RENATA LUMI KANOSHIKI

ANÁLISE DOS FATORES DE VIRULÊNCIA E PROTEOMA DE *Candida tropicalis* SENSÍVEL E RESISTENTE AO FLUCONAZOL

Londrina
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Dissertação apresentada ao Programa de Pós-graduação em Microbiologia, da Universidade Estadual de Londrina, como requisito parcial para obtenção do título de Mestre em Microbiologia.

Orientação: Prof. Dr. Benedito Prado Dias Filho.
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Londrina, 15 de março de 2010.

DEDICO

A meus pais, irmãs, e amigos,
pelo carinho, compreensão e incentivo
em todos os momentos da minha vida.

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“Quanto mais os telescópios forem aperfeiçoados,
mais estrelas surgirão”

Gustave Flaubert

KANOSHIKI, Renata Lumi. **Análise dos fatores de Virulência e proteoma de candida tropicalis sensível e resistente ao Fluconazol.** 2010. 99 f. Dissertação (Mestrado em Microbiologia) – Universidade Estadual de Londrina, Londrina, 2010.

RESUMO

Espécies de *Candida* são agentes oportunistas mais frequentes em infecções fúngicas. Embora *Candida albicans* seja a principal e a mais estudada espécie do gênero *Candida*, espécies não-*albicans* vêm emergindo como importantes agentes de candidíases. Entre as espécies não-*albicans* relata-se *C. tropicalis* como importante agente etiológico de candidemia em pacientes com câncer, principalmente aqueles com leucemia. Em países da América Latina, esta espécie é bastante frequente, mesmo entre pacientes não portadores de câncer, sendo a segunda principal causa de candidemia no Brasil. A alta mortalidade de pacientes infectados por *C. tropicalis* e o surgimento de isolados resistentes a antifúngicos corroboram sua importância clínica. Na literatura relata-se que a exposição de *Candida* spp. a antifúngicos influencia na expressão de fatores de virulência, dos quais se destacam: aderência a tecidos do hospedeiro, morfogênese, formação de biofilme e secreção de enzimas. Haja vista sua importância à patogênese, este trabalho teve como objetivo estudar a influência do fluconazol sobre os fatores de virulência e o proteoma de isolados de *C. tropicalis*. Para tanto, fatores de virulência de *C. tropicalis* em amostras isoladas de cavidade bucal, ponta de cateter e hemocultura foram analisados. Nesta última, os fatores de virulência foram avaliados também após breve exposição ao fluconazol (1 h) e após desenvolvimento de resistência *in vitro*. Ademais, foi realizada análise comparativa do proteoma dos isolados de *C. tropicalis* resistentes e sensíveis ao fluconazol, e comparação de células planctônicas e sésseis. Para a identificação das espécies foram utilizados testes fenotípicos padrões e semi-nested PCR. A concentração inibitória mínima (CIM) para o fluconazol foi determinada por microdiluição em caldo. A atividade de protease foi avaliada em placa utilizando-se meio mínimo contendo caseína de leite 0,6%, enquanto a atividade de fosfolipase e hemolisina foram investigadas em Sabouraud Dextrose Agar com gema de ovo a 4% e sangue de carneiro a 5%, respectivamente. O ensaio de hidrofobicidade da superfície celular (CSH) foi realizado pela técnica de Anil *et al*. (2001). Para a formação de biofilme foram utilizados dois diferentes métodos: avaliação da porcentagem de transmitância bloqueada (%Tbloc) e o ensaio de redução do XTT. A adesão em poliestireno e células de mamíferos também foi analisada. Todas as amostras não apresentaram atividade de fosfolipase e foram capazes de hemolisar parcialmente sangue de carneiro (similar a hemólise do tipo alfa de *Streptococcus* spp.). Diferenças relacionadas à origem das amostras ($p< 0,05$) foram encontradas quanto à produção de biofilme, e hidrofobicidade celular. Nesses ensaios, amostras de cateter e de cavidade bucal apresentaram maior capacidade de produção de biofilme e maior hidrofobicidade celular em relação à amostra de hemocultura. A resistência in vitro só foi obtida para o isolado de sangue, e diferenças significativas ($p< 0,05$) foram detectadas na atividade proteolítica, formação de biofilme e CSH. Em todos os casos, a cepa resistente ao fluconazol apresentou maior atividade para os fatores analisados e a breve exposição ao fluconazol resultou em diminuição da expressão das mesmas. Foi observado também correlação entre formação de biofilme e hidrofobicidade da superfície celular. Na análise proteômica, as proteínas totais foram extraídas por método mecânico e os géis bidimensionais foram corados com Coomassie G-250 coloidal. Após análise comparativa dos géis, 7 e 2 polipeptídeos mostraram-se diferencialmente expressos por células planctônicas das cepas sensível e resistente, respectivamente. Por outro lado, quando os proteomas de células sésseis foram comparados, 1 e 11 polipeptídeos apresentaram expressão diferencial na cepas sensível e resistente, respectivamente. Finalmente, 5 e 7 polipeptídeos foram diferenciamente expressos em células planctônicas e sésseis da cepa sensível ao fluconazol.

Palavras-chave:*Candida tropicalis*. Fatores de virulência. Resistência. Fluconazol.

KANOSHIKI, Renata Lumi. **Analysis of Virulence factors and proteome of Candida tropicalis sensitive and resistant to Fluconazole.** 2010. 99 p. Dissertation (Master´s Degree in Microbiology) – Universidade Estadual de Londrina, Londrina, 2010.

ABSTRACT

Candida species represent the most frequent opportunistic pathogen among the fungal infections. Although *Candida albicans* is the most prevalent and studied species, *Candida* non-*albicans* species have currently emerged. *C. tropicalis* is reported as an important etiologic agent of candidemia in patients with cancer, mainly in patients with leukemia. In Latin America, this species is significantly frequent, even in non-cancer patients, representing the second main cause of candidemia in Brazil. The high mortality in *C. tropicalis* infections and the emergence of antifungal resistant isolates corroborate its clinical importance. It is reported in the literature a correlation between *Candida* spp. antifungal exposure and the expression of virulence factors, which include adhesion to host tissues, morphogenesis, biofilm formation and enzyme secretion. The aim of this study was to investigate the effect of fluconazole on virulence factors and the proteome of *Candida tropicalis*. First, virulence factors of *C. tropicalis* isolated from the oral cavity, catheter tip and bloodstream infection were analyzed. For the blood isolate, the virulence factors were also evaluated after brief exposure to fluconazole (1 h) and after *in vitro* resistance development. Furthermore, we performed comparative proteome analysis of *Candida tropicalis* strains, resistant and susceptible to fluconazole, and planktonic and sessile cells. For the species identification, semi-nested PCR and phenotypic tests were performed. The minimal inhibitory concentration (MIC) of fluconazole was determined by broth microdilution. The proteolytic activity was evaluated in minimum medium plates containing skim milk 0.6%. For phospholipase and hemolytic activity, plates with Sabouraud Dextrose Agar supplemented with egg yolk 4% and sheep blood 5% were used, respectively. The cell surface hydrophobicity (CSH) assay was performed as described by Anil et al. (2001). Relative to the biofilm formation, two different methods were used: the percentage of blocked transmitance (%Tbloc) method, and the XTT reduction assay. Adherence to polystyrene and mammalian ephitelial cells was also analyzed. All isolates and strains were negative to phospholipase production and presented alpha-like hemolysis. Differences related to the isolates origins ($p< 0.05$) were found for biofilm and CSH properties. In these assays, isolates from catheter and oral cavity presented greater capacity of biofilm production, and higher cellular hidrophobicity compared to bloodstream infection sample. The successful *in vitro* fluconazole resistance was obtained only for bloodstream isolate, and significant differences ($p< 0.05$) were found in the proteolytic activity, biofilm formation and CSH. The fluconazole resistant strain presented higher values for the analyzed factors, and the brief fluconazole exposure resulted in reduction of these virulence factors expressions. It was also observed a correlation between biofilm formation and CSH. In proteomics analysis, the total proteins extraction was performed by mechanical method and two-dimensional gels were stained with colloidal Coomassie G-250. After comparison of the gels, 7 and 2 polypeptides were shown to be differentially expressed by planktonic cells of susceptible- and resistant- strains, respectively. On the other hand, when the proteomes of sessile cells were analyzed, 1 and 11 polypeptides showed differential expression in the susceptible- resistant- strains, respectively. Finally, 5 and 7 diferent polypeptides were expressed in planktonic and sessile cells of the fluconazole susceptible strain, respectivelly.

Keywords: *Candida tropicalis*. Virulence factors. Resistance. Fluconazole.

LISTA DE ABREVIATURAS E SIGLAS

ATCC	*American type culture collection*
ºC	grau Celsius
CFU	*Colony forming unit* – Unidade formadora de colônia
CHAPS	Ciclohexilamino dimetilamônio propano sulfonado
CSH	*cellular surface hydrophobicity* – Hidrofobicidade de superfície celular
2D	Eletroforese bidimensional
DMEM	Dulbec's Modified Eagle Medium
DTT	Ditiotreitol
EDTA	ácido etileno-diamino-tetracético
g	grama
h	hora
HCl	ácido clorídrico
IAA	iodoacetamida
IPG	*Immobilized pH gel* – Gradiente de pH Imobilizado
l	litro
mA	miliamper
µg	micrograma
MIC	*Minimum inhibitory concentration* – Concentração inibitória mínima
min	minuto
µl	microlitro
ml	mililitro
mm	milímetro
mM	milimolar
MM	*Minimal médium* – meio minimo
nm	nanômetro
PAGE	*Polyacrylamide Gel Eletrophoresis* – Eletroforese em gel de poliacrilamida
PBS	tampão fosfato-salino
PCR	*polymerase chain reaction* – reação em cadeia pela polimerase

Pz	*Precipitation zone* – zona de precipitação
rpm	rotações por minuto
SDA	Sabouraud Dextrose Agar
SDB	Saubouraud Dextrose Broth
SDS	Dodecil Sulfato de Sódio
TEMED	N,N-tetrametiletilenodiamina
TLCK	N-tosil- L-lisina-clorometil cetona
%T bloc	porcentagem de transmitância bloqueada
V	volts
XTT	2,3-bis (2-metoxi-4-nitro-5-sulfofenil)-5-[(fenilamino)carbonil]-2H-tetrazolium hidróxido
W	Watts

SUMÁRIO

1 REVISÃO BIBLIOGRÁFICA

Leveduras associadas a infecções no homem são ubíquas no ambiente e algumas são habitantes normais do corpo humano. Esses microrganismos são frequentemente oportunistas e causam infecções agudas ou crônicas quando em condições favoráveis no hospedeiro. Espécies de leveduras do gênero *Candida* são os agentes oportunistas mais frequentemente isolados dessas infecções (PERLHOTH *et al*., 2007), sendo responsáveis por cerca de 80% das infecções fúngicas no ambiente hospitalar, constituindo causa relevante de infecções de corrente sanguínea (COLOMBO; GUIMARÃES, 2003; PFALLER; DIEKEMA, 2007).

Espécies de *Candida* são comensais em 30% a 60% de indivíduos saudáveis (HABERLAND-CARRODEGUAS *et al*., 2002). Contudo, quando em condições predisponentes relacionadas ao hospedeiro, esses microrganismos podem causar infecções que variam desde infecções superficiais de mucosa, como candidíase vulvovaginal (CVV) e orofaríngea (COF), até infecções sistêmicas (MAVOR *et al.*, 2005; RANGANATHAN *et al.*, 2008). Entre os fatores que contribuem para o aumento na frequência dessas infecções fúngicas, incluem-se: infecções virais, especialmente as relacionadas ao vírus da imunodeficiência humana; desordens hematológicas, tais como diferentes tipos de leucemias e transplantes; práticas médicas agressivas ou invasivas como cirurgias, implantes e uso de cateteres; tratamentos como radioterapia, quimioterapia, e uso contínuo de antimicrobianos e esteróides. Pacientes sob tratamento de diálise ou que apresentam distúrbios metabólicos e hormonais também estão predispostos à candidíase (KOJIC; DAROUICHE, 2004; PASQUALOTTO *et al*., 2005, ALMIRANTE *et al*., 2006) .

Embora *C. albicans* represente a espécie prevalente entre os isolados de infecções superficiais e invasivas, outras espécies como *Candida parapsilosis*, *Candida*

tropicalis, *Candida glabrata*, *Candida krusei*, *Candida guilliermondii* e *Candida lusitaniae* também mostram-se em evidência quanto à importância clínica. Espécies não-*albicans* respondem por 50% das infecções invasivas por *Candida* spp. (COLOMBO; GUIMARÃES, 2003).

Um aumento progressivo na frequência de candidemia tem sido observado mundialmente, particularmente em pacientes expostos a antibióticos, terapias imunossupressoras ou nutrição parenteral, assim como em pacientes sujeitos a procedimentos médicos invasivos (PLAYFORD *et al*., 2010). Estudos conduzidos em hospitais terciários de diferentes países da Europa e Estados Unidos mostraram que a incidência de candidemia variou de 0,17 a 0,76 (RICHET *et al*., 2002; TORTORANO *et al*., 2004; ALONSO-VALLE *et al*., 2003; VIUDES *et al*., 2002) e 0,28 a 0,96 por 1000 admissões (BANERJEE *et al*., 1991; PITTET; WENZEL, 1995; WISPLINGHOFF *et al*., 2004), respectivamente. No Brasil, estudo mostra índice ainda maior alcançando 1,66 por 1000 admissões (COLOMBO *et al*., 2007). Vale salientar que a candidemia leva a um longo período de hospitalização e sua mortalidade atinge aproximadamente 50% dos pacientes (GUDLAUGSSON *et al*., 2003; HORN *et al.*, 2009).

A incidência crescente dessas infecções e a sensibilidade variada aos agentes antifúngicos utilizados na prática médica são aspectos importantes a se considerar (KAM; LIN, 2002; BARCHIESI *et al*., 2004; PFALLER *et al*., 2004; SAMRA *et al*., 2005). Programas de vigilância sobre resistência a antimicrobianos têm sido conduzidos por diferentes grupos e evidências sugerem que a emergência de infecções invasivas causadas por espécies de *Candida* não-*albicans* resistentes ao fluconazol pode ser um problema crescente em vários centros médicos (CHRYSSANTHOU, 2001; DIEKEMA *et al*., 2002; OSTROSKY- ZEICHNER *et al*., 2003; HAJJEH *et al*., 2004; CUENCA-ESTRELLA *et al*., 2005; TORTORANO *et al*., 2006). Em estudo realizado em um hospital do Japão, foi

observado que dos casos de candidemia entre 1996 a 2007, 62% eram causados por espécies não-*albicans*. Para candidemia causada por *C. albicans* 18 dos 22 casos tinham sido expostos ao fluconazol, enquanto que para as espécies não-*albicans* a relação foi de 36 para 36 (MYOKEN, 2009).

C. tropicalis tem sido relatada como o segundo ou terceiro agente etiológico mais comum de candidemia em pacientes com câncer, sendo sua frequência maior em leucemias e menor em tumores sólidos (WINGARD, 1995; VELASCO; BIGNI, 2008; SABINO *et al.*, 2009). Em países da América Latina, esta espécie é bastante frequente, mesmo entre pacientes não portadores de câncer, e, no Brasil, ela é a segunda principal causa de candidemia (COLOMBO; NUCCI, 2007). Além disso, o seu isolamento de quadros infecciosos está associado à supressão da microbiota pelo uso de antimicrobianos e danos na mucosa gastrointestinal (COLOMBO; GUIMARÃES, 2003). Apesar de sua baixa prevalência (7%-18%) entre as infecções fúngicas disseminadas, essa espécie está associada a uma alta taxa de mortalidade dos pacientes (COSTA *et al*., 2000).

Partridge *et al*. (1971) ao classificar as espécies de *Candida* de acordo com a patogenicidade em ovos embrionados, identificaram *C. albicans* e *C. tropicalis* como as mais invasivas entre as seis espécies estudadas. Em estudo *in vivo*, Arendrup *et al*. (2002) classificaram diversas espécies de *Candida* em relação à taxa de patogenicidade, de acordo com a mortalidade e alterações histológicas no fígado de camundongos infectados por oito espécies de *Candida*. Esses autores separaram estas espécies em três categorias de decrescente patogenicidade. No primeiro grupo foram incluídas as espécies *C. albicans* e *C. tropicalis*, seguido por um grupo intermediário formado por *C. glabrata*, *C. lusitaniae* e *C. kefyr* e no grupo menos patogênico *C. parapsilosis*, *C. krusei* e *C. guilliermondii*.

Para o tratamento de candidíases são utilizados antifúngicos como os derivados azólicos (cetoconazol, itraconazol, fluconazol), principalmente o fluconazol e a

anfotericina B que, apesar de sua toxicidade, é considerada em muitos casos o fármaco de escolha para o tratamento de infecção sistêmica. Além dos fármacos citados, outros como o voriconazol, segunda geração de triazólico, e a caspofungina, equinocandina que atua na parede celular, são disponíveis para o tratamento de infecções fúngicas (GALLIS *et al*., 1990; HARTSEL; BOLARD, 1996).

Na grande maioria das vezes, os isolados clínicos de *C. tropicalis* são sensíveis a anfotericina B e aos triazólicos, entretanto, podem desenvolver rapidamente resistência ao fluconazol e a taxa de isolados clínicos resistentes a esse fármaco tem aumentado significativamente (YANG *et al*., 2004; CHAKRABARTI *et al.*, 2009). Em alguns trabalhos realizados com isolados de candidemia por *C. tropicalis* a taxa de resistência a antifúngicos apresentou valores entre 0% a 12% (DIEKEMA *et al*., 2002; OSTROSKY-ZEICHNER *et al*., 2003; HAJJEH *et al*., 2004; COLOMBO *et al*., 2006).

A ação antifúngica do fluconazol está relacionada com a inibição da biossíntese do ergosterol de origem fúngica, atuando sobre a enzima lanosterol 14-alfa-demetilase, presente na membrana celular, impedindo com isto o crescimento fúngico. Espécies de *Candida* são geralmente sensíveis a este antifúngico. No entanto, o número de isolados clínicos resistentes ao fluconazol tem aumentado significativamente (MYOKEN *et al*., 2004a; MYOKEN *et al*., 2004b; YANG *et al*., 2004). Muitos mecanismos de resistência aos azólicos têm sido descritos para *Candida* spp. como: efluxo aumentado do fármaco por aumento na expressão de transportadores ABC (*ATP-binding-cassete*); transportadores de membrana como os membros da superfamília de facilitadores principais (MFS); e o aumento na expressão de genes envolvidos na biossíntese do ergosterol, tais como *ERG11* que codifica a enzima lanosterol 14-alfa-demetilase ou mutações na sequência desse gene que levam a diminuição da afinidade dos azólicos pelo seu alvo (MORSCHHÄUSER, 2002). No caso de *C. tropicalis* o mecanismo tem sido associado ao aumento na expressão do gene

MDR1 (Ct*MDR1*), que codifica uma proteína do tipo MFS (VANDEPUTTE *et al*., 2005).

A forma de resistência pode ser considerada primária quando ocorre antes da exposição ao fármaco e pode ser secundária quando é desenvolvida em resposta à exposição ao fármaco (WYNN *et al*., 1999). Relacionados à resistência secundária, alguns dados indicam que a ausência de sensibilidade ao fluconazol está associada à exposição prévia ao mesmo e sugerem que esse processo também pode conduzir à resistência cruzada ao voriconazol (COLOMBO *et al*., 2006; MAGILL *et al.*, 2006). Esse mecanismo tardio de resistência parece ser responsável pela emergência de resistência aos azólicos vista nos últimos anos, principalmente em pacientes HIV-positivo em cujo tratamento antifúngico é utilizado principalmente o fluconazol (WHITE *et al*., 2002). Uma vez que certos antifúngicos, como os derivados azólicos, possuem atividade fungistática, as células expostas repetidamente a esses antifúngicos podem se adaptar à pressão por estes exercida, e eventualmente, tornar-se resistentes. Assim, o aumento no uso de azólicos, aliado ao fato destes serem fármacos fungistáticos, aparentemente levou a emergência da resistência aos azólicos (JABRA-RIZK, 2004).

O desenvolvimento da infecção por microrganismos oportunistas pode ser considerado como uma consequência da relação entre a virulência destes agentes e a habilidade do hospedeiro de impedir ou resistir à colonização ou a invasão microbiana (HUBE, 2004). Evidentemente, microrganismos patogênicos possuem mecanismos que permitem a colonização ou infecção do hospedeiro. Muitos destes microrganismos, incluindo *Candida* spp., apresentam fatores de virulência e habilidades estratégicas específicas para colonizar, causar doenças e prevalecer frente aos mecanismos de defesa do hospedeiro.

Os fatores de virulência expressos ou necessários para o desenvolvimento de infecções pode variar de acordo com o tipo de infecção (por ex: mucosa ou sistêmica),

sítio e estágio de infecção e o nível de resposta do hospedeiro. Embora as espécies de *Candida* sejam altamente adaptadas como organismos comensais, estas possuem diversos fatores de virulência associados, tais como: aderência às células e tecidos do hospedeiro, alteração fenotípica (*switching* fenotípico) (CALDERONE; FONZI, 2001) e formação de biofilme (DOUGLAS, 2003). A secreção de enzimas hidrolíticas, como proteases (HUBE; NAGLIK, 2001) e fosfolipases (GHANNOUM, 2000), e a atividade hemolítica (LUO *et al*., 2001), também contribuem para a patogenicidade do microrganismo.

Uma vez que as membranas se constituem de lipídeos e proteínas, estas se tornam grandes alvos para ataque de enzimas. Enzimas proteolíticas extracelulares de fungos saprófitos são secretadas principalmente para fornecer nutrientes às células; porém, fungos patogênicos podem ter adaptado essa propriedade para desempenhar funções especializadas durante o processo de infecção, tal como se observa nas enzimas hidrolíticas produzidas pelos microrganismos que destroem, alteram, ou lesam a integridade da membrana, levando à disfunção e ao rompimento de estruturas do hospedeiro, facilitando a invasão de seus tecidos (VIDOTTO *et al*., 1999; KANTARCIOGLU; YUCEL, 2002; MONOD; BORG-VON ZEPELIN, 2002; NAGLIK *et al*., 2003).

Espécies patogênicas de *Candida* produzem uma variedade de hidrolases da qual se destaca a aspartil-protease secretada (*Sap: secreted aspartic proteinase*). Essas enzimas catalisam quebras nas ligações peptídicas de proteínas variando quanto a sua especificidade e mecanismo de ação (ZAUGG, 2001). Muitas espécies patogênicas de *Candida* possuem genes SAP capazes de produzí-las ativamente (RÜCHEL *et al*., 1992; GILFILLAN *et al*., 1998). Para *C. tropicalis* são descritos quatro genes *SAP* (*SAP1* a *SAP4*), de cujo produto se destaca Sapt1p, enzima predominantemente secretada em presença de solução albumina sérica bovina (BSA) *in vitro* (ZAUGG, 2001).

As Saps de *C. albicans* são produtos de uma família de pelo menos 10

genes (*SAP* 1-10). Sua ativação e expressão ainda não estão totalmente elucidadas, mas as etapas de regulação de proteases podem ser definidas basicamente pelo seguinte processo: os dez genes *SAP* codificam pré-proenzimas mais longas que a enzima madura; a porção N-terminal é clivada por uma peptidase sinal no retículo endoplasmático; no Golgi, o propeptídeo é removido pela proteinase Kex2 *subtilisin-like*, para ativar as proteases antes de serem exportadas via vesículas à superfície celular por secreção ou âncora de glicosilfosfatidilinositol (GPI). Embora, Kex2 seja considerada proteinase regulatória chave para Saps, existem outros caminhos de processamento (TOGNI *et al*., 1996), e pode ocorrer também, extracelularmente, auto-ativação para Sap1-3 e Sap 6 em determinados valores de pH (KOELSCH, *et al*., 2000). A enzima madura possui estrutura tridimensional, provavelmente mantida por resíduos de cisteína conservados, e um motivo típico para todas as proteases aspárticas, incluindo os dois resíduos de aspartato conservados do sítio ativo (HUBE, 1996). As proteínas Sap9 e Sap10 parecem ser representadas como proteínas-GPI ancoradas na membrana celular ou parede celular (CARO *et al.*, 1997).

A capacidade de produção dessas enzimas pode ser relacionada ao nível de virulência do microrganismo. Matriz extracelular e proteínas de superfície como queratina, colágeno, laminina, fibronectina, e mucina são eficientemente degradados por Sap2. Também, diferentes proteínas de defesa do hospedeiro tais como lactoferrina salivar, macroglobulina-a inibidora de protease, macrófagos e imunoglobulinas, incluindo a IgA secretora, podem ser hidrolisadas pela Sap2. Vários autores mostram a relação entre a expressão de membros dessa família de genes por *Candida* spp, e o processo de patogênese (TAYLOR *et al.*, 2005; VILLAR *et al*., 2007). Nos estudos realizados com *C. albicans*, foi observada a atenuação da virulência principalmente para as mutantes de Sap4 a Sap6 (MONOD *et al*., 1994; BORG-VON ZEPELIN *et al*., 1998, 1999). Outros estudos mostram que *SAP1*, *SAP2* e *SAP3* são expressos apenas por células leveduriformes, enquanto que

SAP4-6 se limitam a células filamentosas (HUBE *et al*.,1998; SCHALLER *et al*., 1999 b).

Vários estudos mostram que as Saps podem exercer diferentes funções que incluem: digestão de moléculas para fins nutricionais (MACDONALD, 1984; KVAAL *et al*., 1999); participação no processo de adesão ao tecido do hospedeiro (WATTS *et al*., 1998; BORG-VON ZEPELIN *et al*., 1999; ALBRECHT *et al*., 2006); invasão e degradação de tecidos e órgãos (MORSCHHÄUSER *et al*., 1997; SCHALLER *et al*., 1999 a, b; SCHALLER *et al*., 2000; FELK *et al*., 2002; SCHALLER *et al*., 2003); evasão de mecanismos de defesa do hospedeiro pela destruição de células e moléculas do sistema imune (RÜCHEL, 1986; KAMINISHI *et al*., 1995; GROPP *et al*., 2009) ou resistência à fagocitose por macrófagos (BORG-VON ZEPELIN *et al*., 1998).

As fosfolipases são enzimas que junto com as proteases fazem parte da vasta família de enzimas de *Candida* spp. O termo fosfolipase é utilizado para denominar um grupo heterogêneo de enzimas que compartilham da habilidade de hidrolisar especificamente uma ou mais ligações éster em glicerofosfolipídeos (GHANNOUM, 2000).

Normalmente, as fosfolipases estão envolvidas em muitos eventos biológicos, compreendendo o metabolismo de fosfolipídeo e a transdução de sinais nas células eucarióticas. Em microrganismos patogênicos, no entanto, essas enzimas podem auxiliar no processo de infecção, pois catalisam reações de hidrólise de fosfolipídeos, o principal componente da membrana celular do hospedeiro, facilitando dessa forma a invasão do hospedeiro. Ao hidrolisar uma ou mais ligações éster em glicerofosfolipídeos, estas enzimas levam à desestabilização da membrana e consequente lise de células, prejudicando o hospedeiro (GHANNOUM, 2000). Dessa forma, em *Candida* spp. essas enzimas podem desempenhar papéis importantes na infecção como na adesão a células epiteliais (BARRETT-BEE *et al*., 1985; IBRAHIM *et al*., 1995), na invasão do tecido (PUGH; CAWSON, 1975) e na interferência nos mecanismos de defesa do hospedeiro (BARRETT-

BEE *et al*., 1985). Diversas fosfolipases estão envolvidas nestes processos. Para *C. albicans*, são descritas: fosfolipase A, fosfolipase B, fosfolipase C, fosfolipase D, lisofosfolipase, e fosfolipase transacilase (COSTA *et al*., 1968; PUGH; CAWSON, 1975; BANNO *et al.*, 1985; MCLAIN; DOLAN, 1997). Já em *C. tropicalis*, a atividade de fosfolipase é relativamente baixa quando comparada a *C. albicans*, mas provavelmente participa nos processos de patogenicidade (CLANCY *et al*. 1998). Curiosamente, em estudo de Prakobphol e colaboradores (1994) foi observada adesão específica de *C. tropicalis* a lisofosfolipídeos. E, em experimentos subsequentes usando um inibidor da atividade de lisofosfolipase-transacilase, foi observada uma diminuição na capacidade de adesão de células de *C. tropicalis* (PRAKOBPHOL *et al*., 1997), sugerindo a importância da atividade de fosfolipase em *C. tropicalis* no processo de adesão.

Enquanto muitos estudos são desenvolvidos para a investigação das enzimas hidrolíticas, muito pouco se sabe a respeito da atividade hemolítica exibida por diferentes espécies de *Candida*. A capacidade de organismos patogênicos para a aquisição de ferro no hospedeiro mamífero parece ser de fundamental importância no estabelecimento de infecção (OTTO *et al*., 1992). Nos seres humanos, a maior parte do ferro está localizada intracelularmente como ferritina ou em compostos heme. Já a pequena quantidade de ferro extracelular está ligada a proteínas e transportadores transferrina e lactoferrina. Portanto, como não há essencialmente ferro livre no hospedeiro humano, grande parte dos patógenos o adquire indiretamente de componentes que o contém disponível, como a hemoglobina (OTTO *et al*., 1992). Muitos microrganismos são equipados com um mecanismo que destrói o grupo heme e extrai o elemento ferro do hospedeiro (BÉLANGER *et al*., 1995). Assim, ao mediar essa atividade, essas enzimas são consideradas como um fator de virulência relevante que possibilita ao patógeno sua sobrevivência e infecção no hospedeiro humano (OTTO *et al*., 1992).

A respeito de *Candida* spp., foi observado que *C. albicans* possui um fator hemolítico que permite a aquisição de ferro a partir de eritrócitos do hospedeiro (MANNS *et al*. 1994). Assim como outros microrganismos, *C. albicans* é capaz de utilizar gupos heme e hemoglobina como fontes de ferro. O mecanismo pelo qual o átomo de ferro é extraído da hemoglobina e levado para dentro da célula fúngica é pouco conhecido, mas sabe-se que sua mediação é facilitada por proteína extracelular GPI (glicofosfatidilinositol) ancorada Rbt5 por ligação a grupo heme e hemoglobina cuja função pode também ser relacionada a rápida endocitose da hemoglobina ao vacúolo de *C. albicans* (WEISSMAN; KORNITZER, 2004; WEISSMAN *et al*., 2008).

Ao considerar diferentes microrganismos, facilmente os associamos a suas formas livres unicelulares, em cultura pura. No entanto, na natureza, grande parte dos microrganismos é encontrada em comunidades microbianas denominadas biofilme. Este pode ser definido basicamente como uma comunidade complexa de microrganismos ligados irreversivelmente a um substrato e embebidos em uma matriz de polímeros extracelular produzido pelos mesmos (DONLAN; COSTERTON, 2002).

A formação do biofilme (Figura 1) é um processo dinâmico que pode ser dividido basicamente em adesão, formação inicial, maturação e dispersão. Primeiramente, células livres (planctônicas) aderem-se a superfície, mesmo em ambientes com alto fluxo de líquidos, como em dialisadores ou cateteres. O contato com uma superfície induz a expressão de enzimas microbianas que catalisam, a partir dos nutrientes disponíveis, a formação de substância polimérica extracelular (EPS) e promovem a colonização e proteção das células aderidas (sésseis). Inicialmente, são formadas microcolônias por reprodução clonal das células, que poderão se fundir e formar uma camada de biofilme microbiano sobre a extensão da superfície. A camada, então, se desenvolve com geração de novas células e EPS formando um ambiente favorável ao seu desenvolvimento. Após a maturação do biofilme, por fatores

como estresse ambiental, força de fluxo, lise de células no biofilme; algumas células ou pequenos pedaços do biofilme podem se liberar e colonizar novos sítios. A adesão destes agregados de microrganismos, gerados continuamente por processos de erosão e liberação de células ou pequenos pedaços do biofilme, representa mecanismo eficaz de colonização de novos ambientes (STOODLEY *et al*., 2002).

Durante o processo de maturação, o biofilme aumenta sua densidade celular e substância polimérica extracelular. Assim, as substâncias poliméricas produzidas pelos organismos do biofilme são consideradas o principal elemento estrutural da matriz extracelular do biofilme ao garantir sua integridade mecânica (BRANDA *et al*., 2005). EPS é formado principalmente por polissacarídeos, e variações em suas propriedades químicas e físicas podem conferir alterações no biofilme devido a diferenças na hidrofobicidade e na solubilidade (SUTHERLAND, 2001).

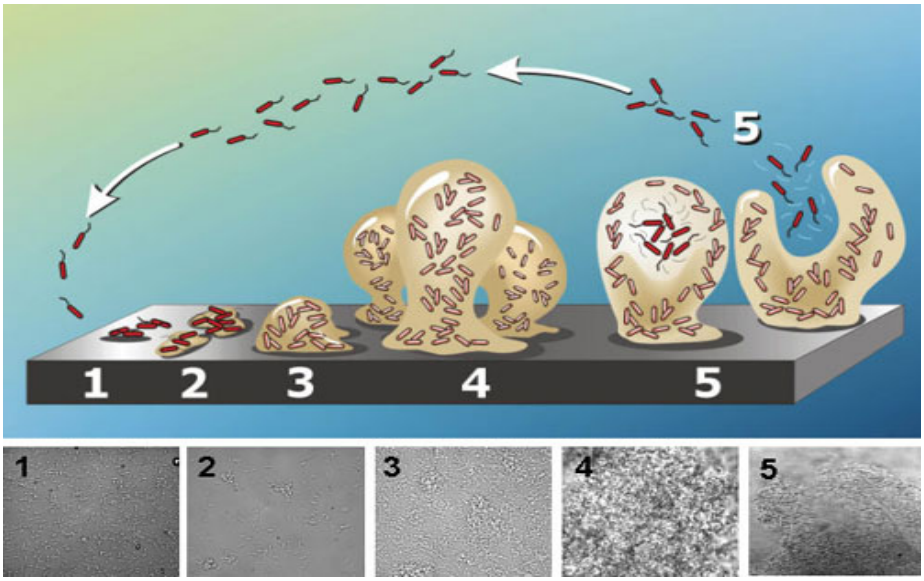

Figura 1 - Representação esquemática de formação de biofilme bacteriano. **1.** Adesão inicial das células planctônicas à superfície. **2.** Produção da matriz polimérica extracelular. As células estão "irreversivelmente" aderidas à superfície. **3** e **4**. Desenvolvimento inicial e maturação da arquitetura do biofilme, respectivamente. **5.** Dispersão das células sésseis para outros sítios de adesão. O painel inferior mostra micrografias eletrônicas de biofilme formado por *Pseudomonas aeruginosa* em superfície de vidro. Fonte: Stoodley *et al*. (2002); Sauer *et al*. (2002).

O biofilme maduro exibe uma complexa estrutura tridimensional formado por uma malha de canais interconectados de curvatura e diâmetro variável que permite a passagem de água do meio para o interior do biofilme, e servem como filtro para a entrada de material particulado. Esse arranjo espacial, além de facilitar o influxo de nutrientes, permite a eliminação de produtos, e o estabelecimento de micro-nichos no biofilme (HAWSER; DOUGLAS, 1994; KUMAMOTO, 2002).

A arquitetura final pode variar dependendo das condições de crescimento, da superfície onde o biofilme é formado e do microrganismo (CHANDRA *et al*., 2001a; SUCI *et al*., 2001). Em *Candida* spp., a formação de biofilme se mostra variável de acordo com as suas diferentes espécies (KUHN *et al.*, 2002a; LI *et al*., 2003; BIZERRA *et al*., 2008). Para o biofilme maduro de *C. albicans* (após 48 h), visualizado em microscopia eletrônica de transmissão, é possível observar uma malha formada por células leveduriformes, hifas, pseudo-hifas e EPS em arquitetura de extensiva heterogeneidade e espessura variável de 25 a mais de 450 μm (HAWSER; DOUGLAS, 1994; CHANDRA *et al*., 2001a; RAMAGE *et al*., 2001). Já em *C. tropicalis* observa-se que durante o desenvolvimento do biofilme as células, em sua maioria, se apresentam na forma filamentosa (6-24 h), e após a maturação uma rede de leveduras e pseudo-hifas formam o biofilme (BIZERRA *et al*., 2008) ao qual se descreve também uma alta capacidade produtiva de substância extrapolimérica mesmo em ambientes estáticos (AL-FATTANI; DOUGLAS, 2006).

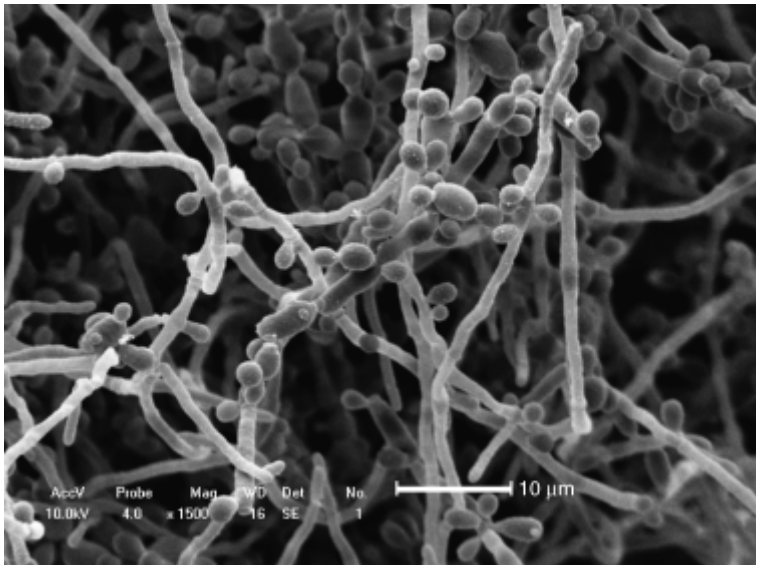

Figura 2 - Microscopia eletrônica de superfície de biofilme maduro de *C. tropicalis* (48 h) em cateter de PVC (Imagem: Bizerra et al., 2008)

Os biofilmes são altamente organizados e as células no interior do biofilme podem apresentar características singulares. A formação estrutural em comunidade e a proximidade entre as células permitem a sinalização célula-célula, ou o "quorum-sensing", fundamentais para o desenvolvimento do biofilme. Essa sinalização entre células permite controlar a superpopulação do biofilme, a competição de nutrientes e tem grandes implicações no processo de infecção, particularmente para a disseminação e para o estabelecimento de infecções em sítios distantes. Para *C. albicans*, o farnesol tem sido descrito como molécula participante do *quorum-sensing*, ao inibir a mudança para a sua forma filamentosa (HORNBY *et al*., 2001). Ao revés, estudos mostram que o tirosol, outra molécula descrita, parece favorecer a formação de formas filamentosas de *C. albicans* (CHEN *et al*., 2004; ALEM *et al*., 2006). Em estudo realizado por Alem e colaboradores (2006), foi observado um aumento da massa celular do biofilme durante o seu estágio inicial de formação, quando exposta ao tirosol. A partir dos resultados, neste mesmo estudo, foi sugerido que a atividade de tirosol seja superior a do farnesol depois de 14 h e é revertida depois de 24h, e que durante estágios finais de formação do biofilme (48 a 72h) ocorra um

aumento significativo da produção do farnesol. Assim sendo, estas duas moléculas, além de importantes ao controle positivo e negativo da morfogênese em *C. albicans*, poderiam desempenhar papel importante no processo de formação do biofilme, ao promover formação de hifas durante os estágios, inicial e intermedário, por estimulação de tirosol, enquanto o farnesol poderia ser importante aos estágios finais de formação, controlando o seu aumento, e estimulando a liberação de células, favorecendo a dispersão e colonização em novas superfícies (ALEM *et al*., 2006).

Devido ao seu grau de complexidade, o biofilme pode ser formado em diferentes tipos de ambientes e superfícies. Na área clinica, por exemplo, biofilmes de *Candida* spp. podem ser formados em dispositivos médicos como próteses, implantes, tubo endotraqueal, cateteres, entre outros, servindo como fonte de infecção (RAMAGE *et al*., 2006). Dessa forma, o biofilme microbiano está associado a uma proporção substancial de infecções humanas, destacando-se entre elas as causadas por fungos do gênero *Candida*. Visto que isolados de *Candida* spp são encontrados com frequência como parte da microbiota humana, o acesso a esses dispositivos se torna fácil, possibilitando a colonização e formação de biofilme (KOJIC; DAROUICHE, 2004). Tal fato associado ao aumento do uso de dispositivos médicos, geralmente em pacientes imunodeficientes pode ser implicado ao aumento de infecções causadas por espécies de *Candida* observado atualmente. Além de infecções relacionadas ao uso de dispositivos médicos contaminados, as infecções podem ser causadas pelo envolvimento direto do biofilme como em endocardites, otite média, prostatite crônica bacteriana, fibrose cística e cárie (DONLAN; COSTERTON, 2002).

Uma propriedade relevante do biofilme para a área clínica envolve o desenvolvimento de resistência a antimicrobianos e à ação do sistema imune. O fenótipo alterado em relação aos antimicrobianos torna esse tipo de infecção de difícil tratamento, constituindo-se fonte permanente de reinfecções (DOUGLAS, 2003). Os antimicrobianos

ministrados ao paciente podem agir sobre as células superiores do biofilme, mas, geralmente, não atinge as camadas profundas que, assim, continuam a servir como fonte de reinfecção. Quando comparadas às células planctônicas, as células presentes no biofilme podem ser 1000 vezes mais resistentes aos agentes antimicrobianos. Muitos trabalhos mostram a resistência do biofilme de *Candida* spp. a vários antifúngicos, incluindo anfotericina B, triazólicos, e flucitosina (BAILLIE; DOUGLAS, 1998, 1999; HAWSER; DOUGLAS, 1995; CHANDRA *et al*., 2001a; KUHN *et al*., 2002b; KUHN; GHANNOUM, 2004; ANDES *et al*., 2004; AL-FATTANI; DOUGLAS, 2006).

A resistência desenvolvida pelo biofilme ainda não é totalmente compreendida, mas um mecanismo multifatorial parece estar envolvido. Alguns trabalhos mostraram diferenças na secreção de carboidratos entre biofilme e células planctônicas que podem contribuir para a resistência das células sésseis (HAWSER; DOUGLAS, 1998; BAILLIE; DOUGLAS, 2000; AL-FATTANI; DOUGLAS, 2006). Outros fatores como a alta densidade celular, a matriz extracelular (impedindo difusão da droga), estado fisiológico da célula (baixo crescimento e baixa nutrição), e expressão de genes de resistência, principalmente os responsáveis pela ativação de bombas de efluxo na membrana da célula, como os transportadores ABC em *C. albicans,* e presença de células com atividade metabólica extremamente baixa ("células persistentes") também contribuem para a resistência (BAILLIE; DOUGLAS, 2000; RAMAGE *et al*., 2002; MUKHERJEE *et al*., 2003; NETT *et al*., 2007). Em *C. tropicalis*, é descrito o aumento na expressão de genes *ERG*11 e *MDR*1 (codifica bomba de efluxo de membrana) em células sésseis resistentes a anfoterecina B e fluconazol, os quais poderiam ser relacionados à resistência do biofilme (BIZERRA *et al*., 2008).

O crescimento em superfícies pode conduzir a diferentes respostas nos microrganismos. Além da formação de biofilme, a invasão celular também é um processo mediado inicialmente pelo contato célula-superfície. Assim, para a colonização ou infecções

dos tecidos do hospedeiro, os microrganismos devem primeiramente se aderir. Esta adesão inicial, dependendo de cada microrganismo, pode ser mediada tanto por fatores não específicos (hidrofobicidade da superfície celular e forças eletrostáticas) quanto por fatores específicos como fímbrias, flagelos e adesinas presentes na superfície celular que reconhecem ligantes, tanto em filmes condicionantes, como em proteínas séricas (fibrinogênio e fibronectina) e fatores salivares (RAMAGE *et al*., 2005).

No caso de fatores específicos de adesão destacam-se para *Candida* spp., as adesinas codificadas por membros da família *ALS* (*agglutinin-like sequence*), Eap1p (produto de *EAP1* – provável adesina de parede celular), e Hwp1p (*hyphal wall protein*). A família gênica *ASL* codifica glicoproteínas de superfície celular envolvidas no processo de adesão de células (HOYER *et al*., 1998a). Para *C. albicans*, já foram descritas cerca de oito diferentes proteínas (Als1p a Als7p e Als9p) das quais somente Als1p e Als5p têm sido estudadas a partir de uma perspectiva funcional. Em *C. tropicalis*, por sua vez, são descritas apenas três proteínas (AlsT1p, AlsT2p e AlsT3p), de sequência incompleta obtidas a partir de homologia às sequências de genes de *C. albicans* (HOYER, 2001; HOYER; HECHT, 2001; ZHAO *et al*., 2007).

Em estudos com Als1p, por ensaios de expressão heteróloga, foi observada sua capacidade de adesão a células de endotélio vascular humano (FU *et al*., 1998; FU *et al*., 2002). Já no caso da proteína Als5p, a partir da expressão heteróloga em *S. cerevisiae* foi observada aderência a vários substratos como colágeno, albumina bovina sérica, e laminina (GAUR *et al*,.1999; GAUR *et al*., 2002). Além disso, estudos mostram que esses genes *ALS* são diferencialmente regulados por condições fisiologicamente relevantes *in vitro*, como em crescimento em meios de culturas diferentes (HOYER *et al*., 1998b) e durante infecção experimental utilizando epitélio oral humano reconstituído (GREEN *et al*., 2004). Especialmente, em um dos estudos relacionados a proteínas Alsp de *C. albicans*, os

resultados além de relacionar a capacidade de adesão das proteínas, também foram sugestivos à capacidade invasiva destas (SHEPPARD *et al*., 2004).

Li e Palecek (2003) identificaram para a Eap1 um gene cuja sequência nucleotídica apresenta homologia com outros genes que codificam proteínas de parede celular. A introdução desse gene em *S. cerevisiae* aumentou significativamente a aderência desses microrganismos à superfície de células epiteliais renais, e a sua deleção em *C. albicans* reduziu a capacidade de adesão ao poliestireno e células epiteliais renais.

O gene *HPW1*, também importante à adesão de espécies de *Candida*, foi primeiramente descrito como gene específico da diferenciação hifa-tubo germinativo. Sabe-se que este gene codifica manoproteínas da superfície externa da célula provavelmente com o seu domínio amino-terminal orientado externamente e seu domínio carbóxi-terminal covalentemente integrado a β-glucanas da parede celular. A sequência do domínio carbóxi-terminal se assemelha ao substrato transglutaminase (TGase). Assim, a Hpw1p serve como substrato para as transglutaminases numa ligação covalentemente estável. Em estudos sobre a Hwp1p, foi possível relacionar sua adesão a células epiteliais bucais (STAAB, *et al*., 1999) e em trabalho realizado por Tsuchimori e colaboradores (2000) foi observado que mutantes de *C. albicans* deficientes de *HWP1* resultam em menor mortalidade em camundongos, e em diminuição da infecção nos rins dos animais infectados, causando, consequentemente, menores danos celulares ao endotélio.

Já quanto aos fatores inespecíficos, a hidrofobicidade da superfície celular (CSH: *cellular surface hydrophobicity*) é considerada uma característica importante para a adesão de células microbianas. A importância desse fator na adesão de células microbianas ocorre pois interações hidrofóbicas tendem a aumentar com a elevação da não polaridade de uma ou ambas as superfícies. Alguns microrganismos, por exemplo, além de serem negativamente carregados, também contém componentes hidrofóbicos em sua superfície que

facilitam a adesão (ROSENBERG, 2006). No caso de fungos como *Candida* spp., a parede celular é a principal estrutura a se estudar a variabilidade na hidrofobicidade. Muitos estudos mostram que a hidrofobidade celular pode variar em razão de mudanças na estrutura das células, como o aumento de fibrilas externamente à parede celular, forma de crescimento leveduriforme e filamentosa (HAZEN, 1989), e mudanças de temperatura (HAZEN *et al*., 1991). Assim, a variação transiente do estado de hidrofobicidade celular poderia permitir às células não infectivas da microbiota invadir o tecido do hospedeiro e a estabelecer infecções em outros sítios do corpo humano. Além disso, a mudança na CSH também é correlacionada à formação do tubo germinativo, adesão a células epiteliais e proteínas da matriz extracelular e organização da superfície celular (HAZEN; HAZEN, 1988; ARENDORF *et al*., 1998). Estudo realizado por Hazen e colaboradores (1991) permitiu observar em candidíase experimental, em camundongos, que células de *C. albicans* hidrofóbicas apresentaram maior resistência à fagocitose em relação a células hidrofílicas, sendo dessa forma a CHS considerada um fator importante na patogênese dessa doença.

Uma vez que possuem grande importância à patogenicidade, os fatores de virulência de *Candida* spp. despertam grande interesse como alvos para o desenvolvimento de novas intervenções terapêuticas contra candidíase (PERFECT, 1996). A inibição da atividade de protease extracelular, por exemplo, poderia possibilitar tanto uma forma de bloqueio em estágios invasivos de candidemia quanto o desenvolvimento de novas formas de tratamento (SYMERSKY *et al*., 1997).

Para o melhor entendimento destes fatores, o emprego de estudos, como a proteômica, ou estudo do proteoma, se mostra de grande valia, pois permite elucidar, através da expressão funcional do genoma, o estado atual de funcionamento do sistema, permitindo dessa forma, o estudo dos processos biológicos, da análise sistemática das proteínas expressas nas células ou tecidos em condições fisiológicas específicas (PEREIRA *et al*.,

2005).

O proteoma tem sido definido como o conjunto de proteínas expresso pelo genoma (WILKINS *et al.*, 1996; LOO *et al.*, 1996). Essa definição restrita implica uma natureza estática do proteoma. Porém, na realidade, o proteoma é altamente dinâmico no processo de expressão quanto a diferentes proteínas, a quantidade de proteínas, estado de modificação, localização e outros fatores, dependendo do estado fisiológico da célula ou do tecido. Portanto, o proteoma reflete o estado celular ou as condições externas encontradas por uma célula, e a sua análise ou proteômica, pode ser visto como um estudo genômico amplo para estudar os estados celulares e determinar os mecanismos moleculares que os controlam (HAYNES *et al*., 1998).

Ainda que vasto banco de dados permita a análise de sequências gênicas de DNA, apenas o estudo destas sequências não é o suficiente para elucidar uma dada função biológica, pois algumas modificações nas proteínas, como isoformas ou modificações pós-traducionais, não são aparentes na sequência de DNA, sendo somente determinadas por metodologias de proteômica. Assim, a proteômica se torna complementar à genômica, já que determina produtos gênicos, ou seja, os agentes ativos nas células (PANDEY; MANN, 2000).

Diversos recursos podem ser utilizados para a caracterização e identificação de proteínas, sendo as técnicas principais da proteômica as baseadas na utilização de eletroforese em gel bidimensional e espectrometria de massa. Os resultados gerados podem permitir, após comparação com sequências de aminoácidos depositados em bancos de dados, o processo de identificação de proteínas (WILDGRUBER *et al*., 2000).

A eletroforese bidimensional (2D) é aplicada para a análise do perfil de expressão quantitativo de proteínas de um dado sistema biológico. Nesta técnica, realiza-se a separação de misturas complexas de proteínas de acordo com o ponto isoelétrico, massa

molecular, solubilidade, e quantidade relativa, gerando um mapa de proteínas em que se pode comparar proteínas pontuais (spots) em diferentes géis. Por isso, considera-se a eletroforese bidimensional um método importante para a análise em ampla escala de conjuntos complexos de proteínas de uma amostra biológica (GÖRG *et at*., 2004).

Em conjunto com a eletroforese bidimensional, a técnica de espectrometria de massa trouxe grandes avanços à proteômica. Esta técnica analítica permite, pela relação massa/carga dos íons resultantes da ionização de peptídeos, determinar qualitativamente e quantitativamente os diversos componentes de uma amostra. Em sequência à análise de proteínas por espectrometria de massa, as proteínas podem ser identificadas se existir um banco de sequências para a sua comparação (AEBERSOLD; GOODLETT, 2001).

Haja vista sua grande importância, a proteômica possui amplo campo de aplicações, dos quais se destacam: a aplicação farmacêutica, pela identificação de novas moléculas bioativas; a identificação e caracterização de marcadores biológicos, que podem ser úteis para o diagnóstico precoce de doenças; e a aplicação na área de biologia molecular e bioquímica por permitir a descoberta de vias metabólicas nas diferentes etapas celulares (PEREIRA *et al*., 2005).

Assim, estudos como a proteômica podem ser grandes aliados à melhor compreensão dos mecanismos de desenvolvimento de infecções e dos diferentes estágios de evolução, bem como ao entendimento das causas para o desenvolvimento de resistência ou diminuição da sensibilidade aos fármacos por certos microrganismos. Análise proteômica, por exemplo, ganha grande aplicação no estudo de proteínas envolvidas na resistência a antifúngicos em *Candida* spp. Como, já, indicam alguns estudos com *C. albicans* resistente ao fluconazol por meio desta referida técnica, proteínas envolvidas no metabolismo energético e proteínas descritas previamente no mecanismo de resistência são encontradas alteradas quando comparadas à amostra parental sensível (YAN *et al.*, 2007; HOEHAMER *et*

al., 2009).

Como mencionado, espécies de *C.* não-*albicans* vêm sendo isoladas com alta frequência e embora, muitas pesquisas sobre esses fatores sejam realizadas com *C. albicans*, muito pouco se sabe a respeito de *C. tropicalis*. Portanto, investigações relacionadas aos mecanismos de patogenicidade de *Candida* spp. e sua resistência aos fármacos são necessárias para melhor compreensão das patologias associadas as mesmas, bem como para o desenvolvimento de estratégias de tratamento mais específicas e eficazes.

2 OBJETIVOS

2.1 Geral

Estudar os fatores de virulência e o proteoma de *C. tropicalis*.

2.2 Específicos

2.2.1 Realizar a identificação molecular das leveduras isoladas de infecção sanguínea, infecção bucal e ponta de cateter;

2.2.2 Determinar a concentração inibitória mínima de fluconazol dos isolados de *C. tropicalis*;

2.2.3 Induzir a resistência *in vitro* de *C. tropicalis* ao fluconazol;

2.2.4 Analisar, quanto à origem dos isolados e quanto à resistência e influência do fluconazol, sobre os seguintes fatores de virulência:

2.2.4.1 Atividade de enzimas hidrolíticas (protease e fosfolipase);

2.2.4.2 Atividade hemolítica

2.2.4.3 Hidrofobicidade celular;

2.2.4.4 Capacidade de adesão a superfície abiótica e biótica;

2.2.4.5 Capacidade de formação de biofilme;

2.2.5 Comparar o proteoma de isolados de *C. tropicalis* sensível e resistente ao fluconazol;

2.2.6 Comparar o proteoma de células planctônicas e sésseis de *C. tropicalis*.

3 REFERÊNCIAS BIBLIOGRÁFICAS

AEBERSOLD, R.; GOODLETT, D.R. Mass Spectrometry in Proteomics. *Chemical reviews*, v.101, p.269–295, 2001.

ALEM, M.A.S; OTEEF, M.D.Y; FLOWERS, T.H.; DOUGLAS, L.J. Production of tyrosol by *Candida albicans* biofilms and its role in quorum sensing and biofilm development. Eukaryotic Cell, v.5, n.10, p.1770-1779, 2006.

AL-FATTANI, M.A.; DOUGLAS, L.J. Biofilm matrix of *Candida albicans* and *Candida tropicalis*: chemical composition and role in drug resistance. *Journal of Medical Microbiology*, v.55, p.999–1008, 2006.

ALONSO-VALLE, H.; ACHA, O.; GARCÍA-PALOMO, J.D.; FARIÑAS-ALVAREZ, C.; FERNÁNDEZ-MAZARRASA, C.; FARIÑAS, M.C. Candidemia in a tertiary care hospital: epidemiology and factors influencing mortality. *European Journal of Clinical Microbiology & Infectious Diseases*, v.22, p. 254–257, 2003.

ANDES, D.; NETT, J.; OSHEL, P.; ALBRECHT, R.; MARCHILLO, K.; PITULA, A. Development and characterization of an *in vivo* central venous catheter *Candida albicans* biofilm model. *Infection and Immunity*, v.72, p.6023–6031, 2004.

ANIL, S.; ELLEPOLA, A.N.B.; SAMARANAYAKE, L.P. The impact of polyene, azole and DNA analogue antimycotics on the cell surface hydrophobicity of *Candida albicans* and *Candida tropicalis* in HIV infection. *Mycopathologia*, v.153, p.179–185, 2001.

ARENDORF, T.M.; BREDEKAMP, B.; CLOETE, C.A.; SAUER, G. Oral manifestations of HIV infection in 600 South African patients. *Journal of Oral Pathology & Medicine*, v.27, p.176–179, 1998.

ARENDRUP, M.; HORN, N.; FRIMODT-MOLLER, N. In vivo pathogenicity of eight medically relevant *Candida* species in an animal model. *Infection*, v.30, p.286–291, 2002.

BAILLIE, G.S.; DOUGLAS, L.J. Effect of growth rate on resistance of *Candida albicans* biofilms to antifungal agents. *Antimicrobial Agents and Chemotherapy*, v.42, p.1900–1905, 1998.

BAILLIE, G.S.; DOUGLAS, L.J. *Candida* biofilms and their susceptibility to antifungal agents. *Methods in Enzymology,* v.310, p.644–656, 1999.

BAILLIE, G.S.; DOUGLAS, L.J. Matrix polymers of *Candida* biofilms and their possible role in biofilm resistance to antifungal drugs. *Journal of Antimicrobial Chemotherapy*, v.46, p.397–403, 2000.

BANERJEE, S.N.; EMORI, T.G.; CULVER, D.H.; GAYNES, R.P.; JARVIS, W.R.; HORAN, T.; EDWARD, J.R.; TOLSON, J.; HENDERSEN, T.; MARTONE, W.J. Secular trends in nosocomial primary bloodstream infections in the United States, 1980-1989. National Nosocomial Infections Surveillance System. *American Journal of Medicine*, v.91, p. 86S–89S, 1991.

BANNO, Y.; YAMADA, T.; NOZAWA, Y. Secreted phospholipases of the dimorphic fungus *Candida albicans*; separation of three enzymes and some biological properties. *Sabouraudia*, v.23, p.47–54, 1985.

BARCHIESI, F.; MARACEI, M.; BALDASSARRI, I.; SPREGHINI, E.; GIANNINI, D.; SCALISE, G. Tolerance to amphotericin B in clinical isolates of *Candida tropicalis*. *Diagnostic Microbiology and Infectious Disease*, v.50, p.179–185, 2004.

BARRETT-BEE, K.; HAYES, Y.; WILSON, R.G.; RYLEY, J.F. A comparison of phospholipase activity, cellular adherence and pathogenicity of yeasts. *Journal of General Microbiology*, v.131, p.1217–1221, 1985.

BÉLANGER, M.; BEGIN, C.; JACQUES, M. Lipopolysaccharides of *Actinobacillus pleuropneumoniae* bind pig hemoglobin. *Infection and Immunity*, v.63, p.656–62, 1995.

BIZERRA, F.C.; NAKAMURA, C.V.; DE POERSCH, C.; SVIDZINSKI, T.I.E; QUESADA, R.M.B; GOLDENBERG, S.; KRIEGER, M.A.; YAMADA-OGATTA, S.F. Characteristics of biofilm formation by *Candida tropicalis* and antifungal resistance. *FEMS Yeast Research*, p.442–450, 2008.

BORG-VON ZEPELIN, M.; BEGGAH, S.; BOGGIAN, K.; SANGLARD, D.; MONOD, M. The expression of the secreted aspartyl proteinases Sap4 to Sap6 from *Candida albicans* in murine macrophages. *Molecular Microbiology*, v.28, p.543–554, 1998.

BORG-VON ZEPELIN, M.; MEYER, I.; THOMSSEN, R.; WURZNER, R.; SANGLARD, D.; TELENTI, A.; MONOD, M. HIV-protease inhibitors reduce cell adherence of *Candida albicans* strains by inhibition of yeast secreted aspartic proteases. *Journal of Investigative Dermatology*, v.113, p.747–751, 1999.

BRANDA, S.S.; VIK, S.; FRIEDMAN, L.; KOLTER, R. Biofilms: the matrix revisited. *Trends in Microbiology,* v.13, p.20-26, 2005.

CALDERONE, R.A.; FONZI, W.A. Virulence factors of *Candida albicans*. *Trends in Microbiology*, v.9, n.7, p.327–335, 2001.

CARO, L.H.; TETTELIN, H.; VOSSEN, J.H.; RAM, A.F.; VAN DEN ENDE, H.; KLIS, F.M. In silicio identication of glycosylphosphatidylinositol-anchored plasma-membrane and cell wall proteins of *Saccharomyces cerevisiae*. *Yeast*, v.13, p.1477–1489, 1997.

CHANDRA, J.; KUHN, D.M.; MUKHERJEE, P.K.; HOYER, L.L.; MCCORMICK, T.; GHANNOUM, M.A. Biofilm formation by the fungal pathogen *Candida albicans*: development, architecture, and drug resistance. *Journal of Bacteriology*, v.183, p.5385–5394, 2001a.

CHANDRA, J.; MUKHERJEE, P.K.; LEIDICH, S.D.; FADDOUL, F.F.; HOYER, L.L.; DOUGLAS, L.J.; GHANNOUM, M.A. Antifungal resistance of Candidal biofilms formed on denture acrylic *in vitro*. *Journal of Dental Research*, v.80, p.903–908, 2001b.

CHEN, H.; FUJITA, M.; FENG, Q.; CLARDY, J.; FINK, G.R. Tyrosol is a quorum-sensing molecule in *Candida albicans*. *PNAS*, v.101, n.14, p.5048–5052, 2004.

CHRYSSANTHOU, E. Trends in antifungal susceptibility among Swedish *Candida* species bloodstream isolates from 1994 to 1998: comparison of the E-test and the Sensititre YeastOne Colorimetric Antifungal Panel with the NCCLS M27-A reference method. *Journal of Clinical Microbiology*, v.39, p.4181– 4183, 2001.

CLANCY, C.J.; GHANNOUM, M.A.; NGUYEN, M.H. In: 36th Annual Meeting of the Infectious Diseases Society of America, 1998: 317.

COLOMBO, A.L.; GUIMARÃES, T. Epidemiologia das infecções hematogênicas por *Candida* spp. *Revista da Sociedade Brasileira de Medicina Tropical*, v.36, n.5, p.599–607, 2003.

COLOMBO, A.L.; NUCCI, M.; PARK, B.J.; NOUER, S.A.; ARTHINGTON-SKAGGS, B.; DA MATTA, D.A.; WARNOCK, D.; MORGAN, J. Epidemiology of candidemia in Brazil: a nationwide sentinel surveillance of candidemia in eleven Medical Centers. *Journal of Clinical Microbiology*, v.44, n.8, p.2816–2823, 2006.

COLOMBO, A.L.; GUIMARÃES, T.; SILVA, L.R.B.F.; DE ALMEIDA MONFARDINI, L.P.; CUNHA, A.K.B.; RADY, P.; ALVES, T.; ROSAS, R.C. Prospective observational study of candidemia in São Paulo, Brazil: Incidence rate, epidemiology, and predictors of mortality. *Infection control and hospital epidemiology*, v.28, p570–576, 2007.

COSTA, A.L.; COSTA, C.; MISEFARI, A.; AMATO, A. On the enzymatic activity of certain fungi. VII. Phosphatidase activity on media containing sheep's blood of pathogenic strains of *Candida albicans* (in Italian). Atti Societa Peloritana Di Scienze Fisiche Mathematische, *Naturali*, v.14; p.93–101, 1968.

COSTA, S.F.; MARINHO, I.; ARAUJO, E.A.P.; MANRIQUE, A.E.I.; MEDEIROS, A.S.; LEVIN, A.S. Nosocomial fungaemia: a 2-year prospective study. *The Journal of Hospital Infection*, v.45, p.69-72, 2000.

CUENCA-ESTRELLA, M.; RODRIGUEZ, D.; ALMIRANTE, B.; MORGAN, J.; PLANES, A.M.; ALMELA, M.; MENSA, J.; SANCHEZ, F.; AYATS, J.; GIMENEZ, M.; SALVADO, M.; WARNOCK, D.W.; PAHISSA, A.; RODRIGUEZ-TUDELA, J.L. *In vitro* susceptibilities of bloodstream isolates of *Candida* species to six antifungal agents: results from a population-based active surveillance programme. *Journal of Antimicrobial Chemotherapy*, v.55, p.194– 199, 2005.

DIEKEMA, D.; MESSER, S.A.; BRUEGGEMANN, A.B.; COFFMAN, S.L.; DOERN, G.V.; HERWALDT, L.A.; PFALLER, M.A. Epidemiology of candidemia: 3-year results from the emerging infection and epidemiology of Iowa organism study. *Journal of Clinical Microbiology*, v.40, p.1298–1302, 2002.

DONLAN, R.M.; COSTERTON, J.W. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clinical Microbiology Reviews*, v.15, n.2, p.167–193, 2002.

DOUGLAS, L.J. *Candida* biofilms and their role in infection. *Trends in Microbiology*, v.11, p.30–36, 2003.

FELK, A.; KRETSCHMAR, M.; ALBRECHT, A.; SCHALLER, M.; BEINHAUER, S.; NICHTERLEIN, T.; SANGLARD, D.; KORTING, H.C.; SCHÄFER, W.; HUBE, B. *Candida albicans* hyphal formation and the expression of the Efg1-regulated proteinases

Sap4 to Sap6 are required for the invasion of parenchymal organs. *Infection and Immunity*, v.70, n.7, p.3689–3700, 2002.

FERRER, J. Vaginal candidosis: epidemiological and etiological factors. *International Journal of Gynecology & Obstetrics,* v.71, p.21–27, 2000.

FU, Y.; RIEG, G.; FONZI, W.A.; BELANGER, P.H.; EDWARDS, J.E.JR.; FILLER, S.G. Expression of the *Candida albicans* gene *ALS1* in *Saccharomyces cerevisiae* induces adherence to endothelial and epithelial cells. *Infection and Immunity*, v.66, p.1783–1786, 1998.

FU, Y.; IBRAHIM, A.S.; SHEPPARD, D.C.; CHEN, Y.C.; FRENCH, S.W.; CUTLER, J.E.; FILLER, S.G.; EDWARDS, J.E. JR. *Candida albicans* ALS1P: an adhesion that is downstream effector of the EFG1 filamentation pathway. *Molecular Microbiology*, v.44, p.61-72, 2002.

GALLIS, H.A.; DREW, R.H.; PICKARD, W.W. Amphotericin B: 30 years of clinical experience. *Reviews of Infectious Diseases*, v.12, p.308-329, 1990.

GAUR, N.K.; KLOTZ, S.A.; HENDERSON, R.L. Overexpression of the *Candida albicans* ALA1 gene in *Saccharomyces cerevisiae* results in aggregation following attachment of yeast cells to extracellular matrix proteins, adherence properties similar to those of *Candida albicans*. *Infection and Immunity*, v.67, p.6040–6047, 1999.

GAUR, N.K.; SMITH, R.L.; KLOTZ, S.A. *Candida albicans* and *Saccharomyces cerevisiae* expressing *ALA1/ALS5* adhere to accessible threonine, serine or alanine patches. *Cell Communication & Adhesion*, v.9, p.45–57, 2002.

GHANNOUM, M.A. Potential role of phospholipases in virulence and fungal pathogenesis. *Clinical Microbiology Reviews*, v.13, p.122–143, 2000.

GILFILLAN, G.D.; SULLIVAN, D.J.; HAYNES, K.; PARKINSON, T.; COLEMAN, D.C.; GOW, N.A.R. *Candida dubliniensis*: phylogeny and putative virulence factors. *Microbiology*, v.144, p.829–838, 1998.

GÖRG, A.; WEISS, W.; DUNN, M.J. Current two-dimensional electrophoresis technology for proteomics. *Proteomics*, v.4, n.12, p.3665–3685, 2004.

GREEN, C.B.; CHENG, G.; CHANDRA, Y.; MUKHERJEE, P.; GHANNOUM, M.A.; HOYER, L.L. RT-PCR detection of *Candida albicans ALS* gene expression in the reconstituted human epithelium (RHE) model of oral candidiasis and in model biofilms. *Microbiology*, v.150, p.267–275, 2004.

GUDLAUGSSON, O.; GILLESPIE, S.; LEE, K.; VANDE BERG, J.; HU, J.; MESSER, S.; HERWALDT, L.; PFALLER, M.; DIEKEMA, D. Attributable mortality of nosocomial candidemia, revisited. *Clinical Infectious Diseases*, v.37, p.1172–1177, 2003.

HABERLAND-CARRODEGUAS, C.; ALLEN, C.M.; BECK, F.M.; BUESCHILING, W.J.; KOLETAR, S.L.; SUNDSTRON, P. Prevalence of fluconazole-resistant strains of *C. albicans* in otherwise healthy outpatients. *Journal of Oral Pathology & Medicine*, v.31, p.99–105, 2002.

HAJJEH, R.A.; SOFAIR, A.N.; HARRISON, L.H.; LYON, G.M.; ARTHINGTON-SKAGGS, B.A.; MIRZA, S.A.; PHELAN, M.; MORGAN, J.; LEE-YANG, W.; CIBLAK, M.A.; BENJAMIN, L.E.; SANZA, L.T.; HUIE, S.; YEO, S.F.; BRANDT, M.E.;WARNOCK, D.W. Incidence of bloodstream infections due to *Candida* species and in vitro susceptibilities of isolates collected from 1998 to 2000 in a population based active surveillance program. *Journal of Clinical Microbiology*, v.42, p.1519–1527, 2004.

HARTSEL, S.C.; BOLARD, J.A. Amphotericin B: new life for an old drug. *Trends in Pharmacological Sciences*, v.17, n.12, p.445–449, 1996.

HAWSER, S.P.; DOUGLAS, L.J. Biofilm formation by *Candida* species on the surface of catheter materials in vitro. *Infection and Immunity*, v.62, p.915–921, 1994.

HAWSER, S.P.; DOUGLAS, L.J. Resistance of *Candida albicans* biofilms to antifungal agents *in vitro. Antimicrobial Agents and Chemotherapy*, v.39, p.2128–2131, 1995.

HAWSER, S.P.; DOUGLAS, L.J. Production of extracellular matrix by *Candida albicans* biofilms. *Journal of Medical Microbiology*, v.47, p.253–256, 1998.

HAYNES, P.A.; GYGI, S.P.; FIGEYS, D.; AEBERSOLD, R. Proteome analysis: biological assay or data archive?. *Electrophoresis*, v.19, n.11, p.1862–71, 1998.

HAZEN, B.W.; HAZEN, K.C. Dynamic expression of cell surface hydrophobicity during initial yeast cell growth and before germ tube formation of *Candida albicans*. *Infection and Immunity*, v.56, p.2521–2525, 1988.

HAZEN, K.C. Participation of yeast cell surface hydrophobicity in adherence of *Candida albicans* to human epithelial cells. *Infection and Immunity*, v.57, p.1894–1900, 1989.

HAZEN, K.C.; BRAWNER, D.L.; RIESSELMAN, M.H.; JUTILA, M.A.; CUTLER, J.E. Differential adherence of hydrophobic and hydrophilic *Candida albicans* yeast cells to mouse tissues. *Infection and Immunity*, v.59, n.3, p.907–912, 1991.

HORNBY, J.M.; JENSEN, E.C.; LISEC, A.D.; TASTO, J.J.; JAHNKE, B.; SHOEMAKER, R.; DUSSAULT, P.; NICKERSON, K.W. Quorum sensing in the dimorphic fungus *Candida albicans* is mediated by farnesol. *American Society for Microbiology*, v.67, n.7, p. 2982–2992, 2001.

HOYER, L.L.; PAYNE, T.L.; HECHT, J.E. Identification of *Candida albicans* ALS2 and ALS4 and localization of Als proteins to the fungal cell surface. *Journal of Bacteriology*, v.180, n.20, p.5334–5343, 1998a.

HOYER, L.L.; PAYNE, T.L.; BELL, M.; MYERS, A.M.; SCHERER, S. *Candida albicans ALS3* and insights into the nature of the *ALS* gene family. *Current Genetics*, v.33, p.451–459, 1998b.

HOYER, L.L.; HECHT, J.E. The *ALS5* gene of *Candida albicans* and analysis of the ALS5p N-terminal domain. *Yeast*, v.18, p.49–60, 2001.

HOYER, L.L. The ALS gene family of *Candida albicans*. *Trends in Microbiology*, v.9, p.179–180, 2001.

HUBE, B. *Candida albicans* secreted aspartic proteinases. *Current Topics in Medical Mycology*, v.7, p.55–69, 1996.

HUBE, B.; RÜCHEL, R.; MONOD, M.; SANGLARD, D.; ODDS, F.C. Functional aspects of the secreted *Candida* proteinases. *Advances in Experimental Medicine and Biology*, v.436, p.339–344, 1998.

HUBE, B.; NAGLIK, J. *Candida albicans* proteinases: resolving the mystery of a gene family. *Microbiology*, v.147, p.1997–2005, 2001.

HUBE, B. From commensal to pathogen: stage and tissue specific gene expression of *Candida albicans*. *Current opinion in microbiology*, v.7, p.336–341, 2004.

IBRAHIM, A.S.; MIRBOD, F.; FILLER, S.G.; BANNO, Y.; COLE, G.T.; KITAJIMA, Y.; JR. EDWARDS, J.E.; NOZAWA, Y.; GHANNOUM, M.A.; Evidence implicating phospholipase as a virulence factor of *Candida albicans*. *Infection and Immunity*, v.63, n.5, p.1993–1998, 1995.

JABRA-RIZK, M.A.; FALKLER, W.A.; MEILLER, T.F. Fungal biofilms and drug resistance. *Emerging Infectious Diseases*, v.10, n.1, p.14–9, 2004.

KAM, L.W.; LIN, J.D. Management of systemic candidal infections in the intensive care unit. *American Journal of Health-System Pharmacy*, v.59, p.33–41, 2002.

KAMINISHI, H.; MIYAGUCHI, H.; TAMAKI, T.; SUENAGA, N.; HISAMATSU, M.; MIHASHI, I.; MATSUMOTO, H.; MAEDA, H.; HAGIHARA, Y. Degradation of humoral host defence by *Candida albicans* proteinase. *Infection and Immunity*, v.63, n.3, p.984–988, 1995.

KANTARCIOGLU, A.S.; YUCEL, A. Phospholipase and protease activities in clinical *Candida* isolates with reference to the sources of strains. *Mycoses*, v.45, p.160–165, 2002.

KOELSCH, G.; TANG, J.; LOY, J. A.; MONOD, M.; JACKSON, K.; FOUNDLING, S.I.; LIN, X. Enzymic characteristics of secreted aspartic proteases of *Candida albicans*. *Biochimica et Biophysica Acta*, v.1480, p.117–131, 2000.

KOJIC, E.M.; DAROUICHE, R.O. *Candida* Infections of Medical Devices. *Clinical Microbiology Reviews*, v.17, n.2, p.255–267, 2004.

KUHN, D.M.; CHANDRA, J.; MUKHERJEE, P.K; GHANNOUM, M.A. Comparison of biofilms formed by *Candida albicans* and *Candida parapsilosis* on bioprosthetic surfaces. *Infection and Immunity*, v.70, p.878–888, 2002a.

KUHN, D.M.; GEORGE, T.; CHANDRA, J.; MUKHERIEE, P.K.; GHANNOUM, M.A. Antifungal susceptibility of *Candida* biofilms: unique efficacy of amphotericin B lipid formulations and echinocandins. *Antimicrobial Agents and Chemotherapy*, v.46, p.1773–1780, 2002b.

KUHN, D.M.; GHANNOUM, M.A. *Candida* biofilms: antifungal resistance and emerging therapeutic options. *Current Opinion in Investigational Drugs*, v.5, p.186–197, 2004.

KUMAMOTO, C.A. *Candida* biofilms. *Current Opinion in Microbiology*, v.5, p.608–611, 2002.

KURTZMAN, C.P.; FELL, J.W. Definition, classification and nomenclature of the yeasts. In: The Yeasts, A Taxonomic Study, 4th ed., p. 3–5. Elsevier Science B.V., Amsterdam. 1998.

KVAAL, C.A.; LACHCKE, S.A.; SRIKANTHA, T.; DANIELS, K.; McCOY, J.; SOLL, D.R. Misexpression of the opaque-phase-specific gene *PEP1* (*SAP1*) in the white phase of *Candida albicans* confers increased virulence in a mouse model of cutaneous infection. *Infection and Immunity*, v.67, n.12, p.6652–6662, 1999.

LI, F.; PALECEK, S.P. *EAP1*, a *Candida albicans* gene involved in binding human epithelial cells. *Eukaryot Cell*, v.2, n.6, p.1266–1273, 2003.

LOO, R.R.O.; STEVENSON, T.I.; MITCHELL, C.; LOO, J.A.; ANDREWS, P.C. Mass-spectrometry of proteins directly from polyacrylamide gels. *Analytical Chemistry*, v.68, p.1910–1917, 1996.

LUO, G.; SAMARANAYAKE, L.P.; YAU, J.Y.Y. *Candida* species exhibit differential *in vitro* hemolytic activities. *Journal of Clinical Microbiology*, v.39, p.2971–2974, 2001.

MACDONALD, F. Secretion of inducible proteinase by pathogenic *Candida* species. *Journal of medical and veterinary mycology*, v.22, p.79–82, 1984.

MANNS, J.M.; MOSSER, D.M.; BUCKLEY, H.R. Production of a Hemolytic Factor by *Candida albicans*, *Infection and Immunity*, p.5154-5156, 1994.

MCLAIN, N.; DOLAN, J.W. Phospholipase D activity is required for dimorphic transition in *Candida albicans*. *Microbiology*, v.143, p.3521–3526, 1997.

MONOD, M.; TOGNI, G.; HUBE, B.; SANGLARD, D. Multiplicity of genes encoding secreted aspartic proteinases in *Candida* species. *Molecular Microbiology*, v.13, p.357–368, 1994.

MONOD, M.; BORG-VON ZEPELIN, M. Secreted proteinases and their virulence mechanism of *Candida albicans*. *Chemical Immunology*, v.81, p.114–118, 2002.

MORSCHHÄUSER, J.; VIRKOLA, R.; KORHONEN, T.K.; HACKER, J. Degradation of human subendothelial extracellular matrix by proteinase-secreting *Candida albicans*. *FEMS Microbiology Letters*, v.153, p.349–355, 1997.

MORSCHHÄUSER, J. The genetic basis of fluconazole resistance development in *Candida albicans*. *Biochimica et Biophysica Acta*, v.1587, p.240–248, 2002.

MUKHERJEE, P.K.; CHANDRA, J.; KUHN, D.M.; GHANNOUM, M.A. Mechanism of fluconazole resistance in *Candida albicans* biofilms: phase-specific role of efflux pumps and membrane sterols. *Infection and Immunity*, v.71, p.4333–4340, 2003.

MYOKEN, Y.; T. KYO; M. FUJIHARA; T. SUGATA; MIKAMI, Y. Clinical significance of breakthrough fungemia caused by azole-resistant *Candida tropicalis* in patients with hematologic malignancies. *Haematologica*, v.89, p.378-380, 2004.a

MYOKEN, Y.; KYO, T.; FUJIHARA, M.; SUGATA, T.; MIKAMI, Y. Clinical significance of breakthrough fungemia caused by azole-resistant *Candida tropicalis* in patients with hematologic malignancies. *Haematologica*, v.89, p.378–380, 2004.b

MYOKEN, Y. Clinical pathogenisis of candidemia caused by non-*albicans candida* species. *Nippon Ishinkin Gakkai Zasshi*, v.50, n.4, p.225–228, 2009.

NAGLIK, J.R.; CHALLACOMBE, S.J.; HUBE, B. *Candida albicans* secreted aspartyl proteinases in virulence and pathogenesis. *Microbiology and Molecular Biology Reviews*, v.67, p.400–428, 2003.

NETT, J.; LINCOLN, L.; MARCHILLO, K.; MASSEY, R.; HOLOYDA, K.; HOFF, B.; VANHANDE, M.; ANDES, D. Putative Role of β-1,3 Glucans in *Candida albicans* Biofilm Resistance, *Antimicrobial Agents and Chemotherapy*, v.51, n.2, p.510–520, 2007.

COLOMBO, A.L; NUCCI, M. Candidemia due to *Candida tropicalis*: clinical, epidemiologic, and microbiologic characteristics of 188 episodes occurring in tertiary care hospitals. *Diagnostic Microbiology and Infectious Disease*, v.58, n.1, p.77–82, 2007.

OSTROSKY-ZEICHNER, L.; REX, J.H.; PAPPAS, P.G.; HAMILL, R.J.; LARSEN, R.A.; HOROWITZ, H.W.; POWDERLY, W.G.; HYSLOP, N.; KAUFFMAN, C.A.; CLEARY, J.; MANGINO, J.E.; LEE, J. Antifungal susceptibility survey of 2,000 bloodstream *Candida* isolates in the United States. *Antimicrobial Agents and Chemotherapy*, v.47, p.3149–3154, 2003.

OTTO, B.R.; VERWEIJ-VAN VUGHT, A.M.; MACLAREN, D.M. Transferrins and heme compounds as iron sources for pathogenic bacteria. *Critical reviews in Microbiology*, v.18, p.217–233, 1992.

PANDEY, A.; MANN, M. Proteomics to study genes and genomes. *Nature*, v.405, 2000.

PARTRIDGE, B.M.; ATHER, M.A; WINNER, H.I. Chick embryo inoculation as a pathogenicity test for *Candida* species. *Journal of Clinical Pathology*, v.24, n.7, p.645–648, 1971.

PASQUALOTTO, A.C.; NEDEL, W.L.; MACHADO, T.S.; SEVERO, L.C. A comparative study of risk factors and outcome among outpatient-acquired and nosocomial candidaemia. *Journal of Hospital Infection*, v.60, p.129–134, 2005.

PEREIRA, A. DOS S.; BICALHO, B.; LILLA, S.; DE NUCCI, G. Desafios da química analítica frente às necessidades da indústria farmacêutica. *Química Nova* v.28, p.S107–S111, 2005.

PERFECT, J.R. Fungal virulence genes as targets for antifungal chemotherapy. *Antimicrobial Agents and* Chemotherapy, v.40, p.1577–1583, 1996.

PFALLER, M.A.; DIEKEMA, D.J.; MESSER, S.A.; BOYKEN, L.; HOLLIS, R.J.; JONES, R.N. *In vitro* susceptibilities of rare *Candida* bloodstream isolates to ravuconazole and three comparative antifungal agents. *Diagnostic Microbiology and Infectious Disease*, v.48, p.101–105, 2004.

PITTET, D.; WENZEL, R.P. Nosocomial bloodstream infections. Secular trends in rates, mortality, and contribution to total hospital deaths. *Clinical Infectious Diseases*, v.155, p.1177–1184, 1995.

PRAKOBPHOL, A.; LEFFLER, H.; FISHER, S.J. Specific adherence of *Candida tropicalis* to lysophospholipids. *Biochemistry*, v.33, p.9496–9503, 1994.

PRAKOBPHOL, A.; LEFFLER, H.; HOOVER, C.I.; FISHER, S.J. Palmitoyl carnitine, a lysophospholipase-transacylase inhibitor, prevents *Candida* adherence *in vitro*. *FEMS Microbiology Letters*, n.151, p.89–94, 1997.

PUGH, D.; CAWSON, R.A. The cytochemical localisation of phospholipase A and lysophospholipase in *Candida albicans*. *Sabouraudia*, v.13, p.110–115, 1975.

RAMAGE, G.; WALLE, K.V.; WICKES, B.L.; LÓPEZ-RIBOT, J. Biofilm formation by C*andida dubliniensis*, *Journal of Clinical Microbiology*, v.39, p.3234–3240, 2001.

RAMAGE, G.; BACHMANN, S.; PATTERSON, T.F.; WICKES, B.L.; LOPEZ- RIBOT, J.L. Investigation of multidrug efflux pumps in relation to fluconazole resistance in *Candida albicans* biofilms. *Journal of Antimicrobial Chemotherapy*, v.49, p.973–980, 2002.

RAMAGE, G.; SAVILLE, S.P.; THOMAS, D.P.; LOPEZ-RIBOT, J. *Candida* Biofilms: an Update. *Eukaryotic Cell*, v.4, n.4, p.633–638, 2005.

RAMAGE, G.; MARTÍNEZ, J.P.; LÓPEZ-RIBOT, J.L. *Candida* biofilms on implanted biomaterials: a clinically significant problem. *FEMS Yeast Research*, v.6, p.979–986, 2006.

RICHET, H.; ROUX, P.; DES CHAMPS, C.; ESNAULT, Y.; ANDREMONT, A. Candidemia in French hospitals: incidence rates and characteristics. *Clinical Microbiology and Infection*, v.8, p.405–412, 2002.

ROSENBERG, M. Microbial adhesion to hydrocarbons: twenty-five years of doing math. *FEMS microbiology letters*, v.262, p.129–134, 2006.

RÜCHEL, R. Cleavage of immunoglobulins by pathogenic yeasts of the genus *Candida*. *Microbiological Sciences*, v.3, p.316–319, 1986.

RÜCHEL, R.; DE BERNARDIS, F.; RAY, T.L.; SULLIVAN, P.A.; COLE, G.T. *Candida* acid proteinases. *Medical Mycology*, v.30, p.123–132, 1992.

SAMRA, Z.; YARDENI, M.; PELED, N.; BISHARA, J. Species distribution and antifungal susceptibility of *Candida* bloodstream isolates in a tertiary medical center in Israel, *European Journal of Clinical Microbiology & Infectious Diseases*, v.24, p.592–595, 2005.

SAUER, K.; CAMPER, A.K.; EHRLICH, G.D.; COSTERTON, J.W.; DAVIE, D.G. *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. *The Journal of Bacteriology*, v.184, n.4, p.1140–1154, 2002.

SCHALLER, M.; HUBE, B.; OLLERT, M.W.; SCHÄFER, W.; BORG-VON ZEPELIN, M.; THOMA-GREBER, E.; KORTING, H.C. *In vivo* expression and localization of *Candida albicans* secreted aspartyl proteinases during oral candidiasis in HIV-infected patients. *Journal of Investigative Dermatology*, v.112, p.383–386, 1999 a.

SCHALLER, M.; KORTING, H.C.; SCHÄFER, W.; BASTERT, J.; CHEN, W.C.; HUBE, B. Secreted aspartic proteinase (Sap) activity contributes to tissue damage in a model of human oral candidosis. *Molecular Microbiology*, v.34, n.1, p.169–180, 1999 b.

SCHALLER, M.; SCHACKERT, C.; KORTING, H.C.; JANUSCHKE, E.; HUBE, B. Invasion of *Candida albicans* correlates with expression of secreted aspartic proteinases during experimental infection of human epidermis. *Journal of Investigative Dermatology*, v.114, p.712–717, 2000.

SCHALLER, M.; BEIN, M.; KORTING, H.C.; BAUR, S.; HAMM, G.; MONOD, M.; BEINHAUER, S.; HUBE, B. The secreted aspartyl proteinases Sap1 and Sap2 cause tissue damage in an *in vitro* model of vaginal candidiasis based on reconstituted human vaginal epithelium. *Infection and Immunity*, v.71, n.6, p.3227–3234, 2003.

SHEPPARD, D.C.; YEAMAN, M.R.; WELCH, W.H.; PHAN, Q.T.; FU, Y.; IBRAHIM, A.S.; FILLER, S.G.; ZHANG, M.; WARING, A.J.; EDWARDS, J.E. JR. Functional and structural diversity in the ALS protein family on the *Candida albicans*. *The Journal of the Biological Chemistry*, v.279, n.29, p.30480–30489, 2004.

STAAB, J.F.; BRADWAY, S.D.; FIDEL, P.L.; SUNDSTROM, P. Adhesive and mammalian transglutaminase substrate properties of *Candida albicans Hwp1*. *Science*,v.283, p.1535–1538, 1999.

STOODLEY, P.; SAUER, K.; DAVIES, D.G.; COSTERTON, J.W. Biofilms as complex differentiated communities. *Annual Review of Microbiology*, v.56, p.187–209, 2002.

SUCI, P.A.; GEESEY, G.G.; TYLER, B.T. Integration of Raman microscopy, differential interference contrast microscopy, and attenuated total reflection Fourier transform infrared spectroscopy to investigate chlorhexidine spatial and temporal distribution in *Candida albicans* biofilms. *Journal of Microbiological Methods*, v.46, n.3, p.193–208, 2001.

SUTHERLAND, I.W. Biofilm exopolysaccharides: a strong and sticky framework. *Microbiology*, v.147, p.3–9, 2001.

SYMERSKY, J.; MONOD, M.; FOUNDLING, S.I. High-Resolution Structure of the Extracellular Aspartic Proteinase from *Candida tropicalis* Yeast. *Biochemistry*, v.36, p.12700–12710, 1997.

TOGNI, G.; SANGLARD, D.; QUADRONI, M.; FOUNDLING, S. I.; MONOD, M. Acid proteinase secreted by *Candida tropicalis*: functional analysis of preproregion cleavages in *C. tropicalis* and *Saccharomyces cerevisiae*. *Microbiology*, v.142, p.493–503, 1996.

TORTORANO, A.M.; PEMAN, J., BERNHARDT, H.; KLINGSPOR, L.; KIBBLER, C.C.; FAURE, O.; BIRAGHI, E.; CANTON, E.; ZIMMERMANN, K.; SEATON, S.; GRILLOT, R. Epidemiology of candidaemia in Europe: results of 28-month European Confederation of Medical Mycology (ECMM) hospital-based surveillance study.

European Journal of Clinical Microbiology & Infectious Diseases, v.23, p.317–322, 2004.

TORTORANO, A.M.; KIBBLER, C.; PEMAN, J.; BERNHARDT, H.; KLINGSPOR, L.; GRILLOT, R. Candidaemia in Europe: epidemiology and resistance. *International Journal of Antimicrobial Agents*, v.27, p.359–366, 2006.

TSUCHIMORI, N.; SHARKEY, L.L.; FONZI, W.A.; FRENCH, S.W.; EDWARDS JR., J.E.; FILLER, S.G. Reduced virulence of *HWP1*-deficient mutants of *Candida albicans* and their interactions with host cells. *Infection and Immunity*, v.68, p.1997–2002, 2000.

VANDEPUTTE, P.; LARCHER, G.; BERGÈS, T.; RENIER, G.; CHABASSE, D.; BOUCHARA, J. MECHANISMS of Azole Resistance in a Clinical Isolate of *Candida tropicalis*. *Antimicrobial agents and chemotherapy*, v.49; n.11, p.4608–4615, 2005.

VIDOTTO, V.; KOGA-ITO, C.Y.; MILANO, R.; FIANCHINO, B.; PONTÓN, J. Correlation between germ-tube production, phospholipase activity and serotype distribution in *Candida albicans*. *Revista Iberoamericana de Micología*, v.16, p.208–210, 1999.

VIUDES, A.; PEMÁN, J.; CANTÓN, E.; UBEDA, P.; LÓPEZ-RIBOT, J.L.; GOBERNADO, M. Candidemia at a tertiary-care hospital: epidemiology, treatment, clinical outcome and risk factors for death. *European Journal of Clinical Microbiology & Infectious Diseases*, v.21, p. 767–774, 2002.

WATTS, H.J.; CHEAH, F.S.H.; HUBE, B.; SANGLARD, D.; GOW, N.A.R. Altered adherence in strains of *Candida albicans* harbouring null mutations in secreted aspartic proteinase genes. *FEMS Microbiology Letters*, v.159, p.129–135, 1998.

WEISSMAN, Z.; KORNITZER, D. A family of *Candida* cell surface haem-binding proteins involved in haemin and haemoglobin-iron utilization. *Molecular Microbiology*, v.53, p.1209–1220, 2004.

WEISSMAN, Z.; SHEMER, R.; CONIBEAR, E.; KORNITZER, D. An endocytic mechanism for haemoglobin-iron acquisition in *Candida albicans*. *Molecular Microbiology*, v.69, p.201–217, 2008.

WHITE, T.C.; HOLLEMAN, S.; DY, F.; MIRELS, L.F.; STEVENS, D.A. Resistance mechanisms in clinical isolates of *Candida albicans*. *Antimicrobial Agents and Chemotherapy*, v.46, n.6, p.1704–1713, 2002.

WINGARD, J.R. Importance of *Candida* species other than *C. albicans* as pathogens in oncology patients. *Clinical Infectious Diseases*, v.20, p.115–125, 1995.

WILDGRUBER, R.; HARDER, A.; OBERMAIER, C.; BOGUTH, G.; WEISS, W.; FEY, S.J.; LARSEN, P.M.; GÖRG, A. Towards higher resolution: Two-dimensional Electrophoresis of *Saccharomyces cerevisiae* proteins using overlapping narrow immobilized pH gradients. *Electrophoresis*, v.21, n.13, p.2610–2616, 2000.

WILKINS, M.R.; PASQUALI, C.; APPEL, R.D.; OU, K.; GOLAZ, O.; SANCHEZ, J.C.; YAN, J.X.; GOOLEY, A.A.; HUGHES, G.; HUMPHERY-SMITH, I.; WILLIAMS, K.L.; HOCHSTRASSER, D.F. From proteins to proteomes-Large-scale protein

identification by 2-dimensional electrophoresis and amino acid analysis. *Biotechnology & Genetic Engineering Reviews*, v.14, p.61–65, 1996.

WISPLINGHOFF, H.; BISCHOFF, T.; TALLENT, S.M.; SEIFERT, H.; WENZEL, R.P.; EDMOND, M.B. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. *Clinical Infectious Diseases*, v.39, p.309–317, 2004.

WYNN, R.L.; JABRA-RIZK, M.A.; MEILLER, T.F. Antifungal drugs and fungal resistance: the need for a new generation of drugs. *Gen Dent*, v.47, p.352–5, 1999.

YANG, Y.L.; HO, Y.A.; CHENG, H.H.; HO, M.; LO, H.J. Susceptibilities of *Candida* species to amphotericin B and fluconazole: the emergence of fluconazole resistance in the *Candida tropicalis*. *Infection Control and Hospital Epidemiology*, v.25, p.60–64, 2004.

ZAUGG, C.; ZEPELIN, M.B.; REICHARD, U.; SANGLARD, D.; MONOD, M. Secreted Aspartic Proteinase Family of *Candida tropicalis*. *Infection and Immunity*, v.69, n.1, p.405–412, 2001.

ZHAO, X.; O.H., S.H.; HOYER, L.L. Unequal contribution of *ALS9* alleles to adhesion between *Candida albicans* and human vascular endothelial cells. *Microbiology*, v.153, p.2342–2350, 2007.

PERLROTH, J.; CHOI, B.; SPELLBERG. Nosocomial fungal infections: epidemiology, diagnosis, and treatment. *Medical Micology*, v.45, p.321–346, 2007.

PFALLER, M.A.; DIEKEMA, D.J. Epidemiology of invasive Candidiasis: a persistent public health problem. *Clinical Microbiology reviews*, v.20, p.133–163, 2007.

RANGANATHAN, K.; REDDY, B.V.R.; KUMARASAMY, N.; SOLOMON, S.; VISWANATHAN, R.; JOHNSON, N.W. Oral lesions and conditions associated with human immunodeficiency virus infection in 400 south indian patients. *Oral diseases*, v.6, p.152–156, 2008.

ALMIRANTE, B.; RODRIGUEZ, D.; CUENCA ESTRELLA, M.; ALMELA, M.; SANCHEZ, F.; AYATS, J.; ALONSO TARRES, C.; RODRIGUEZ TUDELA, J.L.; PAHISSA, A. Epidemiology, risk factors, and prognosis of *Candida parapsilosis* bloodstream infections: case-control population-based surveillance study of patients in Barcelona, Spain, from 2002 to 2003. *Journal of Clinical Microbiology*, v.44, p.1681–1685, 2006.

MAVOR, A.L.; THEWES, S.; HUBE, B. Systemic fungal infections caused by *Candida* species: epidemiology, infection, process and virulence attributes. *Current Drug Targets*, v.6, p. 863–874, 2005.

PLAYFORD, E.G.; NIMMO, G.R.; TILSE, M.; SORRELL, T.C. Increasing incidence of candidaemia: long-term epidemiological trends, Queensland, Australia, 1999-2008. *Journal of Hospital Infection*, p.1–6, 2010.

VELASCO, E.; BIGNI, R. A prospective cohort study evaluating the prognostic impact of clinical characteristics and comorbid conditions of hospitalized adult and pediatric cancer

patients with candidemia. *European Journal of Clinical Microbiology & Infectious Diseases*, v.11, p.1071–1078, 2008.

SABINO, R.; VERISSIMO, C.; BRANDÃO, J.; PARADA, H.; ROSADO, L.; PAIXAO, E.; VIDEIRA, Z.; SAMPAIO, P.; PAIS, C. Epidemiology of candidemia in oncology patients: a 6-year survey in a Portuguese central hospital. *Medical mycology*, p.1–10, 2009.

MAGILL, S.S.; SHIELDS, C.; SEARS, C.L.; CHOTI, M.; MERZ, G.W. Triazole Cross-resistance bloodstream isolates, and implications for antifungal therapy. *Journal of Clinical Microbiology*, v.44, p.529–535, 2006.

HORN, D.L.; NEOFYTOS, D.; ANAISSIE, E.J.; FISHMAN, J.A.; STEINBACH, W.J.; OLYAEI, A.J.; MARR, K.A.; PFALLER, M.A.; CHANG, C.H.; WEBSTER, K.M. Epidemiology and outcomes of candidemia in 2019 patients: data from prospective antifungal therapy alliance registry. *Clinical Infectious Diseases*, v.48, p. 1695–1703, 2009.

CHAKRABARTI, A.; CHATTERJEE, S.S.; RAO, K.L.; ZAMEER, M.M.; SHIVAPRAKASH, M.R.; SINGHI, S.; SINGHI, S.; VARMA, S.C. recent experience with fungaemia: change in species distribution and azole resistance. *Scandinavian Journal of Infectious Diseases*, v.41, p.275–284, 2009.

TAYLOR, B.N.; STAIB, P.; BINDER, A.; BIESEMEIER, A.; SEHNAL, M.; RÖLLINGHOFF, M.; MORSCHHÄUSER, J.; SCHRÖPPEL, K. Profile of *Candida albicans*-secreted aspartic proteinase elicited during vaginal infection. *Infection and Immunity*, v.73, p.1828–1835, 2005.

VILLAR, C.C.; KASHLEVA, H.; NOBILE, C.J.; MITCHELL, A.P.; DONGARI-BAGTZOGLOU, A. Mucosal tissue invasion by *Candida albicans* is associated with E-cadherin degradation, mediated by transcription factor Rim101p and protease Sap5p. *Infection and Immunity*, v.75, p.2126–2135, 2007.

GROPP, K.; SCHILD, L.; SCHINDLER, S.; HUBE, B.; ZIPFEL, P.F.; SKERKA, C. The yeast *Candida albicans* evades human complement attck by secretion of aspartic proteases. *Molecular Immunology*, v.47, p. 465–475, 2009.

ALBRECHT, A.; FELK, A.; PICHOVA, I.; NAGLIK, J.R.; SCHALLER, M.; DE GROOT, P.; MACCALLUM, D.; ODDS, F.C.; SCHÄFER, W.; KLIS, F.; MNOD, M.; HUBE, B. Glycosylphosphatidylinositol-anchored proteases of *Candida albicans* taget proteins necessary for both cellular processes and host-pathogen interactions. *The Journal of Biological Chemistry*, v.281, p.688–694, 2006.

YAN, L.; ZHANG, J.D.; CAO, Y.B.; GAO, P.H.; JIANG, Y.Y. Proteomic analysis reveals a metabolism shift in a laboratory fluconazole-resistant *Candida albicans* strain. *Journal of Proteome Research*, v.6, p.2248–2256, 2007.

HOEHAMER, C.F.; CUMMINGS, E.D.; HILLIARD, G.M.; MORSCHHÄUSER J, DAVID ROGERS P. Upc2p-associated differential protein expression in *Candida albicans. Proteomics,* v.9, p.4726–4730, 2009.

ANEXOS

ANEXO A

Artigo

Putative virulence factors of *Candida tropicalis* isolated from different origins

Putative virulence factors of *Candida tropicalis* isolated from different origins.

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ABSTRACT

Candida species represent the most frequent opportunistic pathogen of fungal infections. Although *Candida albicans* correspond to the most prevalent and studied species, non-*C. albicans* species have currently emerged. Among these non-*C. albicans* species, *C. tropicalis* has been reported as the second or third more common etiologic agent of candidemia in patients with cancer, mainly in those with leukemia. Moreover, the high mortality in patients infected with *C. tropicalis* and the emergence of antifungal resistant strains corroborate its clinical importance. Several virulence factors are related to these yeasts, which include adhesion to host tissues, morphogenesis, biofilm formation and enzyme secretion. Since these factors have eminent importance to pathogenesis, in this work, *C. tropicalis* isolated from oral cavity, catheter and bloodstream infection were analyzed according to the cell surface hydrophobicity (CSH), adhesion, biofilm formation, production of phospholipase, protease and hemolytic activity. Additionally, the samples were tested *in vitro* for the ability to develop fluconazole resistance. In this test, development of fluconazole resistance was successful only in bloodstream infection isolate. All the samples demonstrated alpha hemolysis and no phospholipase production. For proteolytic activity, samples presented intermediate activity. Differences, considering the origin of the samples, ($p< 0,05$) were found for biofilm production and CSH. In these assays, samples from catheter and oral cavity presented greater capacity of biofilm production, and higher cellular hydrophobicity compared to the sample of bloodstream infection. In addition, it was observed a correlation of biofilm and CSH.

Keywords: *Candida tropicalis*; virulence factors; biofilm.

INTRODUCTION

Candida species appear as endogenous commensals in 30% to 60% of healthy individuals (30), however in predisposing conditions of the host, as in systemic diseases and immunosuppression, particularly those related to human immunodeficiency virus (29), these microorganisms can cause infections ranging from superficial to systemic (30, 54, 78). Currently, *Candida* spp is associated to almost 80% of the nosocomial fungal infections, representing relevant cause of bloodstream infections (18).

Besides the prevalence of *C. albicans*, currently, *C.* non-*albicans* species correspond to 50% of the invasive infections by *Candida* spp. In particular, *C. tropicalis* has been reported as the second or third most common etiologic agent of candidemia in cancer patients (82). In Latin America, this species is very frequent and represents the second leading cause of candidemia in Brazil (60).

Several virulence factors are associated with these yeasts, such as adhesion to cells and host tissues, morphogenesis, phenotypic switching (14) and biofilm formation (22). Likewise, the secretion of hydrolytic enzymes also contributes to the pathogenicity of the microorganism. Extracellular proteolytic enzymes of saprophytic fungi are secreted primarily to provide nutrients to cells, however pathogenic fungi could have adapted that property to perform specialized functions during the infective process. The hydrolytic enzymes produced by microorganisms destroy, alter, or harm the integrity of the membrane, leading to dysfunction and the disruption of the host structures, facilitating the invasion of their tissues (42, 55, 58, 80).

Pathogenic species of *Candida* produce a variety of hydrolases. Relating to them, the secreted aspartic proteinase (SAP) (84) has been described as responsible for functions like: digestion of molecules for nutrition (48, 52), invasion and degradation of

tissues and organs (24, 57, 72, 73, 74), evasion of host defense mechanisms through the destruction of cells and molecules of the immune system (41, 68) and resistance to phagocytosis by macrophages (11). These enzymes can be produced by SAP genes in many pathogenic species of *Candida* like *C. tropicalis* for which are described four SAP genes (SAP1 to SAP4) (84). Another enzyme, the phospholipase, hydrolyzes extracellular phospholipids, the main component of the host cell membrane facilitating the invasion by *Candida* spp. (27).

Moreover, species of *Candida* may have hemolytic activity when destroying and extracting the heme iron component of the host (9). Thus, this hemolytic activity is considered an important virulence factor that allows the pathogen infection and their survival in the human host (61).

As important as the enzymes production, the adherence of these microorganisms must be considered in the infection process. In *Candida* spp. the initial adherence is mediated by both non-specific factors (cell surface hydrophobicity and electrostatic forces) and adhesins present on the cell surface (65). For these, are described members of ALS (agglutinin-like sequence) family and Eap1p, produced by EAP1 gene whose expression appears to be also essential to biofilm formation (50).

The importance of biofilm to clinical area is in its involvement with a substantial proportion of human infections and in the resistance to antimicrobials. The different biofilm phenotype difficults the infection treatment and acts as a source of reinfections (22). Compared to planktonic cells, biofilm cells can be 1,000 times more resistant. Furthermore, many studies show the resistance of *Candida* spp. biofilm to several antifungals, including amphotericin B, triazoles, and flucytosine (2, 3, 6, 15, 31, 46).

Nowadays, the increasing incidence of these infections and the resistance to different antifungal agents used in medical practice are relevant (8, 40, 62, 71). The clinical

isolates of *C. tropicalis* are in most cases, sensitive to amphotericin B and triazoles (18), however, *Candida tropicalis* can rapidly develop resistance to fluconazole (8) and the rate of clinical isolates resistant to this drug has increased significantly (19, 83). Some data indicate that the lack of sensitivity to fluconazole is associated with prior exposure to it (19), a mechanism that appears to be responsible for the emergence of azole resistance seen in recent years, especially in HIV-positive patients in whose antifungal treatment is mainly used fluconazole (81).

As mentioned, non-*albicans* species, as *C. tropicalis*, have been isolated with high frequency in different clinical materials. Therefore, research related to mechanisms of pathogenicity of *Candida* spp. and its resistance to drug becomes necessary for better understanding of pathologies related to them, and for the development of more specific and effective treatment. So, in this work, we analyze the virulence factors of *C. tropicalis* isolated from oral cavity, catheter and candidemia. Additionally, the samples were tested *in vitro* for the ability to develop fluconazole resistance.

MATERIALS AND METHODS

Candida tropicalis isolates and growth conditions

This study used samples of *C. tropicalis* isolated from oral cavity, catheter tip and bloodstream infection collected at the Departamento de Análises Clínicas - LEPAC, Universidade Estadual de Maringá, and it was maintained in Sabouraud Dextrose Agar (SDA) at 4 ° C, with monthly samplings.

Molecular identification

The samples were presumptively identified as *C. tropicalis* by classical phenotypic methods (47) and species identification was confirmed by semi-nested PCR as described by Ahmad et al. (2002) (1). Genomic DNA extraction was performed according to Lee et al., (1992) (49).

Fluconazole susceptibility testing

The determination of fluconazole minimum inhibitory concentration (MIC) was determined by broth microdilution according to standards established by the Clinical Laboratory Standard Institute (CLSI, 2002) (59). The reference samples used were: *C. albicans* (ATCC 10231), *C. parapsilosis* (ATCC 22019) and *C. tropicalis* (ATCC 750). According to the CLSI guidelines, the breakpoints for interpretation of the results were MICs $\geq$ 64 μg/ml for resistant isolates, 16- 32 μg/ml for dose-dependent and MICs $\leq$ 8 μg/ml for susceptible.

Induction of fluconazole resistance

For the induction of resistance, the samples were cultured in RPMI-1640 with exposure of increasing concentrations of fluconazole (4 μg/ml, 8 μg/ml, 16 μg/ml, 32 μg/ml, 64 μg/ml) until the detection of resistance using the methodology described before. An initial inoculum of 5×10^3 cells/ml was cultured in medium containing 4 μg/ml of fluconazole at 35 ºC for 24 h. About 1/3 volume of this culture was transferred to another fresh medium with increased concentration of fluconazole, and this procedure was repeated successively to obtain a population of cells resistant to the antifungal drug.

Analysis of virulence factors

Samples from oral cavity, catheter tip and bloodstream infection were evaluated about the production of protease, phospholipase and hemolytic activity. Cell surface hydrophobicity, adherence capacity and biofilm formation of the samples were also determined. The experiments were carried out in duplicate on three different occasions.

Phospholipase

Phospholipase activity was assayed on plates containing SDA pH 4.5 supplemented with egg yolk (4%), NaCl 20 g/l and $CaCl_2$. $2H_2O$ 1 g/l. Previously, the samples were cultivated on Sabouraud broth for 24 h with constant agitation (200 rpm). After the incubation period, the cell density was determined by direct counting of the culture using a hemocytometer counting chamber (Improved Neubauer Chamber). A volume of 10 μl (approximately 1.0×10^6 cells) was inoculated on the surface of the medium. For observation of cell growth and activity of extracellular phospholipase, the plates were incubated at 37 °C for 96 h and checked every 24 h. Isolates with dense white zone of precipitation around the

colony after incubation period were considered positive for phospholipase. The phospholipase activity was estimated by the ratio of the colony diameter and the diameter of the precipitation zone around the colony (Pz), as described in Price et al. (1982) (64) (Pz = 1, null activity; 0.64≤ Pz< 1, low activity; 0.30≤ Pz< 0.64, intermediate activity; Pz< 0.30, high activity).

Protease

Protease activity was determined on plates containing minimal medium pH 6.8 supplemented with skim milk (0.6%). Previously, isolates were cultured at 37 ºC for 18 h in minimal medium (MM) broth (63) supplemented with 0.6% skim milk, pH 4.0, to induce the secretion of enzyme. An inoculum of 10 ul (approximately 1.0×10^6 cells) was placed in holes of approximately 3 mm of diameter drilled in the agar according to Izumi and Yamada-Ogatta (2004) (38). For observation of cell growth and extracellular proteolytic activity, the plates were incubated at 37 °C for 72 h, and checked every 24 h. After the incubation period, the samples that showed a degradation zone around the colony were positive for protease production. The proteolytic activity was estimated by the ratio of colony diameter and the diameter of degradation zone around the colony (Dz), as previously described.

Hemolytic activity

Hemolytic activity was evaluated according to Luo et al. (2001) (51). Subsequent to cell culture, an inoculum of 10 µl (approximately 1.0×10^6 cells) was placed on the surface of blood agar 5% (SDA medium supplemented with 3% glucose and 5% fresh sheep blood). The plates were incubated at 37 ° C over 24- 48 h in microaerophilic chamber. Positive hemolytic activity was indicated by the presence of a distinct translucent halo

around the inoculum site, and was classified as beta-hemolytic in the presence of a translucent halo around the colony (complete hemolysis), and alpha-hemolytic in the presence of a greenish halo (hemolysis incomplete).

Hydrophobicity assay

The hydrophobicity of the isolates was determined according to Anil et al. (2001) (4). Previously, the isolates were grown in Sabouraud broth and incubated for 24 h at 37 °C under constant agitation (200 rpm). Afterward, the yeasts were harvested by centrifugation and washed twice, and the cell density was then adjusted to an absorbance of 0.4 at 660 nm in 5 ml of 50 mM sodium phosphate buffer pH 7.4 containing 0.15 M NaCl. A volume of 1.5 ml of cell suspension was added to two sterile glass tubes (one test and one control). To test tubes, 0.5 ml of xylene were added, and after incubation for 3 minutes in a water bath at 37 °C the tubes were vigorously mixed for 2 min. Subsequent to 10 min under resting, the aqueous phase of the tubes was carefully removed, and the absorbance was measured at 520 nm. The cell surface hydrophobicity was expressed as the percentage of difference in optical density of the aqueous phase of the test compared to control.

Adherence assay

Adherence assay was performed as previously described (39) with some modifications. The isolates were cultured in Sabouraud broth and incubated for 24 h at 37 °C with constant agitation (200 rpm). Subsequently, the yeasts were washed (6,000 rpm for 10 minutes) with sterile NaCl 0.85% in centrifuge tubes, and the cell density was adjusted to $3.0x\ 10^7$ cells/ml in Sabouraud broth. About 20 µl of the cell suspension ($6x\ 10^5$ yeasts) were added to each well of polystyrene microtiter plates (96 wells) containing 180 µl of SDB.

After incubation at 37 °C for 1 h, the microtiter plates were washed with sterile deionized water and metabolic activity was then evaluated using 2,3-bis (2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide (XTT)-reduction assay according to Ramage et al., 2001. A 100 μL aliquot of XTT menadione [0.1 mg/ml XTT, 1 mM menadione (Sigma Chemical Co.)] was added to each well, and the plates were incubated in the dark for 2 h at 37 ºC before spectrophotometric readings at 490 nm with a microtiter plate reader (Universal Microplate Reader ELx 800, Bio-Tek Instruments).

Biofilm production assay

Biofilm production was evaluated in polystyrene microtiter plates (flat-bottomed 96-well) (Techno Plastic Products, Switzerland), as described by Shin et al. (2002) (75). The isolates were grown in Sabouraud medium supplemented with 8% glucose for 24 h at 37 ºC. Subsequently, the cells were washed (6,000 rpm for 10 minutes) with sterile NaCl 0.85% and the cell suspension was adjusted to 3.0x 10^7 cells/ml in Sabouraud broth. An aliquot of 20 μl of the cell suspension were transferred to each well containing 180 μl of SDB. The plates were incubated at 37 °C for 24 h without agitation and washed once with sterile distilled water. Approximately 200 μl of distilled water were added to each well before spectrophotometric readings at 405 nm with a microtiter plate reader (Universal Microplate Reader ELx 800, Bio-Tek Instruments, USA). The biofilm production was calculated according to the percentage of transmittance blocked (%Tbloc= %T value for each test sample was subtracted from the %T value for the reagent blank) and each isolate was scored according to the authors as negative (%Tbloc, < 5), 1+ (%Tbloc, 5 to 20), 2+ (%Tbloc, 20 to 35), 3+ (%Tbloc, 35 to 50), or 4+ (%Tbloc, >50).

Determination of biofilms metabolic activity by reduction of XTT

A second method used to determine biofilm production was performed according to Chandra et al. (2001) (16) with some modifications. Samples were cultured in Sabouraud medium for 24 h at 37 ºC and adjusted to 3.0x 10^7 cells/ml in RPMI medium. An aliquot of 20 µl taken from the culture cell was inoculated in each well containing 180 µl of RPMI medium, they were incubated at 37 °C for 24 h without agitation. The plates were washed once with sterile distilled water and metabolic activity was then evaluated using 2,3-bis (2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide (XTT)-reduction assay according to Ramage et al. (2001) (67). The optical density was measured by spectrophotometric reading 490 nm.

Statistical analysis

The results were analyzed by ANOVA One-way, and comparative analysis of the samples was generated by Tukey test with statistical significance of $p < 0.05$. Correlation of virulence factors was evaluated by Spearman test.

RESULTS

Characteristics of isolates

Phenotypic and molecular tests confirmed the samples as *C. tropicalis*. In the fluconazole susceptibility assay, isolates were classified as susceptible with MIC_{50} values ranging from 2 μg/ml to 4 μg/ml (Table 1).

Induction of fluconazole resistance

Induction of fluconazole resistance was successful only in bloodstream infection isolate, which initial MIC_{50} was 4 μg/ml. The sample was cultured with increasing concentrations of fluconazole [4 μg/ml (1x), 8 μg/ml (2x), 16 μg/ml (4x), 32 μg/ml (3x), 64 μg/ml (3x) and 80 μg/ml (2x)] until the 15th cultivation, when the resistance was detected.

Activity of hydrolytic enzymes

Phospholipase

In all experiments carried out no precipitation zone around the colony could be detected, so all isolates were considered negative to phospholipase activity.

Protease

In this study, all strains demonstrated protease activity. Dz values ranged from 0.46 ± 0.04 to 0.54 ± 0.03 (Table 2) and the protease activity of all isolates was classified as intermediate. Although no significant difference was detected, Dz values were

higher to bloodstream infection isolate.

Hemolytic activity

The hemolytic activity was partial, or alpha-hemolytic, to all isolates, so after 48 h of incubation a greenish halo surrounding the inoculum site was observed. No significant differences were found in the comparison of alpha-hemolysis halo of isolates from different sites.

Cell surface hydrophobicity

Related to the sources of the strains, CSH values were significantly different ($p< 0.05$), and were higher to the oral cavity isolate (71.76± 6.46) and lower to blood culture isolate (46.47± 6.22) (Table 2). CSH value for catheter tip sample was 60.41± 6.99.

Adherence and biofilm formation

In biofilm formation assays, no significant difference between the two methods used was observed. By Shin et al. (2002) (75) method, all isolates were classified as positive for biofilm production on polystyrene surface. Isolates from catheter tip and blood culture were scored as low biofilm producers [1+ (%Tbloc, 5 to 20)], and the oral cavity isolate was scored as high biofilm producer [3+ (%Tbloc, 35 to 50)]. In the XTT-reduction assay the optical density were respectively 0.680 ± 0.038; 0.215 ± 0.045 and 0.703 ± 0.027.

For the adherence assay on polystyrene surface, the isolates optical density ranged from 0.144 ± 0.021 to 0.151 ± 0.055 and no significant difference was observed.

Relationship between the virulence factors expressed by different strains of *C. tropicalis*

Considering the different virulence factors analyzed, significant correlation (p <0.05) was found between biofilm formation and CSH.

In the analysis of the isolates from different origins, there was a significant difference in biofilm formation and CSH. For both tests, oral cavity isolate produced higher values, followed by isolates from catheter and blood culture. Isolates from oral cavity and blood culture also differed to protease assay for which the second had a higher enzyme activity.

DISCUSSION

Over several years has been demonstrated the importance of *Candida* species as the etiologic agent of nosocomial infections. Besides the increase in frequency, new infections caused by these yeasts have been accompanied by greater severity and significant increase in morbidity and mortality. In addition, certain antifungal drugs currently available for these infections treatment have side effects, occasionally they are inefficient and can lead to rapid development of resistance, resulting in profound effects on health.

Although *C. albicans* represents the most common species in serious fungal infections, other species have emerged as important pathogens of these opportunistic infections (45). *C. tropicalis* is an important agent of candidiasis principally in leukemia patients (82). The virulence factors of this species most widely studied are: surface adhesion, hyphal development and protease activity (79). Other factors such as biofilm formation, production of phospholipase, hemolytic activity and cell surface hydrophobicity are also studied (22, 27, 36, 51).

One of the factors investigated in this work was the production of phospholipase. To this factor, all samples of *C. tropicalis* were classified as negative. The same result was observed in a study (69) using the same conditions. However, Clancy et al. (1998) (17) observed that non-*albicans* species produced extracellular phospholipase by colorimetric assays and in medium containing egg yolk. It was observed that 41% of the samples of *C. glabrata*, 50% of *C. parapsilosis*, 70% of *C. tropicalis* and 100% of *C. krusei* were phospholipase detectable. The discrepancy in the activity of phospholipase in *C.* non-*albicans* species observed by different studies may be due to the variation and number of isolates tested, or differences in the preparation of egg yolk plates methodology used to test the production of this enzyme (27). Furthermore, although the method in the plate is

traditional for the assessment of phospholipase enzymatic activity in *Candida* species, this may not be suitable for the identification of fungi isolates with low phospholipase production (26, 27).

Some studies show that the differential expression of certain virulence factors in isolates of *Candida* spp. may be related to their different origins. In a recent study, Li et al. (2003) (50) found no correlation between biofilm formation and origin of samples of *C. albicans* isolated from three different sources: oral cavity of healthy volunteers, vagina of candidiasis patients and the environment. The results suggested a great variability in biofilm formation. However, this variation was also found among isolates from the same source. In contrast, Kuhn et al. (2002) (44) showed by XTT-reduction assay the variation in biofilms isolated from invasive and non-invasive *C. albicans*. In this study, noninvasive isolates demonstrated a higher level of XTT activity than invasive isolates. Our results for biofilm formation, from two methods, were similar to those of Kuhn et al. (2002) (44). We observed that the values for biofilm formation in oral cavity sample were higher than the values for blood culture ($p< 0.01$).

This same difference found between the isolates was also observed for the cell surface hydrophobicity assay performed in this work. Similarly, one study with *C. albicans* samples found a discrepancy of CSH tested with polystyrene microspheres (33, 34), which could indicate a possible differential expression of cell surface hydrophobicity among the samples.

The CSH can be characterized as a manifestation of various molecules on the surface that are involved in virulence. The lack of these molecules may not apparently reduce the CSH, but may affect the virulence by influencing the range of sites to be infected (37). In addition, the CSH cannot be regarded as single virulence factor but, concurrently with others, contributes to virulence (20).

Singleton et al. (2001) (77) demonstrated that incubation of *C. albicans* glucanases eliminate cell wall proteins responsible for the cell surface hydrophobicity. Moreover, these authors have cloned a gene (CSH1) that seems to present homology with other proteins in the cell wall of *C. albicans* and seems to contribute to the cell surface hydrophobicity. CSH1 null mutants resulted in a cell surface hydrophobicity reduction when compared to the wild sample.

It is unknown how this factor specifically affects virulence but hydrophobic cells appear to have greater adherence to host tissue and substrates (including mucins, epithelial cells, endothelial cells and extracellular matrix proteins) and a higher resistance to phagocytosis (5, 21, 28, 35, 53).

The biofilm can occur in response to various signals, such as: high cell density, pH, lack of nutrients and physical environmental stress. The ability of biofilm formation is also related to the type of surface used for the initial attachment. Several authors have shown that microorganisms adhere more easily on hydrophobic and polar surfaces, such as Teflon and other plastics (10, 25, 65).

In the laboratory, a typical model of biofilm formation by fungi involves three steps: attachment, growth and biofilm maturation (7, 15). In a study of different isolates of *C. albicans*, a correlation between biofilm formation and cell surface hydrophobicity was found (50). However, the contribution of CSH in the process of biofilm formation in *Candida* spp. is still unknown. The results of this study reinforced others on which hydrophobicity proved to be an important determinant for biofilm formation.

In previous studies with different *Candida* species was also observed the relationship between adherence to plastic surfaces and cell surface hydrophobicity (23, 32, 43, 70, 76). This relationship was also observed in the study in which knockout of the gene CHS1 resulted in a decrease in measurable cell surface hydrophobicity and in adhesion of *C.*

albicans to fibronectin (77).

Another finding of this work was the *C. tropicalis* ability to rapidly develop fluconazole resistance. Similar experiments have been performed with *C. albicans* using azoles derivatives. In Moran et al. (1997) (56) study, the attempts to generate fluconazole-resistant derivatives from the fluconazole-sensitive *C. albicans* oral sample were unsuccessful. And in experiments performed by Vanden Bossche et al. (1990) (12), with *C. albicans*, the resistance of ketoconazole, another type of azole derivative, slight change in azole susceptibility was detected following 148 sequential subcultures. In contrast, in our study, fluconazole resistance was detected at the 15th subculture.

Although a limited number of *C. tropicalis* samples has been examined in the present study, the results reinforce the relationship between origin of the isolates and expression of virulence factors. The oral cavity sample presented the strongest biofilm formation and showed greater CSH, followed by samples isolated from catheter and blood culture. The samples isolated from blood culture showed higher enzyme activity. In addition, correlation was observed between biofilm formation and cell surface hydrophobicity.

As mentioned, the species C. non-*albicans* have been isolated with high frequency and in despite of the numerous studies developed for *Candida albicans*, little is known about *C. tropicalis*. Therefore, further studies are required to determine what mechanisms are involved in the pathogenicity of *C. tropicalis* strains, what would certainly be of eminent importance for better understanding of the associated pathologies, and for the development of more specific and effective treatment strategies.

REFERENCES

1.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, p.2483–2489, 2002.

2.AL-FATTANI, M.A.; DOUGLAS, L.J. Biofilm matrix of *Candida albicans* and *Candida tropicalis*: chemical composition and role in drug resistance. *Journal of Medical Microbiology*, v.55, p.999–1008, 2006.

3.ANDES, D.; NETT, J.; OSHEL, P.; ALBRECHT, R.; MARCHILLO, K.; PITULA, A. Development and characterization of an *in vivo* central venous catheter *Candida albicans* biofilm model. *Infection and Immunity*, v.72, p.6023–6031, 2004.

4.ANIL, S.; ELLEPOLA, A.N.B.; SAMARANAYAKE, L.P. The impact of polyene, azole, and DNA analogue antimycotics on the cell surface hydrophobicity of *Candida albicans* and *Candida tropicalis* in HIV infection. *Mycopathologia*, v.153, p.179–185, 2001.

5.ANTLEY, P.P.; HAZEN, K.C. Role of yeast cell growth temperature on *Candida albicans* virulence in mice. *Infection and Immunity*, v.56, p.2884–2890, 1988.

6.BAILLIE, G.S.; DOUGLAS, L.J. Effect of growth rate on resistance of *Candida albicans* biofilms to antifungal agents. *Antimicrobial Agents and Chemotherapy*, v.42, p.1900–1905, 1998.

7.BAILLIE, G.S.; DOUGLAS, L.J. *Candida* biofilms and their susceptibility to antifungal agents. *Methods in Enzymology,* v.310, p.644–656, 1999.

8.BARCHIESI, F.; MARACEI, M.; BALDASSARRI, I.; SPREGHINI, E.; GIANNINI, D.; SCALISE, G. Tolerance to amphotericin B in clinical isolates of *Candida tropicalis*. *Diagnostic Microbiology and Infectious Disease*, v.50, p.179–185, 2004.

9.BÉLANGER, M.; BEGIN, C.; JACQUES, M. Lipopolysaccharides of *Actinobacillus pleuropneumoniae* bind pig hemoglobin. *Infection and Immunity*, v.63, p.656–62, 1995.

10.BENDINGER, B.; RIJNAARTS, H.H.M.; ALTENDORF, K.; ZEHNDER, A.J.B. Physicochemical cell surface and adhesive properties of coryneform bacteria related to the presence and chain length of mycolic acids. *Applied and Environmental Microbiology*, v.59, p.3973–3977, 1993.

11.BORG-VON ZEPELIN, M.; BEGGAH, S.; BOGGIAN, K.; SANGLARD, D.; MONOD, M. The expression of the secreted aspartyl proteinases Sap4 to Sap6 from *Candida albicans* in murine macrophages. *Molecular Microbiology*, v.28, p.543–554, 1998.

12.BOSSCHE, V.; MARICHAL, P.; GORRENS, J.; BELLENS, D.; MOEREELS, H.; JANSSEN, P.A.J. Mutation in cytochrome P-450-dependent 14-demethylase results in decreased affinity for azole antifungals. *Biochemical Society*, v.18, p.56–59, 1990.

13.BUDTZ-JORGENSEN, E.; MOJON, E.; RENTSCH, A.; DESLAURIERS, N. Effects of an oral healthy program on the occurrence of oral candidosis in a long-term care facility. *Community Dentistry and Oral Epidemiology*, v.28, n.2, p.141–149, 2000.

14.CALDERONE, R.A.; FONZI, W.A. Virulence factors of *Candida albicans*. *Trends in Microbiology*, v.9, n.7, p.327–335, 2001.

15.CHANDRA, J.; KUHN, D.M.; MUKHERJEE, P.K.; HOYER, L.L.; MCCORMICK, T.; GHANNOUM, M.A. Biofilm formation by the fungal pathogen *Candida albicans*: development, architecture, and drug resistance. *Journal of Bacteriology*, v.183, p.5385–5394, 2001a.

16.CHANDRA, J.; MUKHERJEE, P.K.; LEIDICH, S.D.; FADDOUL, F.F.; HOYER, L.L.; DOUGLAS, L.J.; GHANNOUM, M.A. Antifungal resistance of Candidal biofilms formed on denture acrylic in vitro. *Journal of Dental Research*, v.80, p.903–908, 2001b.

17.CLANCY, C.J.; GHANNOUM, M.A.; NGUYEN, M.H. In: 36th Annual Meeting of the Infectious Diseases Society of America, 1998: 317.

18.COLOMBO, A.L.; GUIMARÃES, T. Epidemiologia das infecções hematogênicas por *Candida* spp. *Revista da Sociedade Brasileira de Medicina Tropical*, v.36, n.5, p.599–607, 2003.

19.COLOMBO, A.L.; NUCCI, M.; PARK, B.J.; NOUER, S.A.; ARTHINGTON-SKAGGS, B.; DA MATTA, D.A.; WARNOCK, D.; MORGAN, J. Epidemiology of candidemia in Brazil: a nationwide sentinel surveillance of candidemia in eleven Medical Centers. *Journal of Clinical Microbiology*, v.44, n.8, p.2816–2823, 2006.

20.CUTLER, J.E. Putative virulence factors of *Candida albicans*. *Annual Review of Microbiology*, v.45, p.187–218, 1991.

21.DE REPENTIGNY, L.; AUMOUNT, F.; BERNARD, K.; BELHUMEUR, P. Characterization of binding of *Candida albicans* to small intestinal mucin and its role in adherence to mucosal epithelial cells. *Infection and Immunity*, v.68, p.3172–3179, 2000.

22.DOUGLAS, L.J. *Candida* biofilms and their role in infection. *Trends in Microbiology*, v.11, p.30–36, 2003.

23.EL-AZIZI, M.; KHARDORI, N. Factors influencing adherence of *Candida* spp. to host tissues and plastic surfaces. *Indian Journal of Experimental Biology*, v.37, p.941–951, 1999.

24.FELK, A.; KRETSCHMAR, M.; ALBRECHT, A.; SCHALLER, M.; BEINHAUER, S.; NICHTERLEIN, T.; SANGLARD, D.; KORTING, H.C.; SCHÄFER, W.; HUBE, B. *Candida albicans* hyphal formation and the expression of the Efg1-regulated

proteinases Sap4 to Sap6 are required for the invasion of parenchymal organs. *Infection and Immunity*, v.70, n.7, p.3689–3700, 2002.

25.FLETCHER, M.; LOEB, G.I. Influence of substratum characteristics on the attachment of a marine pseudomonad to solid surfaces. *Applied and Environmental Microbiology*, v.37, p.67–72, 1979.

26.FU, Y.; IBRAHIM, A.S.; FONZI, W.; ZHOU, X.; RAMOS, C.F.; GHANNOUM, M.A. Cloning and characterization of a gene (*LIP1*) which encodes a lipase from the pathogenic yeast *Candida albicans*. *Microbiology*, v.143, p.331–340, 1997.

27.GHANNOUM, M.A. Potential role of phospholipases in virulence and fungal pathogenesis. *Clinical Microbiology Reviews*, v.13, p.122–143, 2000.

28.GLEE, P.M.; SUNDSTROM, P.; HAZEN, K.C. Expression of surface hydrophobic proteins by *Candida albicans* in vivo. *Infection and Immunity*, v.63, p.1373–1379, 1995.

29.GUARRO, J.; GENE, J.; STCHIGEL, A.M. Developments in Fungal Taxonomy. *Clinical Microbiology Reviews,* v.12, p.454–500, 1999.

30.HABERLAND-CARRODEGUAS, C.; ALLEN, C.M.; BECK, F.M.; BUESCHILING, W.J.; KOLETAR, S.L.; SUNDSTRON, P. Prevalence of fluconazole-resistant strains of *C. albicans* in otherwise healthy outpatients. *Journal of Oral Pathology & Medicine*, v.31, p.99–105, 2002.

31.HAWSER, S.P.; DOUGLAS, L.J. Resistance of *Candida albicans* biofilms to antifungal agents *in vitro*. *Antimicrobial Agents and Chemotherapy*, v.39, p.2128–2131, 1995.

32.HAZEN, K.C.; PLOTKIN, B.J.; KLIMAS, D.M. Influence of growth on cell surface hydrophobicity of *Candida albicans* and *Candida glabrata*. *Infection and Immunity*, v.54, p.269–271, 1986.

33.HAZEN, B.W.; HAZEN, K.C. Dynamic expression of cell surface hydrophobicity during initial yeast cell growth and before germ tube formation of *Candida albicans*. *Infection and Immunity*, v.56, p.2521–2525, 1988.

34.HAZEN, K.C. Participation of yeast cell surface hydrophobicity in adherence of *Candida albicans* to human epithelial cells. *Infection and Immunity*, v.57, p.1894–1900, 1989.

35.HAZEN, K.C.; BRAWNER, D.L.; RIESSELMAN, M.H.; JUTILA, M.A.; CUTLER, J.E. Differential adherence of hydrophobic and hydrophilic *Candida albicans* yeast cells to mouse tissues. *Infection and Immunity*, v.59, n.3, p.907–912, 1991.

36.HAZEN, K.C.; MANDELL, G.; COLEMAN, E.; WU, G. Influence of fluconazole at subinhibitory concentrations on cell surface hydrophobicity and phagocytosis of *Candida albicans*. *FEMS Microbiology Letters*, v.183, p.89–94, 2000.

37.HAZEN, K.C.; WU, J.G.; MASUOKA, J. Comparison of the hydrophobic properties of *Candida albicans* and *Candida dubliniensis*. *Infection and Immunity*, v.69, n.2, p.779–786, 2001.

38.IZUMI, E.; YAMADA-OGATTA, S.F. Análise da atividade proteolítica extracelular de isolados clínicos de *Candida albicans*. Universidade Estadual de Londrina, Monografia de Conclusão de Curso, Ciências Biológicas, 50p., 2004.

39.JIN, Y.; SAMARANAYAKE, Y.H.; YIP, H.K.; SAMARANAYAKE, L.P.Characterization of switch phenotypes in *Candida albicans* biofilms. *Mycopathologia*, v.160, p.191–200, 2005.

40.KAM, L.W.; LIN, J.D. Management of systemic candidal infections in the intensive care unit. *American Journal of Health-System Pharmacy*, v.59, p.33–41, 2002.

41.KAMINISHI, H.; MIYAGUCHI, H.; TAMAKI, T.; SUENAGA, N.; HISAMATSU, M.; MIHASHI, I.; MATSUMOTO, H.; MAEDA, H.; HAGIHARA, Y. Degradation of humoral host defence by *Candida albicans* proteinase. *Infection and Immunity*, v.63, n.3, p.984–988, 1995.

42.KANTARCIOGLU, A.S.; YUCEL, A. Phospholipase and protease activities in clinical *Candida* isolates with reference to the sources of strains. *Mycoses*, v.45, p.160–165, 2002.

43.KLOTZ, S.A.; DRUTZ, D.J.; ZAJIC, J.E. Factors governing adherence of *Candida* species to plastic surfaces. *Infection and Immunity*, v.50, p.97–101, 1985.

44.KUHN, D.M.; CHANDRA, J.; MUKHERJEE, P.K; GHANNOUM, M.A. Comparison of biofilms formed by *Candida albicans* and *Candida parapsilosis* on bioprosthetic surfaces. *Infection and Immunity*, v.70, p.878–888, 2002a.

45.KUHN, D.M.; GEORGE, T.; CHANDRA, J.; MUKHERIEE, P.K.; GHANNOUM, M.A. Antifungal susceptibility of *Candida* biofilms: unique efficacy of amphotericin B lipid formulations and echinocandins. *Antimicrobial Agents and Chemotherapy*, v.46, p.1773–1780, 2002b.

46.KUHN, D.M.; GHANNOUM, M.A. *Candida* biofilms: antifungal resistance and emerging therapeutic options. *Current Opinion in Investigational Drugs*, v.5, p.186–197, 2004.

47.KURTZMAN, C.P.; FELL, J.W. Definition, classification and nomenclature of the yeasts. In: The Yeasts, A Taxonomic Study, C.P. Kurtzman and J.W. Fell (eds.) 4th edn. pp. 3-5. Elsevier Science B.V., Amsterdam. 1998.

48.KVAAL, C.A.; LACHCKE, S.A.; SRIKANTHA, T.; DANIELS, K.; McCOY, J.; SOLL, D.R. Misexpression of the opaque-phase-specific gene *PEP1* (*SAP1*) in the white phase of *Candida albicans* confers increased virulence in a mouse model of cutaneous infection. *Infection and Immunity*, v.67, n.12, p.6652–6662, 1999.

49.LEE, F.J.S. Modified protocol for yeast DNA mini-preparation. *Biotecniques*, v.5, p.677, 1992.

50.LI, F.; PALECEK, S.P. *EAP1*, a *Candida albicans* gene involved in binding human epithelial cells. *Eukaryot Cell*, v.2, n.6, p.1266–1273, 2003.

51.LUO, G.; SAMARANAYAKE, L.P.; YAU, J.Y.Y. *Candida* species exhibit differential in vitro hemolytic activities. *Journal of Clinical Microbiology*, v.39, p.2971–2974, 2001.

52.MACDONALD, F. Secretion of inducible proteinase by pathogenic *Candida* species. *Journal of medical and veterinary mycology*, v.22, p.79–82, 1984.

53.MASUOKA, J.; WU, G.; GLEE, P.M.; HAZEN, K.C. Inhibition of *Candida albicans* attachment to extracellular matrix by antibodies which recognize hydrophobic cell wall proteins. *FEMS Immunology and Medical Microbiology*, v.24, p.421–429, 1999.

54.MAZA, J.L.; ELGUEZABAL, N.; PRADO, C.; ELLACURIA, J.; SOLER, I.; PONTÓN, J. *Candida albicans* adherence to resin-composites restorative dental materials: influence of role human saliva. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology & Endodontics*, v.94, n.5, p.589–92, 2002.

55.MONOD, M.; BORG-VON ZEPELIN, M. Secreted proteinases and their virulence mechanism of *Candida albicans*. *Chemical Immunology*, v.81, p.114–118, 2002.

56.MORAN, P.G.; SULLIVAN, D.J.; HENMAN, M.C.; MCCREADY, C.E.; HARRINGTON, B.J.; SHANLEY, D.B.; COLEMAN, D.C. Antifungal drug susceptibilities of oral *Candida dubliniensis* isolates from Human Immunodeficiency Virus (HIV)-infected and non- HIV-infected subjects and generation of stable fluconazole-resistant derivatives in vitro. *Antimicrobial agents and chemotherapy*, v.41, n.3, p.617–623, 1997.

57.MORSCHHÄUSER, J.; VIRKOLA, R.; KORHONEN, T.K.; HACKER, J. Degradation of human subendothelial extracellular matrix by proteinase-secreting *Candida albicans*. *FEMS Microbiology Letters*, v.153, p.349–355, 1997.

58.NAGLIK, J.R.; CHALLACOMBE, S.J.; HUBE, B. *Candida albicans* secreted aspartyl proteinases in virulence and pathogenesis. *Microbiology and Molecular Biology Reviews*, v.67, p.400–428, 2003.

59.NCCLS - Método de Referência para Testes de Diluição em Caldo para a Determinação da Sensibilidade a Terapia Antifúngica das Leveduras. Norma Aprovada - Segunda Edição. NCCLS document M27-A2 [ISBN 1-56238-469-4]. NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 Estados Unidos, 2002.

60.NUCCI, M.; COLOMBO, A.L. Candidemia due to *Candida tropicalis*: clinical, epidemiologic, and microbiologic characteristics of 188 episodes occurring in tertiary care hospitals. *Diagnostic Microbiology and Infectious Disease*, v.58, n.1, p.77–82, 2007.

61.OTTO, B.R.; VERWEIJ-VAN VUGHT, A.M.; MACLAREN, D.M. Transferrins and heme compounds as iron sources for pathogenic bacteria. *Critical reviews in Microbiology*, v.18, p.217–233, 1992.

62.PFALLER, M.A.; DIEKEMA, D.J.; MESSER, S.A.; BOYKEN, L.; HOLLIS, R.J.; JONES, R.N. *In vitro* susceptibilities of rare *Candida* bloodstream isolates to ravuconazole and three comparative antifungal agents. *Diagnostic Microbiology and Infectious Disease*, v.48, p.101–105, 2004.

63.PONTECORVO, G.; ROPER, J.A.; HEMMONS, L.M.; McDONALD, K.D.; BUFTON, W.F.C. The genetics of *Aspergillus nidulans*. *Advances in Genetics*, v.5, p.141–238, 1953.

64.PRICE, M.F.; WILKINSON, I.A.; GENTRY, L.O. Plate method for detection of phospholipase activity in *Candida albicans*. *Sabouraudia*, v.20, p.7–14, 1982.

65.PRINGLE, J.H.; FLETCHER, M. Influence of substratum wettability on attachment of freshwater bacteria to solid surfaces. *Applied and Environmental Microbiology*, v.45, p.811–817, 1983.

66.RAMAGE, G.; SAVILLE, S.P.; THOMAS, D.P.; LOPEZ-RIBOT, J. *Candida* Biofilms: an Update. *Eukaryotic Cell*, v.4, n.4, p.633–638, 2005.

67.RAMAGE, G.; WALLE, K.V.; WICKES, B.L.; LÓPEZ-RIBOT, J. Biofilm formation by *Candida dubliniensis*, *Journal of Clinical Microbiology*, v.39, p.3234-3240, 2001.

68.RÜCHEL, R. Cleavage of immunoglobulins by pathogenic yeasts of the genus *Candida*. *Microbiological Sciences*, v.3, p.316–319, 1986.

69.SAMARANAYAKE, L.P.; RAESIDE, J.M.; MACFARLANE, T.W. Factors affecting the phospholipase activity of *Candida* species *in vitro*. *Sabouraudia*, v.22, p.201–207, 1984.

70.SAMARANAYAKE, Y.H.; WU, P.C.; SAMARANAYAKE, L.P; SO, M. Relationship between the cell surface hydrophobicity and adherence of *Candida krusei* and *Candida albicans* to epithelia and denture acrylic surfaces. *APMIS*, v.103, p.707–713, 1995.

71.SAMRA, Z.; YARDENI, M.; PELED, N.; BISHARA, J. Species distribution and antifungal susceptibility of *Candida* bloodstream isolates in a tertiary medical center in Israel, *European Journal of Clinical Microbiology & Infectious Diseases*, v.24, p.592–595, 2005.

72.SCHALLER, M.; HUBE, B.; OLLERT, M.W.; SCHÄFER, W.; BORG-VON ZEPELIN, M.; THOMA-GREBER, E.; KORTING, H.C. In vivo expression and localization of Candida albicans secreted aspartyl proteinase during oral. *Journal of Investigative Dermatology*, v.112, p.383-386. 1999.

73.SCHALLER, M.; SCHACKERT, C.; KORTING, H.C.; JANUSCHKE, E.; HUBE, B. Invasion of *Candida albicans* correlates with expression of secreted aspartic proteinases during experimental infection of human epidermis. *Journal of Investigative Dermatology*, v.114, p.712–717, 2000.

74.SCHALLER, M.; BEIN, M.; KORTING, H.C.; BAUR, S.; HAMM, G.; MONOD, M.; BEINHAUER, S.; HUBE, B. The secreted aspartyl proteinases Sap1 and Sap2 cause

tissue damage in an in vitro model of vaginal candidiasis based on reconstituted human vaginal epithelium. *Infection and Immunity*, v.71, n.6, p.3227–3234, 2003.

75. SHIN, J.H.; KEE, S.J.; SHIN, M.G.; KIM, S.H.; SHIN, D.H.; LEE, S.K.; SUH, S.P.; RYANG, D.W. Biofilm production by isolates of *Candida* species recovered from nonneutropenic patients: comparison of bloodstream isolates from other sources. *Journal of Clinical Microbiology*, v.40, n.4, p.1244–1248, 2002.

76. SILVA, T.M.; GLEE, P.M.; HAZEN, K.C. Influence of cell surface hydrophobicity on attachment of *Candida albicans* to extracellular matrix proteins. *Journal of medical and veterinary mycology*, v.33, p.117–122, 1995.

77. SINGLETON, D.R.; MASUOKA, J.; HAZEN, K.C. Cloning and analysis of a *Candida albicans* gene that affects cell surface hydrophobicity. *Journal of Bacteriology*, v.183, n.12, p.3582–3588, 2001.

78. SOLL, D.R. *Candida* commensalism and virulence: the evolution of phenotipic plasticity. *Acta Tropica*, v.81, p.101–110, 2002.

79. SYMERSKY, J.; MONOD, M.; FOUNDLING, S.I. High-Resolution Structure of the Extracellular Aspartic Proteinase from *Candida tropicalis* Yeast. *Biochemistry*, v.36, p.12700–12710, 1997.

80. VIDOTTO, V.; KOGA-ITO, C.Y.; MILANO, R.; FIANCHINO, B.; PONTÓN, J. Correlation between germ-tube production, phospholipase activity and serotype distribution in *Candida albicans*. *Revista Iberoamericana de Micología*, v.16, p.208–210, 1999.

81. WHITE, T.C.; HOLLEMAN, S.; DY, F.; MIRELS, L.F.; STEVENS, D.A. Resistance mechanisms in clinical isolates of *Candida albicans*. *Antimicrobial Agents and Chemotherapy*, v.46, n.6, p.1704–1713, 2002.

82. WINGARD, J.R. Importance of *Candida* species other than *C. albicans* as pathogens in oncology patients. *Clinical Infectious Diseases*, v.20, p.115–125, 1995.

83. YANG, Y.L.; HO, Y.A.; CHENG, H.H.; HO, M.; LO, H.J. Susceptibilities of *Candida* species to amphotericin B and fluconazole: the emergence of fluconazole resistance in the *Candida tropicalis*. *Infection Control and Hospital Epidemiology*, v.25, p.60–64, 2004.

84. ZAUGG, C.; ZEPELIN, M.B.; REICHARD, U.; SANGLARD, D.; MONOD, M. Secreted Aspartic Proteinase Family of *Candida tropicalis*. *Infection and Immunity*, v.69, n.1, p.405–412, 2001.

Table 1 - MICs range for fluconazole activity of the *Candida tropicalis* isolates

Isolate[1]	CIM_{50}
B	2 µg/ml
C	4 µg/ml
H	4 µg/ml

*Isolates analyzed: (B) isolate of the oral cavity, (C) catheter tip, (H) blood culture. The MICs were determined according to NCCLS, 2002 (59).

Table 2 - Putative virulence factors of *Candida tropicalis* isolates tested *in vitro*

	Phospholipase[a]	Protease[a]	Hemolisine[b]	Adherence[e]	Biofilm[d]	Biofilm[e]	CSH[c]
B	-	0.54±0.03	0.27±0.01	0.144±0.021	39.10±4.22	0.703±0.027	71.76±6.46
C	-	0.49±0.04	0.26±0.01	0.151±0.055	18.30±2.43	0.680±0.038	60.41±6.99
H	-	0.46±0.04	0.25±0.02	0.148±0.048	13.63±3.42	0.215±0.045	46.47±6.22

*Isolates analyzed: (B) isolate of the oral cavity, (C) catheter tip, (H) blood culture. The values represent the arithmetic mean and standard deviation of three experiments in duplicate. (-) Phospholipase negative.

a Enzymatic activity of protease and phospholipase determined on plates containing 0.6% skim milk and 4.0% egg yolk respectively. Dz or Pz = 1, null activity; 0.64 ≤ Dz/Pz <1, low activity; ≤ 0:30 Dz/Pz <0.64, intermediate activity; Dz/Pz <0.30, high activity. Dz or Pz index determined by the ratio between colony diameter and the diameter of the zone of degradation / precipitation.

b Hemolytic activity was determined in SDA medium supplemented with 3% glucose and 5% sheep blood. Evaluation of Pz was determined by alpha-hemolytic activity.

c Percentage of cell surface hydrophobicity (CSH) determined by the difference in the optical density of the aqueous phase between test and control.

d Biofilm formation in microtiter plates (96 wells). % TBlocked (percent transmittance blocked) is the amount of light blocked after going through each well. The production of biofilm was evaluated as negative (% T bloc <5), + (% T bloc = 5-20); + + (% T bloc= 20-35); + + + (% Tbloc = 35-50); + + + + (% T bloc ≥ 50).

e Adherence assay and biofilm formation performed by XTT assay. In the adherence assay the yeast cells were incubated for1 hour while in the biofilm formation the incubation was of 24 h.

ANEXO B

Artigo

Effect of fluconazole on putative virulence factors and proteome of *Candida tropicalis*

Effect of fluconazole on putative virulence factors and proteome of *Candida tropicalis*

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INTRODUCTION

Yeasts associated with human infections are ubiquitous in the environment and some of them are commensal inhabitants of human body. These opportunistic microorganisms are often harmless, however under favorable conditions they can cause acute to chronic infections (22). *Candida* species are opportunistic agents most frequently isolated from these infections (37), and represent 80% of the healthcare associated fungal infections (9).

Although *Candida albicans* correspond to the most prevalent and studied species of these yeasts, *Candida* non-*albicans* species have currently emerged and *C. tropicalis* has been reported as the second or third most common etiologic agent of candidemia in cancer patients, mainly in patients with leukemia (41). In addition to the increased incidence *C. tropicalis* is associated to a high mortality rate of patients (11) and can display variable susceptibility to antifungal agents used in medical practice (3, 16, 30, 35).

It has been showed that resistance to fluconazole can be associated with prior exposure to the antifungal and this process can also lead to cross-resistance to voriconazole (10). This mechanism of late resistance could be responsible for the emergence of azole resistance seen in recent years, especially in *Candida* spp. isolated from HIV-positive patients in which fluconazole is the primarily antifungal therapy used (40). Thus, the increased use of azole combined with the fact that these drugs are fungistatic apparently led to the emergence of azole resistance (14, 25, 26, 43).

The fluconazole antifungal effect is related to the inhibition of fungal ergosterol biosynthesis, mainly the enzyme lanosterol 14-alpha-demethylase, present in the cell membrane, preventing the fungal cell growth. *Candida* species are usually susceptible to this antifungal, however many mechanisms such as the increased expression of ABC (ATP-

binding cassette) and MSF (major facilitator superfamily) transporters genes; the increased expression or mutations on the sequence of genes involved in biosynthesis of ergosterol, such as *ERG11* (24), can lead to fluconazole resistance. In the case of *C. tropicalis* the mechanism has been associated with increased expression of MDR1 gene (Ct*MDR1*), which encodes a protein of MSF type (38).

Candida spp. virulence factors have attracted attention as potential targets for the development of new therapeutic interventions against candidiasis (29). Some examples of virulence factors associated with these yeasts are: adhesion to host cells and tissues, morphogenesis, phenotypic switching (6) and biofilm formation (12). Likewise, the secretion of hydrolytic enzymes also contributes to the pathogenicity of the microorganism. These hydrolytic enzymes produced by microorganisms are related to the host tissues invasion (18, 23, 27, 39), and also to the immune system evasion (4, 17, 34). Therefore, in the present study we analyze the influence of fluconazole on the expression of virulence factors in *C. tropicalis* and we examine changes in *C. tropicalis* proteome after *in vitro* fluconazole resistance development.

MATERIALS AND METHODS

Candida tropicalis isolates and growth conditions

C. tropicalis was isolated from bloodstream infection, at the Departamento de Análises Clínicas - LEPAC, Universidade Estadual de Maringá, and it was maintained in Sabouraud Dextrose Agar (SDA) at 4 ° C, with monthly samplings.

Molecular identification

The isolate was presumptively identified as *C. tropicalis* by classical phenotypic methods (19) and the species identification was confirmed by semi-nested PCR as described by Ahmad et al. (2002) (1). Genomic DNA extraction was performed according to Lee et al. (1992) (20).

Fluconazole susceptibility testing

The fluconazole minimum inhibitory concentration (MIC) was determined by broth microdilution according to standards established by the Clinical Laboratory Standard Institute (CLSI, 2002) (28). The reference samples used were: *C. albicans* (ATCC 10231), *C. parapsilosis* (ATCC 22019) and *C. tropicalis* (ATCC 750). According to the CLSI guidelines, the breakpoints for interpretation of the results were MICs≥ 64 μg/ml for resistant isolate, 16- 32 μg/ml for dose-dependent and MICs≤ 8 μg/ml for susceptible.

Induction of fluconazole resistance

For the induction of resistance, the sample was cultured in RPMI-1640 with exposure of increasing concentrations of fluconazole (4 μg/ml, 8 μg/ml, 16 μg/ml, 32 μg/ml, 64 μg/ml) until the detection of resistance using the methodology described before. An initial inoculum of 5×10^3 cells/ml was cultured in medium containing 4 μg/ml of fluconazole at 35 ºC for 24 h. About 1/3 volume of this culture was transferred to another fresh medium with increased concentration of fluconazole, and this procedure was repeated successively to obtain a population of cells resistant to the antifungal drug.

Analysis of virulence factors

To assess the influence of fluconazole on the expression of virulence factors, fluconazole resistant (FLC-resistant) and the susceptible parental strain (FLC-susceptible) incubated previously, for 1h our with subinhibitory fluconazole concentration (2μg/ml) were tested to protease, phospholipase and hemolytic activity, cell surface hydrophobicity, adherence capacity and biofilm formation. After fluconazole exposure, the cells were washed twice before proceeding to the tests. The experiments were carried out in duplicate on three different occasions.

Phospholipase

Phospholipase activity was assayed on plates containing Sabouraud dextrose agar pH 4.5 supplemented with egg yolk (4%), NaCl 20 g/l and $CaCl_2$. $2H_2O$ 1 g/l. Previously, the samples were cultivated on Sabouraud broth for 24 h with constant agitation (200 rpm). After the incubation period, the cell concentration was determined by direct counting of the culture using a hemocytometer counting chamber (Improved Neubauer

Chamber). A volume of 10 µl (approximately 1.0× 10^6 cells) was inoculated in spot on the surface of phospholipase plates. For observation of cell growth and activity of extracellular phospholipase, the plates were incubated at 37 °C for 96 h and analyzed every 24 h. Isolates with dense white zone of precipitation around the colony after incubation period were considered positive for phospholipase. The phospholipase activity was estimated by the ratio of the colony diameter and the diameter of the precipitation zone around the colony (Pz), as described in Price et al. (1982) (32) (Pz = 1, null activity; 0.64≤ Pz< 1, low activity; 0.30≤ Pz< 0.64, intermediate activity; Pz< 0.30, high activity).

Protease

Protease activity was determined on plates containing minimal medium pH 6.8 supplemented with skim milk (0.6%). Previously, isolates were cultured at 37 ºC for 18 h in minimal medium (MM) broth (Pontecorvo et al., 1953) (31) supplemented with 0.6% skim milk, pH 4.0, to induce the secretion of enzyme. An inoculum of 10 ul (approximately 1.0× 10^6 cells) was placed in holes of approximately 3 mm of diameter drilled in the agar according to Izumi and Yamada-Ogatta (2004) (13). For observation of cell growth and extracellular proteolytic activity, the plates were incubated at 37 °C for 72 h, and analyzed every 24 h. After the incubation period, the samples that showed a degradation zone around the colony were positive for protease production. The proteolytic activity was estimated by the ratio of colony diameter and the diameter of degradation zone around the colony (Dz), as described previously.

Hemolytic activity

Hemolytic activity was evaluated according to Luo et al. (2001) (21).

Subsequent to cell culture, an inoculum of 10 µl (approximately 1.0×10^6 cells) was placed on the surface of blood agar (SDA medium supplemented with 3% glucose and 5% fresh sheep blood). The plates were incubated at 37 °C over 24- 48 h in microaerophilic chamber. Positive hemolytic activity was indicated by the presence of a distinct translucent halo around the inoculum site, and was classified as beta-hemolytic in the presence of a translucent halo around the colony (complete hemolysis), and alpha-hemolytic in the presence of a greenish halo (incomplete hemolysis).

Hydrophobicity assay

The hydrophobicity of the isolates was determined according to Anil et al. (2001) (2). Previously, the isolates were grown in Sabouraud broth and incubated for 24 h at 37 °C under constant agitation (200 rpm). Afterward, the yeasts were harvested by centrifugation and washed twice, and the cell density was then adjusted to an absorbance of 0.4 at 660 nm in 5 ml of 50 mM sodium phosphate buffer pH 7.4 containing 0.15 M NaCl. A volume of 1.5 ml of cell suspension was added to two sterile glass tubes (one test and one control). To test tubes, 0.5 ml of xylene were added, and after incubation for 3 minutes in water bath at 37 °C the tubes were vigorously mixed for 2 min. The aqueous phase of the tubes was carefully removed, and the absorbance was measured at 520 nm. The cell surface hydrophobicity was expressed as the percentage of difference in optical density of the aqueous phase of the test compared to control.

Adherence assay

Adherence assay was performed as previously described by Jin et al. (2005) (15) with some modifications. The isolates were cultured in Sabouraud broth and incubated

for 24 h at 37 °C with constant agitation (200 rpm). Subsequently, the yeasts were washed (6,000 rpm for 10 minutes) with sterile 0.85% NaCl, and the cell density was adjusted to 3 x 10^7 cells/ml in Sabouraud broth. An aliquot of 20 µl of the cell suspension (6 x 10^5 yeasts) were added to each well of polystyrene microtiter plates (96 wells) containing 180 µl of SDB. After incubation at 37 °C for 1 h, the microtiter plates were washed with sterile deionized water and metabolic activity was then evaluated using 2,3-bis (2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide (XTT)-reduction assay according to Ramage et al., 2001. A 100 µL aliquot of XTT menadione [0.1 mg/ml XTT, 1 mM menadione (Sigma Chemical Co.)] was added to each well, and the plates were incubated in the dark for 2 h at 37 ºC before spectrophotometric readings at 490 nm with a microtiter plate reader (Universal Microplate Reader ELx 800, Bio-Tek Instruments).

Adherence assay on biotic surface

In this assay, the adherence capacity to VERO cells (kidney of African green monkey) was evaluated. Prior to adherence assay, VERO cells (4 x 10^5 cells/ml) were incubated in the microtiter plates (24 wells) with Dulbecco's Modified Eagle Medium (DMEM) for 48 h in the presence of CO_2 (5%) at 37 °C. Approximately 200 µl of the yeast suspension (2.0 x 10^6 cells/ml) prepared previously by direct counting of the cells using a hemocytometer counting chamber (Improved Neubauer Chamber) were then added. After a period of adherence of two hours at 5% CO_2 and 37 °C the plates were washed with PBS, and 100 µl of Triton X-100 were added to the wells. An aliquot of 20 µl of this suspension were serially diluted (ratio 10) and aliquots (100 µl) corresponding to dilutions 10^{-2}, 10^{-3} and 10^{-4} were plated on Sabouraud Agar 2.0% and incubated at 37 °C for 24 hours. After this period, CFU (colony forming unit) was counted to calculate the percentage of adherence

given by the ratio of the adhered cells after two hours and the initial number of cells inoculated.

Biofilm production assay

Biofilm production was evaluated in polystyrene microtiter plates (flat-bottomed 96-well) (Techno Plastic Products, Switzerland), as described by Shin et al. (2002) (36). The isolates were grown in Sabouraud medium supplemented with 8% glucose for 24 h at 37 ºC. Subsequently, the cells were washed (6,000 rpm for 10 minutes) with sterile 0.85% NaCl and the cell suspension was adjusted to 3.0 x 10^7 cells/ml in Sabouraud broth. An aliquot of 20 µl of the cell suspension were transferred to each well containing 180 µl of SDB. The plates were incubated at 37 °C for 24 h without agitation and washed once with sterile distilled water. Approximately 200 µl of distilled water were added to each well before spectrophotometric readings at 405 nm with a microtiter plate reader (Universal Microplate Reader ELx 800, Bio-Tek Instruments, USA). The biofilm production was calculated according to the percentage of transmittance blocked (%Tbloc=%T value for each test sample was subtracted from the %T value for the reagent blank) and each isolate was scored according to the authors as negative (%Tbloc, < 5), 1+ (%Tbloc, 5 to 20), 2+ (%Tbloc, 20 to 35), 3+ (%Tbloc, 35 to 50), or 4+ (%Tbloc, >50).

Determination of biofilms metabolic activity by reduction of XTT

A second method used to determine biofilm production was performed according to Chandra et al. (2001) (8) with some modifications. Samples were cultured in Sabouraud medium for 24 h at 37 ºC and adjusted to 3.0 x 10^7 cells/ml in RPMI medium. An aliquot of 20 µl taken from the culture cell was inoculated in each well containing 180 µl of

RPMI medium, they were incubated at 37 °C for 24 h without agitation. The plates were washed once with sterile distilled water and metabolic activity was then evaluated using 2,3-bis (2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide (XTT)-reduction assay according to Ramage et al. (2001) (33). The optical density was measured by spectrophotometric reading 490 nm.

Statistical analysis

The results were analyzed by ANOVA One-way, and comparative analysis of the samples was generated by Tukey test with statistical significance of $p < 0.05$.

Proteomics

***Candida tropicalis* growth conditions**

Here, the proteome of planktonic FLC-susceptible *C. tropicalis* were compared to planktonic FLC-resistant and to FLC-susceptible sessile counterparts' cells. In addition, the proteome of sessile cells of FLC-susceptible was compared to sessile cells of FLC-resistant *C. tropicalis* strains. So, planktonic cells were grown in Sabouraud broth for 24 h at 37 °C under constant agitation (200 rpm). The sessile cells were obtained after 24 h of biofilm formation, performed as described previously (36). After growth in 24 wells plates, sessile cells were harvested by gentle scraping with a sterile toothpick.

Total protein extraction

A mechanical technique, adapted from Wong et al. (2007) (42) was used for protein extraction. The yeast cells were harvested and washed two times with sterile PBS

(150 mM NaCl, pH 8.0 phosphate buffer). An equal volume of 1 mm glass beads was added into each tube. After freezing with liquid nitrogen during one minute, the tubes were homogenized in a vortex for three minutes. This cycle was repeated fifteen times, and then the protein extract was separated from cell debris and glass beads by centrifugation at 13,000 rpm for 10 min followed by further centrifugation for 15 min. The protein extract was stored at -20 °C.

Protein quantitation

Protein concentration was determined with the Bradford assay (1976) (5) and the spectrophotometric reading was performed on Shimadzu 1650PC.

Two dimensional PAGE

Prior to isoelectric focusing (IEF), Immobiline DryStrips pH 3–10 non-linear (13 cm) were rehydrated. Samples containing 400 μg of protein were solubilized in a rehydration solution (7 M urea, 2 M, 2% CHAPS, 0.5% ampholyte 'IPG buffer pH 3 NL -10', 0.002% bromophenol blue). After DTT (0.28%) addition, this rehydration solution was applied to the IPG strips for overnight rehydration. The rehydrated IPG strips were then submitted to isoelectric focusing performed on an IPGphor II system (GE Healthcare) at 20 °C using the following 4 stages program: 500 Volts (V) for 1 h, 1000 V for 1 h, 8000 V for 2.5 h, and 8000 for 55 min. Subsequently, the immobilized pH gradient strips were reduced (1% dithioerythritol) and then alkylated (2.5% iodoacetamide) in an equilibration buffer (6 M urea, 75 mM Tris-HCl pH 8.8, 29.3% glycerol, 2% SDS, 0.002% bromophenol blue). The second-dimension SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in SE 600 Ruby unit (GE Healthcare) using 1.5 mm-thick SDS-polyacrylamide gel 10% (30%

acrylamide, 0.8% bisacrilamida, 1.5 M Tris-HCl pH 8.8, 10% SDS, 10% ammonium persulfate, TEMED). Eletrophoresis was maintained at constant conditions of 270 V, 50 W at 10 °C and the current was adjusted to 15 mA/gel for the initial migration and then was increased to 30 mA/gel.

Gel staining

The gels obtained from the second dimension were stained with Coomassie blue G-250 colloidal according to Candiano et al. (2004) (7). Following the separation, the gels were fixed with a solution containing 3% phosphoric acid and 50%ethanol (1h, twice), followed with 2% phosphoric acid solution (1 h) and stained with colloidal Coomassie Blue G-250 (0.1% Coomassie G-250, 2% phosphoric acid, 15% ammonium sulfate, 17% ethanol) during 16 h. The gels were then washed with ultrapure water to remove Coomassie G-250 residues and stored in a solution of 15% ammonium sulfateat 4 °C.

Image analysis

After staining, the 2-D gels images were captured by scanning on an ImageScanner II (GE Healthcare Life Sciences) using LabScan 5.0 software (GE Healthcare) and analyzed on Image Master 2D Platinum 6.0. With this image analysis software, the gels were subjected to automatic spot detection, and after making manual necessary corrections they were processed for volumetric quantification, matching, and statistical analysis (ANOVA). For the comparative gels analysis, 3 gels representative of each condition were analyzed to ensure the accuracy of analyses, so differentially expressed proteins were selected for identification. Spots of interest were excised and subjected to trypsinization.

RESULTS and DISCUSSION

Species identification and induction of fluconazole resistance

Phenotypic and PCR-based methods confirmed the identity of *C. tropicalis*. The MIC_{50} value of fluconazole was 4 μg/ml. For the Induction of fluconazole resistance the sample was cultured with increasing concentrations of fluconazole [4 μg/ml (1x), 8 μg/ml (2x), 16 μg/ml (4x), 32 μg/ml (3x), 64 μg/ml (3x) and 80 μg/ml (2x)] until the 15th cultivation, when the resistance was detected.

Phospholipase, protease and hemolytic activities

In all experiments carried out no precipitation zone around the colony could be detected, so all isolates were considered negative to phospholipase activity. The Dz values corresponding to proteolytic activity of *C. tropicalis* exposed to fluconazole, before and after induction of fluconazole resistance, ranged from 0.36± 0.02 to 0.53± 0.02. For fluconazole resistant isolate, Dz value was significantly higher than the initial isolate ($p< 0.001$), and after 1 h of fluconazole exposure the value was significantly decreased ($p< 0.01$). The hemolytic activity was partial, or alpha-hemolytic, to all isolates, so after 48 h of incubation a greenish halo surrounding the inoculum site was observed. Differences were found on the alpha-hemolysis halo of isolate after fluconazole exposure and of the fluconazole resistant strain. In both cases, the hemolysis halo was minor than the parental strain.

Cell surface hydrophobicity

An increased value of CSH was observed for FLC-resistant compared to

FLC-susceptible *C. tropicalis* ($p< 0.001$). It was also noticeable a decrease in cell surface hydrophobicity after exposure to fluconazole ($p< 0.05$).

Adherence and biofilm formation

In biofilm formation assays, no significant difference between the two methods used was observed. By Shin et al. (2002) method, FLC-susceptible strain was scored as low biofilm producer [1+ (%Tbloc, 5 to 20)]. After induction of fluconazole resistance, biofilm formation was significantly increased ($p< 0.05$) to high in the classification [3+ (%Tbloc, 35 to 50)], but the prior fluconazole exposure had no significant effect to the biofilm formation (Tables 1 and 2). On the adherence assay on abiotic surface, for FLC-susceptible strain exposed to fluconazole andthe fluconazole resistant strain the values were significantly higher than the FLC-susceptible initial blood culture isolate (table 2). The same pattern was observed for the adhesion assay on biotic surface.

Correlation of the virulence factors expressed by different strains of *C. tropicalis*

Considering the different virulence factors analyzed, significant values for correlation were found to biofilm formation and cell surface hydrophobicity ($p< 0.05$). The influence of fluconazole exposure was observed in most of the virulence factors analyzed. In these tests, blood culture wild isolate had significantly lower activity after brief fluconazole exposure, with only higher values in adherence assay. Otherwise, for FLC-resistant strain, the values for most of the virulence factors were higher than the FLC-susceptible counterpart strain. FLC-resistant strain showed higher proteolytic activity, enhanced adherence and biofilm formation, higher CSH, and lower alpha-hemolytic activity.

Proteomics

Despite the relative difficulty of obtaining proteins from fungi, mechanical extraction by freezing enabled the gain of a significant amount of proteins. And, as observed in the two-dimensional gels, the proteins had a good distribution in different isoelectric points as well as in different molecular weights (Figures 1, 2, 3). In this study, an amount of 400 μg protein/gel was used. This amount allowed a good resolution in the gel analysis. Around 400 or more spots were quantified by automatic detection. As mentioned, for each studied condition (planktonic cells and sessile cells of *C. tropicalis* sensitive and resistant to fluconazole) bi-dimensional gels were made. Therefore, only spots present in three replicates were considered for analysis. After matching, the classes were analyzed. In the analysis of *C. tropicalis* cells susceptible and resistant to fluconazole gels, only a few spots were considered significantly different ($p< 0.05$). In most of them, the spot intensity was higher to *C. tropicalis* susceptible gel. However, the analysis of samples of sessile cells resistant and susceptible to fluconazole, the opposite was found. In this case, most of the spots had higher expressive level to the group of sessile cells of resistant isolate. Finally, a comparison was made to sessile and planktonic populations. In this analysis, difference in the spot expressive level were found in twelve spots, five of them more expressive in the population of planktonic cells and seven in the sessile population (Figure 3). In the first comparison of planktonic cells resistant and sensitive (Figure 1), nine potential spots were chosen, two of them more expressive in resistant cells gels. For comparison of isolates of sessile cells resistant and susceptible to fluconazole, eleven spots were selected, one of them more expressive to susceptible cells gels (Figure 2).

REFERENCES

1.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, p.2483–2489, 2002.

2.ANIL, S.; ELLEPOLA, A.N.B.; SAMARANAYAKE, L.P. The impact of polyene, azole and DNA analogue antimycotics on the cell surface hydrophobicity of *Candida albicans* and *Candida tropicalis* in HIV infection. *Mycopathologia*, v.153, p.179–185, 2001.

3.BARCHIESI, F.; MARACEI, M.; BALDASSARRI, I.; SPREGHINI, E.; GIANNINI, D.; SCALISE, G. Tolerance to amphotericin B in clinical isolates of *Candida tropicalis*. *Diagnostic Microbiology and Infectious Disease*, v.50, p.179–185, 2004.

4.BORG-VON ZEPELIN, M.; BEGGAH, S.; BOGGIAN, K.; SANGLARD, D.; MONOD, M. The expression of the secreted aspartyl proteinases Sap4 to Sap6 from *Candida albicans* in murine macrophages. *Molecular Microbiology*, v.28, p.543–554, 1998.

5.BRADFORD, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, v.72, p.248–254, 1976.

6.CALDERONE, R.A.; FONZI, W.A. Virulence factors of *Candida albicans*. *Trends in Microbiology*, v.9, n.7, p.327–335, 2001.

7.CANDIANO, G.; BRUSCHI, M.; MUSANTE, L.; SANTUCCI, L.; GHIGGERI, G.M.; CARNEMOLLA, B.; ORECCHIA, P.; ZARDI, L.; RIGHETTI, P.G. Blue silver: a very sensitive colloidal Coomassie G-250 staining for proteome analysis. *Electrophoresis*, v.25, n.9, p.1327–33, 2004.

8.CHANDRA, J.; MUKHERJEE, P.K.; LEIDICH, S.D.; FADDOUL, F.F.; HOYER, L.L.; DOUGLAS, L.J.; GHANNOUM, M.A. Antifungal resistance of Candidal biofilms formed on denture acrylic *in vitro*. *Journal of Dental Research*, v.80, p.903–908, 2001.

9.COLOMBO, A.L.; GUIMARÃES, T. Epidemiologia das infecções hematogênicas por *Candida* spp. *Revista da Sociedade Brasileira de Medicina Tropical*, v.36, n.5, p.599–607, 2003.

10.COLOMBO, A.L.; NUCCI, M.; PARK, B.J.; NOUER, S.A.; ARTHINGTON-SKAGGS, B.; DA MATTA, D.A.; WARNOCK, D.; MORGAN, J. Epidemiology of candidemia in Brazil: a nationwide sentinel surveillance of candidemia in eleven Medical Centers. *Journal of Clinical Microbiology*, v.44, n.8, p.2816–2823, 2006.

11.COSTA, S.F.; MARINHO, I.; ARAUJO, E.A.P.; MANRIQUE, A.E.I.; MEDEIROS, A.S.; LEVIN, A.S. Nosocomial fungaemia: a 2-year prospective study. *The Journal of Hospital Infection*, v.45, p.69–72, 2000.

12.DOUGLAS, L.J. *Candida* biofilms and their role in infection. *Trends in Microbiology*, v.11, p.30–36, 2003.

13.IZUMI, E.; YAMADA-OGATTA, S.F. Análise da atividade proteolítica extracelular de isolados clínicos de *Candida albicans*. Universidade Estadual de Londrina, Monografia de Conclusão de Curso, Ciências Biológicas, 50p., 2004.

14.JABRA-RIZK, M.A.; FALKLER, W.A.; MEILLER, T.F. Fungal biofilms and drug resistance. *Emerging Infectious Diseases*, v.10, n.1, p.14–9, 2004.

15.JIN, Y.; SAMARANAYAKE, Y.H.; YIP, H.K.; SAMARANAYAKE, L.P.Characterization of switch phenotypes in *Candida albicans* biofilms. *Mycopathologia*, v.160, p.191–200, 2005.

16.KAM, L.W.; LIN, J.D. Management of systemic candidal infections in the intensive care unit. *American Journal of Health-System Pharmacy*, v.59, p.33–41, 2002.

17.KAMINISHI, H.; MIYAGUCHI, H.; TAMAKI, T.; SUENAGA, N.; HISAMATSU, M.; MIHASHI, I.; MATSUMOTO, H.; MAEDA, H.; HAGIHARA, Y. Degradation of humoral host defence by *Candida albicans* proteinase. *Infection and Immunity*, v.63, n.3, p.984–988, 1995.

18.KANTARCIOGLU, A.S.; YUCEL, A. Phospholipase and protease activities in clinical *Candida* isolates with reference to the sources of strains. *Mycoses*, v.45, p.160–165, 2002.

19.KURTZMAN, C.P.; FELL, J.W. Definition, classification and nomenclature of the yeasts. In: The Yeasts, A Taxonomic Study, C.P. Kurtzman and J.W. Fell (eds.) 4th edn. pp. 3-5. Elsevier Science B.V., Amsterdam. 1998.

20.LEE, F.J.S. Modified protocol for yeast DNA mini-preparation. *Biotecniques*, v.5, p.677, 1992.

21.LUO, G.; SAMARANAYAKE, L.P.; YAU, J.Y.Y. *Candida* species exhibit differential in vitro hemolytic activities. *Journal of Clinical Microbiology*, v.39, p.2971–2974, 2001.

22.MANNARELLI, B.M.; KURTZMAN, C.P. Rapid Identification of *Candida albicans* and other human pathogenic yeasts by using short oligonucleotides in a PCR. *Journal of Clinical Microbiology*, v.36, n.6, p.1634–1641, 1998.

23.MONOD, M.; BORG-VON ZEPELIN, M. Secreted proteinases and their virulence mechanism of *Candida albicans*. *Chemical Immunology*, v.81, p.114–118, 2002.

24.MORSCHHÄUSER, J. The genetic basis of fluconazole resistance development in *Candida albicans*. *Biochimica et Biophysica Acta*, v.1587, p.240–248, 2002.

25.MYOKEN, Y.; T. KYO; M. FUJIHARA; T. SUGATA; MIKAMI, Y. Clinical significance of breakthrough fungemia caused by azole-resistant *Candida tropicalis* in patients with hematologic malignancies. *Haematologica*, v.89, p.378–380, 2004.a

26.MYOKEN, Y.; KYO, T.; FUJIHARA, M.; SUGATA, T.; MIKAMI, Y. Clinical significance of breakthrough fungemia caused by azole-resistant *Candida tropicalis* in patients with hematologic malignancies. *Haematologica*, v.89, p.378–380, 2004.b

27.NAGLIK, J.R.; CHALLACOMBE, S.J.; HUBE, B. *Candida albicans* secreted aspartyl proteinases in virulence and pathogenesis. *Microbiology and Molecular Biology Reviews*, v.67, p.400–428, 2003.

28.NCCLS - Método de Referência para Testes de Diluição em Caldo para a Determinação da Sensibilidade a Terapia Antifúngica das Leveduras. Norma Aprovada - Segunda Edição. NCCLS document M27-A2 [ISBN 1-56238-469-4]. NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 Estados Unidos, 2002.

29.PERFECT, J.R. Fungal virulence genes as targets for antifungal chemotherapy. *Antimicrobial Agents and* Chemotherapy, v.40, p.1577–1583, 1996.

30.PFALLER, M.A.; DIEKEMA, D.J.; MESSER, S.A.; BOYKEN, L.; HOLLIS, R.J.; JONES, R.N. *In vitro* susceptibilities of rare *Candida* bloodstream isolates to ravuconazole and three comparative antifungal agents. *Diagnostic Microbiology and Infectious Disease*, v.48, p.101–105, 2004.

31.PONTECORVO, G.; ROPER, J.A.; HEMMONS, L.M.; McDONALD, K.D.; BUFTON, W.F.C. The genetics of *Aspergillus nidulans*. *Advances in Genetics*, v.5, p.141–238, 1953.

32.PRICE, M.F.; WILKINSON, I.A.; GENTRY, L.O. Plate method for detection of phospholipase activity in *Candida albicans*. *Sabouraudia*, v.20, p.7–14, 1982.

33.RAMAGE, G.; WALLE, K.V.; WICKES, B.L.; LÓPEZ-RIBOT, J. Biofilm formation by C*andida dubliniensis*, *Journal of Clinical Microbiology*, v.39, p.3234–3240, 2001.

34.RÜCHEL, R. Cleavage of immunoglobulins by pathogenic yeasts of the genus *Candida*. *Microbiological Sciences*, v.3, p.316–319, 1986.

35.SAMRA, Z.; YARDENI, M.; PELED, N.; BISHARA, J. Species distribution and antifungal susceptibility of *Candida* bloodstream isolates in a tertiary medical center in Israel, *European Journal of Clinical Microbiology & Infectious Diseases*, v.24, p.592–595, 2005.

36.SHIN, J.H.; KEE, S.J.; SHIN, M.G.; KIM, S.H.; SHIN, D.H.; LEE, S.K.; SUH, S.P.; RYANG, D.W. Biofilm production by isolates of *Candida* species recovered from nonneutropenic patients: comparison of bloodstream isolates from other sources. *Journal of Clinical Microbiology*, v.40, n.4, p.1244–1248, 2002.

37.VAN BURIK, J-A.H.; MAGEE, P.T. Aspects of fungal pathogenesis in humans. *Annual Review of Microbiology*, v.55, p.743–772, 2001.

38.VANDEPUTTE, P.; LARCHER, G.; BERGÈS, T.; RENIER, G.; CHABASSE, D.; BOUCHARA, J. MECHANISMS of Azole Resistance in a Clinical Isolate of *Candida tropicalis*. *Antimicrobial agents and chemotherapy*, v.49; n.11, p.4608–4615, 2005.

39. VIDOTTO, V.; KOGA-ITO, C.Y.; MILANO, R.; FIANCHINO, B.; PONTÓN, J. Correlation between germ-tube production, phospholipase activity and serotype distribution in *Candida albicans*. *Revista Iberoamericana de Micología*, v.16, p.208–210, 1999.

40. WHITE, T.C.; HOLLEMAN, S.; DY, F.; MIRELS, L.F.; STEVENS, D.A. Resistance mechanisms in clinical isolates of *Candida albicans*. *Antimicrobial Agents and Chemotherapy*, v.46, n.6, p.1704–1713, 2002.

41. WINGARD, J.R. Importance of *Candida* species other than *C. albicans* as pathogens in oncology patients. *Clinical Infectious Diseases*, v.20, p.115–125, 1995.

42. WONG, S.F.; MAK, J.W.; POOK, P.C.K. New mechanical disruption method for extraction of whole cell protein from *Candida albicans*. *Southeast Asian*, v.38, n.3, 2007.

43. YANG, Y.L.; HO, Y.A.; CHENG, H.H.; HO, M.; LO, H.J. Susceptibilities of *Candida* species to amphotericin B and fluconazole: the emergence of fluconazole resistance in the *Candida tropicalis*. *Infection Control and Hospital Epidemiology*, v.25, p.60–64, 2004.

Table 1 - Putative virulence factors of *Candida tropicalis* isolates tested *in vitro*

	Phoospholipase[a]	Protease[a]	Hemolisine[b]	Adherence[e]	Adherence[e1]	Biofilm[d]	Biofilm[e]	CSH[c]
H	-	0.46±0.04	0.25±0.02	0.148±0.048	36.75±18.98	13.63±3.42	0.215±0.045	46.47±6.22
Hr	-	0.36±0.02	0.28±0.02	0.187±0.077	75.5±6.64	35.87±5.54	0.721±0.036	70.57±6.00
H1h	-	0.53±0.02	0.30±0.02	0.186±0.076	76.94±18.91	13.66±2.22	0.127±0.036	36.06±5.39

*Isolates analyzed: (H) blood culture, (Hr) isolate of blood culture fluconazole resistant and (H1) isolate of blood culture after 1 h of exposure to fluconazole. The values represent the arithmetic mean and standard deviation of three experiments in duplicate. (-) Phospholipase negative.

a Enzymatic activity of protease and phospholipase determined on plates containing 0.6% skim milk and 4.0% egg yolk respectively. Dz or Pz = 1, null activity; 0.64 ≤ Dz/Pz <1, low activity; ≤ 0:30 Dz/Pz <0.64, intermediate activity; Dz/Pz <0.30, high activity. Dz or Pz index determined by the ratio between colony diameter and the diameter of the zone of degradation / precipitation.

b Hemolytic activity was determined in SDA medium supplemented with 3% glucose and 5% sheep blood. Evaluation of Pz was determined by alpha-hemolytic activity.

c Percentage of cell surface hydrophobicity (CSH) determined by the difference in the optical density of the aqueous phase between test and control.

d Biofilm formation in microtiter plates (96 wells). % TBlocked (percent transmittance blocked) is the amount of light blocked after going through each well. The production of biofilm was evaluated as negative (% T bloc <5), + (% T bloc = 5-20); + + (% T bloc= 20-35); + + + (% Tbloc = 35-50); + + + + (% T bloc ≥ 50).

e Adherence assay and biofilm formation performed by XTT assay. In the adherence assay the yeast cells were incubated for1 hour while in the biofilm formation the incubation was of 24 h.

e1 Adherence assay on biotic surface performed with VERO (kidney of African green monkey) cells plates. The adhered cells were plated for later cfu count (colony forming unit) and adherence percentage calculation given by the ratio of adhered cells after two hours and the initial number of cells innoculated.

Table 2 - Comparative analysis of virulence factors in *Candida tropicalis* isolates

	Protease[a]	Hemolisine[b]	Adherence[e]	Adherence[e1]	Biofilm[d]	Biofilm[e]	CSH[c]
HxHr	< 0.001 0.101*	< 0.05 -0.029*	< 0.05 -0.039*	< 0.05 -38.75*	< 0.001 -22.25*	< 0.001 -0.506*	< 0.001 - 24.10*
HxH1	< 0.01 -0.069*	< 0.01 -0.046*	< 0.05 -0.038*	< 0.05 -0.069*	> 0.05	< 0.001 0.088*	< 0.05 10.41*
HrxH1	< 0.001 -0.171*	> 0.05	> 0.05	> 0.05	< 0.001 22.21*	< 0.001 0.594*	< 0.001 34.51*

Isolates analyzed: (H) blood culture, (Hr) isolate of blood culture fluconazole resistant and (H1) isolate of blood culture after 1 h of exposure to fluconazole.

a Enzymatic activity of protease and phospholipase determined on plates containing 0.6% skim milk and 4.0% egg yolk respectively. Dz or Pz = 1, null activity; 0.64 ≤ Dz/Pz <1, low activity; ≤ 0:30 Dz/Pz <0.64, intermediate activity; Dz/Pz <0.30, high activity. Dz or Pz index determined by the ratio between colony diameter and the diameter of the zone of degradation / precipitation.

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*Values for mean difference (expressed only for significant values $p< 0.05$).

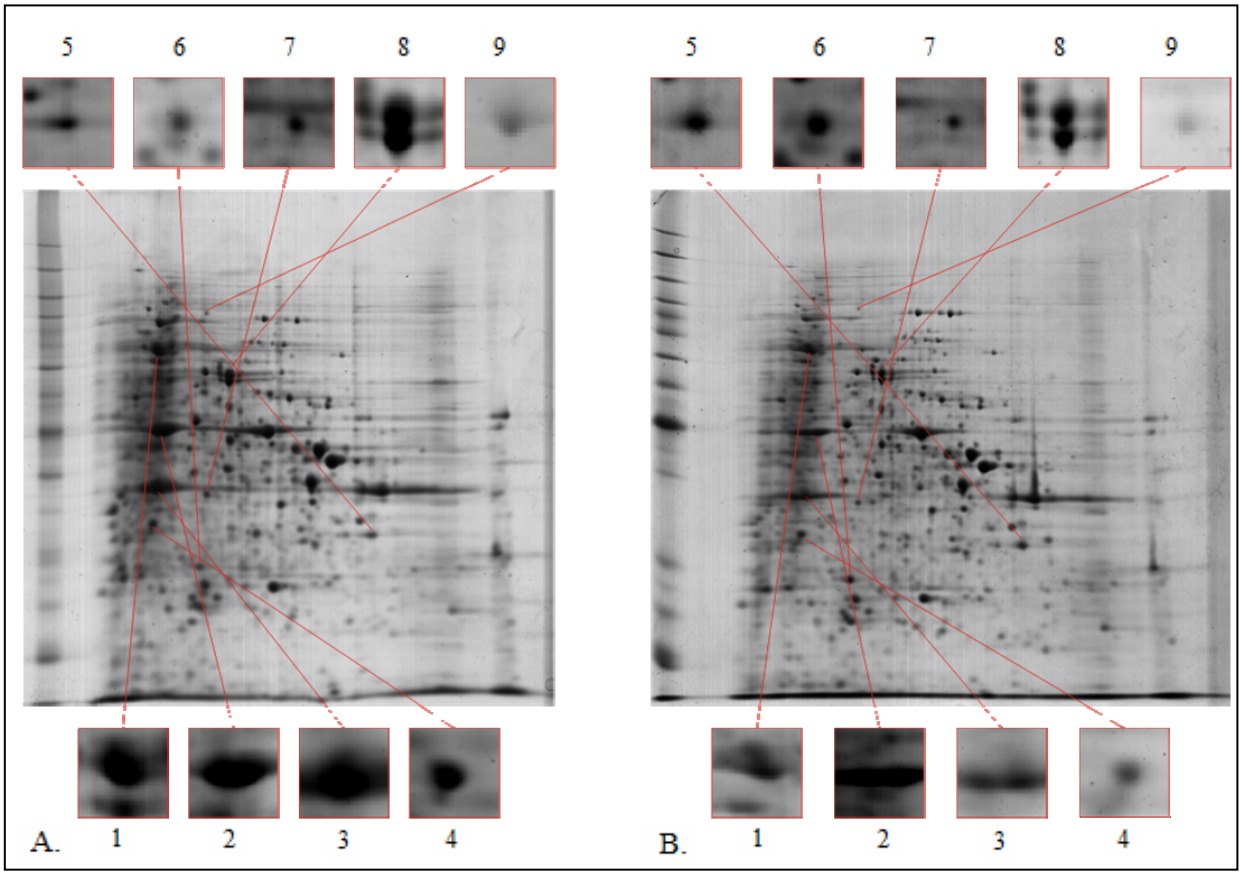

Figure 1 - Comparison between two-dimensional gels of *Candida tropicalis* cells resistant and susceptible to fluconazole. (A.) two-dimensional gel of sensitive cells. The numbers represents significant spots: 1, 2, 3, 4, 7, 8 and 9. (B) two-dimensional gel of resistant cells. Numbers represents the most significant spots: 5 and 6. The sample was isolated from blood infection and subjected to fluconazole resistance induction *in vitro*. Isoelectric focusing was performed on IPG strips pH 3-10 non-linear and SDS-PAGE was performed on polyacrylamide gel 10%.

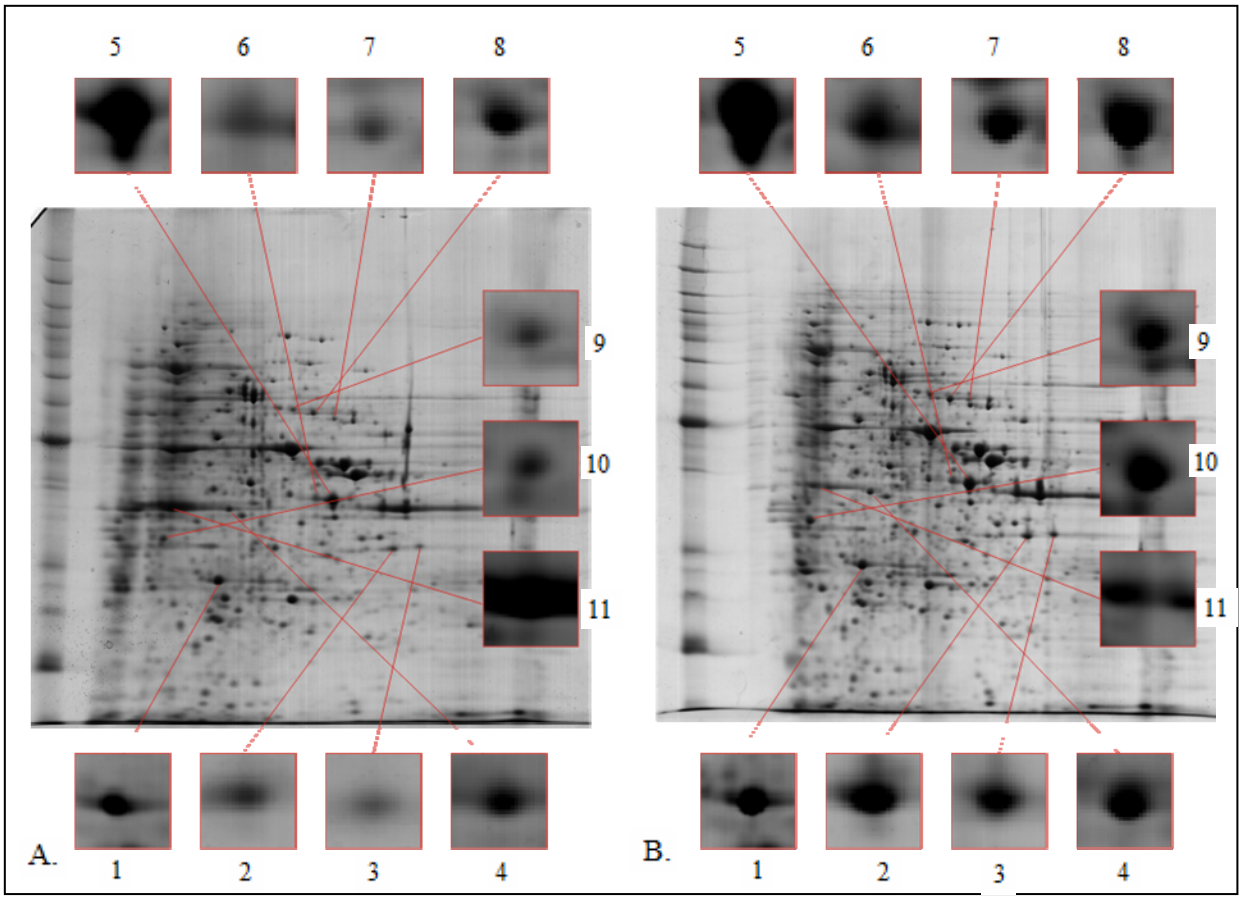

Figure 2 - Comparison between two-dimensional gels of *Candida tropicalis* sessile cells of fluconazole resistant strain and susceptible strain. (A) Two-dimensional gel of sessile cell fluconazole susceptible strain. The number 11 represents the most significant spot. (B) Two-dimensional gel of sessile cell fluconazole reesistant strain. Numbers represents the most significant spots: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 12. The sample was isolated from blood infection and subjected to fluconazole resistance development *in vitro*. Isoelectric focusing was performed on IPG strips pH 3-10 non-linear and SDS-PAGE was performed on polyacrylamide gel 10%.

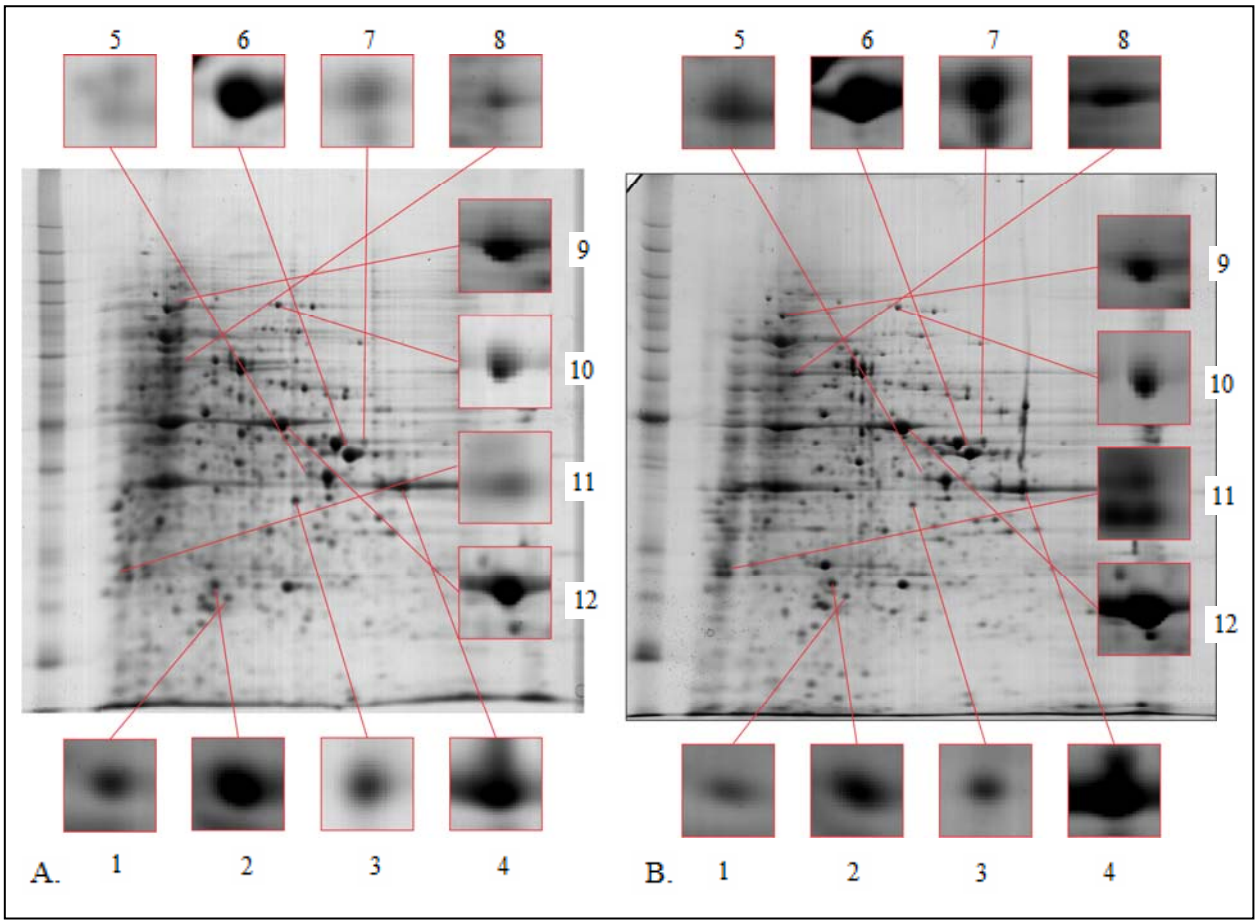

Figure 3 - Comparison between two-dimensional gels of *Candida tropicalis* sessile and planktonic cells. (A.) Two-dimensional gel of planktonic cells. The most significant spots are represented by the numbers: 1, 2, 3, 9 e 10. (B.) Two-dimensional gel of sessile cells. The numbers represents the most significant spots: 4, 5, 6, 7, 8, 11, 12. Isoelectric focusing was performed on IPG strips pH 3-10 non-linear and SDS-PAGE was performed on polyacrylamide gel 10%.