbreaks and 5% will be affected by the recurrent condition (RVVC), which is usually defined as the occurrence of 3 or 4 episodes of VVC in a period of 12 months. The pathogenesis of RVVC in women that do not have apparent predisposing conditions is under investigation.

The main source of vaginal yeasts is the gastrointestinal tract, through a process called endogenous transmission. They are transmitted to the vagina by autoinoculation, where they adapt and evolve themselves, and are able to cause immediate disturbances or form themselves in reservoirs for later infections. The evolution of the infection is helped by a series of predisposing factors of the host, such as: prior colonisation by the yeast and later reduction in the immunological response capacity observed in immunosuppressant diseases, diabetes mellitus, pregnancy and chronic use of corticoids. The use of antibiotics, estrogenic therapy, small traumas through sexual acts, the habit of using tight or synthetic fibre clothes, as well as diet also seem to contribute.

As well as the intrinsic factors of the host, it has also been postulated that the fungus is not merely a passive participant in the infectious process. In this regard, a series of virulence factors has been proposed, which are no more than properties related to the cells of *C. albicans* that confer to them the capacity to produce disease. The following are described as virulence factors: adhesion to inert and biological substrates, formation of germ tubes with the consequent evolution of the filamentous form, phenotype variability ("switching"), genotype variability, production of toxins and extracellular hydrolytic enzymes, antigenic variability, immunomodulation of the defence mechanisms of the host and hydrophobicity of the cell surface.

C. albicans's ability of morphological transition has been suggested as an important virulence factor. The hyphae have a greater capacity to stick to and penetrate human epithelial cells than the blastoconidias, while mutants incapable of producing hyphae lose the virulence of their parents. These transitions represent a response of the fungus to alterations in their environmental conditions and make possible their adaptation to different biological niches, and consequent fungal dissemination in human cells. However, few studies have been carried out with the objective of verifying if there is a relation between the capacity to form germ tubes and the development of the infection.

In spite of the high prevalence of VVC and the extensive use of topical and oral antifungals, little is known about the antifungal resistance of *C. albicans* isolates from vaginal secretions of immunocompetent women. Reports associating the effects of the selective pressure of antifungals on the physiopathogenesis of VVC and the increase of resistant microorganisms are also rare.

Infections by *C. albicans* are directly related to the production of exoenzymes. In fact, these have been placed among the most important virulence factors of this fungus.

Much has been studied about extracellular protease, which is directly implicated to the virulence of *C. albicans*, with mutant species with a deficient or reduced capacity of protease secretion being less virulent than the others. Phospholipase type enzymes are also associated to its virulence.

In the last few years, the importance of understanding the process of microbial adhesion has been understood, particularly at the bacterial level with studies on yeasts, in particular *C. albicans* isolated from vaginal secretions, with a lower density of knowledge. Evidence exists that *C. albicans* may produce more than one adhesive structure, with the adhesive function being primarily attributed to the mannoprotein in the adhesion reactions. Some *in vivo* and *in vitro* studies have been developed to quantify and characterise the adherency of *C. albicans* to cellular and inanimate surfaces. The bonding of *C. albicans* to mucosal surfaces has been described as an important step in the infectious process, particularly in the oral cavity and vaginal mucosa. It is already known that hydrophobic leveduriform cells are more virulent in rats than hydrophilic leveduriform cells. The expression of cellular surface hydrophobicity (CSH) for *C. albicans* has in this way been correlated with increased virulence, probably by stimulating the phenomenons of aderency, resistance to phagocytosis and germination.

In the first work, we intended to correlate the frequency of yeasts and their respective species in asymptomatic women and with different clinical manifestation of VVC; evaluate possible relationships between number of fungus colonies and symptoms of this pathology. All of the women who attended at the Teaching and Research in the Laboratory of Teaching and Research in Clinical Analysis of the State University of Maringa – Paraná - Brazil, within a period of five months, for routine examinations of vaginal secretion, independent of the presence or absence of symptoms of VVC, were included in this study. Of these, women with immunodeficiency or with an infection of the genital tract by another agent were excluded. Yeasts were identified by classical methods and patients subdivided into asymptomatic, VVC and RVVC groups. *C. albicans* was the yeast most frequently isolated (60.0%). Among the non-*C. albicans* yeasts, 61.5% were isolated of the asymptomatic women, 38.7% from patients with VVC and 11.1% of those from patients with RVVC. *C. albicans* was associated with symptoms of VVC and while, the presence of non-*C. albicans* yeasts with asymptomatic women. However, there was no association between the number of fungal colonies and symptoms.

In the second work, the identification of twenty *C. albicans* strains were confirmed molecularly using the sequence of rDNA analysis. Yeasts were incubated in RPMI + fetal

calf serum to analyze germ tubes every two hours, up to 10 hours. *In vitro* sensitivity to fluconazole, itraconazole, ketoconazole, amphotericin B and nystatin was analyzed according to NCCLS-M27-A microdilution assay. Yeast isolated from symptomatic women produced significantly more germ tubes than asymptomatic women ($p<0.05$). However, no significant difference between yeasts from VVC and RVVC occurred ($p>0.05$). Variation between MIC (minimal inhibitory concentration)$_{50}$ and MIC$_{90}$ of tested antifungal agents was slight among isolated yeasts, while no resistant yeasts were detected. Nevertheless, VVC yeasts were more DDS (reduced dose-dependent susceptibility) for nystatin and RVVC were more DDS for ketoconazole. Results suggest that colonization by yeast in the vagina and lack of symptoms may be partially explained by the yeast's sparse capacity to form germ tubes.

The objective of the third work was to evaluate if there is a correlation among the proteinase activity and other virulence factors (biofilm formation, hydrofobicity, hypha formation and cell adhesion) in different *C. albicans* strains isolated from vaginal environment of patients in the three different clinical conditions (asymptomatic women, VVC and RVVC). The virulence factors were determined in standard conditions and compared with the results obtained in presence of the aspartate proteinase inhibitor, pepstatin A. Pepstatin A did not cause any significant effect in the hypha formation, biofilm production and hydrofobicity of isolates in the three different clinical conditions. However, pepstatin A reduced the adherence of *C. albicans* to human surfaces. This results suggest that the secreted fungal proteinases may have auxiliary roles as cellular adhesions.

TITLE:

Correlation of *Candida* species and symptoms among patients with vulvovaginal candidiasis in Maringa, Parana, Brazil

AUTHORS:

Márcia Edilaine Lopes Consolaro[1],Thâmara Aline Albertoni[1], Celina Shizue Yoshida[1], Josmar Mazucheli[2], Rosane Marina Peralta[3],Terezinha Inez Estivalet Svidzinski[1]

ADDRESS:

[1]Division of Medical Mycology, Department of Clinical Analysis – Teaching and Research in Clinical Analysis Laboratory; [2]Department of Statistical; [3]Department of Biochemistry - State University of Maringá, 5790, Maringá, Paraná, Brazil

SUMMARY:

Vulvovaginal candidiasis (VVC) is an infection caused by abnormal yeast growth in the mucosa of the female genital tract which is a commonly diagnosed in gynecology. The aim of this study was to correlate the frequency of yeasts and their respective species in asymptomatic women and with different clinical manifestation of VVC; evaluate possible relationships between number of fungus colonies and symptoms in this pathology. All patients who visited the laboratory within a period of five months, for routine examinations of vaginal secretion, independent of the presence or absence of symptoms of VVC were included in this study. Of these, women with immunodeficiency or with an infection of the genital tract by another agent were excluded. *Candida albicans* was the most frequently yeast isolated (60.0%). Among the non-*C. albicans* yeasts, 61.5% were isolated of the asymptomatic women, 38.7% from patients with VVC and 11.1% of those from patients with RVVC. *C. albicans* was associated with symptoms of VVC and while, the presence of non-*C. albicans* yeasts with asymptomatic women. However, there was no association between the number of fungal colonies and symptoms.

KEY WORDS:

Vulvovaginal candidiasis; non-*C. albicans* yeasts; asymptomatic; recurrence

INTRODUCTION:

Vulvovaginal candidiasis (VVC) is a disease caused by abnormal growth of yeasts in the mucosa of the female genital tract and it is frequently diagnosed in the daily practice of gynecology, with a significant increase during the last years [16]. Approximately 75% of adult women will experience at least one episode of VVC during their lifetime, among which approximately 40 to 50% will experience further episodes and 5% will develop the recurrent type (RVVC). On the other hand, studies show that 20 to 25% of healthy and totally asymptomatic women exhibit positive vaginal secretion cultures for yeasts [12].

According to Sobel [12], 80 to 90% of VVC cases are caused by *Candida albicans*, while 10 to 20% are due to other non-*C. albicans* yeasts (*Candida tropicalis; Candida glabrata; Candida krusei; Candida parapsilosis*). However, it seems that there is an increase in non- *C. albicans* yeast frequency in certain populations [10].

In this study we intend to correlate frequency of yeasts and their respective species in asymptomatic females and those with different clinical manifestation of VVC. The possible relationships between number of fungus colonies and the symptomatology of this pathology were investigated.

MATERIALS AND METHODS:

This study was carried out in the Laboratory of Teaching and Research in Clinical Analysis of the State University of Maringá – Maringá, Paraná – Brazil, during the period of April to August 2002. It was carried out on those females who visited the laboratory for examinations of vaginal secretion, independent of the presence or absence of symptoms of VVC. Of these, women with a previous history of immunodeficiency, including AIDS, or with an infection of the genital tract by another agent were excluded.

All patients answered a standard questionnaire on their symptoms of vulvovaginal candidiasis (vaginal discharge, vulvovaginal itching, vulvovaginal burning sensation, dysuria and dyspareunia) [4, 8]. The characteristic vaginal discharge was classified by the health agent when sample was taken, according to Odds *et al.* [8]. All women involved in the research signed a term of consent in which they declared to know that the collected material would be used for research, as mandatory by the Ethical Commission in Research Involving Humans of State University of Maringá (Reg. 007/2002; Report 013/2002; CI 032/2002).

Cervical and vaginal specimens for the culture were collected with the aid of a disposable vaginal speculum (Vagispec, Brazil) and immediately spread in Petri dishes containing the culture medium Sabouraud Dextrose Agar "SDA" (Difco, USA) supplemented with 50 mg/ml of chloramphenicol (Sigma, USA). The dishes were incubated at 37ºC, for 48-72 hours, then a pool of the grown colonies were subcultured in CHROMagar *Candida*® (Probac-France) to investigate the purity of the culture and the colour of the colonies. From this selective and differential medium the yeasts were identified according to classical methods [5, 6]. The numbers of yeasts colonies isolates were assigned according Odds [8].

Cervico-vaginal samples were also collected for smears in microscope slides, which were dyed by the Gram and Papanicolaou stains for the evaluation of the presence of the yeasts and other infectious agents. Specific microorganisms were identified by microbiological and serological methods.

Patients with positive yeast culture were distributed into three groups: asymptomatic women who presented none or only one symptom out the five mentioned above, VVC women who presented two or more symptoms in a single occurrence in the past of one year, RVVC women who presented two or more symptoms, in three or four occurrences during the year.

Statistical analysis was Mantel-Haenszel's χ^2 test [14]: Prism 3.00 (Graphpad Software, Inc) was used throughout the analysis. A p-value less than 0.05 was regarded as significant.

RESULTS:

One hundred and sixty-one patients with 14 to 66-year-old were evaluated. Positive fungus culture had a frequency of 21.7%, or 35 patients. *C. albicans* was isolated in 60.0% (21/35). The percentage of non- *C. albicans* yeasts may be subdivided into 25.7% of *C. glabata* (9/35); 5.7% *C. parapsilosis* (2/35); 5.7% *Sacharomyces cerevisiae* (2/35); and 2.9% *Trichosporon* sp (1/35). Table 1 shows the relative and absolute frequencies of these species in the three groups of patients analyzed. In addition, according to figure 1, the isolation of *C. albicans* was proportional to the presence of symptoms, and the inverse occurred with non-*C. albicans* yeasts.

According to table 1, Mantel-Haenszel's χ^2 test showed that there is a correlation between the isolated yeast and the presence of symptoms. *C. albicans* was more frequent among the symptomstic women, while non- *C. albicans* were more frequent in asymptomatic ones ($p = 0.03$). These analyses corroborate the trend revealed in table 1, or rather, isolation of *C. albicans* increases in proportion to severity of vulvovaginitis, whereas the opposite occurs in the case of non-*C. albicans* yeasts. Table 2 shows the colony count and their relation with the symptoms of VVC analyzed. Only 33 samples were used in this table and the statistical analysis did not show association between the number of colonies and the five symptoms evaluated in this study.

DISCUSSION:

Yeasts were isolated from the vaginal secretions of 21.7% of the studied women, this finding is in agreement with Bauters *et al.* [2], who isolated yeasts in 20.1%. Abu-Elteen [1] detected yeasts in 48.4% of fertile women and in 51.5% of those with infertility problems. In relation to the species, this research showed a predominance of *C. albicans*, accounting for 60.0% of the total of the yeasts isolated. Verghese *et al.* [15] found *C. albicans* in 40.5%, and Bauters *et al.* [2] in 68.3%.

Candida glabrata was the second most frequently isolated yeast (25.7%), coinciding with the results of other authors [7, 11]. This species is represented in 16.3% and 38.1% of the cases in the studies of Bauters *et al.* [2] and Verghese *et al.* [15], respectively. The isolation rate of *C. glabrata* in the asymptomatic patients was 53.8% (Table 1). These data are similar to those of Oriel *et al.* [9], who found that more than half of the women with *C. glabrata* were asymptomatic. It is not surprising that in asymptomatic women, the ratio of isolation of non-*C. albicans* yeasts was higher than for patients with vaginal symptoms. This is due to the fact that the typical clinical manifestations of infections by these yeasts are characterized by less intensive symptoms than those caused by *C. albicans* [13].

The genus and species of the yeasts isolated from vaginal secretions has been poorly associated to the symptoms of VVC. Dan *et al.* [3], found similar results to those findings in this study, despite that their study was carried out on selected patients with symptoms of VVC. There is a statistical tendency of a high frequency of non-*C. albicans* yeasts in asymptomatic women. On the other hand, *C. albicans* is associated with VVC and RVVC. This seems to be a general tendency, but further studies should be carried out in patients selected and non-selected for clinical aspects of VVC to confirm this tendency.

Table 1 shows that *Saccharomyces cerevisiae* was isolated in two VVC women (2/35). Dan *et al.* [3] reported the same yeast in a single case (1.7%).

Our study did not find association between colony count and the five symptoms valuated in this study (Table 2). This fact is in contrast with results by Odds *et al.* [8] who detected a relationship between number of colonies and vaginal discharge and itching.

This study showed that although the most isolated yeast from vaginal secretions is still *C. albicans*, this is only true for patients with symptoms. The isolation of non-*C. albicans* yeasts was not low (40.0%), but was more frequent in the asymptomatic women, representing 61.5% of the yeasts found in this group. These findings suggest that in the vagina, colonization by yeasts is associated with non-*C. albicans* yeasts and that the evolution to the symptoms of VVC or RVVC would depend, among other factors, on replacement by *C. albicans*. If this idea is correct, the routine practice of carrying out culture identifications of yeasts could contribute to clarifying the challenge of differentiating colonization's of infection by yeasts in vaginal secretions.

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Table 1 Yeasts species isolated from women, according to clinical categories.

Genus and species	Asymptomatic		VVC		RVVC		Total	
	No.	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
C. albicans	5	38,5	8	61,5	8	88,9	21	60,0
C. parapsilosis	1	7,7	0	0	1	11,1	2	5,7
C. glabrata	7	53,8	2	15,5	0	0	9	25,7
S. cerevisiae	0	0	2	15,5	0	0	2	5,7
Trichosporon sp	0	0	1	7,7	0	0	1	2,9
Total	13	100,0	13	100,0	9	100,0	35	100,0

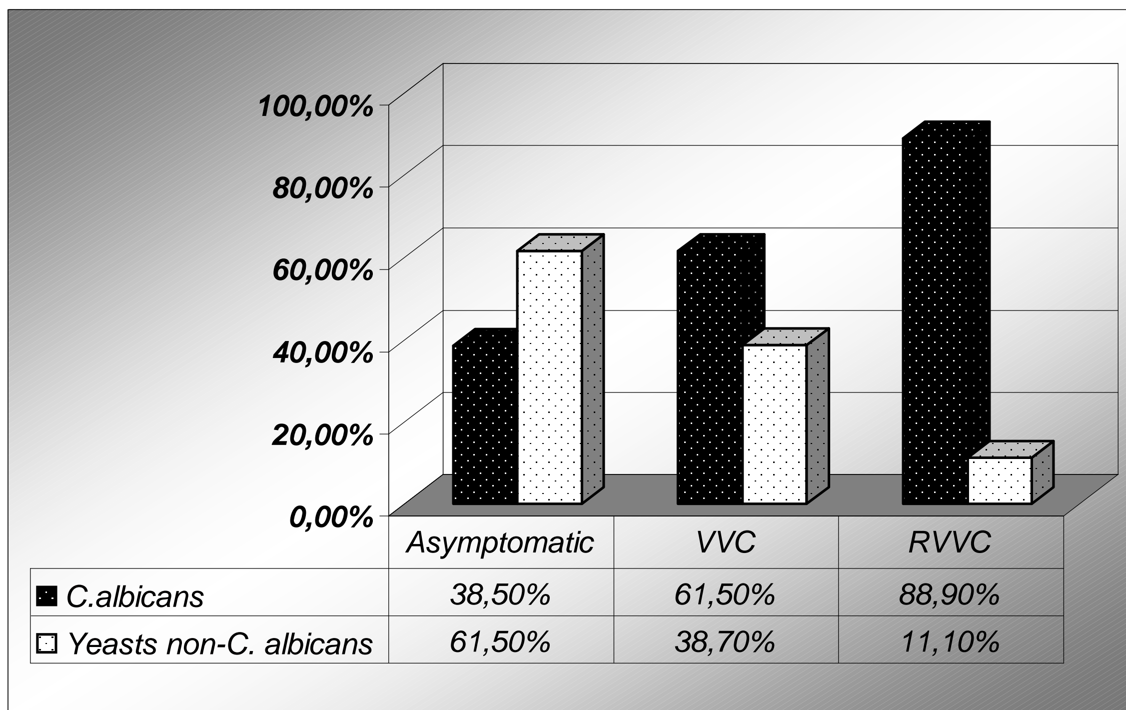

Figure 1 Distribuition of *C. albicans* and non-*C. albicans* yeasts.

Table 2 Colony count and their relationship to the symptoms under analysis.

Symptoms		**1 - 99 colonies (n=11)**	**≥ 100 colonies (n=22)**	**p. value**
Vaginal discharge	Yes	7	12	0.71934
	No	4	10	
Vulvovaginal itching	Yes	2	10	0.24922
	No	9	12	
Burning sensation	Yes	3	12	0.26593
	No	8	10	
Dysuria	Yes	4	4	0.39148
	No	7	18	
Dyspaurenia	Yes	3	9	0.70260
	No	8	13	

***Vulvovaginal candidiasis is associated with the production of germ tubes by* Candida albicans**

M. E. L. Consolaro [1], T. A. Albertoni [1], A. E. Svidzinski [1], R. M. Peralta [2] and T. I. E. Svidzinski [1]

[1]Division of Medical Mycology -Teaching and Research in Clinical Analysis Laboratory, State University of Maringá, Maringá, Paraná, Brazil; [2] Department of Biochemistry - State University of Maringá, Maringá, Paraná, Brazil

Abstract. Twenty *Candida albicans* strains isolated from women attended at the Teaching and Research in the Laboratory of Teaching and Research in Clinical Analysis of the State University of Maringa – Paraná - Brazil, have been analyzed. Yeasts were identified by classical methods and patients subdivided into asymptomatic, VVC (vulvovaginal candidiasis) and RVVC (recurrent vulvovaginal candidiasis) groups. Yeasts were incubated in RPMI + fetal calf serum to analyze germ tubes every two hours, up to 10 hours. *In vitro* sensitivity to fluconazole, itraconazole, ketoconazole, amphotericin B and nystatin was analyzed according to NCCLS-M27-A microdilution assay. Yeast isolated from symptomatic women produced significantly more germ tubes than asymptomatic women ($p<0.05$). However, no significant difference between yeasts from VVC and RVVC occurred ($p>0.05$). Variation between MIC_{50} and MIC_{90} of tested antifungal agents was slight among isolated yeasts, while no resistant yeasts were detected. Nevertheless, VVC yeasts were more DDS (reduced dose-dependent susceptibility) for nystatin and RVVC were more DDS for ketoconazole. Results suggest that colonization by yeast in the vagina and lack of symptoms may be partially explained by the yeast's sparse capacity to form germ tubes. On the other hand, RVVC was not associated with antimicrobial resistance. DDS high frequency for nystatin and ketoconazole indicates that identification, and susceptibility of antifungals tests are important to management of VVC.

Key words: antifungal agents, *Candida albicans*, germ tube, vulvovaginal candidiasis

Introduction

Vulvovaginal candidiasis (VVC) is an infection caused by abnormal growth of yeasts in the mucosa of the female genital tract. It is a frequent diagnosis in the daily practice of gynaecology, with a significant increase during the last year [1]. Around 75% of adult women will experience at least one episode of VVC during their lifetime, among which approximately 40 to 50% will experience further episodes and 5% will develop the recurrent type (RVVC), with at least three symptomatic episodes of vulvovaginitis in one year. On the other hand, studies show that 20 to 25% of healthy and totally asymptomatic women exhibit positive vaginal secretion cultures for yeasts [2].

Candida albicans is the specie most frequently isolated for VVC [1,2] and is considered a dimorphic fungus. *C. albicans* reproduces itself by germination, creating budding cells, called blastospores or blastoconidia, which are associated with normal colonisation. The conversion of yeasts to the filamentous stage (pathogenic) starts with production of germ tubes that result in the formation of hyphae and the formation of pseudohiphae occurs by polarized cell division when yeast cells growing by budding have elongated without detaching from adjacent cells [3].

This ability to morphological transformation has been suggested as an important factor for virulence [4]. Hyphae have a greater capacity to adhere to and penetrate human epithelial cells than blastoconidia [4,5]. Mutants incapable of producing hyphae lose the virulence of their parents [6]. According to Chaffin *et al.* [3] these transformations represent a response of the fungus to alterations in the environment and enable their adaptation to different biological niches, and consequent fungal dissemination in human cells. This reinforces the idea that the ability to form hyphae represents an important function in the pathogenesis of *C. albicans.* However, few studies have been conducted

with the aim of verifying if there is a relation between the capacity to form germ tubes and the virulence of *C. albicans* in VVC, especially considering the different clinical conditions of this disease.

In spite of the high prevalence of VVC and the extensive use of topical and oral antifungals, little is known about the antifungal resistance of *C. albicans* isolates from vaginal secretions of immunocompetent women. Reports associating the effects of the selective pressure of antifungals on the physiopathogenesis of VVC and the increase of resistant microorganisms are also rare [7,8].

The physiopathogenesis of VVC is a complex and multifactorial mechanism, which involves characteristics of the host and of the microorganism. Although some predisposing factors involving the host are well known, the recurring characteristics have not been associated with antifungal resistance, and it is not clear if they can be associated with the virulence of the microorganism. The objective of the present study was to evaluate the ability of *C. albicans*, isolated from different clinical conditions of VVC, to form germ tubes and also their response *in vitro* against azole and polyene antifungals.

Sampling, culturing procedures and identification of yeasts

This research was conducted with 20 samples of *C. albicans* isolates from vaginal secretions of women who attended the Laboratory of Teaching and Research in Clinical Analysis of the State University of Maringá – Paraná - Brazil in the period of April to August 2002. The patients were aged between 14 and 66 years and visited the laboratory for routine examinations. They did not have a previous history of illness associated with immunodeficiency, including AIDS, nor the presence of other infections in the genital tract. All the patients responded to a questionnaire according to Odds *et al.* [5] and Enweani *et al.* [9], about the presence of symptoms (vaginal discharge, vulvovaginal itching, vulvovaginal burning, dysuria, and dyspaurenia). The vaginal discharge was classified by a health professional when the sample was collected, conforming to recommendations by Odds *et al.* [5]. All the women participating in this research signed terms of consent in which they declared they were informed that the material collected would be used for research, as approved by the Ethics Committee for Research Involving Humans at the State University of Maringa – protocol number 013/2002).

Cervico-vaginal samples were collected with the aid of a disposable vaginal speculum (Vagispec – Brazil) and immediately spread with microbiological loop on Sabouraud Dextrose Agar "SDA" (Difco- USA) supplemented with 50 mg/ml of chloramphenicol (Sigma-USA). These petri dishes were incubated at 37ºC, for 48-72 hours, then a pool of the grown colonies were subcultivated in CHROMagar *Candida*® (Probac-France) to investigate the purity of the culture and the colour of the colonies. From this selective and differential medium the yeasts were identified according to classical methods [10,11]. In addition, all of the isolates were confirmed as *C. albicans* using the sequence of rDNA analysis according to Sugita *et al.* [12] (data not shown).

The patients with positive cultures for yeasts were distributed into three groups according to Consolaro *et al*. [13]: asymptomatics: women without symptoms or with only 1 of the 5 symptoms asked about (n=5); VVC: women that showed 2 or more of the symptoms, with only one episode of VVC in a period of 1 year (n=7); RVVC: women that showed 2 or more of the symptoms, with 3 or 4 episodes that occurred in a period of 1 year (n=8).

Formation of germ tubes

A liquid medium containing equal volumes of RPMI 1640 (Sigma-Germany) and fetal calf serum (Laborclin-Brazil) was prepared and sterilised by filtration. Fresh cultured colonies of the **C. albicans** ***were obtained after being spread in SDA (18-24 hours). For every isolate, a suspension in phosphate buffer saline (PBS) containing 5 X 10^7 cells/ml was prepared, and counted by microscopy using a Neubauer chamber. This suspension was added to the medium of RPMI + fetal calf serum in the proportion 1:2 and incubated at 37ºC without agitation. The germ tubes were defined according to the description by Hammer*** **et al.** ***[4]: as a cell bearing a rounded outgrowth with a length greater than or equal to the diameter of the parent cell, not constricted at the base. The percentage of cells that formed germ tubes was determined by microscopy counting after 2, 4, 6, 8, and 10 hours of incubation, and each experiment was conducted in triplicate [14].***

In vitro sensitivity test

C. albicans isolates were tested by NCCLS reference broth, microdilution method (National Committee for Clinical Laboratory Standards, document M 27-A, 1997) [15].

Drugs

The following five drugs were used: fluconazole (Pfizer Inc, New York, NY, USA), itraconazole and ketoconazole (Janssen Pharmaceutical, Titusville, NJ, USA), amphotericin B (Squibb Pharmaceutical, Princeton, NJ, USA) and nystatin (Sigma Pharma-St Louis, MO, USA). Stock solutions were prepared at 10 times the strength of final concentration and diluted with RPMI 1640 (Sigma, Steinheim, Germany), with L-glutamine, without bicarbonate, supplemented with 2% dextrose and buffered to pH 7.0 with 0.165 N- morpholinopropanesulfonic acid (MOPS) to obtain twice the final concentration.

Assay

Yeast isolates were grown on SDA for 48 hours, at 37ºC. The density suspension of cells in sterile distilled water was adjusted by spectrophotometer to a final transmission of 90%, at a wavelength of 530 nm. Suspension was made with a 1:50 dilution in sterile distilled water, followed by a 1:5 dilution in RPMI medium to obtain a two times final concentration. Testing was performed in sterile, flat-bottom 96 well microtiter plates. Volumes of 100 µl of twice the dilutions and 100 µl of twice the inoculum were dispensed into wells. Inoculum size averaged between 0.5 and 2.5 x 10^3 cells/ml. The plates were incubated at 37°C, up to 48 hours. Quality control isolates *Candida parapsilosis* ATCC 22019 were included in each experiment. Growth and sterility controls consisted of two wells from each row.

Endpoint MICs

The minimum inhibitory concentration (MIC) for azoles was defined as the first well with a significant growth reduction (approximately 50%) when compared to that of positive control. In the case of amphotericin B and nystatin, it was defined as the lowest concentration capable of inhibiting any visual growth. MIC_{50} and MIC_{90} were defined as MIC for 50% and 90% of isolates, respectively. Isolates with MICs between 16 and 32 μg/ml for fluconazole (FLU), 8 to 32 μg/ml for nystatin (NYST) and 0.25 to 0.5 μg/ml for itraconazole (ITR) and ketoconazole (KET) were reduced (dose-dependent) susceptibility (DDS). Isolates with MICs ≤ 8 μg/ml for FLU, ≤ 4 μg/ml for NYST, ≤ 0.125 μg/ml for ITRA and KET, and ≤ 1 μg/ml for AmB, were susceptible. MICs ≥ 64 μg/ml for FLU and NYST, ≥ 1 μg/ml for ITR and KET, and ≥ 2 μg/ml for AmB, were resistance endpoints for antifungal agents [15].

Statistical analysis

Statistical analysis was performed by Student's *t*-test. The Mann Whitney's and Kruskal-Wallis' non-parametric methods were used when appropriate. Prism 3.00 (Graphpad Software, Inc) was used throughout the analysis. A *P*-value less than 0.05 was regarded as significant.

Formation of germ tubes

Table 1 shows the mean of the triplicated experiments as the percentage of germ tube formation in the three different clinical conditions studied, up until 10 hours of incubation.

The statistical analysis showed that the mean percentage of germ tube formation differed according to the clinical conditions of the patients. Yeasts isolated from the VVC and RVVC groups produced more germ tubes than those from the asymptomatic women ($p<0.05$). However, there was no significant difference between the yeasts of the VVC and RVVC groups, nor in the formation of germ tubes in the different periods of incubation for the three clinical conditions.

Figure 1 shows the formation of germ tubes in each sample of *C. albicans* for the asymptomatic, VVC and RVVC clinical conditions respectively, in the different periods of incubation.

MIC

The MIC_{50} and MIC_{90} values of the five antifungal agents tested on the *C. albicans* isolates are shown in Table 2.

C. albicans DDS was observed for nystatin and for ketoconazole in 9 (45.0%) and 8 (40.0%) patients respectively. Two isolates of *C. albicans* (40.0%) were identified as DDS for nystatin and one (20.0%) for ketoconazole in asymptomatic patients. For isolates of

VVC patients, 2 (28.6%) were DDS for nystatin and 2 (28.6%) for ketoconazole and nystatin. Of the yeasts isolated from RVVC patients, 1 (12.5%) was DDS for nystatin, 2 (25.0%) DDS for nystatin and ketoconazole, and 3 (37.5%) DDS for ketoconazole (Table 3).

Discussion

In this study, the formation of germ tubes was low in the yeasts from the group of asymptomatic patients when compared with those of the VVC and RVVC groups (see Table 1 and Figure 1). This data reinforces the idea that the presence of yeasts in the vagina as microbiota is related to the smaller capacity of these yeasts to form germ tubes, and is in agreement with the following facts: a) it is already known that the ability of *C. albicans* to change its cellular morphology from blastoconidial form to hyphae contributes to the adherence of the fungus in the host epithelium [16,17,18] and to the penetration of their tissues [19]; b) yeasts may be present in the vagina, despite the absence of clinical symptoms, in around 30% of women, predominantly in the blastoconidial form and usually in small numbers [20,21]. The larger percentage of germ tube formation in the yeasts of patients with VVC reinforces the evidence that pseudohyphae and hyphae are frequently identified in symptomatic vulvovaginal candidiasis [20].

Figure 1 also shows that some yeasts, such as those of n.1 and n.3 (VVC), n.1 and 3 (RVVC), despite having originated from symptomatic conditions, show a lower percentage of germ tube formation than the other symptomatic samples, demonstrating that the formation of germ tubes must not be the only event involved in the clinical progression of vaginitis for *C. albicans*. This is in agreement with Sobel *et al.* [16], who suggested that the capacity of *C. albicans* to produce hyphae seems to be an important factor for virulence but is not essential in the pathogenesis of VVC.

The methodology used in the present study, based on Vilela *et al.* [14], had the purpose of evaluating the kinetics of germ tube production with 10 hours of incubation and evaluations every 2 hours. The statistical analysis did not show a significant increase in the percentage of germ tubes formed from 2 hours to 10 hours, different from Vilela *et al.* [14] who observed increase until 8 hours of incubation. Actually *C. albicans* morphogenesis is influenced by several environment factors like pH, serum, nitrogen level and temperature, and it is regulated by multiple signals and signalling pathways. We observed that the necessary period to induce germ tube is strongly variable, from 0.5 hours to 8 hours [4, 22, 23]. However, it seems more efficient in the beginning of the incubation. Although progress has been achieved in the recent years, the mechanisms governing these morphogenetic conversions are still not fully understood. It is possible that the vaginal isolates were induced to germinate efficiently and synchronously until 2 hours. Similarly Hube *et al.* [22] observed that in 120 minutes, 90% of the cells were transformed in hypha.

It seems that the tendency of a patient with VVC to develop RVVC is not associated with the resistance of the microorganism to antifungal agents and consequently to the inefficiency of treatment. In fact, the variation between MIC_{50} and MIC_{90} of the antifungal agents studied against the yeasts isolated from the three groups of patients was very small (Table 2). In spite of this, some observations are important and deserve to be discussed.

The yeasts of the VVC group were the ones that displayed the most DDS for nystatin, while those of the RVVC group mainly displayed DDS for ketoconazole. The isolates of asymptomatic women displayed the lowest frequency of DDS for nystatin as well as ketoconazole and a resultant greater sensitivity to the antifungals tested.

In this study, none of the yeasts displayed resistance to the antifungals, corresponding with the findings of Ribeiro *et al.* [24]. According to Sobel [25], the

majority of cases of VVC (90-95%) were caused by sensitive samples of *C. albicans*. Other studies failed to consider resistance to antifungal drugs as a mechanism responsible for RVVC caused by *C. albicans* [26]. These results emphasise that the impairment of local immunity mediated by cells must have a more important function in the pathogenesis of RVVC than the resistance to antifungal drugs [20,26,27,28].

In conclusion, this was one of the first studies to demonstrate the greater capacity of yeasts isolates of RVVC and VVC to form germ tubes than those of asymptomatic patients. It was also observed that recurrences were not associated with antimicrobial resistance and that the majority of the symptomatic samples showed themselves DDS for nystatin and/or ketoconazole. This leads us to suggest that laboratory tests including the identification of the fungus species and the execution of susceptibility tests to antifungals would have increasing importance in the management of VVC.

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Table 1. Percentage of *C. albicans* cells forming germ tubes, up to 2-10 h incubation, in three different clinical conditions.

Clinical conditions	Time (h)				
	2	4	6	8	10
Asymptomatic	3.4 ± 0.8	2.2 ± 0.5	3.7 ± 0.6	3.3 ± 0.7	3.5 ± 0.6
VVC	9.7 ± 2.1	10.9 ± 2.2	16.9 ± 2.4	10.9 ± 2.4	11.4 ± 2.6
RVVC	15.7 ± 3.7	15.3 ± 1.8	15.3 ± 3.2	14.5 ± 1.8	15.7 ± 2.4
*Mean (SEM)					

***means in triplicate; standard error mean**

Table 2. Susceptibility profile of *C. albicans* isolates from vaginal secretion, against five antifungal agents.

Drug	All isolates*		Asymptomatic		VVC		RVVC	
(μg/ml)	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
Fluconazole	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Itraconazole	0.03	0.06	0.06	0.06	0.03	0.03	0.03	0.03
Ketoconazole	0.125	0.25	0.125	0.125	0.125	0.25	0.25	0.25
Amphotericin B	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Nystatin	4	8	4	8	4	4	4	8

*** Arithmetical means of the all isolates**

Table 3. MICs of five antifungal drugs against 20 *C. albicans* isolates

Number the yeasts isolates	Fluconazole MIC	Itraconazole MIC	Ketoconazole MIC	Amphotericin B MIC	Nistatin MIC
Asymptomatic					
3	0,5	<0,25	<0,25	0,5	8[a]
6	0,25	<0,25	<0,25	0,5	8[a]
8	0,25	<0,25	<0,25	0,25	4
10	1	<0,25	0,5[a]	0,25	4
18	0,5	<0,25	<0,25	0,5	4
CVV					
2	0,5	<0,25	0,25[a]	0,5	8[a]
4	0,5	<0,25	<0,25	0,5	8[a]
7	0,25	<0,25	<0,25	1	8[a]
9	0,5	<0,25	<0,25	0,25	4
15	0,5	<0,25	0,25[a]	0,5	8[a]
17	0,25	<0,25	<0,25	0,5	4
19	0,25	<0,25	<0,25	0,25	4
CVVR					
1	0,5	<0,25	<0,25	0,5	8[a]
5	0,5	<0,25	0,25[a]	0,5	8[a]
11	0,5	<0,25	<0,25	0,25	4
12	0,5	<0,25	0,25[a]	0,5	4
13	0,5	<0,25	<0,25	0,5	4
14	0,5	<0,25	0,25[a]	0,5	4
16	0,5	<0,25	0,25[a]	0,5	8[a]
20	0,25	<0,25	0,25[a]	0,5	4

[a]DDS isolate

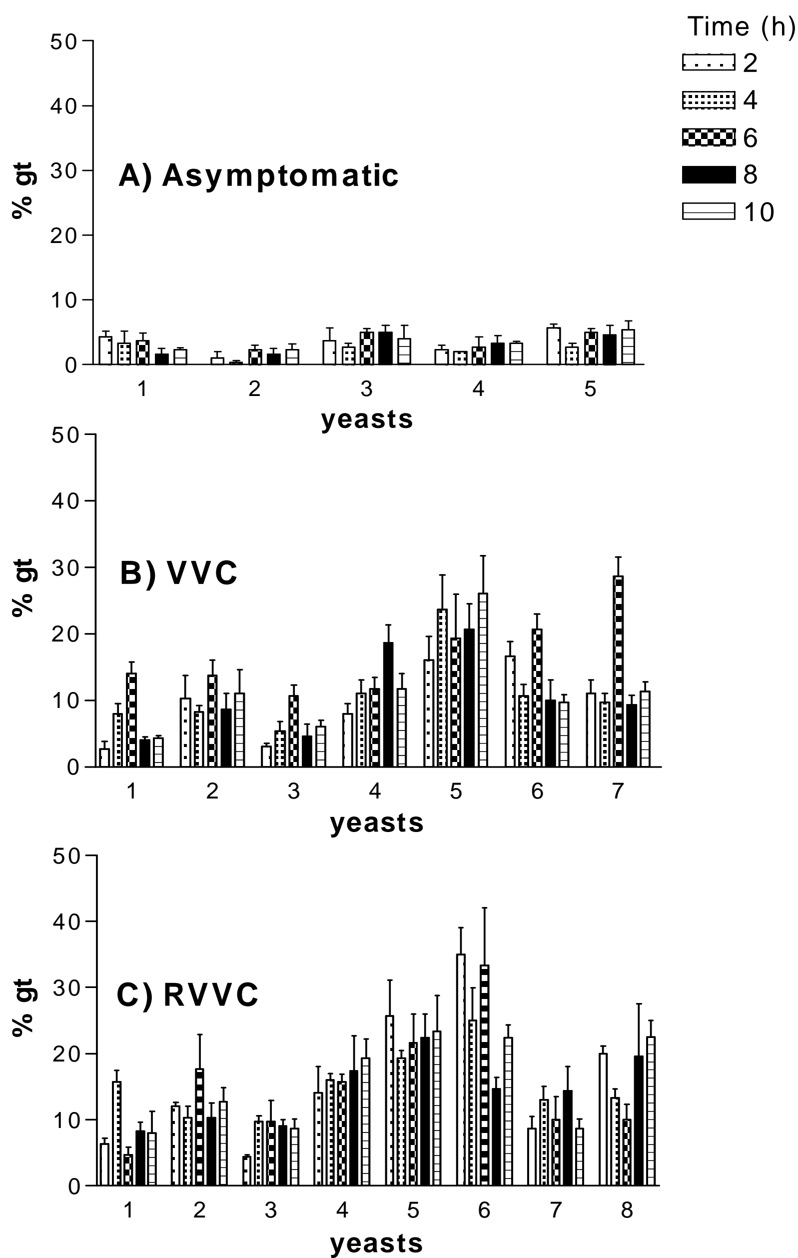

Figure 1. The percentage of yeasts that produced germ tubes (% gt) after 2, 4, 6, 8, and 10 hours of incubation, in 20 samples of *C. albicans* according to the clinical condition of the patients: A) Asymptomatic group (isolates 1-5); B) VVC group (isolates 1-7); C) RVVC

group (isolates 1-8). The vertical bars represent the standard deviation obtained between the results of every isolate in every incubation time.

Effect of pepstatin A in the virulence factors of *Candida albicans* strains isolated from vaginal environment of patients in three different clinical conditions

[1]Consolaro, M.E.L., [1]Svidzinski, T.I.E., [2]Peralta, R.M.

[1]Departamento de Análises Clínicas, [2]Departamento de Bioquímica
Universidade Estadual de Maringá, 87020-900, Maringá, PR, Brazil.

ABSTRACT

The aspartate proteinase inhibitor pepstatin A has been used to study a possible correlation among proteinase activity and other virulence attributes of *C. albicans* strains isolated from vaginal environment of patients in three different clinical conditions: asympthomatic, vulvovaginal candidiasis (VVC) and recurrent vulvovaginal candidiasis (RVVC). Pepstatin A did not cause any significant effect in the hypha formation, biofilm production and hydrofobicity of isolates in the three different clinical conditions. However, pepstatin A reduced the adherence of *C. albicans* to human surfaces. This result suggests that the secreted aspartate proteinases (Saps) of this fungal pathogen may have auxiliary roles as cellular adhesions.

Key words: adherence, aspartate proteinase, *Candida albicans*, virulence factors, pepstatin A.

INTRODUCTION

Candida albicans is a dimorphic yeast which exists as a comensal organism in the oral cavity, gastrointestinal tract, female genital tract and occasionally on the skin. Under certain circumstances, usually associated with a compromised host immune system, *C. albicans* and related species can become pathogenic, causing oral, vaginal and/or sistemic candidiasis (Odds, 1994).

Putative virulence factors of *C. albicans* include cell wall adhesions, phenotypic switching, hypha formation, thigmotropism and the secretion of hydrolytic enzymes (Cutler, 1991, Odds, 1994, Gow, 1996, Lo et al., 1997).

Since the first description of production of proteinase by *C. albicans* (Staibe, 1969), the enzyme has been a major focus for studies of the virulence of *Candida* species. These secreted aspartate proteinases are characterized by an acidic optimum pH (Ruchel et al., 1982), a wide substrate specificity and sensitivity to the inhibitor pepstatin A, a hexapeptide from *Streptomyces* (Davies, 1990).

C. albicans proteinase activity is associated with a family of 10 aspartic proteinase (Saps) isoenzymes, with molecular weights between 35 and 50 kDa, encoded by the genes SAP1-10 (Monod et al., 1994, 1998, Felk et al., 2000). All Saps are inhibited by pepstatin A. The Saps of *C. albicans* are essential for growth when protein is the sole nitrogen source (Hube et al. 1994). In addition, Sap production appear to be associated with other putative virulence factors of *C. albicans* including hyphal formation, adhesion and phenotypic switching, which highlights the complexity of Sap involvement in *C. albicans* pathogenicity (Naglik et al., 2003).

Recent studies using proteinase inhibitors and Sap-disrupted or Sap-overexpressing mutants have compellingly demonstrated that Saps contribute to the virulence of *C. albicans*. Inhibition of Saps with the aspartic proteinase inhibitor pepstatin A prevent the initial penetration of *C. albicans* through mucosal surfaces, but not the dissemination of the fungus once the cells had already reached the blood vessels (Fallon et al., 1997). Moreover,

pepstatin A prevent *C. albicans* invading and causing tissue damage in oral, vaginal and skin experimental infection models (Schaller et al., 1999).

Experimental studies have suggested that elevated Sap production is an important aspect of *C. albicans* interaction with host tissue and strain virulence (De Bernardis et al., 2001). It has been demonstrated that *C. albicans* isolated from patients with vulvovaginal candidiasis are significantly more proteolytic than those from healthy carriers (Cassone et al., 1987).

Candida albicans is the most important species of yeast in the female genital tract. About 20 to 25 % of healthy and completely asymptomatic women present positive vaginal secretion cultures for this fungus. Around 75% of adult women have at least one episode of vulvovaginal candidiasis (VVC) during their life, with prevalence of *C. albicans* in 70-90 % (Chong et al., 2003). Some of these patients will experience episodes again and 5 % will suffer recurrent VVC (RVVC) (Marrazzo, 2003). VVC is caused by the transformation of the yeasts from coloniser status (asymptomatic) to infectious agents (symptomatic vaginitis) (Ziarrusta, 2002). However, the factors responsible for this transformation and the mechanisms that result in the pathological effects of *C. albicans* are poorly understood (Fidel et al., 2004).

The present study investigated a possible correlation among protease activity and other virulence factors (hypha formation, hydrofobicity, biofilm formation and cell adhesion) in different *C. albicans* strains isolated from vaginal environment of patients in three different clinical conditions in the Laboratory of Teaching and Research in Clinical Analysis of the State University of Maringá, PR, Brazil.

MATERIALS AND METHODS

Sampling, culturing procedures and identification of yeasts

This research was conducted with 19 samples of *C. albicans* isolates from vaginal secretions of women who attended the Laboratory of Teaching and Research in Clinical Analysis of the State University of Maringá, PR, Brazil in the period of April to August 2002. The patients were aged between 14 and 66 years and visited the laboratory for routine examinations. They did not have a previous history of illness associated with immunodeficiency, including AIDS, nor the presence of other infections in the genital tract. All the patients responded to a questionnaire according to Odds et al., (1988) and Enweani et al., (2001) about the presence of symptoms (vaginal discharge, vulvovaginal itching, vulvovaginal burning, dysuria and dyspaurenia). The vaginal discharge was classified by a health professional when the sample was collected, conforming to recommendations by Odds et al., 1988. All the women participating in this research signed terms of consent in which they declared they were informed that the material collected would be used for research, as approved by the Ethics Committee for Research Involving Humans at the State University of Maringa – protocol number 013/2002).

Cervico-vaginal samples were collected with the aid of a disposable vaginal speculum (Vagispec, Brazil) and immediately spread with microbiological loop on Sabouraud Dextrose Agar "SDA" (Difco, USA) supplemented with 50 mg/ml of chloramphenicol (Sigma, USA). These Petri dishes were incubated at 37 ºC, for 48-72 hours, then a pool of the grown colonies were subcultivated in CHROMagar *Candida*® (Probac, France) to investigate the purity of the culture and the colour of the colonies. From this selective and differential medium the yeasts were identified according to classical methods (Laroni, 1995, Luo et al., 2001). All of the isolates were confirmed as *C. albicans* using the sequence of rDNA analysis according to Sugita et al., (2002).

The patients with positive cultures for yeasts were distributed into three groups (Consolaro et al., 2004): asymptomatics: women without symptoms or with only 1 of the 5 symptoms asked about (n=4); VVC: women that showed 2 or more of the symptoms, with

only one episode of VVC in a period of 1 year (n=7); RVVC: women that showed 2 or more of the symptoms, with 3 or 4 episodes that occurred in a period of 1 year (n=8).

Production of proteinases and inhibition of proteinase activity by pepstatin A.

The production of extracellular proteinase activity was determined by the bovine serum albumin plate method (Ruchel et al., 1982), with incubation for 96 hours at 37 °C. The presence of enzyme was detected by the formation of a clear zone of hydrolysis around the colony. The enzymatic activity (Pz) was measured by ratio between the diameter of the colony and the diameter of the colony plus the zone of precipitation. The tests were conducted in duplicate on 3 different occasions. The isolate *C. albicans* 12A, donated by the Institute of Biomedical Sciences – University of São Paulo, was used as a positive control. The *C. albicans* strains isolates proteinase activities were determined also in presence of Sap inhibitor, pepstatin A. Pepstatin was prepared in methanol and added in the medium to give the final concentrations of 0.5, 0.75 and 1.0 μM. A possible effect of methanol in the proteinase activities was checked by the addition of equal volumes methanol in the cultures.

Formation of germ tubes

A liquid medium containing equal volumes of RPMI 1640 (Sigma-Germany) and fetal calf serum (Laborclin-Brazil) was prepared and sterilised by filtration. Fresh cultured colonies of the C. albicans were obtained after being spread in SDA (18-24 hours). For every isolate, a suspension in phosphate buffer saline (PBS) containing 5 X 10^7 cells/ml was prepared, and counted by microscopy using a Neubauer chamber. This suspension was added to the medium of RPMI plus fetal calf serum in the proportion 1:2 and incubated at 37ºC without agitation. The germ tubes were defined according to the description by Hammer et al., (2000) as a cell bearing a rounded outgrowth with a length greater than or equal to the diameter of the parent cell, not constricted at the base. The percentage of cells that formed germ tubes was determined by microscopy counting after 3 hours of incubation, and each experiment was conducted in triplicate (Vilela et al., 2002).

Determination of cell surface hydrofobicity (CSH)

C. albicans cells cultivated on SDA plates for 24 h at 25°C were transferred to tubes containing 2 ml of Sabouraud Dextrose Broth (SDB), and maintained for 24 hours at 25° C. Cells were collected by centrifugation (3,000 rmp for 5 minutes), washed three times in phosphate-urea-magnesium buffer (PUM) and standardized to 10^{8} yeast/ml. Three ml of suspension cell were transferred to glass tubes (13 x 100 mm) and mixed with 0.4 ml of n-hexadecane. The mixtures were maintained at 30° C for 10 minutes. The tubes were agitated twice for 30 seconds with 5 seconds of interval. The absorbance of two phases were determined at 520 nm (Doyle and Rosemberg, 1990). The hydrophobicity was expressed as the percentage reduction in optical density of the test suspension compared with control. Thus, the greater the change in absorbance, the greater the shift in yeast from the bulk medium to the interface, ie, the more hydrophobic the yeast strain. Suspensions without n-hexadecane were used as the negative controls. Each assay procedure was performed on three separate occasions with quadriplicate determinations each time (Sweet et al., 1987).

Determination of biofilm production by spectrophotometric method.

Sabouraud dextrose broth (SDB) was prepared from powered Sabouraud broth (Merck) according to the manufacturer's instructions, except for supplementation with 60 g of glucose per liter (final glucose concentration, 80 g/liter or 8%). Organisms were grown for 24 h at 35 °C on Sabouraud dextrose agar plates (BBL), and saline-washed suspensions of each strain of *Candida albicans* were prepared. The turbidity of each suspension was adjusted to the equivalent of 3 X 10^{7} CFU/ml using a Neubauer haemocytomer chamber. Next, each well of microtitration plates was inoculated with aliquots of 20 μl of yeast cell suspension and 180 μl of SDB. Plates were then incubated at 35 °C for 24 hours without agitation. The culture medium was removed by suction. 200 μl of distilled water were added and the absorbance of mixture determined at 450 nm. The %T value for each test sample was subtracted from the %T value for the reagent blank to obtain a measure of the

amount of light blocked when passing through the wells (%T $_{bloc}$). Each isolate was tested at least twice (Shin et al., 2002).

Determination the adherence of human vaginal mucosa epithelial (VME) cells

Adhesion of *C. albicans* to human VME cells was studied by a modification of methods described previously (Williams et al., 1999, Moragues et al., 2003). Human VME cells were collected from the vagina of 1 healthy adult volunteer by gently rubbing sterile swabs over the surface of the mucosa. The experiment used cells from the only one volunteer. The swabs were subsequently agitated in 2 ml of phosphate buffered saline (PBS; 0.01 M, pH 7.2) to recover the epithelial cells. The cell suspension was centrifuged (1000 g for 15 minutes) and the cell pellet washed three times in PBS. The VME cells were examined by light microscopy to ensure was no colonization by yeasts. The human VME cells were counted using an improved Neubauer haemocytometer chamber and ressuspended in PBS to 10^5 cells/ml. All *C. albicans* cells was cultured in yeast nitrogen base médium (Difco, USA) supplemented with 50 mM galactose at 37°C for 24 hours, and washed three times in PBS. Yeasts cells were identified as described above and ressuspended in PBS at a concentration of 10^7 cells/ml.

Equal volumes (0.5 ml) of human VME cells (10^5 cells/ml) and *Candida* (10^7 cells/ml) were mixed and incubated on a shaker (30 rpm) at 37°C for one hour. The epithelial cells this mix were then washed twice with PBS by centrifugation at 1,000 X g for 10 minutes. Air-dried smears of the sedimented human VME cells were made on glass slides and were initially stained with crystal violet and then Papanicolaou stain. Adhesion was assayed microscopically (x 125 magnification) by counting the total number of adherent yeasts to 100 human VME cells. The procedure was repeated in triplicate on four separate occasions by a single observer.

Determination of effect of pesptatin A on germ tube formation, biofilm formation, adherence of human vaginal mucosa epithelial and hydrofobicity.

To study the effect of pespstatin A on germ tube formation, biofilm formation, adherence of human vaginal mucosa epithelial and hydrofobicity, experiments were conducted using 1.0 µM pepstatin (final concentration). Each assay was conducted in parallel with two controls: standard conditions and with the addition of equal volume of methanol used to prepare the pepstatin solution.

Statistical analysis

The obtained data were submitted to ANOVA and compared by Tukey´s test ($p < 0.05$) using the statistical pack program GrapPad Prism® (Graph Pad Software, San Diego, USA).

RESULTS

The effect of pespstatin on proteinase activity was shown in FIG. 1. To all *C. albicans* strains, the addition of pepstatin inhibited the proteinase activity. Apparently, the *C. albicans* strains proteinase activities isolated from asymptomatic women were more resistant to inhibition by pepstatin than those isolated from patients with VVC and RVVC. Compared to the control, the addition of methanol did not cause any significant alteration in the proteinase activity ($p > 0.05$). A final concentration of pepstatin of 1.0 μM was chose for further experiments.

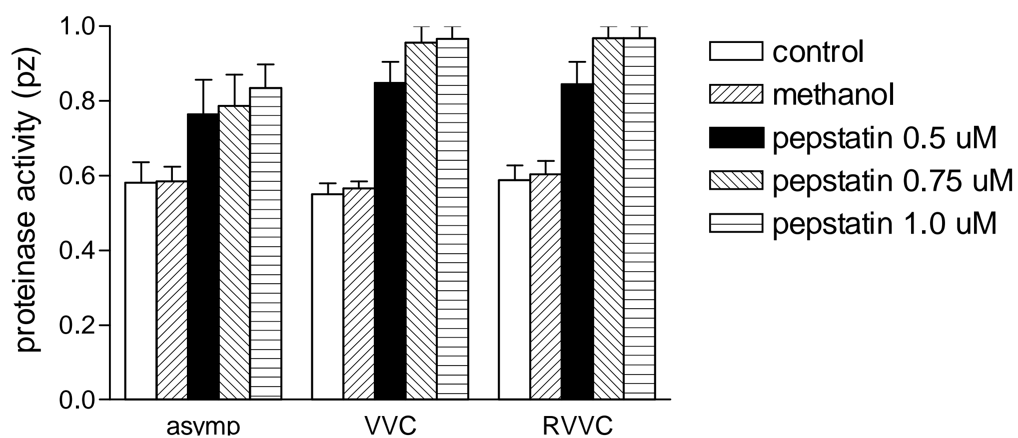

Figure 1. Effect of pepstatin A on proteinase activity from *C. albicans* isolated from from vaginal environment of the patients in three different clinical conditions: asympthomatic women (asymp), VVC, and RVVC.

The effect of pepstatin on germ tube formation, biofilm formation, hydrofobicity and on adhesion of *C. albicans* to human vaginal mucosa ephitelial cells is presented in FIG 2. The statistical analysis showed that the mean percentage of germ tube formation differed according to the clinical conditions of the patients (FIG 2A). Yeasts isolated from the VVC and RVVC groups produced more germ tubes than those from the asymptomatic women ($p<0.05$). However, there was no significant difference between the formation of germ tubes in the absence or presence of pepstatin ($p>0.05$).

In relation to hydrofobicity, no significant difference was observed among strains isolated from different clinical conditions. The addition of pepstatin did not change the hydrofobicity of cells (FIG 2B).

The effect of pepstatin in biofilm formation is showed in FIG 2C. A tendency of higher biofilm formation was evidenced in the isolates from VVC and RVVC. However, the biofim formation was not significantly altered by pepstatin ($p>0.05$).

A significant reduction in adherence was achieved by pepstatin among isolates from three clinical conditions (FIG 2D). The presence of pepstatin yielded a reduction in adherence of 53.1, 48.7 and 59.9%, respectively to isolated from asymptomatic patients, VVC and RVVC.

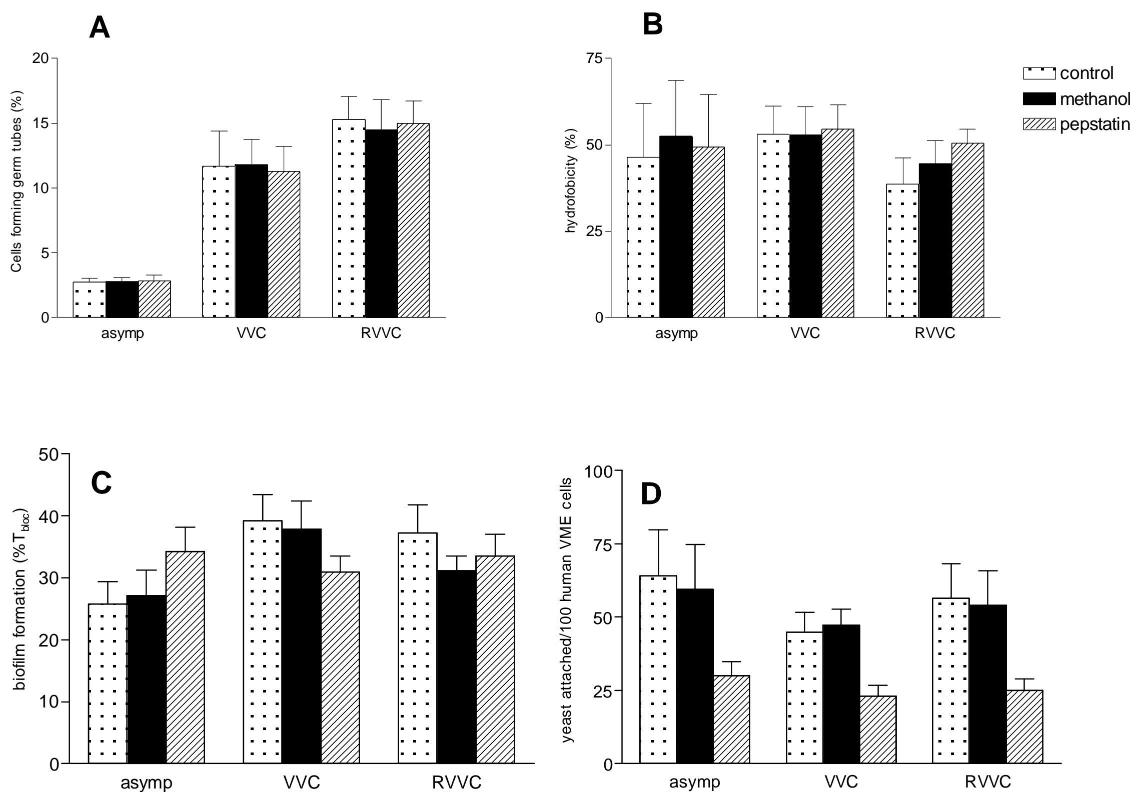

Figure 2. Effect of pepstatin A in the formation of germ tubes (A), hydrofobicity (B), biofilm formation (C) and on adherence in of human vaginal mucosa epithelial (VME) cells (D) of *C. albicans* strains isolated from vaginal environment of the patients in three different clinical conditions.

DISCUSSION

Extracellular proteinases of saprophytic microorganisms are primarily secreted to breakdown or decompose complex materials into nutrients available to the cells or to compete with other environmental bacteria, parasites or fungi (Cunnigham and Agard, 2004). However, pathogenic microorganisms appear to have adapted this biochemical property to fulfil a number of specialized functions during the infective process in humans, animals and plants. Direct virulence functions include hydrolysing proteins of host cell membranes to facilitate adhesion and tissue invasion, or damaging cells and molecules of the host defence system to avoid or resist anti-microbial attack (Klemba and Goldberg, 2002, Monod and Borg-von Zepelin, 2002, Peschel, 2002).

Candida albicans virulence and pathogenicity is complex and it is belivied to be correlated to different virulence factors. The production of a secreted family of proteinases indicates that these virulence enzymes have evolved and adapted to the animal/human host to achieve maximum benefits for *C. albicans* at multiple infection sites. It was postulated that Sap production appears to be a highly regulated process, which probably reflects their complex transcriptional co-regulation with other virulence attributes of *C. albicans* and the multiple functions the proteinases possess *in vivo,* including biofilm formation, adhesion, dimorphism and phenotypic switching (Naglik et al., 2004).

In this work *C. albicans* strains isolated from vaginal environmental were used to study a possible correlation among proteinase activity with other *C. albicans* properties possibly involved in its virulence. The inhibition of proteinase activity with pepstatin resulted in a significant reduction of adherence in VME cells, however, no alteration in biofilm formation, hydrofobicity and germe tube formation could be demonstrated.

Although both yeast and hyphal cells of *C. albicans* are found in infected host tissues, it is widely believed that hyphal formation is important for the invasive properties (Felk et al., 2002). This fact was confirmed in this work, where a higher germ tube formation was found in *C. albicans* isolated from vaginal environment in women with VVC and RVVC than in asymptomatic women (FIG 2A). However, no correlation between proteinase activity and germ tube formation could be established. In a study using

C. albicans strains isolated from the oral cavity, it was found a correlation between high phospholipase activity and germ tube formation (Vidotto et al., 1999).

In relation to hydrofobicity, all isolates of *C. albicans* exhibited low levels (<50%) of CSH, without any significant difference of CSH among strains isolated from different clinical conditions. The addition of pepstatin did not change the hydrofobicity of cells (FIG 2B). The expression of CSH by *C. albicans* has been correlated with increased virulence compared to cell surface hydrofobicity (Antley and Hazen, 1988, Glee et al., 1995). How CSH specifically influences virulence is unknown, but hydrophobic cells compared to hydrophilic cells are more adherent to host and inanimate substrata (including mucin, epithelial cells, endothelial cells, and extracellular matrix proteins), more resistant to phagocytosis and more germination competent (Antley and Hazen, 1988, Glee et al., 1995, Masuoka et al., 1999). Our data showed no significant difference in expression of CSH in the yeasts isolated from patients in different clinical conditions and pepstatin did not cause any alteration in CSH ($p>0.05$), what suggest that the presence of active proteinase was not directly involved in hydrofobicity of cells.

Our results showed no significant difference in the biofilm formation by yeasts isolated from different clinical conditions and the fact of no significant alteration was caused by the presence of pepstatin (FIG 2C), the proteinase activity is not directly involved in the biofilm formation. Although bacterial biofilms and their role in disease have been investigated in detail, much less is known about fungal biofilms (Douglas, 2003, Ramage et al., 2002). The structure of a mature *C. albicans* biofilm produced *in vitro* after 48 h incubation consist of a dense network of yeasts, hyphae, and pseudohyphae (Jabra-Rizk et al., 2004). The two consequences of biofilm growth with profound clinical implications are the markedly enhanced resistance to antimicrobial agents and protection from host defenses, the main reasons why biofilm-associated infections are frequently refractory to conventional therapy (Chandra et al., 2001).

Our data permitted to conclude there is a correlation between proteinase activity and adherence of *C. albicans* strains (FIG 2D). This results coincide with those obtained by other investigators where the use of Sap inhibitor pepstatin A caused a reduction in the adherence of yeast cells in various cell types (Ray and Payne, 1988, El-Magharabi et al., 1990, Ollert et al., 1993). The use of Sap null mutant strains of *C. albicans* also showed a

correlation between proteinase activity and adherence (Watts et al., 1998). The fact of pepstatin A reduced the number of yeasts adhering to VME cells, suggest that enzymatic activity of Saps is important in the formation of adhesive bonds. However, it is important to remember that adhesion of *C. albicans* is know to be a complex, multifactorial property dependent on a multiplicity of recognition systems and surface receptors such as surface integrin-like molecules (Hostetter, 1994), associated to general biophysical properties of the cell such as cell surface hydrofobicity (Hazen et al., 1991) and electrostatic charge (Jones and O´Shea, 1994, Klotz, 1994) are involved in the capability of adherence, and such properties were not focused in this work.

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